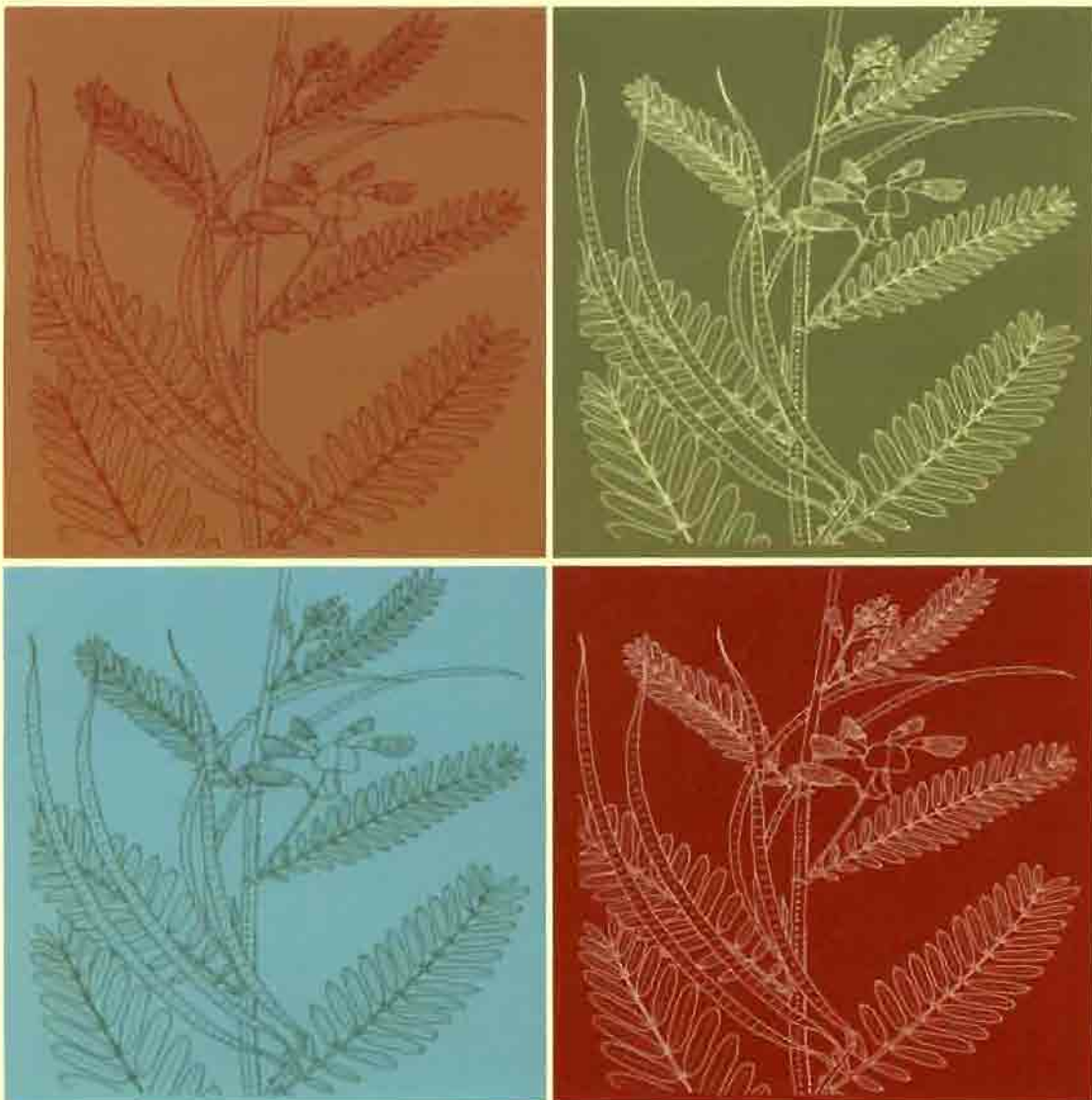**CTA**

Sesbania rostrata

Actes - Proceedings
Dakar, Sénégal - 1989
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ORSTOM

Centre Technique de Coopération Agricole et Rurale ACP/CEE

Technical Centre for Agricultural and Rural Cooperation ACP/EEC

Sesbania rostrata

CONGRES INTERNATIONAL

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DAKAR, :   3AL - 1989

ORSTOM

ACTES - PROCEEDINGS

Préface

Découvert en 1979 comme fixateur d'azote à nodules caulinaires, Sesbania rostrata est devenu un modèle d'étude pour de nombreuses équipes de par le monde. Au niveau fondamental, la symbiose est originale: elle fait intervenir deux genres bactériens, Rhizobium et le nouveau genre Azorhizobium; le mode d'infection des tiges et des racines est particulier, de même que le mode de régulation de la nodulation et de la fixation d'azote. Au niveau appliqué, Sesbania rostrata, du fait de sa croissance exceptionnelle et de son efficacité vis à vis de la fixation d'azote, présente des potentialités indéniables comme engrais vert, qui sont du reste déjà mises à profit dans certains pays.

Une meilleure compréhension des mécanismes de la symbiose pourrait permettre la manipulation éventuelle de cette association pour le bénéfice de l'agriculture, notamment dans les pays en voie de développement. Ceci explique l'intérêt d'un nombre croissant de pays du monde entier via à vis des Sesbania sp. et de Sesbania rostrata en particulier.

Après une décennie de recherches sur Sesbania rostrata couvrant des domaines très variés allant de l'agronomie à la biologie moléculaire et menées dans toutes régions du globe, il était devenu urgent d'organiser une rencontre des scientifiques concernés pour rassembler les données éparses, confronter les besoins des agronomes aux possibilités actuelles de la recherche fondamentale et convenir des orientations prioritaires à privilégier. Ainsi plus de 50 scientifiques de 18 pays ont pris part à ce premier colloque international sur Sesbania rostrata qui concrétise neuf ans de recherches pluridisciplinaires sur Sesbania rostrata, et qui marque le début d'une collaboration internationale par la mise en place de réseaux d'informations et d'échanges de matériel biologique.

Six sessions avec 26 présentations orales et une session de posters ont su retenir l'intérêt des participants venus des universités, des instituts de recherche et des secteurs privés, s'intéressant aux recherches fondamentales et à leurs applications éventuelles.

Le congrès de Dakar n'aurait pu avoir lieu sans la participation des diverses institutions, organismes de recherche et universités, ainsi que l'assistance des deux institutions locales organisatrices.

Nous remercions en particulier pour leur soutien:

▲ *Le Centre Technique de Coopération Agricole et Rurale (CTA), -Le Ministère Français des Affaires Etrangères,*

▲ *Le Ministère Français de la Coopération,*

▲ *La DIVA (Département pour l'Information et la Valorisation) de l'Institut Français de Recherche Scientifique pour le Développement en Coopération (ORSTOM),*

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▲ *The International Society for Plant Molecular Biology,*

▲ *l'Institut Senegalais de Recherches Agricoles (ISRA), ainsi que toutes les personnes qui ont contribué à faire de ce congrès une réussite et spécialement Madame Anna Marthe Konte qui a participé à la mise en forme de cet ouvrage.*

Les éditeurs.

Preface

In 1979 Sesbania rostrata was recognised as a N_2-fixing legume with stem nodules. Since that time it has become a model for teams all over the world to study. Basic research is interested in its special symbiotic effect which involves two bacterial genera, Rhizobium, and a newly described genus, Azorhizobium. The mode of stem and root infection and the mode of N-fixation are unusual. For applied research, Sesbania rostrata, because of its exceptional growth and efficient N-fixing capacity has undeniable potentials as green manure. These potentials have already been developed in certain countries.

A better understanding of the mechanisms of symbiosis might facilitate manipulations that would render this association useful to agriculture, in particular in the developing countries. This explains the interest of a growing number of countries throughout the world in the Sesbania species, especially in S. rostrata.

After some ten years of research on S. rostrata, conducted in all the regions of the world, in fields as varied as agronomy and molecular biology, the time was ripe for organising a seminar for "Sesbania scientists" who thus could pull together all their uncoordinated data, compare present day agronomic needs with the capabilities of basic research and set out priority lines of action. The first international colloquium of S. rostrata thus brought together more than 50 scientists from 18 countries to give tangible expressions to nine years of multi-disciplinary research on S. rostrata and to inaugurate international cooperation by the establishment of networks on information and on exchanges of biological materials.

Six sessions, with 26 oral presentations and a poster session, attracted participants from universities, research institutes and the private sector, all interested in basic research and the related applications.

The Dakar conference could not have been held without inputs from various institutions, research organisations and universities and without assistance from the two local organising institutions.

We gratefully acknowledge support from:

▲ *The Technical Centre for Agricultural and Rural Cooperation,*

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The Editors.

Résumé

Sesbania rostrata, le sujet de ce colloque international, est une plante légumineuse annuelle possédant des propriétés remarquables. Le taux de croissance prodigieux de S. rostrata est de grand intérêt. Il est lié à sa capacité de former des nodules fixateurs d'azote atmosphérique, non seulement sur les racines, mais aussi au niveau de sites prédéterminés sur la tige (primordiums de racines latérales). Sesbania rostrata croît durant la saison des pluies en Afrique de l'Ouest, ce qui correspond à l'été sous les latitudes du nord. Ainsi qu'en témoignent les chercheurs présents à ce colloque, le prodigieux potentiel fixateur des légumineuses à nodules de tige permet à Sesbania rostrata d'enrichir l'azote combiné dans le sol Sénégalais à raison de 250 kg-atomes N par hectare. Des travaux effectués en laboratoire et en serre ont en outre démontré que le taux de fixation d'azote de Glycine max (soja) (la plante de référence parmi les plantes légumineuses fixatrices d'azote) paraît insignifiant en comparaison avec celui de S. rostrata.

Ce colloque international a été organisé par B. Dreyfus (ORSTOM, Dakar, Sénégal) avec le soutien financier du CTA (Centre Technique de Coopération Agricole et Rurale, fondé par la Communauté Européenne pour la coopération avec les pays en voie de développement). Le désir des organisateurs était de fournir un forum où la recherche de base en biologie moléculaire végétale pourrait être confrontée avec la recherche sur les applications à l'agriculture de subsistance des pays en voie de développement. On espérait ainsi atteindre deux buts. Premièrement, ceux d'entre nous engagés dans la recherche fondamentale pourraient montrer son accessibilité et son utilité aux autres chercheurs et deuxièmement, ceux d'entre nous qui étudient les applications à l'agriculture auraient l'occasion de formuler de façon explicite les besoins réels des agriculteurs. En effet, il est nécessaire pour les chercheurs en biologie moléculaire végétale de comprendre leurs problèmes afin qu'ils puissent développer des stratagèmes permettant d'isoler les gènes importants. A l'avis des participants, ces deux buts ont été atteints et le colloque fut un grand succès. Par contre, la question du support financier de la recherche biologique fondamentale au profit des agricultures de subsistance n'a pas été abordée.

La biologie moléculaire végétale est généralement perçue en tant que discipline qui crée des technologies utilisables par les agricultures intensives, pratiquées principalement par les pays développés pour profit financier. Naturellement, à l'ouverture du colloque, il fut difficile pour les chercheurs appliqués de supplanter le concept que la biologie moléculaire végétale ne présentait pas d'intérêt pour les types d'agricultures dépourvus de hautes technologies. Dans son exposé, M. van Montagu (Plant Genetic Systems at Rijksuniversiteit Gent, Belgique) a détruit ces idées préconçues. Il a rappelé que la biologie moléculaire végétale n'était encore qu'une science naissante, et que, pareille à toutes les autres sciences naissantes, son coût élevé provenait du manque d'efficacité et d'exactitude. Avec le perfectionnement des technologies, les ressources nécessaires à la biologie moléculaire végétale seront simplifiées et les coûts relatifs en temps et en énergie seront par conséquent diminués. M. van Montagu a encouragé les chercheurs à garder leur optimisme dans le dialogue qui allait suivre.

Durant les premières sessions scientifiques l'intérêt s'est porté sur le non moins remarquable partenaire symbiotique de S. rostrata, les bactéries du genre Azorhizobium, décrit récemment. C'est à ces bactéries que la plante doit sa croissance rapide dans les sols tropicaux pauvres. Azorhizobium, a souligné B. Ludwig (University of California, Santa Cruz, USA), est le seul prokaryote connu qui, parmi les divers genres d'organismes fixateurs d'azote, est capable de fixation bi-phasique, aussi bien au profit de sa propre croissance bactérienne qu'au profit de la plante hôte. Un des intérêts du groupe de Ludwig est de comprendre les processus de régulation génétique et de foncitons enzymatiques qui permettent à Azorhizobium de convertir l'azote molécu-

laire en ammoniaque soit en fonction de l'assimilation d'ammoniaque en acides aminés et pro-
téines (durant la croissance bactérienne), soit indépendamment de cette assimilation durant la
symbiose (transfer d'ammoniaque à la plante). Dans ce dernier cas les organelles bactéroïdes
d'Azorhizobium (et de tous les autres Rhizobiacees) existent en tant qu'"usines" productrices
d'ammoniaque dont les besoins énergétiques sont satisfaits par la photosynthèse. Ludwig ainsi que
F. de Bruijn (Max Planck Institut für Zuchtungsforschung, Cologne, RFA) ont proposé que la
régulation du gène nifA d'Azorhizobium (qui code un activateur de transcription) est à la base du
contrôle de la fixation bi-phasique d'azote. J. Denarié (CNRS-INRA, Toulouse, France) a ajouté
que D. Kahn et ses collègues à l'institut ont identifié un groupe de gènes de Rhizobium meliloti qui
contrôlent l'expressions de nifA dans cet organisme en fonction d'O_2. Curieusement, dans ce
groupe, l'un des deux gènes qui codent des activateurs de transcription est homologue au gène nifA
lui-même.

Par suite, Denarié a présenté un exposé sur la génétique moléculaire des gènes de nodulation (nod)
chez l'endosymbiont de luzerne, Rhizobium meliloti. Ces organismes servent de modèle de la
symbiose chez les légumineuses tempérées. Denarié a proposé que la spécificité vis-à-vis de l'hôte
est déterminée par l'interaction entre les gènes nod, communs à tous les Rhizobiums, et les gènes
hsn, spécifiques pour chaque espèce de Rhizobium. M. Holsters (Rijksuniversiteit Gent, Belgique)
a décrit l'identification des gènes communs de nodulation chez Azorhizobium. Ces gènes sont
activés par une apigénine substituée par un trisaccharide, cette substance étant excrétée par les
primordiums de tige de S. rostrata. Holsters a également démontré que le gène nodD de Rhizobium
NGR 234, une souche qui interagit avec plusieurs hôtes, est capable de transactiver les gènes
communs nod d'Azorhizobium en présence d'apigénine. Ainsi des souches dee Rhizobium isolés
par B. Dreyfus qui forment strictement des nodules de racine ont été rendus capables de former des
nodules de tiges grâce à l'introduction du gène nodD de Rhizobium NGR 234.

La recherche fondamentale sur S. rostrata décrite par H. Trinh (Rockefeller University, New York
City, USA) porte sur la morphogenèse in vitro d'organes similaires à des nodules. Ces organes ont
été obtenus par croissance de protoplastes individuels cocultivés avec Azorhizobium. Trinh a
démontré que ces organes contiennent un périderme, un cortex avec des rhizobiums intracellu-
laires et un tissu vasculaire bien organisé. Un tel système de nodulation in vitro présente des
avantages considérables pour l'étude du développement du nodule. T. Bisseling (Université Agro-
nomique, Wageningen, Pays-Bas) a décrit les produits spécifiques aux nodules qui sont formés
durant le développement des plantes-hôtes, aussi bien chez les légumineuse tempérées, exempli-
fiées par Pisum sativum (pois), que chez les légumineuses tropicales, exemplifiées par Glycine max
(soja). Bisseling a démontré que plusieurs de ces "nodulines" sont produites après contact des
plantes avec les produits génétiques de Rhizobium (dans le cas de P. sativum) ou de Bradyrhizo-
bium (dans le cas de G. max). Le groupe de Bisseling a également déterminé les temps d'expression
des gènes de nodulines ainsi que les sites cytologiques où les nodulines agissent. P. de Lajudie
(ORSTOM, Dakar, Sénégal) a présenté ses progrès dans l'identification des nodulines de S. rostra-
ta. Plusieurs chercheurs ont reporté que, bien que S. rostrata soit aisément transformée avec des
souches d'Agrobacterium tumefaciens ou A. rhizogenes, des plantes transgéniques de S. rostrata
(ainsi que de nombreuses autres légumineuses) n'ont pas encore été obtenues.

Les sessions dédiées à la recherche sur les applications à l'agriculture ont mis en évidence l'utilité
de S. rostrata en tant que fourrage et "engrais vert". H. Diara (ANDRAO, Sénégal) et I. N'Doye
(Ministère du Plan et de la Coopération, Dakar, Sénégal) ont montré les bienfaits de S. rostrata
pour la culture du riz au Sénégal. J.K. Ladha (International Rice Research Institute, Philippines) a
confirmé leurs succès avec ses résultats en rhiziculture dans l'Asie du Sud-Est. Aux Philippines,
dans des climats qui incluent une saison de pluies, la croissance de S. rostrata sur les parcelles
expérimentales était abondante. Comme en Afrique de l'Ouest, où S. rostrata est indigène, l'incor-
poration des plantes au sol a augmenté la teneur en azote fixé et a considérablement amélioré le
rendement du riz, lorsque la céréale était cultivée dans le sol ainsi enrichi. Ladha a présenté un
nombre de stratagèmes ingénieux que les chercheurs du IRRI ont développé afin d'exploiter le
potentiel fourrager de S. rostrata. K. Moulongoy (IITA, Ibadan, Nigéria) a décrit les techniques
employées dans les cultures mixtes de S. rostrata et de riz ou maïs dans les différentes régions du
Nigéria. Moulongoy a montré que, grâce au taux de croissance rapide de S. rostrata, les parties

apicales de la plante peuvent être coupées avec des moyens méchaniques sans endommager la culture vivrière avoisinante. Les débris ainsi dissémés ont enrichi le sol en azote et amélioré le rendement des rhizicultures en basses altitudes; en altitudes plus élevées (où les sols sont plus secs) le rendement du maïs était moins impressionant, sans doute à cause des exigences en eau de S. rostrata. Plusieurs autres chercheurs venant d'autres pays d'Afrique, du Madagascar, du Sri Lanka, du Bengladesh, du Népal et de la Chine ont exprimé leur enthousiasme pour l'utilisation de S. rostrata dans leurs pays respectifs.

Bien que l'éloge de S. rostrata fut générale, Y. Dommergues (ORSTOM, France) a rappelé aux participants que cette plante légumineuse présente certains problèmes pour le génie génétique ou les sélections traditionnelles. Dans son environment naturel, S. rostrata fleurit durant la photopériode décroissante. Par conséquent, l'utilité des souches sauvages est réduite sous les tropiques équatoriaux (latitude 10) où les plantes fleuriraient prématurément à cause de la photopériode trop courte. D'autre part, les exigences en eau de S. rostrata sont très importantes et la plante n'est pas adaptée aux climats où la saison des pluies ne coïncide pas aveac la photopériode favorable. Néanmoins, à l'avis de Dommergues, ces limitations peuvent être surmontées à l'aide du génie génétique ainsi que par les méthodes de sélection traditionelles. Paralellement, Dommergues a encouragé la recherche appliquée sur les différentes espèces d'Aeschynomene, légumineuses qui elles aussi forment des nodules de tige. Enfin, il a porté l'attention des participants sur les propriétés désirables de certains nouveaux cultivars de S. rostrata, notamment, une résistance accrue aux parasites, tels les nématodes, un taux de croissance supérieur dans des sols pauvres en minéraux et une valeur nutritionnelle accrue pour le fourrage.

Par suite, M. van Montagu (Rijksuniversiteit Gent, Belgique) et N-H. Chua (Rockefeller University, New York City, USA) ont parcouru les différentes technologies développées par les biologistes moléculaires végétaux afin de produire des plantes transgéniques. Deux cas de succès technologiques ont été présentés: la résistance des plantes aux parasites et aux herbicides. Van Montagu a expliqué en détail deux méthodes pour produire des plantes résistantes: premièrement, l'expression de toxines de choix, telles que l'endotoxine de Bacillus thuringiensis et deuxièmement, l'expression de résistance aux pesticides ou herbicides (tels la phosphinotricine). La phosphinotricine est un inhibiteur spécifique de glutamine synthétase, elle est donc innofensive, du moins en théorie, aux fourrageurs des plantes, l'homme en particulier. Des plantes transgéniques de tabac porteuses de phosphinotricine acétyl transférase de Streptomyces (le gène bar) ont été obtenues. Leurs niveaux de résistance à la phosphinotricine sont suffisamment hauts pour employer les plantes en agriculture. Chua a présenté les résultats de ses travaux sur la cross-protection virale dans les plantes transgéniques qui par suite deviennent résistantes au virus mosaïque de concombre (un agent à large spectre d'infection) et au virus X de pomme-de-terre. La méthode de Chua consiste à développer des plantes transgéniques porteuses des gènes de la capsule du virus; ces gènes sont transcrits d'une manière constitutive dans les deux directions possibles ("sens" et "anti-sens"). L'inoculation avec le virus adéquat a demontré que les plantes transgéniques porteuses des gènes exprimant les protéines de la capsule ont acquis la résistance au virus. Par contre, malgré les résultats encourageants obtenus auparavant, la résistance des plantes porteuses des gènes "anti-sens" n'était que marginale. Ces exemples de génie génétique ont employé des espèces de Nicotinia (tabac) qui sont particulièrement bien adaptées aux manipulations techniques requises. Van Montagu et Chua ont souligné qu'étant donné la nouveauté technologique, leurs projets n'ont pu être réalisés qu'avec un support financier substantiel. Il est évident que la culture de tabac est profitable dans les pays développés où les gouvernements contrôlent les prix. Néanmoins, les deux chercheurs ont rappelé que la généralisation et la simplification des méthodes sont inévitables, ce qui rendra le génie génétique accessible aux cultures de subsistance ainsi qu'aux cultures de rapport existant dans les pays en voie de développement

Pour une première réunion entre chercheurs avec des orientations très différentes le colloque fut un grand succès. Les participants ont souhaité voir deux améliorations dans les réunions à venir. Premièrement, certaines présentations n'ont pas suffisammente tenu compte des intérêts des deux groupes de chercheurs. On espère qu'avec la prise de conscience des disciplines scientifiques de chacun, les futures présentations seront mieux adaptées aux colloques diversifiés. Deuxièmement, à cause du désir des participants d'utiliser au maximum l'opportunité du dialogue entre groupes

scientifiques si différents, plusieurs chercheurs ont présenté une quantité d'information excessive étant donné le temps accordé à chacun. Les participants ont grâcieusement accepté cette "surcharge", sans doute à cause de l'incertitude quant aux interactions à venir. D'ailleurs, le besoin de présenter tous les résultats dans le temps accordé est un syndrome général de toutes les réunions scientifiques.

Il faut espérer que ce colloque ne soit qu'un début d'un vrai dialogue durable entre biologistes moléculaires végétaux et chercheurs intéressés aux applications à l'agriculture de subsistance. Certainement, le véritable défi consiste à matérialiser ce dialogue en créant des programmes de recherche et bien sûr des types d'agriculture de subsistance plus productifs.

R.A. Ludwig

Synopsis

The subject of this International Colloquium, Sesbania rostrata is an annual tropical legume with properties among which are a prodigious growth rate correlating with the capacity to form N_2-fixing nodules on stem lateral-root primordia. Indigenous to West Africa, S. rostrata grows during the wet season, which is summer in northern latitudes. As the applied scientist attending this Colloquium enthusiastically attested, because of the astonishing rates of N_2 fixation on stem-nodulated plants, S. rostrata can augment soil-N with an average yield of 250 kg-atoms N per hectare in Senegal. When tested in the laboratory or greenhouse, stem-nodulated S. rostrata plants exhibit N_2 fixation rates that dwarf those of effective-nodulated Glycine max (soyabean), the benchmark N_2-fixing legume.

Organized by B. Dreyfus (ORSTOM, Dakar, Senegal) and financed by CTA (Agricultural and Rural Technical Centre, endowed by the European Community for cooperation with developing countries), this International Colloquium was convened in order to confront headlong basic plant molecular research with applied research in the subsistence agricultures of developing countries. It was the hope of the organizers that in so doing, (1) those of us working in fundamental research might convey both its accessibility and utility to applied researchers, and (2) applied researchers might explicitly formulate the needs of subsistence farmers so that basic plant molecular biology researcher might comprehend the problems and design strategies to begin the hunt for relevant genes. In these two aspects, the participants judged the Colloquium a resounding success. Left undiscussed was the question of who will support fundamental plant molecular biology research targeted to the subsistence agricultures of developing countries.

As was the case at the outset of this Colloquium, plant molecular biology is most often perceived as a discipline creating technologies relevant to resource-intensive agricultures, primarily practised by developed countries, as for-profit enterprises. As such, it was difficult for many applied scientists to transcend the preconception that plant molecular biology might not truly be relevant to low-to-moderate technology agriculture. In his overview, M. van Montagu (Plant Genetic Systems and State University of Ghent, Belgium) sought to dispel these preconceptions. He pointed out that as is the case for most nascent sciences, plant molecular biology in resource-intensive because to a great extent it is still an inefficient, inexact science. With the perfection of streamlined technologies, Van Montagu asserted that plant molecular biology resource requirements would be simplified and relative costs in both time and energy correspondingly decreased. He urged that both basic molecular biology and applied agricultural scientists be optimistic and begin the dialogue.

In the scientific sessions, attention was first focused on S. rostrata's equally remarkable symbiotic partner, bacteria of the newly described genus Azorhizobium, to which the plant owes its avid growth in poor tropical soils. As pointed out by R.A. Ludwig (University of California, Santa Cruz, USA), Azorhizobium is the only characterized prokaryote, among many diverse N_2-fixing genera, capable of bi-modal N_2 fixation. Azorhizobium can fix N_2 for the benefit of either its own (bacterial) growth processes or those or its host plant. Ludwig's group has sought to understand the underlying processes of gene regulation and enzyme function that allow Azorhizobium to convert N_2 to ammonium such that ammonium production is coupled to its assimilation into amino acids and proteins (for bacterial growth) or, alternatively, is decoupled from these processes and is shunted to the host plant. In the latter case, as is true with all bona fide rhizobia, bacteroid organelles exist as ammonium factories fuelled by host plant photosynthate. Both Ludwig and F. de Bruijn (Max Planck Institute for Breeding Research, Cologne, GFR) pointed out that regulation of the Azorhizobium nifA gene (itself encoding a transcriptional activator) expression is like to mediate bi-modal N_2 fixation. J. De'narie (CNRS-INRA, Toulouse, France) remarked that D. Kahn and co-workers in his institute have identified a cluster of Rhizobium meliloti genes that regulate nifA

gene expression in that organism in response to O_2. Intriguingly, among the salient genes, two encode transcriptional activators, one of which exhibits homology with nifA itself.

De'narie' also outlined the molecular genetics of nodulation (nod) genes in the bona fide rhizobia that nodulate temperate legumes, using as his model Rhizobium meliloti, the alfalfa endosymbiont. Dénarié asserted that interaction between the nod genes, common to all Rhizobium species, and the hsn genes, specific to each Rhizobium species, mediated host specificity. M. Holsters (State University of Ghent, Belgium) described the identification of Azorhizobium common nodulation genes and their activation by a trisaccharide-substituted apigenin, excreted by S. rostrata lateral root primordia. She reported that the nodD gene from Rhizobium NGR234, a strain with broad host-specificity, could transactivate Azorhizobium common nod genes in the presence of apigenin. Indeed bona fide Rhizobium strains isolated by Dreyfus and able to nodulate only S. rostrata roots also nodulated stems when carrying the Rhizobium NGR234 nodD gene.

In work with S. rostrata, H. Trinh (Rockefeller University, New York City, USA) described evidence for the morphogenesis of nodule-like organs from growth of isolated protoplasts co-cultivated in tissue culture with Azorhizobium. The resulting nodule-like organs contained a peridermis, a cortex containing intercellular rhizobia, and organized vascular tissue. Such an in vitro nodulation system offers great potential for studies of nodule development. T. Bisseling (Agricultural University of Wageningen, the Netherlands) presented a developmental overview of nodule-specific gene products encoded by host p;ants for both the Pisum sativum (pea) temperate legume and Glycine max (soyabean) subtropical legume model systems. Bisseling demonstrated that several such "nodulins" are produced after exposure to Rhizobium (for pea) and Bradyrhizobium (for soyabean) gene products; his group has been able to elucidate both the time course of differential nodulin gene expression during nodulation, as well as cytological targets of nodulin activities. P. de Lajudie (ORSTOM, Dakar, Senegal) reported progress in identifying S. rostrata nodulins. Several researcher reported that S. rostrata was easily transformed with either Agrobacterium tumefaciens or A. rhizogenes strains, as is the case with many legumes, transgenic plants have not yet been produced.

In the applied agricultural sciences session, both H. Diara (WARDA, Senegal) and I. N'Doye (Ministry of Scientific Research Planning and Cooperation, Senegal) remounted the benefits for rice culture in Senegal using S. rostrata as "green manure". J.K. Ladha (International Rice Research Institute, the Philippines) echoed successes with rice cultivation in South East Asia. As is the case in Senegal and other West African environments where it is indigenous, S. rostrata flourished in test plots in the Philippines in wet season climates and, when ploughed under, both augmented total soil-N as well as gave substantially increased yield of rice, when the crop was planted subsequently. Ladha reported on a number of ingenious strategies that IRRI scientists have developed to exploit the green manure capabilities of S. rostrata. K. Mulongoy (IITA, Ibadan, Nigeria) reported on intercropping techniques developed to exploit S. rostrata green manure capabilities with rice and maize crops in various locations in Nigeria. In alley cropping systems, Mulongoy found that owing to its faster growth, S. rostrata can be mechanically sheared at a height not yet reached by the principal crops. The dispersed litter provided increased soil-N and better rice yields in lowland trails; gains in upland (dry) soil for maize were less impressive, owing to intense S. rostrata water requirements. Furthermore, applied agricultural researchers from other African countries, as well as Madagascar, Sri Lanka, Bangladesh, Nepal, and China expressed enthusiasm for the potential utility of S. rostrata in their agricultures.

Despite the chorus of praise for S. rostrata, Y. Dommergues (ORSTOM, France) reminded participants of its limitations, knowledge of which he deemed important for future genetic engineering and traditional plant breeding. As a plant that flowers in declining photoperiods in indigenous environments, S. rostrata wild races have limited utility in the equatorial tropics (10 latitude) where they flower prematurely in shorter photoperiod conditions. Moreover, because it demands constant water, S. rostrata wild races are ill suited for climates where wet seasons and appropriate photoperiods do not coincide. Dommergues felt that both these limitations could be overcome by genetic engineering and/or traditional plant breeding. He also encouraged the applied agricultural scientists to expand green manure experiments with Aeschynomene spp., which also form stem nodules.

He also enumerated as desirable, new S. rostrata properties cultivars that showed increased resistance to pests such as soil nematodes, enhanced growth in mineral stressed soils, and increased nutritional value for use as forage.

M. van Montagu and N.H. Chua (Rockefeller University, New York City, USA) presented overviews of the technologies developed by plant molecular biologists to produce transgenic plants. They also provided examples of technological successes, specifically pest-resistant and herbicide-resistant plants (Van Montagu) and virus-resistant plants (Chua). Van Montagu elaborated two strategies to producing pest-resistant plants, (i) expression of targeted pest toxins such as Bacillus thuriengensis endotoxins and (ii) expression of pesticide/herbicide, such a phosphinotricin, resistance. Phosphinotricin is a targeted inhibitor of glutamine synthetase and thus, in theory, minimally affect foragers (such as man) of sprayed plants. When transgenic tobacco plants were produced carrying the Streptomyces phosphinotricin acetyl transferase (bar gene), plants proved resistant at phosphinotricin doses appropriate to field use. Chua presented evidence that resistance to viruses, such as cucumber mosaic virus, a broad spectrum agent, and potato virus X can be engineered in transgenic plants by viral crossprotection. In this strategy, transgenic plants were constructed carrying constitutively-expressed viral coat protein and "antisense" coat protein genes. When challenged with the appropriate virus, transgenic plants carrying cognate expressed coat protein proved virus resistant. Despite previous encouraging results, incorporation of viral "antisense" coat protein genes in transgenic plants proved less successful. These examples of successful genetic engineering to produce resistant plants were carried out with Nicotiana (tobacco) species, which are presently most amenable to the experimental manipulations involved. However, as both Van Montagu and Chua noted, because such projects remain resource intensive, both projects were carried out with substantial commercial support, tobacco being a profitable crop (if only in developed countries with government price supports). Again both scientists stressed that as these technologies are streamlined and disseminated, they will become more accessible to both subsistence and for profit crops in developing countries.

While the Colloquium was pronounced eminently successful by participants as a first meeting between scientists with different orientations, participants noted two areas in which future colloquia might be improved. First, while speakers had been requested to address their remarks not to their colleagues but to the scientists of the alternative camp, in some cases presentation were indistinguishable from made to colleagues. The participants hope that, with a better awareness of each other's scientific disciplines, more appropriate presentation might occur at future meetings. Second, in an effort to make maximum use of the rare opportunity to convene disparate groups of scientists (whose future interactions are uncertain), participants proved graciously receptive to presentations whose content exceeded time constraints. However participants noted that the "present-every-result-in-allotted-time" syndrome seems to be a malaise endemic to scientific meetings in general.

At the close of the Colloquium, participants hoped that a dialogue between plant molecular biologists and applied agricultural scientists studying subsistence agricultures had indeed begun. Participants noted that the challenge of this Colloquium is now to meld this dialogue into new research programmes and, ultimately, more productive subsistence agricultures.

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Session I

Molecular genetics of *Azorhizobium*

Molecular genetics of *Azorhizobium phenotypic* and genotypic studies on tropical rhizobia leading to the characterization of *Azorhizobium caulinodans*

M. Gillis [1], and J.L. Garcia [2] and B. Dreyfus [3]

I. Introduction

The genus *Rhizobium* (which means etymologically that which lives in the roots') was created in 1889 (7) for those bacteria which nodulate the roots of leguminous plants, wherein these bacteria live as endosymbionts.

Since then, their classification has been changed considerably from a system based mainly on plant cross inoculation groups (8,13) to a scheme based on results involving large parts if not total bacterial genomes (2, 9, 10). In the first edition of Bergey's Manual of Systematic Bacteriology, Jordan (14) distinguishes within the rhizobia two genera, *Rhizobium* and *Bradyrhizobium* with respectively 3 and 1 species. The comparison of ribosomal RRNA'S by cataloguing (18) or DNA-rRNA hybridizations (3, 6, 10) has proven to be an excellent tool to study intergeneric and even more remote relationships of bacteria. DNA-rRNA hybridizations have been used to unravel in more detail the inter- and intrageneric relationships with and within *Rhizobium* and *Bradyrhizobium* (10). Both genera belong in rRNA superfamily IV, but are further removed from each other than they are from other genera in this rRNA superfamily. *Rhizobium* is more closely related to *Agrobacterium*; *Bradyrhizobium* to *Rhodopseudomonas palustris* and *Nitrobacter*. *R. loti* occupies a separate position and the taxonomic position of *R. fredii* and rhizobia isolated from *Galega* species has been determined. One stemnodulating *Sesbania* strain was preliminary included in this study (10): it appeared to occupy a separate position in rRNA superfamily IV.

The aim of our work was to determine the exact taxonomic structure and status of the stem-nodulating *Sesbania* strains and to reveal their closest relatives by a polyphasic approach, involving different modern methods allowing differentiation on different taxonomic levels. The methods used were: numerical taxonomy of phenotypic features, comparison of the SDS gelelectrophoretic wholecell protein patterns, total DNA-DNA hybridizations, DNA-rRNA hybridizations and determination of their % (G+C).

The results lead to the proposal of a new genus for the *Sesbania* root- and stem- nodulating bacteria *Azorhizobium*; this new genus contains one species *Azorhizobium caulinodans*.

II. Phenotypic results

A total of 20 strains isolated from stem nodules of *S. rostrata* were compared by methods of numerical taxonomy with 9 fast growing rhizobia strains isolated from root nodules of different *Sesbania* species, with 20 other strains of the genus *Rhizobium* and with 17 strains of the genes *Bradyrhizobium*. The latter groups contained both own isolates from different *Acacia* species and from *Leucaena leucocephala* from Senegal. We compared 221 characters including 151 sole carbon sources. The results of the complete linkage cluster analysis (Figure 1) reveal three clusters corresponding to *Rhizobium*, *Bradyrhizobium* and *Azorhizobium*, which constitutes a homogeneous phenon, distinct from the other two, although a bit closes to the latter.

The fast growing root-nodulating strains from tropical *Acacia* and *Leucaena* species belong together with the *Sesbania* root-nodulating strains in the *Rhizobium* cluster in which we distinguish 4 subphena. Further studies with more tropical rhizobia (unpublished results from K. Kersters and B. Dreyfus) confirm and even extend this heterogeneity since more strains were found in the 4 subphena but also new subphena were detected. The *Bradyrhizobium* cluster contains 2 subphena. The features differentiating the stem-nodulating strains (*Azorhizobium*) from *Rhizobium* and *Bradyrhizobium* were determined. The most striking presence of one lateral flagellum when grown in liquid medium, their colony morphology, their generation time, the lack of sugar assimilation (except glucose). Moreover the *Sesbania* stem-nodulating bacteria con fix N_2 in culture under microaerobic conditions and grow at the expense of this fixed N_2 (4). This seems to be (together with the stem-nodulating capacity) an important discriminative character between *Azorhizobium*, *Rhizobium* and *Bradyrhizobium*, although some strains belonging to *Rhizobium* have been described as showing some degree of ex-planta nitrogen-fixing ability (1, 15). When compared with the stem-nodulating *Sesbania* strains the nitrogenase activity and oxygen tolerance are the highest for the latter strains (30 nmol of C_2H_2 produced per mg of protein per min, and an oxygen tolerance up to 9 nmol).
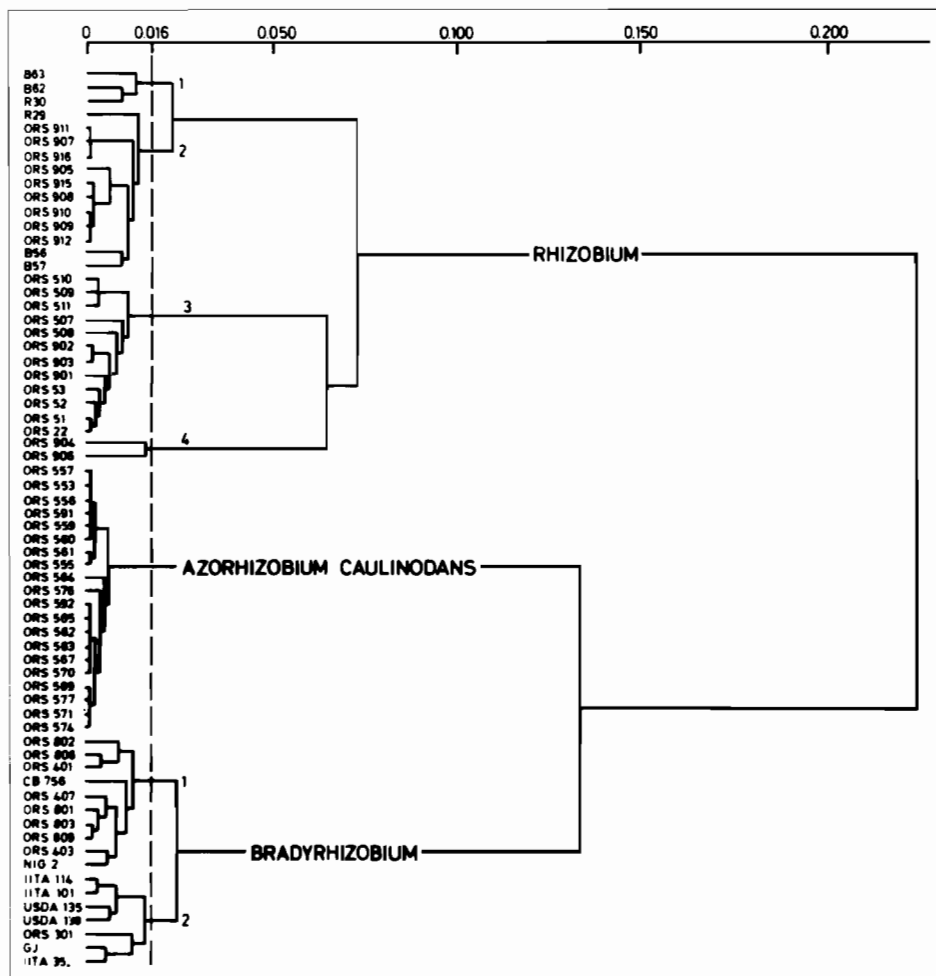
Fig. 1. Dendrogram showing the results of the complete linkage cluster analysis of phenotypic features.

All strains from the *Azorhizobium* cluster formed effective stem and root nodules. Among the *Sesbania* root-nodulating *Rhizobium* strains, 2 strains formed pseudonodules on the stems of *Sesbania rostrata*. Two strains (ORS 609 and ORS 611), isolated from root nodules of *S. cannabina* and *S. grandiflora* were capable to form effective nodules on both roots and stems, but did not fix N_2 in culture. Both strains were not included in the phenotypic analysis but our genotypic results indicated that they were authentic rhizobia.

III. DNA:rRNA hybridization results

Initially, we hybridized DNA from representative strains belonging to the *Rhizobium* phenon (ORS 52 and ORS 22) and the *Sesbania* stem-nodulating phenon (ORS 571) with 3 appropriate rRNA

probes available in our research group. The results (10) located ORS 22 and ORS 52 in the *Rhizobium-Agrobacterium* rRNA complex in rRNA superfamily IV. Strains ORS 571 was located at the bifurcation level (70.5 C) of the *Bradyrhizobium-Rhodopseudomonas palustris* rRNA branch and the *Beijerinckia* rRNA branch. Theoretically the other taxa from this $T_{m(e)}$ level can be more closely related to strain ORS 571: *Methylobacterium*, *Xanthobacter*, *Rhodopseudomonas acidophila* and *Rhodopseudomonas viridis*. In order to unravel these relationships, we prepared a [³H]-labelled rRNA probe from strain ORS 571 and hybridized it with DNA's from other members of the stem-nodulating *Sesbania* phenon and with DNA's from the above mentioned possibly related bacteria.

The results are shown in a $T_{m(e)}$ dendrogram (Figure 2), representing part of the rRNA superfamily IV.

The most useful and significant parameter of a DNA:rRNA hybrid is its $T_{m(e)}$-value (3, 6, 10, 17), because this parameter is a measure of the base sequence similarity between rRNA cistrons and has a decisive taxonomic significance. Our results show that the *Sesbania* stem-nodulating bacteria constitute a separate rRNA subbranch on the *Rhodopseudomonas palustris-Bradyrhizobium* rRNA branch. Four representative strains constitute a very narrow cluster ($T_{m(e)}$ from 80.8 to 81.6 C) belongs also on this subbranch, and this genus is thus the closest relative of the *Sesbania* stem-nodulating strains.

These strains cannot be included in *Xanthobacter* because a difference in $T_{m(e)}$ of 4 C can indeed reflect an intergeneric relationship, provided that sufficient phenotypic arguments are available to differentiate both genera. Since this condition was indeed fulfilled we consequently proposed a separate genus rank for the *Sesbania* stem-nodulating strains. At the moment of this conclusion only 2 species were described in *Xanthobacter* (16) and we made our differentiating table [Table 4 in (5)] according to the available data. Recently (11), a third species *X. agilis* has been described and *X. flavus* was emended (12). The type strain of *X. agilis* has since then also been hybridized with the [³H]-rRNA probe from strain ORS 571, it has a $T_{m(e)}$ value of 77 C, indicating that it is also a member of the *Xanthobacter* rRNA cluster. When the phenotypic results of the revised genus *Xanthobacter* were compared with these of the *Sesbania* stem-nodulating cluster, we still found enough features to differentiate both genera. The revised differentiating table will be published elsewhere (M. Gillis, J.L. Garcia and B. Dreyfus, manuscript in preparation).

Strains ORS 609 and ORS 611 have $T_{m(e)}$ values of 79.7 C versus the rRNA probe from *R. meliloti* NZP 4009 showing that they are members of the *Rhizobium-Agrobacterium* rRNA complex.
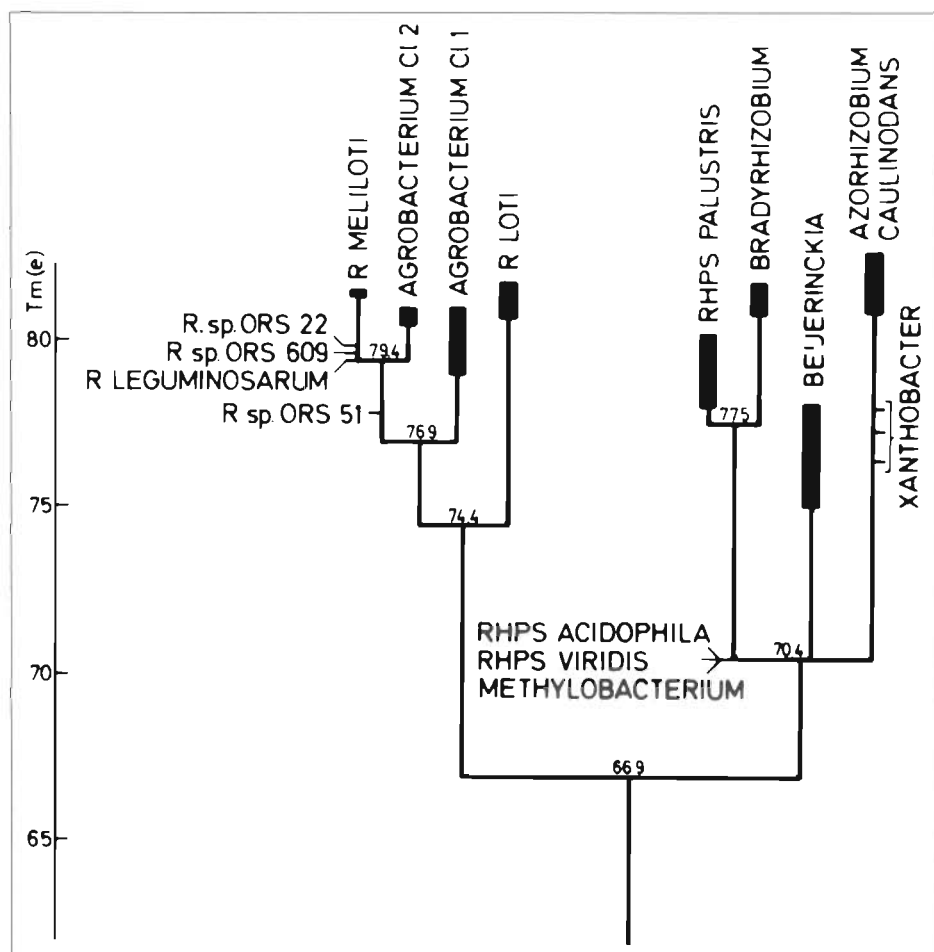
Fig. 2. Simplified T_{m(e)} cistron similarity dendrogram of part of rRNA superfamily IV.

IV. Comparative sds gel-electrophoresis of whole-cell proteins and DNA-DNA hybridizations

Four strains of the stem-nodulating *Sesbania* strains have almost identical protein electrophoregrams (Figure 3) indicating that they constitute indeed a very homogeneous cluster. High percentages of total DNA:DNA binding (95%) were indeed found between representative strains of the *Sesbania* stem-nodulating bacteria showing that these strains belong genotypically in one species.

V. Conclusions

▲ 1.Tropical rhizobia are heterogeneous and more phenotypic and genotypic re-

search is necessary to unravel their relationships.

▲ 2.We propose a new genus and new species for the stem-nodulating *Sesbania* strains because the DNA:rRNA hybridization results show clearly that these strains constitute a separate rRNA subbranch and do not belong in *Rhizobium* nor in *Bradyrhizobium*. *Xanthobacter* (3 species) appears to be

their closest relative, from which they are phenotypically sufficiently different to deserve a separate generic rank.

▲ 3.Because the capability of fixing high amounts of N2 and growing at the expense of this fixed N2, while free living, is a quite discriminative feature of the new genus we proposed to name it *Azorhizobium*.

▲ 4.Within this genus we found genotypically and phenotypically only one species, which we named *caulinodans* according to its stem-nodulating capacity.

The type strain is *Azorhizobium caulinodans* ORS 571 which was deposited in the collection of the Laboratory of Microbiology in Gent (LMG 6465). The G+C content of the DNA is 66.5 mol %. The complete description of the new genus and species can be found in (5).

Acknowledgments
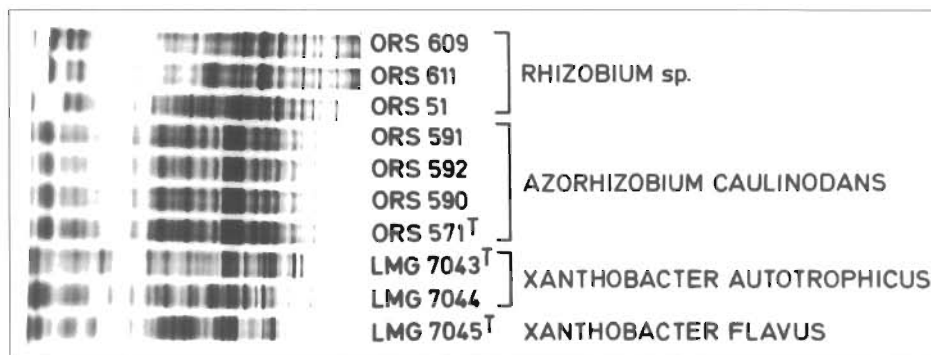
Fig. 3. Normalized SDS-polyacrylamide gel electrophoretic patterns of four Azorhizobium caulinodans strains, three Rhizobium sp. and three Xanthobacter strains.

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Symbiotic genes of *Rhizobium meliloti*

*J. Dénarié and D. Kahn *.*

Rhizobium meliloti strains are highly specific and nodulate only a limited number of temperate legumes belonging to the genera *Medicago, Melilotus* and *Trigonella*. They have not been reported so far to induce the formation of nitrogen-fixing nodules on tropical legumes. Moreover these bacteria elicit nodule formation just on roots, not on stems, and they are not able to fix nitrogen in free-living conditions. So why have we been asked to talk about the symbiotic genes of *R. meliloti* in this book? Probably because *R. meliloti* is the legume symbiont which has been the most thoroughly studied to date, from the genetical point of view, and can thus serve as a reference for comparison with nitrogen-fixing bacteria having different physiological and symbiotic behaviours such as *Azorhizobium*. This review will summarize recent results concerning *R. meliloti* genes involved in the control of nodule formation (infection, nodule initiation and host range) and of symbiotic nitrogen fixation.

1. Infection and nodulation

In the *Medicago sativa/R. meliloti* symbiosis the developmental steps leading to root-nodule formation can be schematically divided as follows: root hair curling, formation of infection threads within root hairs, growth of the threads towards the inner cortex of the root, initiation of a nodule meristem in the root cortex and nodule organogenesis (14). What are the *R. meliloti* genes which control these steps? What are the genetic determinants of the host-specificity of infection and nodulation?

1.1 Common and specific nod genes

Introduction of the pSym megaplasmid of *R. meliloti* into *Agrobacterium tumefa-*

ciens results in the transfer of the ability to nodulate alfalfa: the genetic information required for nodule initiation and host range is located on pSym. A pSym clone of about 30 kb confers the ability to nodulate alfalfa to *R. leguminosarum, R. trifolii* and *A. tumefaciens* (14, 33). Genetic and physical analysis of this region has revealed three clusters of nodulation (*nod*) genes. The first one contains two operons divergently transcribed (*nodD1* and *nodABC*). The *nodABC* genes have been called common *nod* genes because they are physically and functionally conserved among *Rhizobium* species (25, 35). The second one contains at least two operons *nodFEG* and *nodH* which are also divergently transcribed; mutations in these genes cannot be fully completed by *nod* genes from other *Rhizobium* species (13, 14, 33, 35). Between these two clusters is located a 6 kb fragment (region II) which contains a series of *nod* genes which have nog yet been analyzed at the molecular level (14). Another *nod* (or *efn*) locus, controlling the infection process, has been recently mapped elsewhere on the pSym, close to a reiteration of genes controlling nitrogen fixation (45, 48).

nodABC are required for root hair curling and seem to be involved in nodule initiation (14, 25, 35). *nodFE* and *nodH* are required for root hair curling and infection thread formation on homologous hosts and they control root hair curling negatively on heterologous hosts; they are genetic determinants of host range (14, 33). Region II determines the kinetics of infection and nodulation (14).

The *nodH* product (= NodA) is present in the bacterial cytosol whereas NodC is membrane bound. A monospecific polyclonal antiserum against NodA does not alter nodulation whereas an anti-NodC serum inhibits nodulation (see 55). The ami-

no acid sequence of the putative products of the species-specific *nod* genes show an homology between NodF and an acyl-carrier protein, between NodG and a ribitol dehydrogenase, and reveal a unique sequence of four contiguous proline residues in NodH (13, 33). However the *nod* sequence and product studies have not yet allowed to propose a working hypothesis on how the Nod products might mediate infection and nodulation.

A quite different physiological approach, proposed by Van Brussel and co-workers (59, 62), could provide tools for studying the mode of action of *nod* genes. The *nodABC* genes of *R. leguminosarum* have been shown to determine the production of extracellular symbiotic factors of low molecular weight which can be detected by bioassays such as deformation of roots or root hairs (59, 62). We have recently shown that *nodH*, the major host-range gene of *R. meliloti* , determines the production of an alfalfa-specific extracellular signal (Faucher, C., F., Maillet, F., Rosenberg, C., Van Brussel, A.A.N. Truchet, G. and J. Dénarié, submitted for publications). This signal is heat-stable and has a low molecular weight. Thus, specific as well as common *nod* genes might determine infection, nodulation and host-specificity via the production of extracellular signals.

1.2 Regulation of nod genes

Gene fusion studies have revealed that the transcription of *nodABC* requires an activator protein, the *nodD* product, as well as plant exudate (39). The inducer molecule isolated from alfalfa exudate is a flavone called luteolin (44). Luteolin also activates expression of *nodFEG* and *nodH* (F. Maillet and C. Rosenberg, personal communication).

In addition to the first *nodD* gene described (*nodD1*) two other copies of *nodD* have recently been mapped on pSym:
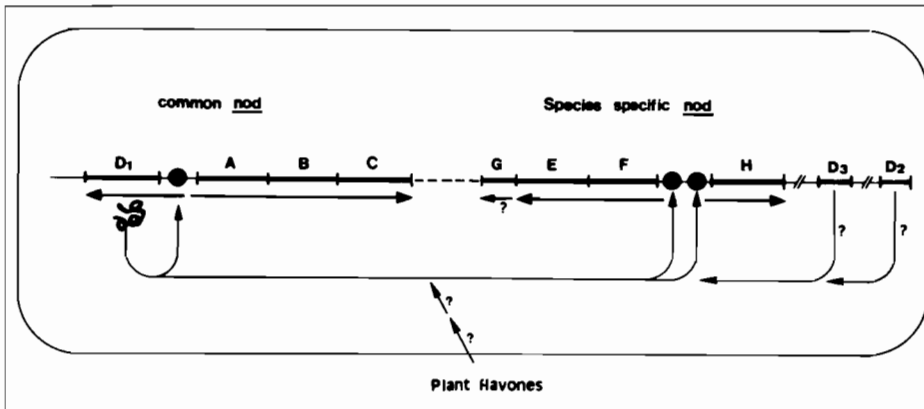
Fig 1. Regulation of R. meliloti nod gene expression
(from references 13, 26, 31a, 39, 44 and 52).

nodD3 (about 20 kb from *nodD1*) and *nodD2* (about 80 kb from *nodD1*). *nodD2* and *nodD3* have been shown to be functional and to control nodulation (26; 31a).

A conserved sequence called the *nod* box, is present upstream *nodABC*, *nodFE* and *nodH* (13, 52); this sequence is involved in the expression of nodulation function (52). Thus, the *nod* genes, common and specific, seem to be part of a regulon involving activator proteins (NodDs), a consensus sequence in promoter regions, and a plant flavonoid signal (see Fig. 1). Similar *nod* regulons have also been described in *R. leguminosarum* (22, 56) and *R. trifolii* (46).

The regulation of nodulation genes can be host specific since the *nodD* regulatory activities of various *Rhizobium* species exhibit a different responsiveness to different sets of plant flavonoids and exudates (32, 56). Thus the host range of *R. meliloti* can be determined at at least two levels: (i) a set of regulatory genes (*nodD*) which activate the *nod* genes differently according to plant signals, and (ii) a set of species-specific genes *nodFEG* and *nodH* whose products control specific interactions with plants.

1.3 exo and ndv genes

Genes controlling infection have also found elsewhere than on pSym: on the chromosome or on a second megaplasmid. *R. meliloti* Exo⁻ mutants, deficient in the production of acidic exopolysaccharide, form Fix⁻ nodules on alfalfa (36). No infection thread is formed and no bacterium is released into the host cells; a small num-

ber of bacteria can be seen between cells in the nodule cortex. Mutations in the *R. meliloti ndvB* homologous to the *chvB* locus of *A. tumefaciens* exhibit the same symbiotic phenotype. This locus could be implicated in the production of cyclic (1-2) ß-glucan (18). These results suggest that: (i) exopolysaccharides are required for infection thread formation, and (ii) nodule formation does not require the presence of bacteria within host cells and could therefore be induced at a distance by a bacterial signal.

Whereas the two *ndv* loci and two *exo* loci are located on the, chromosome, four loci are located on a second megaplasmid. Thus intriguingly two megaplasmids of similar size (around 1500 kb) carry symbiotic genes (21). It is worth to note that some mutations in these non-pSym genes can be complemented by *Agrobacterium* (chromosomal) DNA (8, 18). In contrast to *nod* genes, exo and ndv genes seem not to require a plant signal for their expression.

2. Symbiotic nitrogen fixation

Nitrogen fixation genes from *Rhizobium* fall into two classes: *nif* genes are defines as genes homologous to *Klebsiella pneumoniae nif* genes (16) whereas *fix* genes are the other genes necessary from nitrogen fixation *in planta*. Therefore *fix* genes may encode a variety of biochemical functions, from functions directly involved in the biochemistry of nitrogen fixations to functions more related to the biology of the *Rhizobium*-legume interaction, such as

the differentiation of bacteroids, bacteroid metabolism and signal exchanges between the symbiotic partners. Here we will briefly survey our present knowledge of these genes in *R. meliloti* with references to the *nif* genes from *K. pneumoniae*. Because the genetics and the regulation of *nif* and *fix* genes are significantly different in the *Bradyrhizobium* genus we will point out some of the differences between the *R. meliloti* and de *Bradyrhizobium japonicum* genes.

2.1 The nif region

The *nif* genes from *R. meliloti* were first identified because of the strong conservation of the *nifH* gene among prokaryotes which allowed the detection and cloning of *R. meliloti nifH* by hybridization with a *K. pneumoniae nifH* probe (54). Hybridization showed that nitrogenase genes are located on the pSym megaplasmid (3, 51). Further studies demonstrated that other *nif* and *fix* genes are clustered with *nifH* (10, 53). The *nif-fix* cluster is composed of two divergently transcribed regions separated by 1.9 kb of symbiotically unessential DNA (9). The leftward cluster includes *nifHDKE* whereas the rightward cluster contains *fixABCXnifAB* (Fig. 2). In addition *nifN* has been located close to *nodD1* (1).

The structural genes of nitrogenase are organized in an operon *nifHDK* as in *K. pneumoniae*. This is unlike *B. japonicum* nitrogenase genes that are organized in two operons *nifH* and *nifDK* (30). The *nifB*, *nifEN* and *nifV* genes from *K. pneumoniae* are involved in the synthesis of the nitrogenase iron-molybdenum cofactor (FeMoCo). Among these, *nifB*, *nifE* and *nifN* have been identified in *R. meliloti* (1, 6, 29, 60). *nifA* is the central *nif* regulatory gene in *K. pneumoniae* and has been characterized in *R. meliloti* and in *B. japonicum*. Its function and regulation is discussed below in more detail.

The *fixABCX* operon contains the only genes in the *nif-fix* cluster clearly not related to *K. pneumoniae nif* genes (19). These genes are found in all *Rhizobium* and *Bradyrhizobium* species and in the non symbiotic aerobic nitrogen fixer *Azotobacter vinelandii* (19, 27). Although the biochemical functions of the products of this ope-

ron have not been determined, it has been shown that in *B. japonicum* the *fixA* and *fixB* operons are required for nitrogen fixation *ex planta* (27). The *fixX* protein clearly has a structure of a bacterial ferredoxin. It has been suggested that the operon is involved in electron donation to nitrogenase. This hypothesis is plausible since electron transport is a major biochemical difference between aerobic and anaerobic nitrogen fixation, and because the *K. pneumoniae* anaerobic electron transfer genes *nifF* and *nifJ* seem to lack in *Rhizobium*.

2.2 The fix region

Another *fix* cluster has been identified on the *R. meliloti* pSym at about 200 kb from the *nif* region. It extends over 12.5 kb and consists of two *fix* operons on either side of a 5 kb *fix* region (the "*fixN* cluster") which is duplicated elsewhere on the pSym close to a *nod* locus (4,5, 48). The nucleotide sequence of the whole *fix* cluster has been completed recently and we summarize here the analysis of *fixGHIS*, an operon highly conserved among fast-growing *Rhizobia* (34; Kahn, D., David, M., Domerque, O., Daveran, M.L., Ghai, J., Hirsh, P. and Batut, J., submitted for publication).

The four proteins, FixG, FixH, FixI and FixS are predicted to be transmembrane proteins (20). FixG contains two cysteine clusters typical of bacterial ferredoxins, CysxxCysxxCysxxxCys, and is therefore likely to be involved in a redox process. FixI is homologous with cation ATPases of the P-type (43), most particularly with the KdpB catalytic subunit of the K$^+$-ATPase from *Escherichia coli* but also eukaryotic Ca^{2+}, Na$^+$/K$^+$ and H$^+$ pumps. This homology extends over all the functionally important cytoplasmic domains of the various P-type ATPases. This finding implies a role for a specific cation in symbiotic nitrogen fixation. Because prototrophic growth is unimpaired in *fixI* mutants, the FixI cation pump probably has a specifically symbiotic function. We hypothesize that FixG, FixH and FixS participate in a transmembrane complex (as is the case of the KdpABC ATPase from *E. coli*), so that

a redox process catalyzed by FixG would be coupled to the FixI cation pump.

2.3 Regulation of nif and fix genes

Because *R. meliloti* fixes nitrogen only during symbiosis and in the presence of numerous nitrogen compounds, including ammonium excreted by *Rhizobium* itself, the regulation of nitrogen fixation genes is likely to be different from that of free-living nitrogen fixers such as *K. pneumoniae* which fix nitrogen only in conditions of nitrogen starvation. However regulation of *nif* genes in *Rhizobium* does share some features with that in *K. pneumoniae*, most probably positive regulation by the *nifA* gene product. An excellent review of this topic has been published recently (28).

2.3.1. nifA

The *K. pneumoniae nifA* gene product is an essential positive regulator of the structural *nif* operons (16). It is induced under nitrogen deficiency by the *ntrC* gene product, which controls diverse nitrogen catabolic operons. Action of both NifA and NtrC as positive regulators requires a specific sigma factor, the *ntrA* gene product (31, 37, 42). *K. pneumoniae nifA* is co-transcribed with *nifL*, whose product inactivates the NifA protein in the presence of low concentrations of combined nitrogen or oxygen (28). Similarly, *ntrC* is cotranscribed with *ntrB*, whose product modulates the activity of NtrC according to the nitrogen status of the cell (40). Thus *nif* genes are stringently regulated by a cas-

cade system responding to nitrogen at a global level (the *ntrBC* system) and to nitrogen and oxygen at a specific level (the *nifLA* system).

A functional homologue of *K. pneumoniae nifA* has been characterized in various *Rhizobium* and *Bradyrhizobium* species (7, 23, 58, 61). In all cases NifA appears to be an essential symbiotic activator of structural *nif* genes. As in *K. pneumoniae*, the action of NifA requires the NtrA sigma factor (49). However in *R. meliloti ntrC* appears to be unneccessary for nitrogen fixation *in planta* (57). How then is *nifA* expression regulated in *R. meliloti*? One answer stems from the recent demonstration that *R. meliloti nifA* is not regulated *ex planta* by combined nitrogen as it is in *K. pneumonia* by combined nitrogen as it is in *K. pneumoniae* but by oxygen (15). This makes sense physiologically since the endosymbiotic environment is nitrogen-rich and microaerophilic (2). Therefore potential regulatory genes might be involved in regulation by oxygen rather than by nitrogen (see Fig. 2).

2.3.2. fixLJ

We have now discovered a new regulatory locus, *fixLJ*, which positively regulates *nifA* in symbiosis (11). Therefore *R. meliloti nif* genes are subject to a cascade regulation such that *fixLJ* activate *nifA* which in turn activates the other *nif* operons. This parallels the *nif* regulatory cascade in *K. pneumoniae* with *fixLJ* substituting for ntrBC in their *nif* activating function.

Fig. 2. The nif regulon of R. meliloti (from references 1, 12, 13, 49, 57, 58 and 61)

fixS was previously named fixX (34) but had to be renamed after the description of the fixABCX operon (19)

Furthermore *fixLJ* regulate other *fix* genes not related to *nif* such as genes of the *fixN* cluster. Therefore *fixLJ* are general regulators of various symbiotic nitrogen fixation genes in *R. meliloti*.
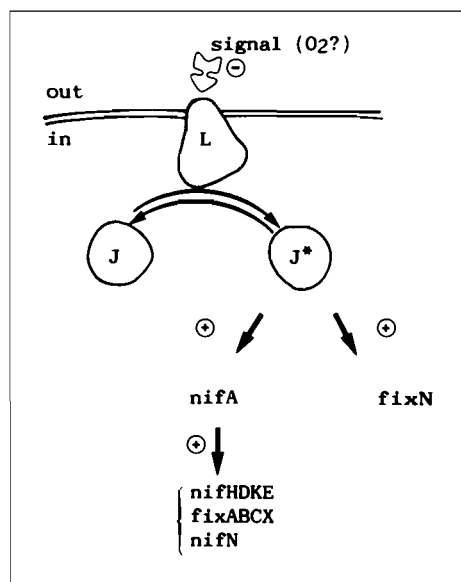

Fig. 3. Current model of the regulation of nif and fix genes in R. meliloti (11)

Sequence analysis of *fixLJ* (11) shows that FixL and FixJ belong to a family of two-component regulatory systems widely distributed amongst prokaryotes and responsive to the cell environment (50). Interestingly, this family includes NtrB (41) and NtrC (17), the positive regulators of *nifA* in *K. pneumoniae*. FixL has features of a transmembrane protein and belongs to the "sensor class" while FixJ belongs to the "regulator class" of Ronson *et al.* (50). According to the current model proteins of the sensor class recognize an environmental stimulus and transmit a signal to proteins of the regulator class. This interaction modulates the activity of the regulators probably by covalent modifications as has been shown for NtrC (40). Therefore we propose that FixL senses a symbiotic signal and transduces it to FixJ, which in turn activates transcription of *nif* and *fix* genes (11).

2.3.3. Regulation by oxygen

The expression of *nifA* (15) and *fixN* can be induced in pure culture in microaerophilic conditions and this expression depends on *fixLJ* (11). One working hypothesis is that FixL could sense the oxygen concentration. Alternatively oxy-

gen regulation could be mediated by another regulatory system that would act in conjuction with *fixLJ*. In the latter hypothesis FixL would sense another symbiotic signal yet to be identified.

In *K. pneumoniae* oxygen regulation of *nif* genes does not operate mainly at the level of *nifA* expression but at the level of *NifA* activity via NifL (38). A potential equivalent of *nifL* has not been found in any *Rhizobium* or *Bradyrhizobium* species. Little is known of the modulation of NifA activity in *Rhizobium*. Evidence indicates that the *B. japonicum* NifA protein is sensitive to oxygen by itself (24), which could obviate the need for a NifL protein in this species.

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Biochemistry and genetics of nitrogen fixation in *Azorhizobium caulinodans* strain ORS571

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Introduction

The fast growing *Rhizobium* strain ORS571, now designated under the species name of *Azorhizobium caulinodans* (10), was isolated from stem nodules of the tropical legume *Sesbania rostrata*. The strain forms nodules on both stems and roots of the host plant (8, 11, 36) and grows in the free-living state at the expense of N_2 as sole nitrogen source (9, 15). The latter property suggested that biochemistry and genetics of nitrogen fixation in this particular strain could be investigated using techniques developed in free-living diazotrophs such as *Klebsiella pneumoniae*. The nitrogenase complex could be purified from free-living diazotrophically-grown organisms. In addition, it was possible to isolate Nif⁻ mutants on nitrogen-free solid medium. This permitted to identify genes involved in the nitrogen fixation process, *in planta* and *ex planta*, to characterize their products and functions and to compare their organization with that of other diazotrophs, including *Klebsiella pneumoniae* and other *Rhizobia*.

The nitrogenase complex

Growth under nitrogen-fixing conditions

Strain ORS571 proliferates in the free living state with either ammonia or N_2 as sole nitrogen source(9, 15). Under both conditions, it requires three vitamins, biotin, nicotinic acid, and panthotenic acid (15). Nicotinic acid which is used as a growth factor under conditions of ammo-nia assimilation, can be utilized as a nitrogen source in the absence of ammonia (14, 22). Consequently, under nitrogen fixing conditions, strain ORS571 requires about ten times more nicotinic acid (i.e. 0.3 mM) than when grown in the presence of ammonia. It was estimated that under nitrogen-fixation conditions, nicotinic accounts for about 1/10 of the total growth observed (14). This suggested that N_2 is preferentially used over nicotinic acid as a nitrogen source.

Nitrogenase activity is depressed under microaerobic conditions. For cultures in flask or on solid medium, a 3% O_2 concentration in the gas phase was found optimum (9, 15). For cultures in fermentors it was necessary to regulate the oxygen concentration using an O_2 electrode so that dissolved oxygen in the medium was maintained at about 15 M (26). Under these conditions, a doubling time of 6 h and a nitrogenase specific activity of 40 units (mg protein)$^{-1}$ in whole cells was regularly obtained (26). This oxygen concentration was higher than the value of 9 M reported by Gebhardt *et al.* (17). It is possible that this apparent discrepancy was due to differences between our use of batch cultures at maximum growth rate, while Gebhardt *et al.* (17) performed continuous cultures at dilution rates of 0.1 or less.

Purification and properties of nitrogenase

Nitrogenase has been isolated from a variety of diazotrophs (12). In all cases, it has been fractionated into two protein components, a Mo-Fe protein or component 1, a tetramer comprised of two different subu-nits, and a Fe protein or component 2, a dimer made of a single type of subunit (12).

The nitrogenase, purified from fermentor grown cells of *A. caulinodans* ORS571, was resolved into two protein components resembling those obtained from other diazotrophs (26).

Component 1, a Mo-Fe protein, had a M_r of 219,000 and contained 1.2 g-atoms Mo mole^{-1} and 20.5 g-atoms acid-labile S mole^{-1}. It consisted of two types of subunits of M_r 56,000 and 59,000. The specific activity (nmol product formed min^{-1} (mg protein)$^{-1}$ of component 1, when assayed in the presence of an optimum concentration of component 2 (molar ratio 1:40), it was 1,250 for acetylene reduction and 1,090 for hydrogen evolution.

Component 2, a Fe protein, had a M_r of 74,000 and contained 3.1 g-atoms Fe mole^{-1} and 3.1 g-atoms acid-labile S mole^{-1}. It consisted of a single type of subunit of M_r 36,000. The specific activity of component 2, when assayed in the presence of an optimum concentration of component 1 (molar ratio 1:1) was 1,700 for acetylene reduction and 1,305 for hydrogen evolution.

"Switch-off" by ammonia

The addition of NH_{4+} or glutamine to N_2-fixing cultures resulted in a rapid and almost total inhibition of nitrogenase activity (26). The inhibitory effect of NH_{4+} on nitrogenase activity *in vivo* was reminiscent of the "switch-off/on" effect first described and now well documented in photosynthetic bacteria (see 21, 37). The effect of NH_{4+} and glutamine addition to nitrogen fixing suspension of bacteroids was also examined. Acetylene reduction

by bacteroids suspension is supported by lactate (35). As shown in Figure 1, with a bacteroid suspension in LSO lactate containing medium (15), "switch-off" of nitrogenase activity was observed only in the presence of glutamine but not in the presence of ammonia (25). In photosynthetic bacteria, it has been proposed that glutamine could be the true effector in nitrogenase switch-off and that ammonia should be metabolized through glutamine synthatase (37). Moreover, the observation that ammonia has no effect on bacteroid nitrogenase is in agreement with the report of Kleiner (24) that there is no ammonium transport in bacteroids and with the finding of Donald and Ludwig (5), showing that bacteroids had no glutamine synthetase activity.
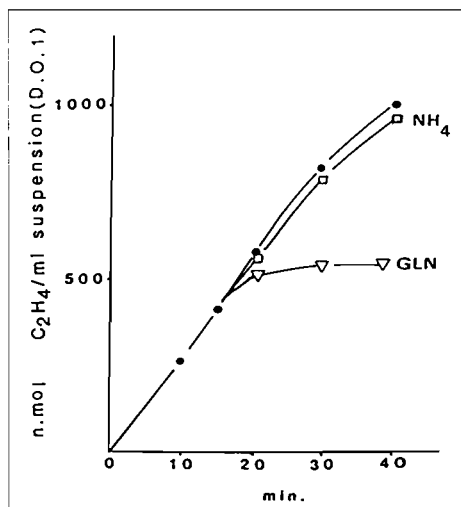
Figure 1 : Nitrogenase switch-off
in bacteroids isolated from root nodules
of Sesbania rostrata inoculated with strain
ORS571. Bacteroids were incubated
in lactate LSO medium, NH_4^+ and glutamine
were added at 10 mM final concentration.

In photosynthetic bacteria, the Fe protein is inactivated by attachment of a modifying group, consisting of adenine diphosphoribose (32). An O_2-sensitive membrane bound activating enzyme, that requires Mn^{++} for activity, has been shown to remove the modifying group (21, 37). This enzyme is present in cells grown on any nitrogen source, even ammonia. Nitrogenase components 1 and 2 isolated from strain ORS571 "ammonia-shocked" bacteria were purified to homogeneity. As in photosynthetic bacteria the enzyme present in N_2-growing cells was called ^{N2}ase

A and the enzyme present in the ammonia-shocked cells was called N_2ase R. NH_{4+} "switch-off" resulted in a specific inactivation of the Fe-protein, the activity of which decreased from 1,700 units (mg protein)$^{-1}$ in N_2ase A to 280 units (mg protein) $^{-1}$ in N_2ase R (26). In contrast to *Rhodospirillum rubrum* (21), SDS-gel electrophoresis failed to resolve the inactive Fe protein of strain ORS571 into two bands. When ammonia shocked bacteria, were labelled with ^{32}P phosphate or with ^3H adenine, no isotope incorporation was detected in Fe-protein (25). In addition, attempts to purify an activating factor, similar to that described in photosynthetic bacteria was unsuccessful.

To our knowledge, this is the first documented report of the existence of a "switch-off" of nitrogenase by ammonia in a *Rhizobium* strain, though the mechanism was not elucidated. This control occurs also when strain ORS571 is living in symbiosis with the host plant, and might be a general property of Rhizobia.

Organisation of nitrogen-fixing genes

Hybridization with heterologous probes

The organization of the *nif* genes was first analyzed by hybridization with *K. pneumoniae nif* probes. Monology was limited to *nifHDK*, the structural genes for nitrogenase (15) and *nifE* (28), which is involved in the formation of the iron-molybdenum cofactor. A cluster containing *nifHDKE* was identified. The four genes are contiguous on a 6.3 kb *Sal*1-*Bam*HI fragment. They are all in the same orientation and in the same order as in *K. pneumoniae* (Figure 2).

A second copy of *nifH (nifH2)* unlinked to the *nifHDKE* cluster, was cloned from a bank of *Pst*I fragments in pHE3 by *in situ* colony hybridization using *nifH* copy 1 as a probe (Figure 2, 29).

Homology to *R. japonicum fixABC* genes (16) was detected in the 9.5 kb *Pst*I fragment carrying *nifH2*. Homology to *fixA*
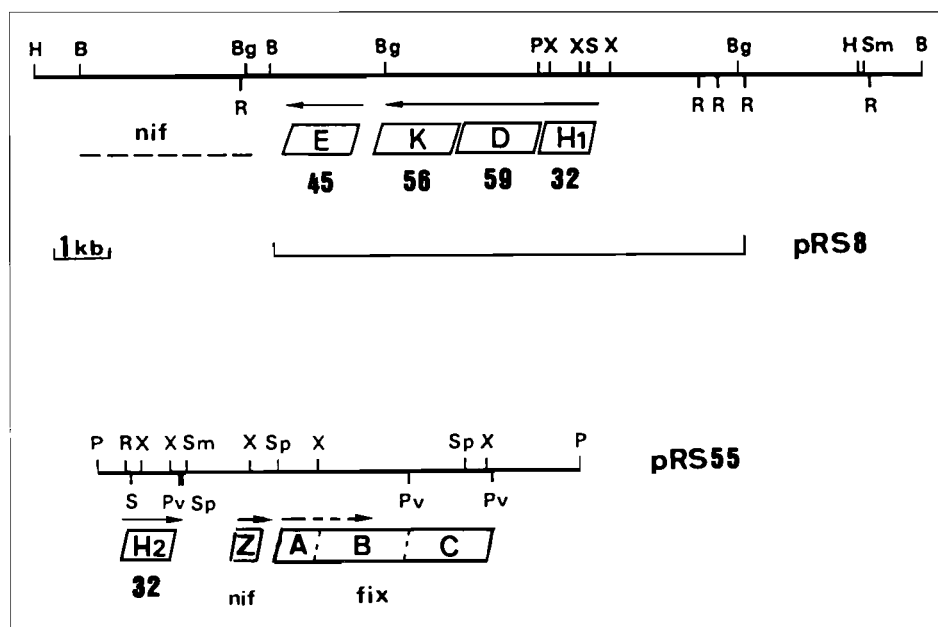

Figure 2 : Organization of the two nif clusters containing nifH1 and nifH2 in strain ORS571.
Restriction sites: B: BamH1; Bg: Bg1II; H: HindIII; P: PstI; Pv: PvuI; R: EcoRI; S: Sal1;
Sm: SmaI; Sp: SphI; X: XhoO. Localization of the nif and fix genes and the direction
of transcription are indicated by boxes and arrows, respectively.
The localization of the nif region adjacent to nifE is indicated by - - -.
M_r are expressed in kb. pRS8 is described by Denèfle et al. (1987)
and pRS55 by Norel & Elmerich (1987).

was found within the 1.4 kb *Xho*I fragment adjacent to *nifH2* and the *fixBC* region was localized in a 3.3 kb XhoI fragment adjacent to *fixA* (Figure 2). This localization is in agreement with that observed by Donald *et al.* (7). Consequently, *fixA* is not localized near *nifH1* as previously reported (30).

Genetic analysis of the nifHDKE cluster

Mutagenesis was performed on ORS571 *nif* DNA fragments cloned in pRS8 (Figure 2), using either Tn5 insertions or *in vitro* substitution of a *nif* fragment by a kanamycin cartridge (3). The mutations were selected in *E. coli* and transferred into ORS571 genome using two techniques. One involved the suicide vehicle pSUP202 (34) which cannot replicate in strain ORS571. The second method was according to Ruvkun and Ausubel (33). The *nif* fragments were cloned in the broad host range vector pRK290, and the mutations were transferred into ORS571 genome after surinfection with pPH1, a plasmid incompatible with PRK290.

Tn5 insertions in *nifH*, *nifD*, *nifK* and *nifE* abolished nitrogen fixation in *Rhizobium* ORS571 *in planta* and *ex planta*. A deletion of the 4 kb *Bam*HI fragment adjacent to *nifE* led also to a Nif⁻, Fix⁻ phenotype, suggesting the existence of a *nif* region adjacent to *nifE* (3). By random mutagenesis using plasmid pVP2021, Donald *et al.* (6) isolated insertion mutants Nif⁻ phenotype. Mapping of the mutants in the *nifHDKE* region is in agreement with our data (7).

The transcriptional organization of the *nifDKE* cluster was examined by genetic complementation and product analysis (3). Though genetic complementation observed was low, our results were in agreement with the existence of three transcription units: *nifHDK*, *nifE* and a new *nif* region adjacent to *nifE*. These three transcription units were also described by Donald *et al.* (7). Moreover, polypeptide analysis was consistent with this organization (3). In the wild type, at least 10 polypeptides present under conditions of nitrogen fixation and absent under conditions of ammonia assimilation were characterized by their M_r and pI, after

two-dimension gel electrophoresis. Beside the nitrogenase subunits, products of *nifHDK* of M_r 36,000, 59,000 and 56,000 respectively, the product of *nifE* was tentatively identified, as a polypeptide of M_r 46,000 and pI 6.3. The new *nif* gene adjacent to *nifE* has not been precisely located. This gene is involved in component 1 maturation since nitrogenase activity of crude extract from a deletion mutant of this region was restored by addition of pure component 1 (3). This new gene cannot correspond to *nifB* which has been cloned by Donald *et al.* (7) and mapped elsewhere.

Consequently, it might correspond to *nifN*.

Nucleotide sequence analysis of the two nifH coding regions

By DNA hybridization, two copies of *nifH* were detected (28). The complete nucleotide sequence of *nifH1* and *nifH2* was established (29). Only six nucleotides were found to be changed between *nifH1* and *nifH2*, which both encoded a polypeptide of 296 amino acids with a single change at position 282, a serine in *nifH1* and a threonine in *nifH2*. At position 102, arginine in the sequence Gly-Arg-Gly-Val-Ile,Thr might be, as in *Rhodospirillum rubrum* the site of "switch-off" inactivation.

Compared with other *nifH* genes, the hightest S_{AB} was found with *Bradyrhizobium japonicum* and *Rhizobium* sp. Parasponia.

Upstream from the initiation codon, a sequence, identical in the case of *nifH1* and very similar in the case of *nifH2*, to the *nif* consensus sequence (1, 4) was found (Figure 3). In addition, homology with sequences upstream from *Klebsiella pneumoniae nif* promotors, which affect *nifA* mediated activation (2), was also found in both cases (28). It remains to be determined whether the slight difference between the two ORS571 *nifH* genes

consensus sequences might account for a different regulation of their expression.

The two copies of nifH are functional

A deletion mutant of *nifH1* was constructed by *in vitro* substitution of the two *Xho*I fragments, overlapping *nifH*, by a kanamycin cartridge, and subsequent recombination in ORS571 genome. The resulting 57182 strain, displayed 30 % of wild type nitrogenase activity in whole cells (Table 1) and in crude extract (3). In this strain, the Km^r gene is in the same orientation as *nifDK*. The Nif⁺ phenotype of the mutant could be explained by assuming that the *nifDK* products are transcribed from the *Km* promoter and that the strain contains another functional copy of *nifH*. The mutant was also Nod⁺ Fix⁺. This observation strongly suggested that *nifH2* was functional.

A deletion of *fH2* (strain 57171) was constructed (29). The resulting strain also led to a Nif⁺ phenotype, indicating that *nifH1* was functional (Table 1). However, a double deletion mutant for *nifH1* and *nifH2* (strain 57171) was Nif⁻ *ex planta* and *in planta*, as shown in Table 1. Thus, no functional *nifH* gene other than *nifH1* and *nifH2* is present in ORS571. Moreover, strains 57171 and 57182 had a nitrogenase specific activity *ex planta* which was 70% and 30% respectively of that observed with the wild type strain. This suggested that, in the wild type strain, *nifH1* accounts for about 70% of nitrogen fixation *ex planta* and *nifH2* for 30%. An unexpected result was the divergence obtained *in planta*, since nitrogenase specific activity of strain 57182 was about 80%, whereas strain 57171 the nitrogenase activity was only 15% and the inoculated plantlets looked poor. This strongly suggested that the optimal expression of

Strain	Relevant genotype	Nitrogen specific activity %	
		ex planta	in planta
ORS571	wild	100	100
57182	nifH1	30	80
57171	nifH2	70	15
57172	nifH1 - nifH2	< 0.1	< 0.1

Table 1 : Nitrogenase activity of ORS571 nif mutants.

nifH1 and of *nifH2* was dependant on different physiological conditions.

Reiteration of *nifH* was shown in several nitrogen-fixing species, e.g. *Rhizobium phaseoli, Rhodopseudomonas capsulata, Azotobacter chroococcum* (see 29). In *A. chroococcum, the second nifH* copy is transcribed under molybdenum deficiency and it encodes the Fe-protein subunit of the vanadium nitrogenase (23). In oRS571, the two *nifH* copies are functional in the presence of molybdenum. The precise role of each gene remains to be determined, particularly in the nodule where maximum activity requires *nifH2*.

The fixABC region, identification of nifO

Using probes from *R. japonicum*, genes homologous to *fixABC* were detected. Deletion of the region adjacent to *nifH2* and covering the *fixABC* region led to a Nif⁻ phenotype, suggesting that the fixABC genes might be involved in nitrogen fixation in the free-living state. Moreover, 5 Nif⁻ mutants (5701, 5750, 5752, 5755 and 5756), obtained after EMS mutagenesis were complemented by pRS55, a plasmid containing the ORS571 *fixABC* region (Figure 2). One of them, strain 5701, has been previously described (15). The 5 mutants were also Nod⁺ and Fix⁻ suggesting that they corresponded to genes involved in nitrogen fixation *ex planta* and *in planta* (27).

In order to localise the mutations carried by these mutants, Tn5 insertions were selected in pRS55 and the resulting plasmids were used to perform genetic complementation. The mutants appeared to belong to three complementation groups. Mutant 5750 mapped in fixC, mutant 5701 mapped in fixA and mutants 5752, 5755 and 5756 corresponded to a new gene which was designated *nifO*. NifO was localized upstream from *fixA*, between two Tn5 insertions distant of 0,6 kb (27).

The existence of *nifO* was confirmed by Tn5 site-directed mutagenesis and by DNA sequencing. A DNA fragment of 1 kb containing *nifO* and the beginning of *fixA* was sequenced (Kaminski, unpublished results). *NifO* likely corresponds to an

nifH1	-196 to -180	CTGGA**CCACCCG**TTGCA		
nifH2	-169 to -153	ATGCGA**CGG**TCG**TTGCT**		
fixA	- 59 to - 42	TTGGTACGACAC**TTGCT**		
nifO	-118 to -112	CTGGCACGGGCA**TTGCT**		

Figure 3: Promoter regions of nifH1, nifH2, nifO, and fixA genes of strain ORS571. Positions upstream from the traduction initiation codon is indicated in base numbers. Bold letters indicate homology with nif promoter consensus sequence.

Strain				
ORS571	wild type	70	68	71
5740	nifK	< 0.1	48	< 0.1
5701	fixA	1.3	1.5	1.9
5750	fixC	6.2	6.9	25
5752	nifO	1.4	1.8	1.3

Table 2 : In vitro biochemical complementation of mutants with ORS571 nitrogenase purified components.

RS1 and RS2 stand for nitrogenase purified components 1 and 2 from strain ORS571.

open reading frame of 350 bp, it is preceded by a AGGAG ribosome binding site 8 bp upstream from the initiation codon. This new *nif* gene and *fixA* are transcribed separately from promoters that share striking similarities with the consensus established for *nif* promoters, and which was also found for the two *nifH* genes (Figure 3). The complete nucleotide sequence of *fixABC* was established in *R. meliloti* (13). The sequence revealed, downstream from *fixC*, the existence of an open reading frame of 297 bp, corresponding to a new gene designated *fixX* (13), which is also present in *R. leguminosarum* (18). This gene is different from *nifO*.

The precise function of *fixABC* is not known as yet. Our results and those of Donald *et al.* (7) suggest that there are required for nitrogen fixation *in planta* and *ex planta*. The same findings were obtained in *Bradyrhizobium japonicum* (19). It has been proposed, that in *R. meliloti* (13) and *B. japonicum* (19), the three genes could be involved in electron transport to nitrogenase. If so, the mutants of ORS571, should be cryptic as observed for *nifF* and *nifJ* in *Klebsiella pneumoniae* (20). Thus, one should expect to observe nitrogenase activity in crude extract when dithionite is the electron donor. Crude extracts of the 5 Nif⁻ mutants were prepared. As reported in Table 2, *nifO* (5752) and *fixA* (5701) mu-

tants did not show any nitrogenase activity and activity was not restored by addition of purified nitrogenase components (Kush, unpublished results). This result was previously observed with purified nitrogenase components from *K. pneumoniae* in the case of 5701 (15). Thus, it is unlikely that *fixA* and *nifO* play a role in electron transport to nitrogenase. The point mutant in *fixC* retained 10% nitrogenase activity, which was increased to 35% after addition of purified Fe-protein (25). As the mutant produced amount of nitrogenase polypeptides comparable to the wild type, it is proposed that *fixC* could play a role in the formation of functional Fe-protein.

Conclusion

Biochemical and genetic analysis of the two regions containing *nifH1* and *nifH2*, in *A. caulinodans* ORS571, revealed the existence of 10 genes involved in nitrogen fixation both in the free-living state and under symbiotic conditions. Four of these genes encode nitrogenase components 1 and 2, namely *nifD, nifK, nifH1* and *nifH2*. *NifE* and an unidentified gene located downstream from *nifE* are involved in maturation of component 1, whereas *fixC* might play a role in maturation or functioning of component 2. Functions of *nifO*,

fixA and *fixB* remain to be established. Two other *nif* genes have been identified, namely *nifB* involved in maturation of component 1 (7) and *nifA* involved in *nif* regulation (7, 31).

In our laboratory more than 15 Nif⁻ mutants have been isolated after EMS mutagenesis (15, 27). Six of them are complemented by plasmid containing the region carrying *nifH1* (5740) or *nifH2* (5701, 5750, 5752, 5755, 5756). Three are complemented by plasmid clones containing other regions. Among these, strain 5751, which did not synthesize any of the *nif* polypeptides (3) is likely to be an interesting regulatory mutant.

Taking into account the ten genes described here, *nifB*, *nifA* and the three mutants not fully characterized, one can consider that more than 15 genes required both *ex planta* and *in planta* should exist in ORS571. It remains to determine if all of them are present in other rhizobia and if, in strain ORS571, some genes are specific for nitrogen fixation under free-living conditions.

Résumé

Etude biochimique et génétique de la fixation de l'azote chez *Azorhizobium caulinodans* ORS571

La souche d'*Azorhizobium caulinodans* ORS571, isolée à partir de tiges de la légumineuse *Sesbania rostrata*, possède la propriété exceptionnelle pour un *Rhizobium* de se développer *ex planta* aux dépens de l'azote moléculaire. Cette propriété a été exploitée pour étudier la biochimie et la génétique de la fixation de l'azote en utilisant les techniques applicables aux fixateurs libres.

La nitrogénase a été purifiée à partir de cellules cultivées en fermenteur. Les propriétés physicochimiques des deux protéines de la nitrogenase sont voisines de celles des enzymes purifiés à partir d'autres fixateurs d'azote. La nitrogénase de la souche ORS571 est soumise *in vivo* au phénomène de "switch-off" après addition d'ammoniaque au milieu de culture. Le phénomène est dû, comme chez les bactéries photosynthétiques, à une inactivation de la protéine 2 alors que la protéine 1 demeure pleinement active.

Deux régions portant des gènes *nif* ont été caractérisées. La première contient *nifHDK*, *nifE* et un cinquième gène non identifié. La seconde contient une autre copie de *nifH*, les gènes *fixABC* et un nouveau gène, appelé *nifO*. La séquence nucléotidique des deux copies de *nifA* a été établie. Les deux gènes ne diffèrent que par six nucléotides et un seul acide aminé. De plus, en amont du codon d'initiation de chaque gène, on a trouvé des séquences qui s'alignent sur les consensus établis pour les promoteurs *nif*. Des souches délétées de l'une ou l'autre des deux copies sont Nif⁺ Fix⁺, ce qui suggère que les deux gènes sont fonctionnels *ex planta* et *in planta*, bien que leur niveau d'expression soit différent.

Acknowledgements

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Nodulation genes
of *Azorhizobium caulinodans* strain ORS571

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Introduction

Azorhizobium caulinodans strain ORS571 (4), isolated from aerial nodules on the stem of *Sesbania rostrata*, is distinct from the *(Brady)Rhizobium* species by its taxonomic position and its capacity for both symbiotic and free-living nitrogen fixation, the latter associated with assimilation of the fixed nitrogen for growth. Infection and nodule induction by ORS571 occur at the site of root primordia that are abundantly present all along the *Sesbania rostrata* stem. The presence of these root primordia is a particular characteristic of this *Sesbania* species. Because of the dormant root-like nature of the infection sites, the term "stem nodulation" might be considered a misnomer but to our feeling it simply and adequately describes the topology of appearance of these nodules. The topology of the infection site may be the major reason why stem nodulation by ORS571 and subsequent symbiotic N2 fixation are little influenced by the presence of fixed nitrogen sources in the soil.

Infection of the stem-located root primordia by ORS571 occurs via a crack entry mechanism (16). The same is probably true when ORS571 nodulates the roots of *S. rostrata* (B. Dreyfus, unpublished results). From root nodules found in *nature* on *S. rostrata*, fast-growing *Rhizobium* strains have been predominantly isolated (4), whereas *A. caulinodans* ORS571 is the major species found in stem nodules. Most of the so-called "root" strains did not nodulate *S. rostrata* stems at all; only in some cases pseudonodules were observed. Thus, two major types of bacterial strains can induce effective nodules on the same host plant, but only the *A. caulinodans* strains are capable in nature of efficiently

interacting with the stem-located root primordia. The aim of our work is to identify ORS571 genes that are involved in the early interaction with its host plant and that are essential for nodulation or contribute to the efficiency of nodulation. By studying the nature of the functions encoded by such genes, we hope to learn about the special features of ORS571 that allow its interaction with the stem-located root primordia of the *S. rostrata* host.

In this paper we present an overview of our work on ORS571 nodulation genes. As many of the data presented are fairly recent, we apologize for having to refer for more detailed information to a number of papers in preparation.

Identification of the two Nod loci in the ORS571 genome

As ORS571 does not seem to harbor any plasmids that could be candidates to carry symbiotic genes, our approach has been to carry out a random Tn5 mutagenesis of the ORS571 genome and to screen mutants for defective symbiotic phenotypes. Two thousand Tn5-containing ORS571 derivates were thus tested on *S. rostrata* roots. Two mutants (Nod⁻1 and Nod⁻2) were Nod⁻, i.e. unable to form nodules on either stems or roots of *S. rostrata* (17). For each of these mutants we could isolate a complementing cone (pRG70 and pRG20, respectively) from a gene bank of wild-type ORS571 DNA in the wide-host range vector pLAFR1. The Tn5 insertion in the mutants allowed the selection of clones containing the mutated DNA segments. From the comparison of the physical maps of the mutated regions it followed that the Nod⁻1 and Nod⁻2 mutations are located in two separate loci of the genome (Nod locus *1* and Nod locus 2), neither of which is

closely linked to the *Nif1* locus in which the *nifKDH* genes coding for the structural subunits of the nitrogenase are located (3, 10).

Next we asked whether any of these two Nod loci of ORS571 showed DNA homology to the common *nodABC* genes of *Rhizobium meliloti* (9). This set of three contiguous genes is structurally and functionally conserved in *Rhizobium* and Bradyrhizobium species. Under low-stringency hybridization conditions, a *nodC*-specific probe (pJS209; ref. 8) hybridized to the 12.7-kb *Eco*RI fragment of clone pRG70 in which the Nod⁻1 Tn5 insertion was mapped. No homology was detected in pRG20. The observed *nodC* homology in Nod locus *1* led us to determine the nucleotide sequence of this DNA region (see further).

Preliminary analysis of Nod locus 2

By site-specific mutagenesis we delimited the essential nodulation region of Nod locus 2 to a 0.8-kb DNA segment wherein the Nod⁻2 Tn5 insertion is located (Fig. 1). Inoculation of *S. rostrata* roots with the Nod⁻2 mutant and with the Nod⁻ deletions Δ1 and Δ2 (Fig. 1) causes slight swellings at the lateral root bases. Upon stem inoculation occasionally very small pseudonodules can be observed. All three mutants can be complemented for normal nodulation upon introduction of the clone pRG20 which contains the wild-type Nod locus 2. A striking feature of the three mutant strains is their extremely rough colony morphology when growing on solid, defined medium with lactate and/or succinate as the C-source, indicating that perhaps an early step in the biosynthesis of polysaccharides is impaired. Whether this lack of
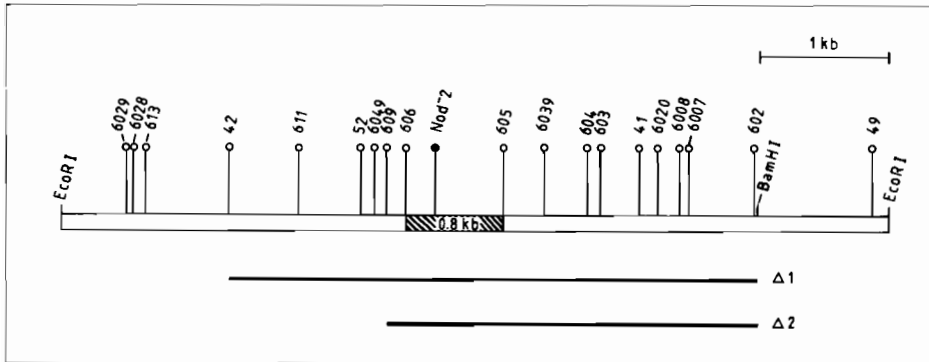
Fig. 1. Nod locus 2: Tn5 insertions and deletions. Black dot, Nod⁻; white dot, Nod⁺.
The deletions Δ1 and Δ2 are Nod⁻.

(exo)polysaccharide production is the direct or the major cause of the inability to nodulate remains to be investigated.

Nod locus 1: site-specific mutagenesis and DNA sequence

A map of Nod locus *1* is presented in Figure 2. In order to delimit the essential nodulation genes we have created two large deletions to the left and to the right of the original Nod⁻ Tn*5* insertion. The deletions were constructed in the clone pRG70 by recombinant DNA techniques and, via *Escherichia coli* as an intermediate host, introduced into ORS571 for homogenotization. The deletion Δ3 has no effect on the nodulation behaviour of ORS571. The deletion Δ4 does not interfere with the efficiency of nodule induction (Nod⁺), but the nodules degenerate soon after they have developed. In-between Δ3 and Δ4 remains a 3.8-kb stretch of DNA containing essential nodulation genes. Indeed, several Tn*5* insertions in this region completely abolish the nodulation capacity or ORS571 (Fig. 2). The Tn*5* insertions were originally isolated in *E. coli* in the clone pRG701 (the 12.7-kb *Eco*RI fragment of Fig. 2, cloned in pBR325) using the Δ::Tn*5* mutagenesis method (1), homogenotized in the ORS571 genome followed by screening of the homogenotes for nodulation phenotype. The Nod⁻ Tn*5* insertions 70 and 86 coincided with that part of Nod locus *1* that shows homology to the *nodC* gene of *R. meliloti*.

After subcloning of adequate DNA fragments we started to determine the nucleotide sequence of the 3.8-kb region

delimited by the deletions Δ3 and Δ4. We shall briefly summarize the major conclusions that we can yet derive from this analysis. The actual sequences and sequencing strategy will be described elsewhere (Goethals *et al.*, in preparation). As schematically indicated in Figure 2, three candidate open-reading frames are present in Nod locus *1* that show homology to the *nodABC* genes of *R. meliloti* and that occur in the same order. Their direction of translation (from right to left in Fig. 2) coincides with the direction of transcrip-

tion of these genes as deduced from the orientation of *lacZ* fusions in this region (see further). They correspond roughly in size to the *R. meliloti* genes: *ORFA* is 591 nucleotides long and shows an overall homology with *R. meliloti nodC* of 56% at the nucleotide level and 56% at the deduce amino acid level; *ORFB* is 633 nucleotides long, has an homology of 41% at the nucleotide level and 40% at the deduced amino acid level with *R. meliloti nodB*. The third ORF, *ORFC*, is 1188 nucleotides long, shows an overall homology of 52% at the nucleotide level with *R. meliloti nodC* and the deduced amino acid sequence presents 50% homology to the *R. meliloti nodC* gene product. We are now extending our sequencing analysis further upstream from *ORFA* in order to screen these sequences for the presence of a "*nod* box", the conserved promoter sequence that precedes the operons in *R. meliloti* and related strains and that are implicated in the activation of expression of these operons (see further)(12). Surprisingly, a sequence that bears some resemblance to the "*nod* box" was detected approximately 170 nucleotides upstream from the possi-
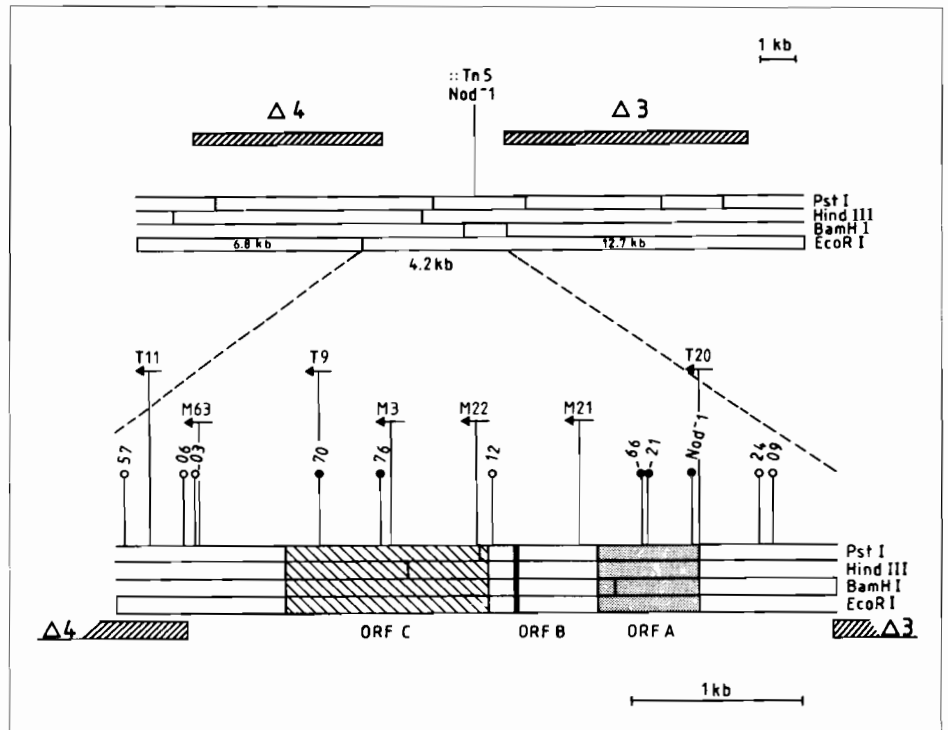

Fig. 2. Nod locus 1 of ORS571. ORFA, ORFB, and ORFC are three open-reading frames, present in the nucleotide sequence and weakly homologous to the nodA, nodB and nodC genes of R. meliloti. The black bar in ORFB indicates the position of a "nod box" (see Fig. 2). The arrows indicate lacZ fusions created by MudIIPR13 (M) or Tn3-HoHo1 (T) that are inducible by naringenin. The circles indicate Tn5 insertions: black, Nod⁻; white, Nod⁺. Δ, deletions.
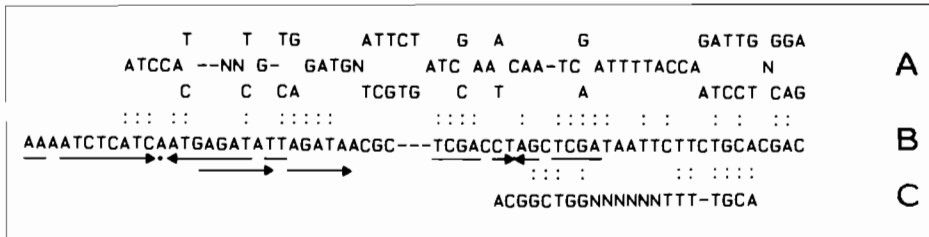
```
       T     T  TG      ATTCT    G  A      G        GATTG GGA
     ATCCA --NN G-  GATGN     ATC AA CAA-TC ATTTTACCA     N          A
       C     C  CA      TCGTG    C  T      A         ATCCT CAG
     ::: ::    :  : :::::         ::::  :  : ::::: : :   : :  ::
   AAAATCTCATCAATGAGATATTAGATAACGC---TCGACCTAGCTCGATAATTCTTCTGCACGAC  B
                                         ::: :         :: ::::: 
                                  ACGGCTGGNNNNNNTTT-TGCA                 C
```

Fig. 3. Nucleotide sequence of the "nod box" present at 170 bp upstream from ORFC in A. caulinodans ORS571 (B), compared to the "nod box" consensus sequence (A; ref. 13) and the nifH promoter from R. meliloti (C; ref. 15). The arrows indicate direct repeats or palindromes.

ble *ORFC* initiation codon (i.e. in *ORFB*; Fig. 2). This sequence, presented in Figure 3, also shows some homology to the *nif* operons and the *R. meliloti nifH* gene and that is typical for *nifA*-activated promoters (6).

When we compare our sequencing data with the results of the Tn*5* mutagenesis (Fig. 2) we see that three Nod⁻ mutations (Nod⁻1, Nod⁻21, and Nod⁻66) are located within *ORFA*, two Nod⁻ mutations (Nod⁻70 and Nod⁻76) are located in *ORFC*. The *ORFC* gene, however, is the only Nod locus *1* gene of which we can at present state that it is absolutely essential for nodulation. Indeed, mutations downstream from *ORFC* do not interfere with the nodule induction by ORS571. Tn*5* insertions in *ORFC*, however, may exert a polar effect on the expression of the *nodC* gene (see further). Therefore, it cannot be concluded that the Nod⁻ phenotype of Tn*5* insertions in *ORFC* is solely due to the absence of the *nodA* gene product.

Nod locus 1: regulation of gene expression

In *Rhizobium* species the common *nod* and the host-specific nodulation (*hsn*) genes are expressed in the presence of root exudate of the host plant. Different flavonoid or related molecules have been identified as the inducing factors of the root exudate (for a review, see 11). A group of regulatory bacterial genes, the structural conserved *nodD* genes, are essential for this plant-induced gene expression. The *nodD* gene can occur in one or more related copies (depending on the bacterial species) and the diversification of the *nodD* sequence leads to a family of related regulatory proteins with different affinities for specific flavonoids. The specificity of this interaction is one of the factors that contribute to the host specificity of the *Rhizobium*/legume symbiosis.

We made use of *lacZ* fusions to monitor the expression of Nod locus *1* genes of ORS571 by the production of the *lacZ*-encoded ß-galactosidase (Tomekpe *et al.*, in preparation). The 12.7-kb *Eco*RI Nod*l* fragment, cloned in PBR325, respectively PRK290, was mutagenized in *E. coli* with the transposable element MudIIPR13 (P. Ratet, personal communication), respectively Tn*3*-HoHo1 (14). These elements may create transcriptional or translational *lacZ* fusions.

Insertion mutation in Nod locus *1* were subsequently introduced into strain ORS571 and screened for ß-galactosidase activity in the presence or the absence of luteolin, a flavonoid that can induce the *nod* genes in a number of *Rhizobium* species. *LacZ* species. *LacZ* fusions that were inducible by luteolin were found to be located in *ORFA*, *ORFB* and in *ORFC*. Also downstream from *ORFC* genes are present, the expression of which is inducible by luteolin. All these inducible fusions

were oriented in the same way, indicating the same direction of transcription (from right to left in Fig. 2) for the genes in which they are inserted. Next, we screened a series of commercially available flavonoids and related molecules for induction of the *lacZ* fusion in ORS571 and obtained the highest levels of induction of the *lacZ* fusion in ORS571 and obtained the highest levels of induction using the flavanone naringenin in a concentration range of 5 to 20 µM. Some quantitative measurements of ß-galactosidase activities measured upon induction with naringenin of *lacZ* fusions in several of the Nod locus *1* genes are presented in Table 1.

We also measured the effect of a Tn*5* insertion upstream from a *nodC-lacZ* fusion on the expression of this fusion. A Tn*5* insertion I21, present in *ORFA*, prevented the naringenin-inducible expression of the *nodC-lacZ* fusion, indicating a transcriptional linkage of *ORFA* and *ORFC* (data not shown).

An inducing flavonoid in *Sesbania rostrata* root exudate

As expected *Sesbania rostrata* rootlets produce a factor that can induce the expression of the "common *nod*" genes of ORS571. This could easily be demonstrated by *in vivo* assays with a Nod locus *1*-*lacZ* fusion of ORS571 and using the blue color produced by degradation of 5-bromo-4-chloro-3-indolyl-ß-D-galactoside (X-gal) as an indication for ß-galactosidase activity. An example of such a test is presented in Figure 4. Interestingly, a similar phenomenon of Nod locus *1*-*lacZ*

ORS571 with plasmid	lacZ in	ß-galactosidase activities (U)	
		-NAR	+NAR
pRG290-12::M6	nod?	22	650
pRG290-12::M3	nodC	11	205
pRG290-12::M22	nodC	17	240
pRG290-12::M21	nodB	18	470
pRG290-12::T20	nodA	23	1680

Table 1 : ß-galactosidase activities with and without induction of ORS571 strains carrying lacZ fusions (see Fig. 2) in the 12.7-kb EcoRI fragments of Nod locus 1, cloned in the vector pRK290 (2).

B-galactosidase unites were measured (7) 12 hours after addition of 10 µM naringenin (NAR) to early log phase cells growing in LSR medium (17).
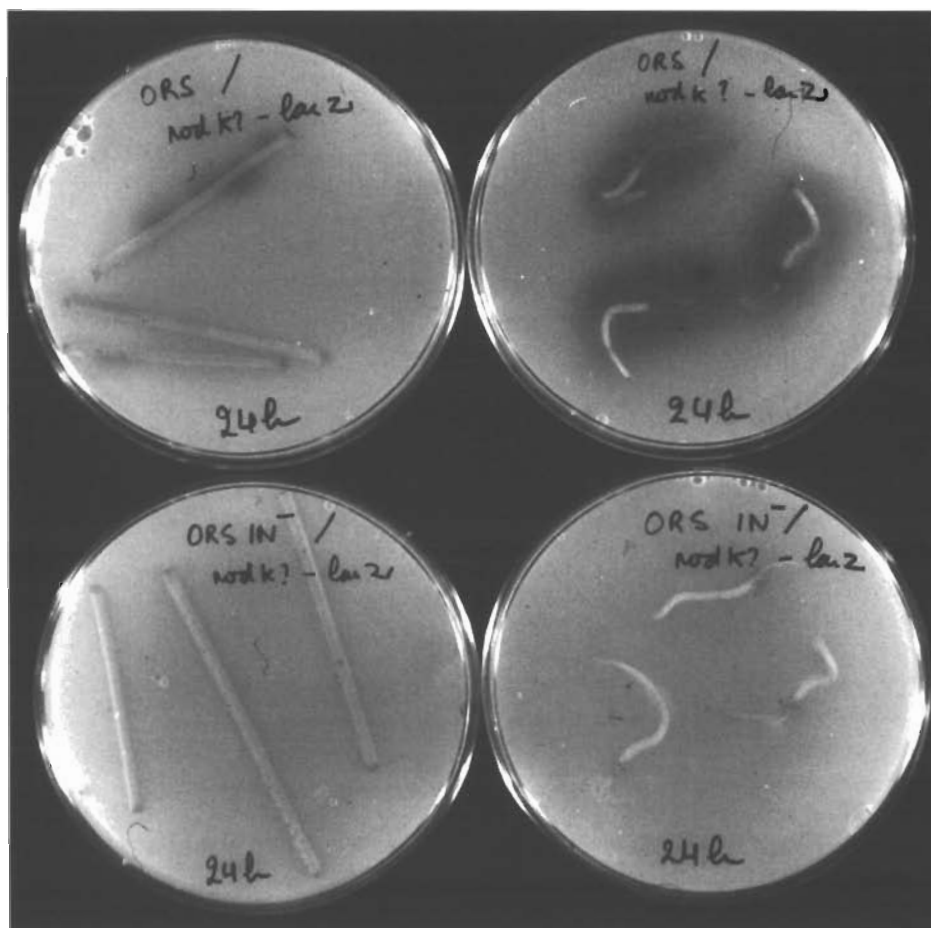
Fig. 4. Production of a nod gene-inducing factor
by Sesbania rostrata stem pieces (left), and rootlets (right).

gene induction was observed around the root primordia on *S. rostrata* stem pieces (Fig. 4).

The plant pieces put on LSR agar medium with X-gal, were overlayered with soft agar containing indicator bacteria, i.e. either the wildtype ORS571 strain with the plasmid pRG290-12::T20 (NodK?-*lacZ*; Table 1) (upper row) or the IN⁻ mutant of ORS571 (lower row) with the same reporter plasmid.

In order to purify the molecule(s) responsible for the gene induction we are preparing exudate from aseptically germinating *S. rostrata* seeds. This exudate is fractionated by fast-protein liquid chromatography (FPLC) using a reversed-phase C18-column. A major peak of biological activity (induction of a *nodC-lacZ* and a *nodA-lacZ* fusion in ORS571 was tested) eludes between 14% and 15% acetonitrile, 0.1% trifluoroacetic acid.

The biologically active fraction is being purified to homogeneity by further reversed-phase chromatography and the structure of the inducing molecule will be determined using spectroscopic methods (U.V., FAB-mass and nuclear magnetic resonance).

Nod locus 3 of ORS571: a positive regulatory locus for nod gene induction

As the expression of *nod* genes in *Rhizobium* species in the presence of host plant exudate is known to be dependent on the product of a regulatory gene, *nodD*, we wondered whether a similar situation exists in ORS571. When total *Eco*RI-digested DNA of ORS571 was hybridized at low stringency to a radioactively labelled probe of the internal amino-terminal region of the *R. meliloti nodD1* gene (5), a large number of *Eco*Ri fragments lit up. Therefore, we rather focused on functional approaches, e.g. searching for ORS571

clones from the pLAFR1 gene bank that would allow the induction by naringenin of an ORS571 *ORFC-lacZ* fusion in the *Agrobacterium tumefaciens* strain C58C1Rif^R. An *ORFC-lacZ* fusion was introduced in *A. tumefaciens* as a clone in the plasmid vector pGV910 (R. Deblaere, personal communication) which is compatible with pLAFR1. A pLAFR1 clone, pRG100, was found that allows *nodC-lacZ* induction in the presence of naringenin; this capacity was ascribed to a 16-kb *Eco*-RI fragment of pRG100.

In a second approach we isolated a spontaneous mutant of ORS571 which was no longer capable of induction of Nod locus *1-lacZ* fusions in the presence of naringenin (ORS571-IN⁻). This mutant was derived from strain ORS571-63 (with a *lacZ* fusion downstream from *ORFC*) and picked up as a pale-blue colony amongst dark-blue ones on solid medium with naringenin and X-gal. The IN⁻ mutant was no longer induced by naringenin nor in the presence of *S. rostrata* plantlets (Fig. 4). Upon inoculation of *S. rostrata* roots or stems it showed a delayed nodulation phenotype and the stem nodules that appeared were less homogeneous in morphology than the normal, very regular, beadlike nodules induced by the wild-type strain. The IN⁻ mutant could be complemented for all its mutant phenotypes by the plasmid pRG910-16 which carried the 16-kb *Eco*-RI fragment of ORS571 that allows naringenin induction of *ORFC-lacZ* in *A. tumefaciens* (data not shown). Thus, our two approaches came together and indicated the presence of a positive regulatory function necessary for the induction of the Nod locus *1* genes. For further characterization of this function we carried out a Tn5 mutagenesis of the clone pRG910-16, introduced a population of mutated clones in ORS571-IN⁻, and screened for colonies that were no longer induced by naringenin. From the mapping of such mutations we learned that they were all located in a 1-kb DNA segment which we called Nod locus 3. This locus does not show any detectable DNA homology in hybridization experiments to the regulatory gene *nodD* of *R. meliloti*.

Summary and discussion

Two mutants of ORS571, incapable of inducing nodules on the host plant *Sesbania rostrata*, led to the identification of two distinct Nod loci in the *Azorhizobium caulinodans* genome. Nod locus *1* contains functions analogous to the common *nodABC* genes of (brady)-rhizobia. Nod locus *2* is probably involved in (exo)polysaccharide production. Nod locus *1* was studied in more detail. Sequence analysis showed the presence of three open-reading frames corresponding to the *nodA*, *nodB*, and *nodC* genes of *Rhizobium meliloti*, occurring in the same relative order and transcribed in the same direction. Sequences related to the regulatory gene *nodD* of *R. meliloti* are not present in Nod locus *1*. The *nodC* gene of ORS571 is the only Nod locus *1* gene of which we are sure that it is essential for nodulation. Tn5 insertions upstream from ORF1 may be Nod⁻ by a polar effect on *nodC* expression. The importance of *ORFA* and *ORFB* gene products in nodulation remains therefore to be investigated. By creating *lacZ* fusions in Nod locus *1* genes we showed that these genes are normally silent; their expression is induced in the presence of *Sesbania rostrata* rootlets and stem-located root primordia as well as by commercially purchased naringenin.

The presence of a Tn5 insertion in ORFA *in cis* upstream from a *nodC-lacZ* fusion abolishes the naringenin or plant-inducible expression of this fusion, indicating that the genes *A*, *B*, and *C* may be transcriptionally coupled in an operon transcribed from *A* to *C*. Surprisingly, a sequence showing some homology to the promoter consensus sequence of the operons of (brady)rhizobia (*nod* box) is present approximately 170 bp upstream from *ORFC* of ORS571. However, the "early" expression of the ORS571 *nodC* gene, induced in the presence of the host plant, does not seem to involve this putative promoter, as evidenced by the polar effect of a *nodA* Tn5 insertion on the expression of the downstream genes under these conditions. At present we are investigating further the role of this putative promoter sequence and trying to define environmental conditions in which it may be activated.

Genetic approaches led to the identification of yet another Nod locus in the ORS571 genome (Nod locus *3*) which, although not strictly essential, contributes to the efficiency of nodulation by providing a function that is required for the induction of the Nod locus *1* genes in the presence of the host plant. Nod locus *3* shows no homology to the *nodD1* gene of *R. meliloti*. A mutation in Nod locus *3* causes severe delay of root and stem nodulation.

We think that the capacity of ORS571 to express its common *nod* genes upon interacting with an inducing molecule produced by stem-located root primordia is an important factor in the efficiency of induction of aerial nodules. In this line of thought we may put forward the hypothesis that the "root strains" associated with *S. rostrata* may be incapable or less well capable of inducing aerial nodules because their *nod* genes are induced by other classes of flavonoids that are present in *S. rostrata* or root exudate but not in the dormant root primordia on the stem. We have started to test this hypothesis by studying *nod* gene induction and nodulation behaviour of a number of "root strains".

In conclusion we hope by the continuation of these experiments to sort out the different factors that characterize the early interaction between *Azorhizobium caulinodans* and the *Sesbania rostrata*, leading to the establishment of the aerial symbiosis. This knowledge may be useful in view of designing approaches to create aerial nodules on other host plants.

Acknowledgements

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Session II

Regulation of N2 Fixation

Regulation of nitrogen metabolism in *Rhizobium*

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1. Introduction

Bacteria of the genus *Rhizobium* can infect roots of leguminous plants and induce the development of root nodules. The nodules are specialized structures in which some cells contain a large number of *Rhizobium* bacteria.

These, called bacteroids, are modified in their morphology and physiology as compared to the free-living forms and, most important, their multiplication is controlled. Bacteroids in many cases show a reduction in cell wall thickness and rigidity, and changes in the outer membrane (2, 32, 41). The DNA content per bacteroidal nucleoid may be greater than that of free-living *Rhizobium*, by 1.6- up to 8-fold (4). Some changes in bacteroid DNA density, denaturation temperatures, molecular weight and kinetic complexity have also been described (45). The relative content of the symbiotic plasmid per total genome appears to decrease in bacteroids as compared to free-living *R.leguminosarum* (C. Vierny and M. Iaccorino, unpublished observation). Also the DNA content per cell, the rate of protein synthesis, the RNA/DNA ratio, and the RNA/protein ratio decline with maturity of bacteroids (5, 13).

Bacteroids isolated from nodule cells are difficult to culture on normal media and a low frequency (0.5%) of colony formation is usually observed (46,47). These colonies probably form from immature rather than from fully differentiated bacteroids (50). Supplementation of the growth medium with sucrose to prevent osmotic lysis causes an increase in the number of colonies (42). Thus, it looks like that *Rhizobium* bacteria differantiate in the nodule into spheroplas like structures and that af-

ter a certain stage this differentiation is irreversible. These events are accompanied by a block in bacterial division and, while it is not clear if this block is the cause or the effect of the cell wall modification, it is certainly an important characteristic of the symbiotic behaviour of *Rhizobium*: if cell division did not stop bacteria would be pathogenic. Since bacteroids do not divide, regulation of their metabolism might require special mechanisms as compared to free-living bacteria.

The symbiosis should be viewed as a metabolic complementation. The plant provides energy in the form of photosynthate; the bacteroids make ATP from these carbon compounds trough a respiration taking place at a very low oxygen tension and synthesize NH_3, which is assimilated by the plant. Lack of photosynthate, experimentally imposed by darkening plants or by harvesting, results in decreased nitrogen fixation (49), and may be a consequence of the decreased ATP concentration in bacteroids.

The use of fixed carbon by nodules is intensive: in *Pisum* it was found that about 30% of the carbon fixed is consumed by the root nodules, while this value was about 10% in lupin (34,38). Photosynthate appears to be translocated to the nodules in the form of sucrose, but it is most probably taken up by bacteroids in the form of dicarboxylic acids (43).

In the study of regulation of nitrogen metabolism in *Rhizobium* three different physiological states should be considered:

▲ a) free-living bacteria, which assimilate various nitrogen sources, depending on the soil or laboratory conditions;

▲ b) bacteria actively growing in the plant: these originate from a single cell up to 10^9 daughters; the assimilate from the plant as yet unknown nitrogen sources;

▲ c) fully differentiated bacteroids; these have little or no protein synthesis, a high content of nitrogenase and probably of ancillary proteins; they assimilate N_2 and convert it into NH_3.

2. Nitrogen metabolism

Bacteria prefer ammonium ions as a nitrogen source and therefore in nitrogen metabolism we consider NH_4^+ as the central molecule from which cell constituents (mostly amino acids) originate. Specific nitrogen sources different from NH_4^+, like N_2, nitrate, a single amino acid etc., all give rise to NH_4^+ before being assimilated in cell constituents. Before studying nitrogen metabolism, therefore, an understanding of ammonium transport is crucial.

2.1. Bacterial ammonium transport

Charged molecules need specific transport systems to cross bacterial membranes: for example, an *Escherichia coli* strain mutated in all transport system for a specific amino acid is completely impermeable to it (27). Small uncharged molecules like NH_3 rapidly pass membranes by unspecific diffusion. Since:

$$NH_3 + H^+ \text{----} NH_4^+ \text{ (p}K_a\text{ 9.25)}$$

then, even at neutral pHs, when ammonium salt is added as a nitrogen source to a minimal medium, NH_3 will be taken up with a consequent acidification of the me-

dium. So for example, in the case of *R.leguminosarum* the pH of the minimal medium (6.3) decreases to 5 when ammonium chloride is the nitrogen source and growth stops, while no pH change is observed if glutamate is the nitrogen source (M. Rossi, unpublished observations.
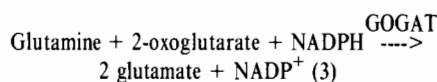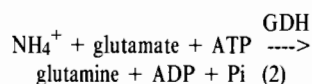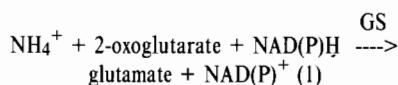
Changes in the pH of the extra- or intracellular medium might explain the widespread toxicity of ammonium salts in all higher organisms, including plants. As reviewed by Kleiner (28), besides unspecific diffusion of NH_3, transport systems for NH_4^+ have been described in many microorganisms and most of them are repressed by the substrate. Kleiner's hypothesis is that NH_3 will diffuse rapidly outside the cell when it is produced inside (from N_2, NO_3^- or from the catabolism of a specific substance) and the transport carrier is needed to maintain the desired intracellular concentration of NH_3. When high concentrations of NH_3 are provided outside, no carrier is needed and this would explain its repression.

In the *Rhizobium* symbiosis plant assimilation of NH_3 produced by nitrogen fixation is efficient because: (a) bacteroid's assimilation is negligible due to a block in protein syntheses (see above); (b) inward transport systems for NH_4^+ are repressed (22, 29); and (c) a specific glutamine synthetase is derepressed in the eukaryotic part of the nodule (24), probably to drive NH_3 diffusion towards the plant and to prevent NH_3 toxicity. The bacterial glutamine synthetase is not needed, but, if present, it might be beneficial to buffer the NH_3 pool in case of a sudden increase.

2.2. Ammonia assimilation

Two main pathways of ammonium assimilation exist in bacteria: via glutamate dehydrogenase (GDH; EC 1.4.1.3) (reaction 1) and via glutamine synthetase (GS; EC 6.3.1.2) and glutamate synthase (GOGAT; 1.4.1.13) (reactions 2 and 3). The end product of both pathways is glutamate, which is used directly for protein synthesis, but most of it acts as an amino group donor in various amino transferase reactions, thus giving rise to the other amino acids. Glutamine is used for protein synthesis and, in addition, serves as a source of nitrogen

atoms in the biosyntheses of all purine and pyrimidine nucleotides, of glucosamine-6-phosphate, of p-aminobenzoic acid and of nicotinamide derivatives. Therefore glutamine synthetase activity is still needed when the GDH pathway is operative, since it links the assimilation of NH_3 with biosynthetic pathways leading to the formation of proteins, nucleic acids, complex polysaccharides, and various coenzymes.

$$NH_4^+ + \text{2-oxoglutarate} + NAD(P)H \xrightarrow{GS} \text{glutamate} + NAD(P)^+ \text{ (1)}$$

$$NH_4^+ + \text{glutamate} + ATP \xrightarrow{GDH} \text{glutamine} + ADP + Pi \text{ (2)}$$

$$\text{Glutamine} + \text{2-oxoglutarate} + NADPH \xrightarrow{GOGAT} \text{2 glutamate} + NADP^+ \text{ (3)}$$

GDH has a high K_m for ammonia and is thus only operative at high ammonia concentrations. GS/GOGAT is the major route of ammonia assimilation when ammonia is limiting. In enteric bacteria, the use of these two pathways is genetically controlled in response to the availability of ammonia. These enzymes form part of a general nitrogen control system (see 23 and 33 for reviews) which includes a number of enzymes involved in the breakdown of nitrogenous compounds. When ammonia is present at high concentrations the genes for GS and other enzymes under nitrogen control are repressed, while GDH is induced. The converse occurs when ammonia is limiting, giving the cell the capability to use a variety of nitrogen sources. Additonally, GS activity is controlled biochemically in response to ammonia. In the presence of ammonia, adenyl groups are reversibly attached to GS, causing inactivation.

2.2.1. The nitrogen control system

Modulation of expression of the intracellular concentration of GDH and GS/GOGAT is accompanied by modulation of the inducibility of other pathways. When ammonia is limiting and other specific conditions are encountered, the genes for nitrogen fixation (*nif*), or those for histidine utilization (*hut*), or for proline utilization (*put*) etc., are induced. In most studies the level of expression of *glnA*, the structural gene for GS, has been used to monitor the modulation of the two pathways for

ammonia assimilation. The GS level, fully induced in bacteria growing in a poor nitrogen source, decreases when ammonia is used, and decreases even further in a medium containing a rich nitrogen source (like ammonia) and a poor carbon source. Thus, bacteria adjust the potential of expression of the nitrogen control system to the ratio of N/C, namely the ratio of glutamine/2-oxoglutarate. When the intracellular concentration of glucose catabolites is suddenly increased (for example by adding glucose to a culture growing on pyruvate) there is an increase in the need for ammonia assimilation, and viceversa. Therefore the general nitrogen control system links nitrogen and carbon metabolism, which may be severely imbalanced under specific situations. Thus, for example, when histidine is used as a sole source of carbon and nitrogen in *Rhizobium*, bacteria accumulate up to 100 mM NH_4^+ in the medium (12).

Promoters of genes regulated by the nitrogen control system have a similar structure and are characterized by the consensus sequence CTGGYAYR-N4-TTGCA extending approximately from -26 to -10 relative to the transcription-initiation site (see 23, for references). This consensus sequence defines *ntr* for promoters and it is different from that of the other *E.coli* promoters. The *ntr* promoters require a specific sigma factor for RNA polymerase, sigma-60, the product of the *ntrA* (also called *glnF* or *rpoN*) gene. They also require the product of the *ntrC* (*glnG*) gene for expression. This protein, NtrC, is an activator of gene expression at all *ntr* promoters except at those of the *nif* genes, where a more specific protein, NifA, is the activator (15). The activity of NtrC is regulated by NtrB, the product of the *ntrB* (*glnL*) gene, which catalyzes a phosphorylation and dephosphorylation of NtrC (35). It appears that NtrB controls the formation and breakdown of NtrC-phosphate. The activity of NtrB is modulated by P_{II}, the product of the *glnB* gene: in the presence of P_{II} NtrB catalyzes the removal of phosphate from NtrC-phosphate thus preventing transcription at *ntr* promoters. The P_{II} protein becomes inactive when uridylylated by the product of the *glnD* gene, GlnD, a protein catalyzing two post-translation modifications of the P_{II} protein, namely

uridylylation or de-uridylylation. Finally GlnD activity is regulated by the intracellular glutamine/2-oxoglutarate ratio. Thus, ammonia starvation, which results in a low ratio, causes a conversion of P_{II} to P_{II}-UMP; this causes a NtrB-catalyzed phosphorylation of NtrC to NtrC-phosphate, which activated transcription of *ntr* promoters. Growth with ammonia excess or a poor carbon source causes an increase n the glutamine/2-oxoglutarate ratio thereby increasing the ratio P_{II}/P_{II}-UMP. Therefore, a change in the growth medium induces a chain of events going from a change in the intracellular glutamine/soxoglutarate ratio, to a change in GlnD activity, to a change in the P_{II}/P_{II}-UMP ratio, to a change in NtrB activity, to a change in the NtrC/NtrC-phosphate ratio and ultimately a change in transcription rate at *ntr* promoters.

2.2.2. Regulations of GS activity

Glutamine synthetase is a dodecamer composed of identical subunits and has a molecular weight of approximately 600,000. GS activity is modulated by several different mechanisms (21) including the interconversion of inactive to active configurations in response to variations in the concentrations of divalent cations, the cumulative feedback inhibition by a number of different end products of glutamine metabolism and a post-translational modification, namely attachment of a single adenyl residue to a specific tyrosine residue on each of the subunits. When the enzyme becomes fully adenylylated as a consequence of a mutation the strain is a glutamine auxotroph, thus confirming the *in vivo* role of adenylelation in regulation of GS activity.

Adenylylation and de-adenylylation are two different activities of the same protein, ATase, the product of the *glnE* gene. ATase activity is regulated by the P_{II} protein described above, which activated adenylylation while P_{II}-UMP is essential for de-adenylylation. Uridylylation and de-uridylylation of P_{II} are two different activities of the *glnD* gene product, which is in turn regulated by the intracellular glutamine/2-oxoglutarate ratio. Therefore, in a bacterium growing with a specific source of carbon, adenylylation and de-adenylylation of GS are controlled by the concentration of ammonia in the growth medium. When the ammonia concentration becomes low, 2-oxoglutarate will accumulate in the cells and glutamine will be in short supply. Under this condition, adenylylation by ATase-P_{II} is inhibited and conversion of P_{II} to P_{II}-UMP is stimulated; the result is conversion of GS-AMP to the active, non adenylylated GS. Conversely, when the ammonia concentration becomes high, the level of 2-oxoglutarate is reduced by its conversion to glutamate, and the level of glutamine increases; this results in adenylylation of GS.

3. Ammonia assimilation in Rhizobium spp.

In free-living *Rhizobium* spp. the level of GDH, GS and GOGAT differs with the strains and the culture media (6, 31, 37). It has been reported that at least some *Rhizobium* spp. are poor at assimilating ammonia and have a marked preference for an organic nitrogen source such as L-gluatamate (31). However, several groups have been able to grow *Rhizobium* on ammonia as the sole nitrogen source provided that the medium was well buffered (26, 40). In Table 1 are reported the growth rated of *E.coli* and of *R.leguminosarum* in minimal medium supplemented with different nitrogen sources. It can be seen that, in agreement with Poole et al. (1987), *R.leguminosarum* shows a significant growth rate in ammonia. However, the relative growth rates in NH_4^+ versus glutamate or glutamine are very differen when *E.coli* is compared to *R.leguminosarum*. While ammonia is the preferred nitrogen source for *E.coli*, this marked preference is not shown by *R.leguminosarum* and other *Rhizobium* spp.. We assume that, while the difference in growth rates between bacteria grown in glutamate and glutamine may be partially due to permeability, this is not true for NH_4^+ and therefore the slow duplication time of *R.leguminosarum* relative to *E.coli* in NH_4^+ may be related to the *in vivo* efficiency of the pathways for ammonia assimilation.

One way to obtain evidence for the presence of a pathway is to study the phenotype of strains carrying specific mutations. An enteric bacterium mutated in *gltB*, the structural genen for GOGAT (19), does not grow on only of several nitrogen sources like arginine, proline, histidine etc., but it will grow if excess NH_4^+ is added to the medium because it uses the GDH pathway. Therefore, a *Rhizobium* mutant lacking GOGAT will allow to test if the GS/GOGAT and the GDH pathways are present. Mutants of different wild type isolates of *R.meliloti* (1, 37) and of *Bradyrhizobium japonicum* (30) lacking GOGAT were isolated and their phenotype shows that the GS/GOGAT pathway is operational. Some of these strains failed to grow on NH_4^+, thus showing that the GDH pathway is missing in some, but not all, the tested strains. A plasmid coding for *E.coli* GDH was introduced in one of the latter strains (30) and in this way aquired the ability to grow in NH_4^+. An analogous experiment should be performed with a wild type *Rhizobium* to check if the relatively slow growth rate in NH_4^+ reported in Table 1 is due to an inefficient GDH pathway.

Wild type *E.coli* end *R.leguminosarum* biovar *viceae*, strain RCC1001 were grown in RMM minimal medium (25) at 37C and 30 C, respectively. NH_4Cl, glutamine and sodium glutamate were added at 1 mg/ml, final concentration. When NH_4Cl was the nitrogen source 100 mM MOPS (pH 7.2) was added to the medium

Nitrogen source	Duplication times (min)	
	E. coli	*R. leguminosarum*
NH_4^+	45	318
Glutamine	138	220
Glutamate	294	276

Table 1 : Growth rates of E.coli and R.leguminosarum in minimal medium supplemented with different nitrogen sources.

and was essential to achieve the indicated growth rate.

Two forms of glutamine synthetase, GSI and GSII have been demonstrated in all species tested of the genus *Rhizobium* and *Agrobacterium* (19). GSI has been shown to be similar to the single GS of enteric bacteria. It is a polymeric enzyme consisting of 12 identical subunits of Mr 59,000, is relatively heat stable, and can be adenylylated (11). In contrast, GSII is made of smaller subunits, Mr 36,000, which are heat labile and not known to be modified after translation. These proteins are products of different genes (8, 10, 18, 44). GSI and GSII can be easily assayed in crude extract on the basis of their heat stability: GSII is completely inactivated after 30 min. incubation at 55C, while GSI is stable. Using extracts of *R.leguminosarum* containing both GS forms we showed that GSI is adsorbed to Blue-Sepharose, while GSII is not. Repeated adsorption/elution experiments showed that one form does not generate the other, thus confirming that they should be considered isozymes. GSI can be eluted from Blue-Sepharose with AMP and purified 80-fold. When the enzyme is purified from *R.leguminosarum* grown in NH4Cl it is found to be 90% adenylylated. Treatment with snake venom phosphodiesterase causes a decrease in adenylylation to a value of 20%. The percentage adenylylation is measured using the transferase activity of GS (17). An assay for the biosynthetic activity (3) on them same samples shows that adenylylation inhibits the forward reaction of GS, similarly to what happens for enteric GS and contrary to what reported for GSI of *Rhizobium* spp. ANU289 (26).

Adenylylation of GSI appears to have an *in vivo* significance since the following values were obtained when *R.leguminosarum* was grown in different nitrogen sources: 90% when grown in glutamine, 19% in glutamate and NH4+ and 50% in nitrate. Moreover, when glutamate grown cells were centrifuged and resuspended for 2 hrs in a medium containing either nitrate of NH4+, the level of adenylylateion became 5% and 98%, respectively.

We reported (10) the cloning and complete nucleotide sequence of the *R.le-guminosarum glnA* gene, the structural gene for GSI. The deduced amino acid sequence of this DNA shows 56%, 63% and 68% homology with the deduced amino acid sequences of *Anabaena*, *E.coli* and *Azospirillum brasilense* GSs respectively, and most of it is in boxes of identical amino acids. The molecular weight of the deduced amino acid sequence is 52,040, in good agreement with that obtained from pure GSI. Antiserum raised against pure GSI immunoprecipitates a radioactive band obtained in a minicells experiment from the DNA region sequenced. Upstream of the *glnA* gene *R.leguminosarum* and of *B.japonicum* (8) is not present a complex regulatory sequence as that reported for *glnA* of enteric bacteria (see, for example 14).

The levels of GSI activity change very little with nitrogen sources in *R.leguminosarum* (26 and our unpublished observations). Instead, GSII levels change dramatically, from a specific activity of 2 (nmoles/min/mg protein in the transferase assay) when glutamine or NH4+ are the nitrogen sources, to about 1,000 when grown on glutamate or nitrate (our unpublished observations). When glutamate grown cells were treated with 10 mM NH4Cl there was a rapid removal of GSII activity suggesting an active mechanism (26). Similar observations were reported for several *Rhizobium* spp. by Fuchs and Keister (1980). With a similar protocol we find that GSII is reduced after 2 hrs of incubation in NH4Cl to 16% of the control and that addition of chloroamphenical prevents this effect. Thus, it appears that the decrease in GSII activity observed after addition of NH4Cl might be the consequence of a post-translational modification and that this requires protein synthesis. If confirmed, this complex mechanism of regulation might be related to the fact that a non-dividing bacteroid cannot achieve repression of a gene product by dilution among daughter cells.

The gene of *B.japonicum* coding for GSII has been cloned and sequenced (7). Comparison of the deduced amino acid sequence to that of other GSs showed limited homology to the bacterial GSs but extensive homology to a GS of *Phaseolus vulgaris* roots, thus suggesting that the gene coding for GSII might be of eukaryotic origin. Upstream of the gene coding for GSII there is a good consensus sequence for an *ntr* promoter (9).

Not much is known yet about the significance of the enzymes for ammonia assimilation with respect to the symbiotic phenotype of *Rhizobium* spp.. As described above, GDH is missing in several strains and perhaps not very active in others. This might cause a slow growth rate in NH4Cl versus other nitrogen sources when compared to enteric bacteria (see Table 1), but it does not affect the Nod and Fix phenotype. Strains defective for GOGAT are Nod+ and Fix+. GSI insertion mutants of *R.meliloti* (44) are Gln+ in the free-living form, probably because GSII expression substitutes for GSI; they are Nod+ and Fix+. Mutants of *B.japonicum* lacking either GSI or GSII are Gln+, Nod+, Fix+; double mutants are Gln-, Nod- (9). *Azorhizobium caulinodans* ORS571 contains only one GS and a mutant devoid of it is Gln- Fix- (16).

Although GSI is adenylylated, nothing is known yet on the presence of an ATase in *Rhizobium* spp.. We reported (10) of a DNA sequence coding for a putative protein which shows a striking similarity to the PII protein of *E.coli*. This sequence is upstream of *glnA*. Therefore, while *glnB* and *glnA* are unlinked in enteric bacteria, a *glnB*-like gene is contiguous to *R.leguminosarum*. A similar sequence appears to be present upstream of *glnA* in *B.japonicum* and *A.brasilense*. No rho-independent transcription-termination signal is present between *glnB* and *glnA* and a preliminary S1 mapping experiment indicates the presence of a *glnB-glnA* transcript as well as of *glnA* transcript. Results with Northern experiments confirm this conclusion. Since the *glnB*-like sequence is expressed in a minicells experiment in *E.coli* we inserted it in an appropriate vector to make a fused protein, which was purified to homogeneity. With this protein preparation we raised an antiserum and assayed crude extracts of *R.leguminosarum* by immunoblot. The antiserum reacts only with native gels giving a doublet of two very close bands (may be the uridylylated and non uridylylated form of the protein), which are present in extracts of *R.leguminosarum*

grown in glutamine or glutamate or nitrate, while they are very faint when grown in ammonium. Upstream of *glnB* and very close to the transcription-initiation site we find the consensus sequence for an *ntr* promoter which might be regulated by ammonium.

4. The nitrogen control system in *Rhizobium* spp.

Not very much is known about the regulation of nitrogen metabolism in *Rhizobium* spp., but the progress is being quite rapid. Consensus sequences for *ntr* promoters have been described (see 23 for a review) and therefore an *ntrA*-like gene should exist.

Szeto et al. (1987) identified an *R.meliloti* *ntrC*-like gene on the basis of interspecies cross-hybridization with the *E.coli* *ntrC* gene. DNA sequence analysis showed that this gene is homologous to the *ntrC* gene of *K.pneumoniae* and *Bradyrhizobium sp.* *(Parasponia)(36) and that an* ntrB-like gene is situated directly upstream from *R.meliloti* *ntrC*. The cloned sequence was used to generate a mutant and this showed that the *ntrC* gene is required for growth in a poor nitrogen source (nitrate) as it is the case for a *K.pneumoniae* *ntrC* mutant. The latter is also Nif⁻, while the *R.meliloti* *ntrC* mutant is Nod⁺Fix⁺, thus indicating the presence of separate regulatory pathways for regulation of nitrogenase and of the other genes for nitrogen metabolism. An *ntrC* gene has been isolated from *A.caulinodans* ORS571 by interspecies hybridization and an insertion mutant has been constructed (39). Analysis of the phenotype indicates that in *A.caulinodans* *ntrC* is essential for efficient derepression of the *nif* genes and proper nodule development in contrast to the situation in *R.meliloti*.

A *glnB*-like gene was isolated from *R.leguminosarum* and it is described above, but no mutant is available yet.

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Regulation of *Azorhizobium* ORS571 N2 fixation

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The novelty of Azorhizobium ORS571

Azorhizobium ORS571 is a novel organism that exhibits properties of both diazotrophic bacteria (those able to utilize N_2 as sole N-source for growth), such as *Klebsiella pneumoniae*, as well as endosymbiotic N_2-fixing bacteria, such as *Rhizobium meliloti*, which fixes N_2 only during symbiosis with *Medicago sativa* (alfalfa). ORS571 also exhibits a unique ecological niche, inhabiting nodules on stem lateral root primordia (stem nodules) elicited on the host plant, *Sesbania rostrata*. In fact, both ORS571 N_2-fixing capabilities are manifested during *S.rostrata nodulation*. A single *Azorhizobium* can infect an *S. rostrata* lateral root primordium to elicit a nodule containing 10^9 bacteria. From where does all the N for this enormous proliferation of cell mass emanate?

An infected *S.rostrata* plant is N-depleted and can supply only a limited amount of N for bacterial growth. Nor do stem-infecting bacteria have access to rhizosphere-N, available to root-infecting bacteria. As a consequence, atmospheric N_2 is the N-source of last recourse. In fact, if an *S.rostrata* plant is inoculated with an *Azorhizobium* ORS571 culture, N_2-fixation can be detected 24-48 hours later. In contrast, in the orthodox *R.meliloti*-alfalfa symbiosis, N_2 fixation does not commence until some weeks after inoculation, until nodule maturation and differentiation if rhizobia into "bacteroids" occur. In this organelle form, rhizobia cease to divide and fix N_2 exclusively for net plant growth processes. During bacterial proliferation, N_2 fixation is thus coupled to assimilation of ammonium, so produced,

into bacterial amino acids and proteins; after bacteroid differentiation, N_2 fixation is decoupled from the assimilation of ammonium, which is instead excreted into the host plant cortical cell cytoplasm for assimilation into plant amino acids and proteins.

The Ntr system regulates expression of genes encoding N-metabolic functions, including N2-fixation

In the prioritized list of potential N-sources for bacterial growth, N_2 is at the bottom owing to the extreme stabilty of its NN triple bond. Broadly grouped, bacteria distinguish primary N-sources, such as glutamine or ammonium, from secondary N-sources, including most amino acids, nitrate, and N_2. As a consequence, enzymes utilizing a broad spectrum of secondary-N compounds are only biosynthesized upon depletion of primary-N. Bacteria possess a nitrogen-regulatory (Ntr) system to activate the expression of genes encoding secondary N-utilization enzymes during primary-N depletion. This Ntr system is composed of three elements: (i) NtrA, a dissociable subunit of RNA polymerase (the enzyme which transcribes genes into mRNA) that directs RNA polymerase to the secondary-N genes, (ii) NtrB, an environmental monitor protein that assesses relative N-availability in the cell, and (iii) NtrC, an NtrB-dependent gene activator protein that stimulates transcription of secondary-N genes by NtrA-containing RNA polymerase. Remarkably, the Ntr regulatory system is

configured similarly among a broad spectrum of bacteria.

Apparently because this system works well for regulation of gene-sets involved in N-metabolism, it has been duplicated many times for other gene-sets, such as those encoding outer membrane proteins, bacterial endospore formation, and dicarboxylate transport, all having nothing to do with N-metabolism (7).

The different modes of N2 fixation in model organisms K.pneumoniae and R.meliloti

For diazotrophic *K.pneumoniae*, N_2 fixation is conducted anaerobically to minimize deleterious effects of O_2. Therefore, *K.pneumoniae* has an additional version of the Ntr system specifically adapted to only allow *nif* gene expression in the absence of O_2. For this regulation, *K.pneumoniae* has enlisted the NifL and NifA gene products; NifL is marginally homologous to NtrB, and NifA is highly homologous to NtrC. NifL is the O_2 environmental monitor protein and NifA is the cognate gene activator. These two systems form a "cascade". Under N-depletion, NtrA+NtrB+NtrC activate the expression of NifLA. Under, in turn, O_2-depletion, NifL+NifA activate expression of the nitrogenase structural genes. In this way, the *K.pneumoniae* nitrogenase structural genes are only activated under both N- and O_2-depletion (5).

In endosymbiotic *R.meliloti* bacteroids, N_2 fixation is conducted microaerobically. Here in the legume nodule, leghemoglobin buffers O_2 and thus prevents free O_2 from reaching levels deleterious to N_2 fixation.

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In fact, D. Kahn and colleagues (see Dénarié et al, this volume) have identified a pair of *R.meliloti* genes encoding FixL and FixJ products that seem both analogous and homologous to *K.pneumoniae* NifL and NifA, even though *R.meliloti* has its own NifA product! *R.meliloti* then, seems to have some sort of "double O_2 protection" gene cascade with which to regulate activation of nitrogenase structural genes. Because *R.meliloti* has not been shown to conduct proliferative N_2 fixation, the activation of its nitrogenase structural genes seems to be independent of the Ntr system. Indeed, *R.meliloti* Ntr⁻ mutants incite alfalfa nodules with normal N_2 fixation activity. Somewhat confusingly, in very sensitive assays for *R.meliloti* nitrogenase structural gene activation, an effect of the Ntr system on the cloned genes has been observed (5). To what extent this observation is biologically important remains to be determined.

Azorhizobium ORS571 conducts synergistic N_2 fixation and nicotinate oxidation

In the face of these two alternatives, the report (3) that *Azorhizobium* ORS571 conducts both proliferative N_2 fixation in aerobiosis as well as endosymbiotic N_2 fixation in microaerobiosis captivated and astonished the N_2 research community. As a first clue, large amounts of nicotinate were found to be required for *Azorhizobium* ORS571 proliferative N_2 fixation in air (4). This observation was subsequently linked to another unusual *Azorhizobium* property: strain ORS571 is an NAD^+ auxotroph as isolated from the wild! Because the inability to produce a vital cofactor (NAD^+) seems hopelessly disadvantageous, this finding also confounded the research community. Nevertheless because *Azorhizobium* ORS571 flourishes upon infection of *S.rostrata*, NAD^+ precursor(s) must be supplied to growing bacteria. Indeed, as first demonstrated in pea (1), legumes synthesize nicotinate, the vitamin precursor to NAD^+ in leaves and transport nicotinate systemically to roots. Infecting *Azorhizobium* ORS571 bacteria presumably tap into this

transported nicotinate, which they take up avidly in culture. However, *Azorhizobium* ORS571 not only converts nicotinate to NAD^+, it also catabolizes nicotinate, on which it can grow as sole N-source.

We subsequently discovered that *Azorhizobium* ORS571 can fix N_2 aerobically during the catabolism of nicotinate, which scavenges intracellular O_2 (6). Thereupon, nicotinate serves as O_2 sink, creating on O_2-depleted intracellular environment in an otherwise aerobic milieu!. In support of this theory, *Azorhizobium* ORS571 mutants unable to use nicotinate as sole N-source for growth were isolated. Such mutants also proved unable to fix N_2 in culture (Nif⁻) and in *S.rostrata* nodules (Fix⁻). Therefore, upon inoculation of *S.rostrata*, *Azorhizobium* ORS571 proliferates using both nicotinate and N_2 as N-sources. From reconstruction experiments in culture that attempt to mimic *in planta* proliferation, we have compared *Azorhizobium* ORS571 growth on nicotinate as N-source under either N_2 or argon atmospheres supplemented with various amounts of O_2. Inferring the effective O_2 concentration *in planta*, we estimate that 90% of proliferating *Azorhizobium* assimilated-N (*in planta*) is derived from N_2; 10 % is derived from nicotinate.

Organization of the Azorhizobium ORS571 nif genes

Interestingly, the *Azorhizobium* ORS571 *nif* genes are dispersed among at least four loci (2), in contrast to *K.pneumoniae*, where all *nif* genes are tightly clustered (5). Although the significance of this observation is unclear, we speculate that one reason for the ORS571 *nif* gene dispersal is differential gene regulation. Of the characterized ORS571 *nif* gene-loci, locus 1 holds the largest number of genes, including the *nifA* regulatory gene (2).

Regulation of and by the Azorhizobium ORS571 nifA gene

We hypothesized that, as well as mediating control of nitrogenase structural gene

expression, the *Azorhizobium nifA* gene must also mediate O_2 control, i.e. assure that nicotinate oxidation is operating to deplete intracellular O_2 before allowing expression of nitrogenease structural genes. Correspondingly, *Azorhizobium* NifA⁻ mutants incite *S.rostrata* nodules that prematurely senesce; in these nodules infected cortical cells show profound necrosis. To assess this hypothesis in detail, we have determined the nucleotide sequence of the *Azorhizobium nifA* gene (Loroch et al, in press). From this sequence, we have deduced the code for the NifA protein and thus its amino acid sequence. Like other decoded *Azorhizobium* and *Bradyrrhizobium* NifA proteins, the *Azorhizobium NifA* gene shows two possible, in-frame translation starts spaced some 36 amino acid codons apart. Nine to fourteen nucleotide pairs upstream from the putative transcriptional initiation sites, an NtrA-like binding site (GGCTGATCCCTCGG-CA) has been identified. As has been confirmed by detailed studies of the cloned *Azorhizobium nifA* gene using gene-fusions, this gene is therefore under Ntr control. Centered around a site some 44 nucleotide pairs upstream of this same *Azorhizobium nifA* transcriptional start site is a palindromic sequence of 14 nucleotide pairs (TTTGATXXXXATCAA, where X is any nucleotide) that is found at the identical position in the *E.coli* genes encoding nitrite reductase, nitrate reductase, and fumarate reductase, all of which are expressed anaerobically. These anaerobic genes are under the control of the *fnr* activator gene. This suggests both that the Fnr protein interacts with DNA upstream from cognate regulated genes at corresponding sites and that *Azorhizobium* has an *fnr* homolog. This would allow further activation of the *Azorhizobium nifA* gene once effective anaerobiosis had been achieved (as during nicotinate oxidation). The exact DNA site upstream from the *Azorhizobium nifA* gene at which the NtrC protein might interact in unclear; it may compete for binding at the Fnr consensus nucleotide sequence.

Based on these and other results, we present models for the regulation of the *Azorhizobium nifA* gene (FIGS. 1,2,3 for three different physiological conditions, all corresponding to bacterial proliferation.
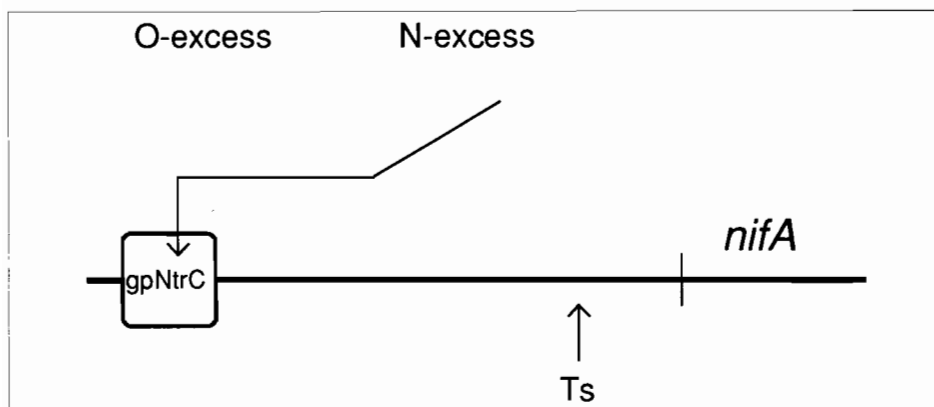
Fig. 1. Model for regulation of Azorhizobium gene expression under physiological conditions
of N-excess leading to nifA repression. No N$_2$ fixation occurs under this condition.
Abbrevations for proteins, indicated as ellipses, are given in the text.
(+) refers to gene activation and (-) to gene repression.
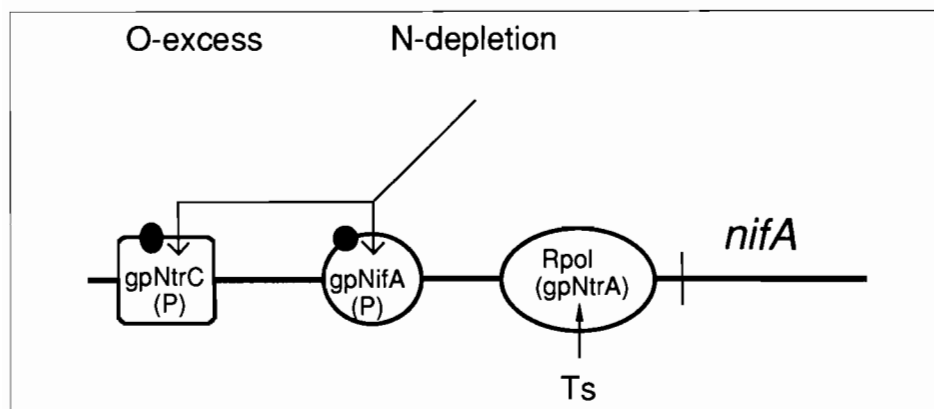*Numbers (-42, -25, -10) refer to positions relative to an inferred nifA transcriptional startpoint.*

Fig. 2. Model for Azorhizobium nifA gene expression under physiological conditions
of N-depletion and O$_2$ excess. No N$_2$ fixation occurs under these conditions.
Legend follows that of FIG. 1. Additional abbrevations: RPOL, RNA polymerase, σ70,
RNA-polymerase sigma subunit of 70 kDa. Proteins (ellipses) supplemented
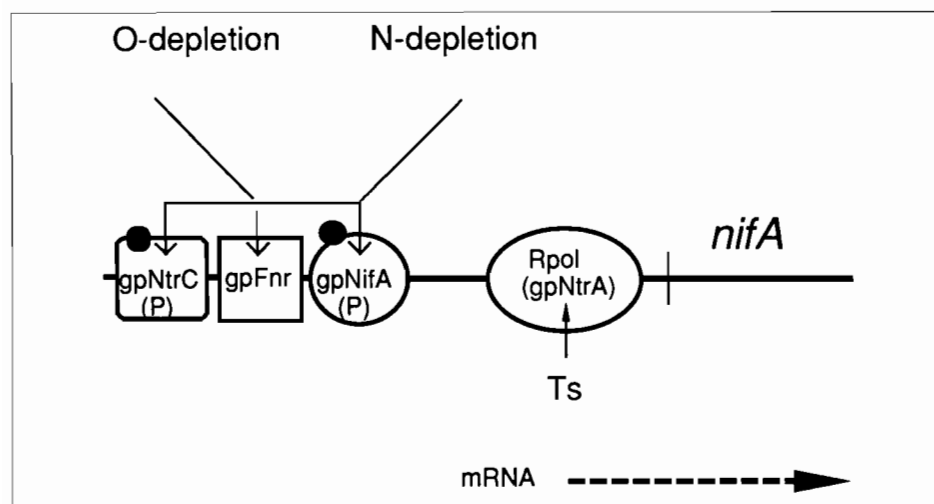*with closed circles imply phosphorylated proteins, e.g. NtrC(P).*

Fig. 3. Model for Azorhizobium nifA gene expression under physiolocal conditions
of N-depletion and O$_2$-depletion. N$_2$ fixation occurs under these conditions.
Figure legend follows that of FIG.2. Additional abbrevations: (ox), Oxidized; (red), reduced.

This model, as operative in the three different physiological states indicated, suffices well enough for proliferative N$_2$ fixation. However, because *Azorhizobium ntr* mutants yield *S.rostrata* nodules with delayed N$_2$ fixation proficiency, during bacteroid N$_2$ fixation in mature nodules, Ntr control of *nifA* gene expression is overridden. Differential control of *nifA* expression during proliferative and bacteroid N$_2$ fixation processes might help explain the unique capability of *Azorhizobium* ORS571 to exhibit bi-modal N$_2$ fixation.

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Régulation de la fixation N2 d'*Azorhizobium* ORS571

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La nodulation de *Sesbania rostrata* par *Azorhizobium* ORS571 inclut un minimum de deux modes de fixation de N2. Le premier mode est prolifératif: au début du processus de nodulation, la fixation de N2 est reliée à l'assimilation bactérienne d'ammoniaque permettant la croissance d'*Azorhizobium* à l'intérieur de la plante hôte. Le deuxième mode est altruiste: dans les nodules matures, la fixation de N2 est découplée de l'assimilation bactérienne d'ammoniaque, celle-ci étant exportée des bactéroïdes au profit de la croissance de la plante hôte. Les gènes de fixation de N2 (*nif*) d'*Azorhizobium* ORS571 sont portés par au moins quatre régions du chromosome. L'ensemble de ces gènes constituent un régulon dont la transcription, durant la fixation proliférative de N2, est sous le contrôle des gènes régulateurs d'azote (*ntr*) qui servent de moniteur de niveaux intracellulaires d'azote disponible. Dans les nodules mûres, le système *Ntr* ne contrôle plus les gènes *nif* d'*Azorhizobium*, permettant aux bactéroïdes de réduire l'azote moléculaire dans un micro-environment riche en azote fixé. L'expression des gènes *nif* d'*Azorhizobium* est également sensible au niveau intracellulaire d'oxygène. Ce deuxième contrôle est exercé par l'intermédiaire de NifA, un activateur de transcription des autres gènes d'*Azorhizobium*. La protéine NifA est inactivée lorsque le niveau d'oxygène est suffisamment élevé pour nuire au processus de fixation de N2. L'ensemble de ces mécanismes régulateurs donnent à *Azorhizobium* ORS571 la capacité unique pour un *Rhizobium* de fixer l'azote moléculaire aussi bien pour sa propre croissance qu'au profit de la plante hôte.

Le caractère unique d'Azorhizobium ORS571

Azorhizobium ORS571 est un organisme unique possédant de propriétés caractéristiques de bactéries diazotrophes (par exemple, *Klebsiella pneumonieae*), qui sont capables d'utiliser N2 en tant que seule source d'azote pour leur croissance, mais aussi des propriétés de bactéries fixatrices endosymbiotiques, telles que *Rhizobium meliloti*, qui fixe N2 uniquement au cours de la symbiose avec *Medicago sativa* (luzerne). *Azorhizobium* ORS571 occupe une niche écologique particulière: les bactéroïdes résident dans des nodules situés sur les primordiums de racines latérales sur la tige de la plante hôte *Sesbania rostrata*. Les deux modes de fixation de N2 d'ORS571 sont manifestement impliqués dans la nodulation de *S.rostrata*. En effet, un seul *Azorhizobium* est capable d'infecter un primordium de racine de *S.rostrata* suivi de développement d'un nodule contenant plus de 10^9 bactéries. Une telle prolifération de masse cellulaire demande un apport d'azote non-négligeable. Son origine soulève des questions importantes.

Au moment de l'infection par *Azorhizobium*, *S.rostrata* est pauvre en azote fixé et ne peut fournir qu'une quantité limitée de N pour la croissance bactérienne. D'autre part, contrairement aux bactéries nodulatrices de racines, *Azorhizobium* qui infecte la tige de la plante n'a pas accès à l'azote du rhizosphère. Par conséquent, la seule source d'azote disponible est l'azote atmosphérique. Ainsi on observe que l'infection de *S.rostrata* avec *Azorhizobium* ORS571 est suivie de fixation de N2 dans les 24 à 48 heures. La situation est bien différente dans le cas de la symbiose classique entre *R.meliloti* et la luzerne où la fixation de N2 n'est évidente que plusieurs

semaines plus tard, après la formation de nodules mûres et la différentiation de *Rhizobium* en bactéroïdes. A ce stade d'organelles, la division cellulaire de *Rhizobium* est arrêtée et l'azote moléculaire est fixé pour les processus de croissance de la plante strictement. Par contre, chez *Azorhizobium* ORS571 la fixation de N2 est bi-phasique: durant la prolifération d'ORS571, la fixation de N2 est couplée à l'assimilation d'ammoniaque en acides aminés et protéines bactériennes; après la différentiation en bactéroïdes la fixation de N2 n'est plus liée a l'assimilation, l'ammoniaque étant exportée dans le cytoplasme des cellules corticales où elle est assimilée en acides aminés et protéines végétaux.

Le système Ntr contrôle l'expression des gènes impliqués dans le métabolisme d'azote, en particulier la fixation de N2

L'azote atmosphérique contient une liaison chimique triple extrêmement stable et constitue par conséquent la source d'azote la moins favorable pour la croissance bactérienne. Les bactéries ont la capacité de distinguer entre deux larges groupes de sources d'azote: les sources primaires d'azote, telles la glutamine et l'ammoniaque, et les sources secondaires d'azote, telles la plupart des acides aminés, le nitrate et le N2. On observe, par exemple, que les enzymes nécessaires à l'utilisation des sources secondaires d'azote ne sont synthétisées qu'après l'épuisement des sources primaires. Afin d'activer la synthèse des enzymes nécessaires à l'utilisation des sources secondaires d'azote, les

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bactéries possèdent un système de régulation d'azote (Ntr). Ce système Ntr est composé de trois éléments: NtrA, NtrB et NtrC. NtrA est une sous-unité de l'ARN polymérase: le complexe NtrA-ARN polymérase est nécessaire à la transcription des gènes d'utilisation des sources secondaires d'azote. NtrB sert de moniteur de l'environnement et détermine la disponibilité de l'azote intracellulaire. NtrB peut activer NtrC qui, à son tour, stimule la transcription par la NtrA-ARN polymérase des gènes d'utilisation des sources secondaires d'azote. Il est intéressant de noter que le système régulateur Ntr est similaire chez un grand nombre d'espèces de bactéries.

Ce système régulateur des gènes du métabolisme de l'azote semble présenter des avantages importants car il a été fidèlement reproduit pour d'autres groupes de gènes qui ne jouent aucun rôle dans le métabolisme de l'azote. Plusieurs exemples ont été décrits impliquant les protéines de la membrane externe, la formation d'endospores bactériens et le transport de dicarboxylates (7).

Les différent modes de fixation N_2 dans les deux organismes modèles *K.pneumoniae* et *R.meliloti*

Dans le cas du diazatrophe *K.pneumoniae*, la fixation de N_2 a lieu dans des conditions strictement anaérobiques ce qui élimine l'effet néfaste de l'oxygène. Le système Ntr de *K.pneumoniae* a été modifié afin d'empêcher l'expression des gènes *nif* en présence d'oxygène. Ce système Ntr inclut deux protéines supplémentaires: NifL, qui présente une homologie marginale avec NtrB, et NifA, qui est fortement homologue à NtrC. NifL agit en tant que moniteur d'oxygène dans l'environment et NifA est l'activateur des autres gènes *nif*. Les deux régulateurs agissent en "cascade". Lorsque l'azote fixé est épuisé, NtrA, NtrB et NtrC activent la synthèse de NifL et NifA. A condition d'avoir simultanément épuisé l'oxygène, NifL et NifA, à leur tour, activent la synthèse des autres gènes *nif*. Grâce à ces deux groupes de régulateurs,

les gènes de structure de la nitrogénase de *K.pneumoniae* ne sont activés qu'en l'absence d'azote fixé et d'oxygène (5).

Dans les bactéroïdes endosymbiotiques de *R.meliloti*, la fixation de N_2 a lieu dans des conditions microaérobiques. L'oxygène est tamponné par la leghémoglobine présente dans le nodule de la plante et l'oxygène libre est effectivement réduit à un niveau qui n'affecte pas la fixation de N_2. D. Kahn et ses collaborateurs (cf. Denarié et al., ce volume) ont identifié deux gènes de *R.meliloti* qui codent les protéines FixL et FixJ homologues et analogues aux protéines NifL et NifA de *K.pneumoniae*. Etant donné que *R.meliloti* possède une véritable protéine NifA, la découverte de D. Kahn suggère que *R.meliloti* possède une cascade de gènes qui offrent une protection double contre l'excès d'oxygène. L'activation des gènes *nif* est apparemment indépendante du système Ntr, du fait que *R.meliloti* ne fixe pas le N_2 d'une manière proliférative. On observe, par exemple, que les mutants Ntr⁻ de *R.meliloti* sont capables de former des nodules avec un niveau normal de fixation de N_2. Bien qu'il a été démontré que le système Ntr exerce un effet sur des gènes clonés, la signification biologique de cette observation est toutefois discutable.

La fixation d'azote et l'oxydation de l'acide nicotinique sont des processus synergistiques chez Azorhizobium ORS571

Si l'on compare les deux modes alternatifs de fixation de N_2 décrits plus haut aux caractéristiques d'*Azorhizobium* ORS571, on comprend la surprise et l'enthousiasme de la communauté scientifique suite à la description de ce nouvel organisme capable de fixer l'azote aussi bien d'une manière proliférative dans des conditions aérobiques que d'une manière endosymbiotique dans des conditions microaérobiques (3). Curieusement, la fixation proliférative dans des conditions aérobiques n'a lieu qu'en présence de vastes quantités d'acide nicotinique (4). Une deuxième observation est encore plus

étonnante: la souche sauvage d'ORS571 est un auxotrophe pour la nicotinamide-adénine-dinucléotide (NAD+). A première vue, l'incapacité de produire un tel cofacteur vital présente un désavantage immense pour la bactérie. Néanmoins, *Azorhizobium* ORS571 prospère une fois à l'intérieur de *S.rostrata* ce qui indique que des précurseurs de NAD^+ sont fournis aux bactéries proliférantes. Il est reconnu que les légumineuses, le pois par exemple (1), synthétisent dans leurs feuilles un précurseur de NAD^+, l'acide nicotinique, qui est ensuite transporté de façon systémique jusque dans les racines. Il est probable qu'au cours de l'infection, *Azorhizobium* ORS571 utilise l'acide nicotinique ainsi transporté. Toutefois, la conversion de l'acide nicotinique en NAD^+ ne représente qu'une des voies métaboliques d'*Azorhizobium* ORS571, étant donné que la bactérie est capable de croître en utilisant uniquement l'acide nicotinique comme seule source d'azote.

Nous avons montré qu'*Azorhizobium* ORS571 est capable de fixer l'azote moléculaire en présence d'oxygène à condition de cataboliser simultanément l'acide nicotinique. L'acide nicotinique servant de "trappe" à oxygène, le niveau intracellulaire d'oxygène est ainsi épuisé. Afin de vérifier cette hypothèse, nous avons obtenu des mutants incapables d'utiliser l'acide nicotinique en tant que seule source d'azote. Ces mutants ont simultanément perdu la capacité de fixer l'azote en culture (nif⁻) et dans les nodules de *S.rostrata* (Fix⁻). La prolifération d'*Azorhizobium* ORS571 à l'intérieur de *S.rostrata* requiert apparemment aussi bien l'acide nicotinique que N_2 en tant que sources d'azote. Nous avons essayé de reconstituer la prolifération *in planta* d'*Azorhizobium* ORS571 en comparant la croissance en culture avec l'acide nicotinique en tant que source d'azote en présence de N_2 ou d'argon et de différents niveaux d'oxygène. En estimant la concentration effective d'oxygène *in planta*, nous avons déduit que 90% ou plus d'azote assimilé par les bactéries proliférantes provient de N_2 et 10% ou moins provient de l'acide nicotinique.

L'organisation des gènes nif dans le chromosome d'Azorhizobium ORS571

Les gènes *nif* d'*Azorhizobium* ORS571 sont portés par au moins quatre régions du chromosome (2), ce qui représente une organisation bien différente de celle de *K.pneumonia* où tous les gènes *nif* sont rassemblés en un groupe (5). Bien que la raison n'en soit pas claire, il est possible que cette dispersion des gènes permet une régulation différentielle de leur expression. Le groupe I des quatre régions caractérisées inclut le plus grand nombre de gènes *nif*, y compris le gène régulateur *nifA*.

Régulation du gène nifA d'Azorhizobium ORS571

Les caractéristiques uniques d'*Azorhizobium* ORS571 nous ont conduit à formuler l'hypothèse suivante: le gène *nifA* d'*Azorhizobium* remplit au moins deux fonctions régulatrices: premièrement, le contrôle direct de l'expression des gènes de structure de la nitrogénase et deuxièmement, la médiation du contrôle par l'oxygène, c'est-à-dire l'activation des gènes de structure de la nitrogénase seulement dans des conditions ou l'oxygène est épuisé grâce au catabolisme de l'acide nicotinique. Les mutants NifA⁻ d'*Azorhizobium* ont un phénotype qui confirme cette hypothèse: l'infection de *S.rostrata* avec ces mutants produit des nodules qui sénescent prématurément, les cellules corticales infectées subissant une nécrose profonde. La première étape pour tester notre hypothèse fut de déterminer la séquence nucléotique du gène *nifA* d'*Azorhizobium* et de déduire la séquence d'acides aminés de la protéine correspondante (Loroch et al., à paraître). La séquence du gène inclut deux sites plausibles d'initiation séparés par 36 codons d'acides aminés; une situation similaire se présente chez *Rhizobium* et *Bradyrhizobium*. Les sites d'initiation sont précédés par un site d'attachement possible de NtrA (GGCTGATCCCTCGGCA). Le contrôle du gène *nifA* par le système Ntr a d'ailleurs été clairement démontré à l'aide de fusions introduites dans le gène
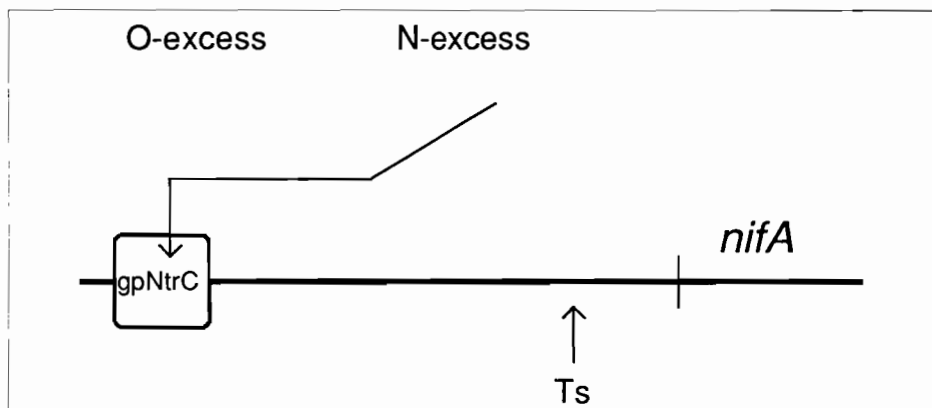

Figure 1 : Modèle pour la régulation de nifA d'Azorhizobium dans des conditions physiologiques où l'azote fixé abonde. La fixation d'azote moléculaire est réprimée. Les protéines sont représentées par des ellipses et les abréviations sont expliquées dans le texte. Les nombres -42, -25 et -10 indiquent les positions des sites relatifs au site d'initiation de nifA déduit d'après la séquence nucléotique.
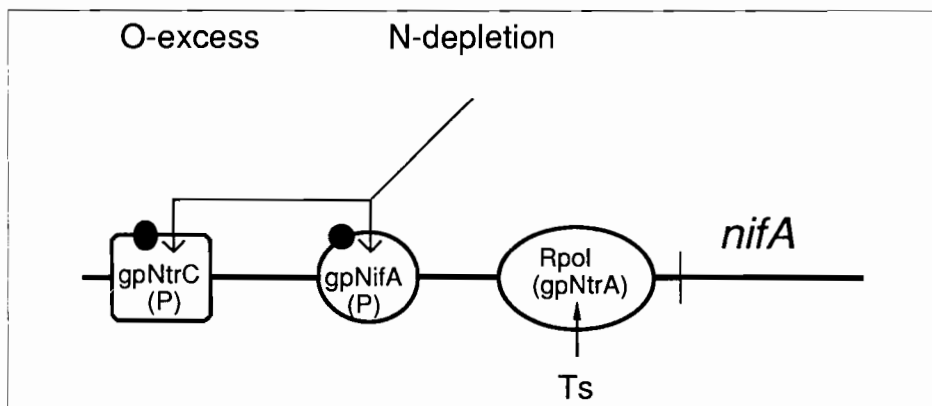

Figure 2 : Modèle pour l'expression de nifA d'Azorhizobium dans des conditions physiologiques où l'azote fixé est épuisé, mais l'oxygène abonde. La fixation d'azote moléculaire est réprimée. Les symboles et abréviations sont ceux de la figure 1. Abréviations supplémentaires: RPOL, ARN polymérase; sigma 70; sous-unité sigma de 70 kDa. de l'ARN polymérase. Les protéines (représentées par des ellipses) avec des cercles fermés indiquent des phosphorilations (par exemple, (NtrC(P)).
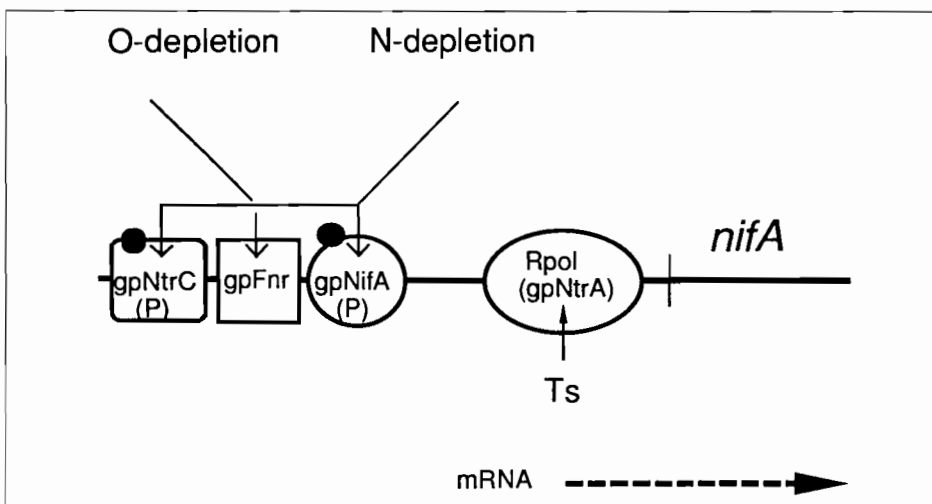

Figure 3 : Modèle pour l'expression de nifA d'Azorhizobium dans des conditions physiologiques où l'azote fixé aussi bien que l'oxygène sont épuisés. L'azote moléculaire est fixé par la nitrogénase. Les symboles et abréviations sont ceux de la figure 2. Abréviations supplémentaires: (ox), oxidé; (red), réduit.

cloné, centré sur le 44ème nucléotide précédant le site d'initiation, présente un intérêt particulier. Il s'agit d'une séquence palindromique de 14 nucléotides (TTTGATXXXXATCAA, X étant variable) se trouvant dans une position identique aussi bien chez *Azorhizobium* que chez *E.coli*, où elle précède certains gènes exprimés dans des conditions anaérobiques (nitrite réductase, nitrate réductase et fumarate réductase). L'expression de ces gènes anaérobiques est sous le contrôle d'un gène activateur d'*E.coli*, le gène *fnr*. La protéine Fnr interagit avec des séquences d'ADN précédant ces gènes de structure. Il est fort possible que le site identifié chez *Azorhizobium* interagit pareillement avec un homologue de Fnr. Ainsi la transcription du gène *nifA* d'*Azorhizobium* serait également activée par une protéine différente de NtrC répondant exclusivement à l'épuisement d'oxygène intra-cellulaire provoqué par l'oxydation de l'acide nicotinique. Le site d'ADN qui interagit avec NtrC n'a pas encore été identifié; il n'est pas exclus que la protéine soit en compétition pour l'attachement au site consensus de Fnr.

Les figures 1,2, et 3 illustrent notre modèle de la régulation du gène *nifA* d'*Azorhizobium* dans trois différentes conditions physiologiques de croissance bactérienne.

Ce modèle permet d'expliquer la fixation proliférative de l'azote moléculaire dans les trois conditions physiologiques indiquées. Toutefois, les mutants Ntr⁻ d'*Azorhizobium* forment des nodules de *S.rostrata* qui fixent l'azote moléculaire, bien qu'avec un délai significatif. Le contrôle Ntr est apparemment supplanté dans les nodules matures. Il est possible que la régulation différentielle de l'expression de *nifA* durant la prolifération bactérienne et durant la fixation des bactéroïdes est à la base de la capacité unique d'*Azorhizobium* ORS571 de fixer l'azote moléculaire dans des contextes très différents.

Remerciements

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Oxygen protection and oxygen requirement for C2H2 reduction by Sesbania nodules and bacteroids

*J. Rigaud, J.C. Trinchant, A. Puppo and L. Dimitrijevec * *

Introduction

The tropical legume *Sesbania rostrata* is characterized by profuse nodulation along the stem and on the roots [6]. The stem nodules induced by specific fast-growing *Azorhizobium caulinodans* exhibit a very high capacity to fix nitrogen [7]. In comparison to the root nodules, their carbon supply is increased by photosyntheses in chloroplasts located in their cortex [8] but the generation of O_2 could modify the O_2 environment of bacteroids. As recently demonstrated [3], C_2H_2 reduction by bacteroids required an adequate O_2 flux to be maximal. Moreover the well-known O_2 sensitivity of nitrogenase [13] confirms the general role of O_2 as limiting factor for the nitrogen fixation process. Since the involvement of SOD in the protection of leghemoglobin against autooxidation [14] and more generally in the general nitrogen fixation process [15] has been proposed, it was of interest to examine whether a specific strategy occurred in *Sesbania* about O_2 toxicity. Moreover, O_2 limited conditions can be imposed to *Sesbania* nodules during the rainy season when the plants thrive in temporarily flooded areas and the maintenance of significant levels of nitrogen fixation under these conditions will be investigated.

Materials and methods

Nodules

The seeds of *Sesbania rostrata* were pre-germinated for 24 h at 30 C [6], transferred into a vermiculite-sand mixture and inoculated with *Azorhizobium caulinodans* strain ORS 571. The plants grew in a glasshouse and received a nitrogen-free mineral solution as previously described [18]. After 3 weeks, the stems were sprayed with a suspension *Azorhizobium*, and inoculation was repeated each week as the stem elongation proceeded. Nodules emerged, 5 days after inoculation were harvested at different ages ranging from 15 to 60 days.

Soybean (*Glycine max* Merr. cv. Kador) and French-bean (*Phaseolus vulgaris* L. cv. Contender) seeds were surface-sterilized for 30 min in Ca-hypochlorite (3.5% W/V) and sown and cultured under the conditions described for *Sesbania rostrata*. Inoculation was done with *Bradyrhizobium* strain 1809 and *Rhizobium leguminosarum* biovar *phaseoli* strain 9-6 respectively. In both cases, nodules were 25 to 30 days old when collected.

The flooding conditions were realized in placing the pots during 72 h in containers filled with water. At that time, *Sesbania* and soybeans were 7 to 8 and 4 to 5 weeks old respectively. The immersion of attached stem nodules was obtained after setting around the stem, a 20 cm long rubber jacket, clipped at the bottom and filled with water.

Bacteroid preparations

Sesbania stem nodules (15-20 g FW) were crushed anaerobically and bacteroids were washed twice with sodium phosphate buffer (50 Mm, Ph 7.4), containing 2 Mm-MgSO4 and 0.3 M-sucrose. After centrifugation, chloroplasts sedimented at the upper part of the pellet of bacteroids were scraped away. The complete eliminiation of chloroplasts was achieved by centrifugation on self generated Percoll gradients in conditions described elsewhere [16].

Laboratory cultured bacteria

Azorhizobium strain ORS 571 was grown at 30 C under air ($0.3\ l.min^{-1}$) in a 6 ; Biolafitte fermenter containing 4 1 YLS medium [9]. *R.leguminosarum* biovar *phaseoli* and *Bradyrhizobium* were grown under the same conditions on a medium described by Kennedy *et al.* [12].

Nitrogen fixation assays

Activity of attached nodules was measured by C_2H_2 reduction under conditions previously described [17]. The upper parts (3 cm) of roots and the lower parts (5 cm) of stems bearing nodules of similar age were selected for these assays. Bacteroid incubations were conducted with a gaz phase containing O_2 or with purified leghemoglobin as O_2 carrier in conditions previously described [4, 22].

SOD activity

All extracts were tested for SOD activity as previously described [14, 16] and, when required, chlorophyll was removed by extraction with acetone. One unit of SOD activity is defined as the amount that inhibits nitro blue tetrazolium photoreduction by 50% [2].

Measurement of O2 *consumption*

This was performed in incubations containing different dissolved O_2 concentrations and conducted, without gas phase, in the 4 ml chamber of an O_2 electrode as described previously [5].

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Partial purification of LDH

Crude extracts were prepared after disruption of bacteria and bacteroids in an Aminco French pressure cell under conditions previously described [20, 21]. The same purification procedure was applied to the red supernatant resulting from the centrifugation of stem nodule homogenetes in order to purify their cytosolic LDH.

LDH assays

For pyruvate reduction, the reaction volume (2 ml) contained 0.5-100 Mm-sodium pyruvate and 0.25 mM-NADH in sodium phosphate buffer (0.1 Ml, Ph 8). For lactate oxidation, incubation mixtures (2 ml) received 0.5 to 100 mM-lithium lactate 0.5 mM -NAD in Tris/HCl buffer (0.1 M, pH 8.6). One unit (U) is defined as the amount of enzyme that oxidizes or reduces 1 mol pyriding nucleotide.min^{-1}.mg^{-1} protein.

Lactate estimation

Nodules homogenates were submitted to a 5% TCA precipitation and lactate was measured by the colorimetric method of Barker [1].

Results

C2H2 reduction by stem and root nodules of Sesbania

C2H2 reduction was followed throughout the plant development with attached nodules both on roots and stems. Specific activity of stem nodules was maximal for 40-days old nodules and remained unchanged for the following 15 days (Fig. 1A). During this period, activity of stem nodules was at least 50% higher than that of root nodules whose activity sharply declined when they were older that 40 days.

During nodule development, SOD activity of the two types of bacteroids rose up to 35-40 days; a high level was maintained

for bacteroids issued from stem nodules, whereas a rapid decline was observed for those from roots (Fig. 1B). Optimal activities were not significantly different, reaching 38 and 36 U.mg^{-1} protein, respectively.

Gel electrophoresis of bacteroids extracts showed that SOD activity was due to two different enzymes exhibiting relative mobilities of 0.13 (Fe-SOD) and 0.34 (Mn-SOD) as previously reported [16]. On the other hand, *Azorhizobium* was equipped with both Fe and Mn SODs exhibiting a general activity of 44 U.mg^{-1} protein.

Oxygen consumption by Azorhizobium and bacteroids

O2 uptake was determined using two different dissolved O2 concentrations in a range (25 to 75 M) where bacteroid nitrogenase was usually active. Addition of exogenous substrates such as glucose did not induce a significant enhancement of O2 uptake by free living bacteria in comparison with endogenous reserves
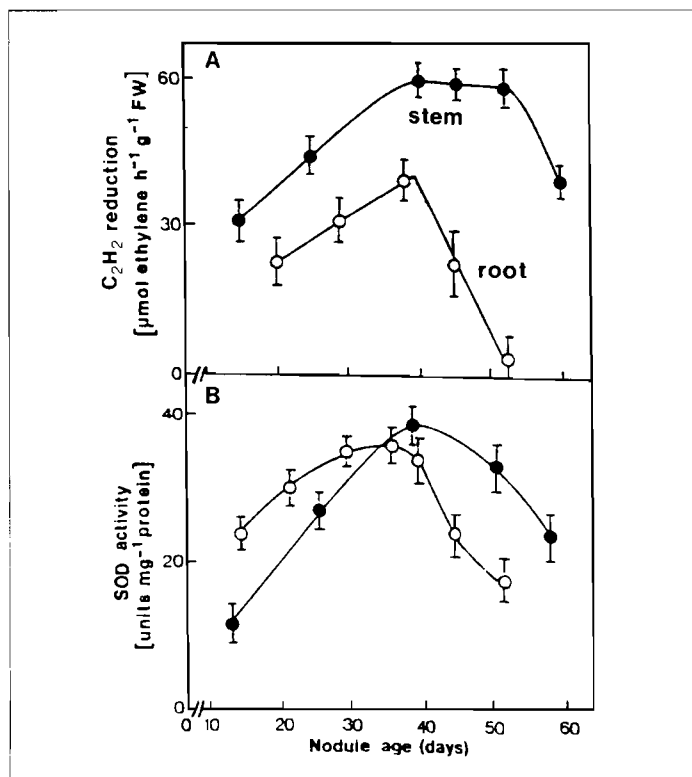

Figure 1 : C2H2 reduction by attached nodules (A) and DOS activities of their bacteroids (B) in relation to nodule age. C2H2 reduction was determined in stem (●) and root (O) nodules and SOD activities in the corresponding bacteroids. Vertical bars show the standard deviations of five experiments. SOD activities of root and stem nodule bacteroids
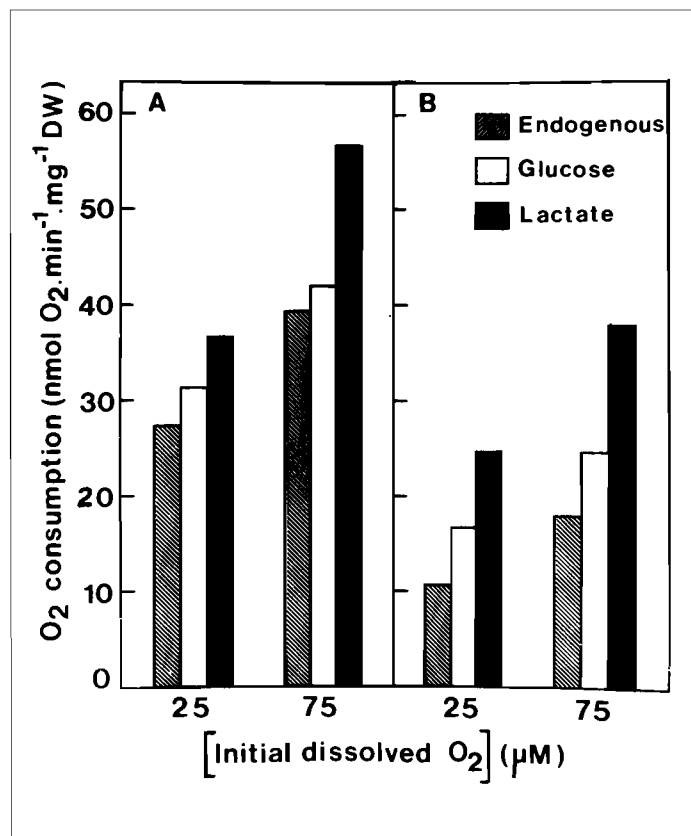

Figure 2 : O2 consumption by Azorhizobium (A) and stem nodule bacteroids (B) under different O2 concentrations. Experiments without a gas phase were performed at 25 C and the energy-yielding substrates were 10 mM.

(Fig. 2A). In contrast the stimulation exerted by lactate at the two O_2 tensions confirmed its efficiency, explaining its use as a carbon source in the culture medium [9].

Oxygen requirements for C_2H_2 reduction by Sesbania bacteroids

Experiments were conducted in incubations with gas phase where O_2 was freely dissolved in the medium. *Sesbania* bacteroids required the addition of carbon substrates to stimulate C_2H_2 reduction as the endogenous reserves were only slightly active (Fig. 3A).

Low O_2 tensions (5 to 15 mm Hg) were able to stimulate bacteroid C_2H_2 reduction in the presence of glucose. A wider range of pO_2 can support this activity with succinate as energy yielding substrate with an optimum at 30 mm Hg. Lactate appeared also able to provide energy for nitrogen fixation, with the same efficiency that glucose and succinate but for intermediate pO_2 values between those defined for these substrates.

Similar studies were realized with bacteroids isolated from *Phaseolus*, another legume routinely studied in the laboratory.

As shown in figure 3B, O_2 requirement for bacteroid C_2H_2 reduction with glucose or succinate were the same than those observed for *Sesbania*. In contrast, lactate was completely inefficient to support C_2H_2 reduction. Similar rates of optimal C_2H_2 reduction were reached by the bacteroids from both plants in the presence of active energy-yielding substrates: 50-55 $nmol.mg^{-1}$ (Fig. 3A, B).

When O_2 was supplied to *Sesbania* bacteroids by oxyleghemoglobin (Fig. 4), the characteristic response to each carbon was maintained: low O_2 concentrations (1-20 nM) favoured glucose efficiency whereas succinate was active for the highest O_2 concentrations (30-50 nM) and lactate, inducing the greatest activity, required intermediary O_2 concentrations.

LDH activity in bacteria, bacteroids and nodule cytosol

In crude extracts of both bacteria and bacteroids, the nucleoproteins and NADH oxidase activity were removed by a protamine sulphate precipitation without affecting the level of LDH activity. Successive ammonium sulphate precipitations showed localization of LDH in the 40-60 % saturation fraction with a purification of

about 50-fold, confirming previous results obtaines with *R.meliloti* LDH [21]. K_m values of different LDH preparations were determined using pyruvate and lactate as substrates; the results are summarized in Table 1. LDH extracted from *Azorhizobium* exhibited a very poor affinity for pyruvate but a very high one for lactate, both in the free-living forms and the bacteroids.

LDH activity was detected in the cytosol of stem nodules of *Sesbania* and root nodules of *Phaseolus* and *Glycine* (Table 1.). As with the bacteria, a 50-fold purification was achieved for this cytosolic LDH. The *Sesbania* LDH exhibited a higher affinity for pyruvate (K_m = 2.5 mM) than for lactate (K_m = 98 mM). In contrast LDH from *Phaseolus* and *Glycine* exhibited a very poor affinity for both pyruvate and lactate [21].

Dehydrogenase activities in O_2 limited conditions

Since the existence of alcoholic fermentation pathway was previously reported by Tajima & LaRue (1982) in soybean nodules, alcohol dehydrogenase activity was determined in stem nodules of *Sesbania* and in the bacteroids. As shown in Table 2, neither aldehyde reduction nor ethanol
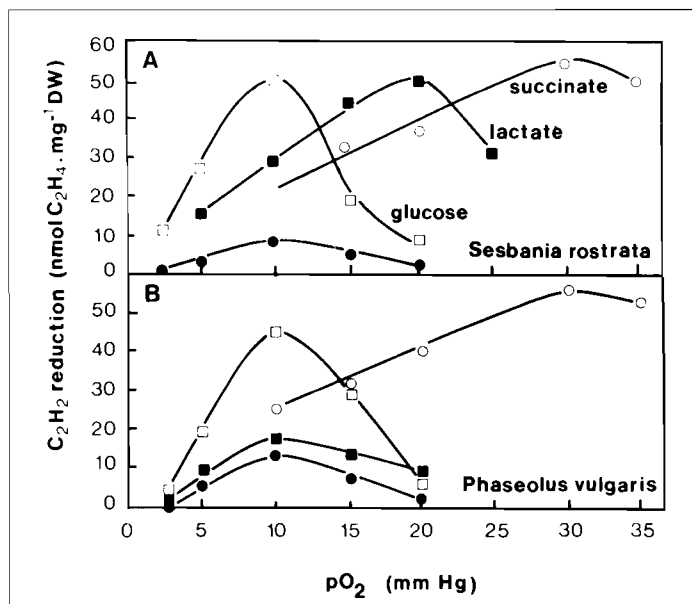

Figure 3. : Effect of increasing pO_2 in the gas phase upon C_2H_2 reduction by bacteroids isolated from Sesbania stem nodules or French-bean root nodules. Incubation mixtures (1 ml) contained bacteroids (9 mg DW) and substrates (50 mM): glucose (□), lactate (■), succinate (O) or nil (●) in sodium phosphate buffer (25 mM, pH 7.4). Assayed at 25 C with shaking (140 rpm).
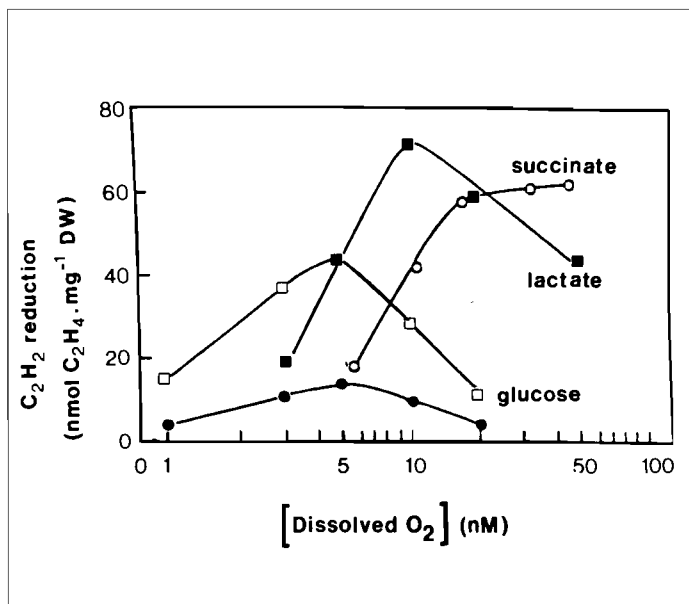
Figure 4 : C_2H_2 reduction by bacteroids isolated from stem nodules of Sesbania at low dissolved O_2 concentrations. Incubations (3 ml), carried out at 25 C without a gas phase, contained oxyleghemoglobine (105 M), bacteroids (2.8 mg DW) and substrates (10 mM): glucose (□), lactate (■), succinate (O) or nil (●) in sodium phosphate buffer (25 mM, pH 7.4).

LDH source		K_m (mM)	
		Pyruvate	Lactate
Bacteria			
- *Azorhizobium*		66	0.082
- *Rhizobium legumosarum* biovar *phaseoli*		0.095	60
- *Bradyrhizobium*		0.090	72
Bacteroids isolated from			
- *Sesbania rostrata*		58	0.075
- *Phaseolus vulgaris*		0.120	80
- *Glycine max*		0.102	56
Cytosol			
- *Sesbania*	stem nodules	2.5	98
- *Phaseolus*	root nodules	65	72
- *Glycine*	root nodules	78	85

Table 1 : K_m values of LDH extracted from different Rhizobia and their corresponding bacteroids, and from the cytosol of stem nodules of Sesbania rostrata and root nodules of Glycine max and Phaseolus vulgaris.

		Stem nodule cytosol		Stem nodule bacteroids	
		Control	After flooding	Control	After flooding
ADH	Acetyldehyde red	-	0.38±0.07	-	-
	Ethanol ox	-	-	-	0.26±0.07
LDH	Pyruvate red	0.28±0.05	0.44±0.08	-	-
	Lactate ox	-	-	0.70±0.08	1.52±0.15

Table 2 : Effect of flooding upon alcohol and lactate dehydrogenase activities in the cytosol of Sesbania stem nodules and crude extracts of bacteroids. Activities are expressed as M pyridine nucleotide oxidized or reduced per mg protein per min and values are means SEs of three measurements.

oxidation occured in nodule cytosol and in bacteroids under usual conditions (control). Under O_2 restricted conditions induced by flooding, a high level of ADH in reducing acetaldehyde occured in nodule cytosol of *Sesbania* whereas ADH oxidizing ethanol was detected in bacteroids. Concerning LDH activity, whose existence and characteristics were reported in Table 1, it was strongly enhanced both in cytosol and in bacteroids by the flooded conditions.

As a result of these dehydrogenase activities, the level of lactate in the stem nodules was 50% increased by nodule immersion (150 instead of 100 $nmol.g^{-1}$ nodule FW)

and the level of ethanol reached 200 $nmol.g^{-1}$ nodule NW.

Conclusions

We did not detect remarkable differences between *Azorhizobium* and the nodule bacteroids of *Sesbania* concerning both the types of SOD and the level of their total activity. Since a correlation between SOD activity and O_2 consumption is generally observed [11], this fact is in favour of a large O_2 demand by the microbial partner. The respiratory experiments confirmed this view since bacteroid O_2 uptake was only slightly lower than that of *Azorhizobium* in spite of marked differences

occuring in their O_2 environment in the nodule central tissue and the fermenter. On the other hand, the SOD contents in the stem and root bacteroids were not significantly different in spite of the existence of a O_2 evolving photosynthetic system: this can be explained both by the presence of an active Cu-Zn SOD in the stem nodule chloroplasts and by the compartmentation of these organelles in the cortex of these nodules [8]. Thus it appears that SOD may protect the nitrogen fixation process by scavanging, with a remarkable efficiency, any O_2^- generated during the vigourous O_2 consumption of bacteroids.

Nevertheless, this strongly suggests the presence, in the nodules, of enzymes (like catalase, peroxidase and glutathione peroxidase) able to remove H_2O_2 formed by SOD and of processes avoiding the existence of free transition metals, to prevent generation of hydroxyl radicals, which are considered as the most toxic O_2 derived species [10].

Oxygen concentration inducing optimal C_2H_2 reduction by *Sesbania* bacteroids was directly related to the nature of the carbon source available. As previously observed with soybean [22] and with French-bean (Fig. 3B), carbohydrates such as glucose were able to support C_2H_2 reduction by bacteroids but only for low O_2 tensions contrasting with organic acids like succinate, active for a wider range of O_2 concentrations.

A new finding concerns the specific role of lactate, unable to support C_2H_2 reduction by either French-bean bacteroids (Fig. 3B) or by soybean bacteroids (results not given), but acting as energy-yielding substrate for *Sesbania* bacteroids. It supported C_2H_2 reduction with a similar rate to that observed with glucose or succinate in the absence of an O_2 carrier, and was particularly efficient when O_2 was provided at very low concentrations by oxyleghemoglobin. The O_2 tensions required for optimal efficiency with lactate could be predicted by the model of Bergersen and Trinchand [3], which takes into account results obtained under experimental conditions similar to those used in the present study where O_2 was also supplied to French-bean bacteroids, free or bound to

leghemoglobin. It made it possible to determine the required O_2 concentration around a bacteroid, for optimal C_2H_2 reduction. This was shown to be directly related to the rate of O_2 consumption for a given substrate. For example, bacteroid O_2 uptake was about 3.3 times higher with succinate than with glucose [21], and a similar ratio of 3:1 was observed between the O_2 tension inducing optimal C_2H_2 reduction with these substrates. Since O_2 uptake by bacteroids was 1.8 times higher with lactate than with glucose [21], the model predicted an O_2 tension of 18 mm Hg to ensure optimal C_2H_2 reduction in the presence of lactate. Experimental data pointed to about 20 mm Hg (Fig. 3A).

The efficiency of lactate in supporting C_2H_2 reduction proved to be in direct relation to the presence of an active LDH in the bacteroids of Sesbania stem nodules. However, the reduction of pyruvate was in general favoured in R.meliloti, R.leguminosarum biovar phaseoli and Bradyrhizobium (free-living bacteria and symbiotic forms) rather than lactate oxidation, as evidenced by the low K_m values for pyruvate. In contrast, LDH extracted from Azorhizobium and stem nodule bacteroids exhibited a high affinity for lactate, confirming the use of this molecule as a carbon source in the culture medium of bacteria [9]. Moreover, the presence of significant quantities of lactate in the cytosol of the host cells of stem nodules makes possible its utilization as an energy source by bacteroids in vivo. Lactate synthesis resulted in the existence of an active LDH in the nodule cytosol exhibiting a high affinity for pyruvate, and was strongly enhanced under O_2 limited conditions imposed by flooding (Table 2). In the same way alcoholic fermentation, commonly present in the soybean nodules [19] was not detected in Sesbania nodules. However, O_2 limited conditions obtained in the laboratory by flooding induced a high ADH activity with ethanol accumulation in the nodule cytosol of Sesbania. Thus, this tropical legume appeared in a priviledged situation when O_2 restricted conditions were temporarily imposed during the rainy season, since two fermentative pathways took place to provide energy for maintaining a substantial level of nitrogen fixation.

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Molecular biology of the *Azorhizobium caulinodans* ORS571 Sesbania rostrata symbiosis: bacterial and plant aspects

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Introduction

The symbiosis between *Azorhizobium caulinodans* ORS571 (19) and its host *Sesbania rostrata* (2) represents an interesting system for studying the molecular basis of bacterium-plant interactions due to the following unique characteristics: First, *A. caulinodans* induces effective nitrogen fixing nodules both on the stem and the root of *S. rostrata* (18). Second, the formation and nitrogen fixation capacity of the stem nodules is relatively insensitive to elevated concentrations of combined nitrogen in the soil (17). Third, *A. caulinodans* is capable of fixing nitrogen efficiently in the free living state *and* of dinitrogen dependent growth both in culture and on plates (20).

Moreover, this symbiotic system is of great interest from an "applied" point of view. Nodulated *S. rostrata* plants have been found to be very useful as green manure in tropical agriculture, especially when intercropped with rice, which has led to dramatic crop yield increases (41; see other contributions this volume).

We have selected the following aspects of this symbiotic system for our studies:

▲ 1. The structure, function and regulation of nitrogen fixation (*nif, fix*) and assimilation (*asm*) genes of *A. caulinodans* in the free living versus symbiotic state.

▲ 2. The ultrastructure of stem- and root nodules induced by wild type and mutant strains of *A. caulinodans*.

▲ 3. The structure and function of a heme biosyntheses (*hemA*) gene of *A. caulinodans*.

▲ 4. The structure and regulation of leghemoglobin (*lb*) genes of *S. rostrata*.

▲ 5. The development of a tissue culture, regeneration and transformation system for *S. rostrata*.

In this paper we will briefly summarize our progress in each of these areas.

Regulation of nitrogen fixation (nif, fix) genes

The capacity of *A. caulinodans* to sustain dinitrogen dependent growth in culture (chemostat), as well as on plates, has greatly facilitated physiological and genetic studies on the nitrogen fixation process and its regulation. It has prompted us and others to use the (molecular) genetic techniques, developed for studying the diazotroph *Klebsiella pneumoniae*, for the analysis of *A. caulinodans* (see also Elmerich et al. and Ludwig et al., this volume). Moreover, it has facilitated a proper comparison of *nif (fix)* gene regulation in the free living versus symbiotic state, which has been difficult in *(Brady)rhizobium* species due to the inability of these bacteria to grow in dinitrogen as primary N-source.

Nitrogen fixation by *A. caulinodans* in the free living state is strictly controlled in response to the concentration (and "quality") of combined nitrogen sources and (dissolved) oxygen in the medium (8, 9, 16, 20, 25, 31, 39, 40). This is analogous to the situation in the diazotroph *K. pneumoniae* (see 23), except that nitrogen fixation and

dinitrogen dependent growth of *A. caulinodans* can take place at a much higher oxygen concentration (up to 3% O_2 in the gas phase) and temperature (37 C). In *K. pneumoniae*, *nif* gene expression is mediated by a *nif* specific (*nifLA*) and a general nitrogen control (*ntr*) system (see 23).

Using heterologous DNA probes from *K. pneumoniae*, we have identified, cloned and characterized the *nifA* and *ntrC* equivalent gene from *A. caulinodans* (8, 39). Transposin Tn5 induced (5) mutations in the *nifA* and *ntrC* loci of *A. caulinodans* have been constructed. Their free living nitrogen fixation (Nif) phenotype has been determined by analysing colony morphology (growth) on nitrogen-free plates, incubated at 37 C under a 3% O_2 plus 97% N_2 gas mixture, and by measuring acetylene reduction activity (ARA) of cultures grown under the same conditions. Their symbiotic nitrogen fixation (Fix) phenotype has been determined by examining the vigour (size; leaf morphology) of stem- and root nodulated *S. rostrata* plants and by measuring the ARA's of entire nodulated plants or detached nodules. *NifA* mutants are strictly Nif⁻ Fix⁻, due to their inability to transcriptionally activate the other *nif (fix)* genes (39, 40). *NtrC* mutants exhibit a severely reduced level of nitrogen fixation in culture (10-15% of wild type levels; Nif$^{-/+}$), as well as in nodules (Fix$^{-/+}$), which also appears to be due to insufficient transcriptional activation of *nif(fix)* genes (39, 40). The Fix$^{-/+}$ phenotype of the *ntrC* is unusual. After six weeks the stem- and root nodules are ineffective (Fix⁻; 39) and their ultrastructure is identical to that of nodules induced by Fix strains (C.H. Wong, K. Pawlowski, M.

Schneider. J. Schell and F.J. de Bruijn, in preparation). After twelve weeks a few fully effective nodules are formed, which are ultrastructurally similar to normal Fix$^+$ nodules and are not due to reversion of the *ntrC* mutation (39; C.W. Wong et al., op. cit.).

Thus, at a certain stage of nodule development *ntr* control of *nif(fix)* expression appears to be "by-passed", resulting in a delayed Fix$^{-/+}$ phenotype; the nodulated plant, however, never regains the vigour of a plant nodulated with wild type *A. caulinodans* bacteria (39). In contrast to the *nifA* mutants, the *ntrC* mutants have a pleiotropic phenotype since *ntrC* strains are also severely disturbed in growth on a number of other nitrogen sources, such as arginine and nitrate (39, 40).

Thus, at first glance, the *nif* regulatory circuitry in *A. caulinodans*, in the free living state, appears to resemble that found in *K. pneumoniae*. A *nif* specific regulatory gene (*nifA*) controls *nif* expression, while a general nitrogen regulation gene (*ntrC*) controls *nifA* gene expression as well as other genes (operons) involved in the catabolism of amino acids and nitrate (8, 39). In the symbiotic state, the role of *nifA* in *nif* gene expression is analogous to that reported for (*Brady*)*rhizobium* species (21, 46), but that of *ntrC* is unusual, since, in contrast, *ntrC* mutants of *Rhizobium meliloti* are Fix$^+$ (46).

To examine how the *ntrC* gene is described above (8, 38) affects *nif(fix)* expression, we have constructed a translational *nifA-lacZ* (ß-galactosidase) gene fusion and measured ß-gal activity in different genetic backgrounds and under different physiological conditions (9). *NtrC* appears to be directly involved in the regulation of the *nifA* promoter, since a two-to three fold reduction of *nifA-lacZ* expression was observed in a *ntrC* mutant strain. However, this is not sufficient to account for the 6-10 fold reduction in nitrogenase activity observed in *ntrC* mutant strains (39). *NifA* expression is repressed (non-activated) in the presence of high concentrations of combined nitrogen sources (100 fold) or oxygen (air, 21% O$_2$; 5 fold), even in a *ntrC* minus background (9). This suggests that the gene we have identified (39) is not

sufficient to mediate complete nitrogen control of *nif* expression in *A. caulinodans* (40, 9).

Regarding oxygen control of *nifA* expression, two additional observations are relevant. First, the expression of the *A. caulinodans nifA-lacZ* fusion is repressed 100 fold by inhibitors of DNA gyrase, such as coumermycin (9). Secondly, the determination of the DNA sequence of the entire *nifA* gene has revealed the presence of a highly conserved (promoter) DNA element found in the promoters of genes induced during anaerobiosis in *Escherichia coli* and controlled by the *fnr* (fumarate nitrate reduction) regulatory system (9). These observations suggest that DNA supercoiling in the *nifA* promoter, and/or activation by (a) *fnr* like regulatory protein(s), may play an essential role in *nifA* activation and lends further support to the finding in various N$_2$-fixing organisms that O$_2$ control of *nif* expression is mediated via regulation of the *nifA* promoter (9, 14, 29, 34). They also confirm the notion that *nif* regulation in *A. caulinodans*, in the free living and symbiotic state, is subject to a complex cascade regulation mechanism, involving several regulatory loci and complex promoter elements in the *nif(fix)* genes (9; see also below), which appears to be a "hybrid" between regulatory circuitries observed in strict diazotrophs and strictly symbiotic nitrogen fixing organisms.

Structure, function and regulation of nitrogen assimilation (asm) genes

The ability of *A. caulinodans* to fix nitrogen in the free living state *and* to grow on this fixed nitrogen as a primary N-source (20) suggests a coupling of the nitrogen fixation and assimilation processes, as found in *K. pneumoniae*. Like most other members of the *Rhizobiaceae* (36), *A. caulinodans* utilizes the glutamine synthetase (GS) - glutamate synthase (GOGAT) pathway for N-(ammonium) assimilation (16). Therefore it was reasonable to assume that a coordinate regulation of GS (GOGAT) and *nif* expression might exist in *A. caulinodans*, as found in *K. pneumoniae* (see 23, 33). In the latter organism, the *ntr* system (see above) is responsible

for this coordinate regulation (6, 7, 23). However, we have found that this is not the case in *A. caulinodans* role since *ntrC* mutants exhibit normal (wild type) levels of GS and GOGAT activity (25, 39).

Most members of the *Rhizobiaceae* examined thus far contain (at least) two distinct GS forms (GSI, II; see 36) and corresponding distinct genes (*glnA, glnII*; 11, 12, 13, 42, 44). Of these genes, the *glnII* but not the *glnA* gene, appears to be *ntr* controlled (11, 42). *A. caulinodans* again appears to constitute an exception, since it does not have GSII activity (16) or the corresponding *glnII* gene (10). To further investigate the structure, function and regulation of the N-assimilation genes the GS (*glnA*) and GOGAT (*glt*) loci of *A. caulinodans* have been cloned, mutagenized and the phenotype of *glnA* and *glt* mutations analysed (8, 9, 15, 16, 25). Interestingly, both *glnA* (GSI$^-$) and *glt* (GOGAT$^-$) mutants are not only incapable of assimilating ammonia (Asm$^-$), but also strictly Nif$^-$ (8, 9, 15, 16, 25). Moreover, they are Fix$^-$ (8, 9, 25), suggesting that the inability to assimilate combined nitrogen sources leads to repression of nitrogenase synthesis or activity in nodules as well in the free living state. This is in contrast with observations made with (brady-) rhizobial species, where *glnA* (GSI), *glnII* (GSII) or *glnA glnII* (GSI$^-$II$^-$, glutamine auxotrophic) mutant strains are Fix$^+$ (11, 12, 13, 27, 44). The Nif$^-$ phenotype of the *A. caulinodans glnA* and *glt* mutants in culture can be attributed to "ammonium-swichoff" of the nitrogenase syntheses of nitrogenase enzyme (31), repression via the *ntr* system (8, 39, 40), or both. Irrespective of the exact mechanism, it appears to be due to the accumulation of combined nitrogen products (ammonium), in the medium of genotypically *nif*$^+$ but *asm*$^-$ bacterial cultures. This has been shown by examining the acetylene reduction actively (ARA) of mixed cultures. Co-cultivation of *glt* bacteria with a *nif* *asm*$^+$ *A. caulinodans* strain (such as *nifHDK* strain carrying a mutation in the structural gene for nitrogenase), was found to lead to a significant amount of ARA activity (Nif$^+$ phenotype) in mixed cultures (25), due to the ability of the *nif* *asm*$^+$ bacteria to assimilate the fixed nitrogen produced by the

nif⁺ asm⁻ strain and thereby to prevent nitrogenase repression.

Thus, although coordinate regulation of the *nif*, *glnA* and *glt* genes by the *ntr* system could not be demonstrated, it is clear that both nitrogen fixation and assimilation processes must be simultaneously functional to allow dinitrogen dependent growth. The exact mechanism involved remains unclear. The Fix⁻ phenotype of *glnA* and *glt(Asm⁻)* strains is rather puzzling. It is difficult to imagine that ammonium feedback repression of nitrogenase biosynthesis or activity would be the primary cause of this phenotype, since in the symbiotic state all rhizobia carry out nitrogen fixation in the bacteroid state, coupled to ammonium export to the plant cytoplasm, without nitrogenase inhibition. Maybe the establishment of an effective nitrogen fixing nodule by *A. caulinodans* on *S. rostrata* requires "free living" nitrogen fixation and assimilation by the invading bacteria while proliferation intercellularly (9) and this process is aborted in nodules by Asm⁻ strains.

Ultrastructure of nodules induced by wild-type and Fix⁻ strains of *A. caulinodans* on *S. rostrata*

The development of an effective, nitrogen fixing nodule clearly involves the coordinated interaction between the bacteria and plant genomes. A number of (regulatory) signals from both symbiotic partners are likely to be required to activate the bacterial and plant genes involved in nodule ontogeny and functioning. It has therefore been of interest to investigate the plant response to ineffective bacterial strains by examining the ultrastructure of nodules induced by rhizobial mutants disturbed in nodule formation or nitrogen fixation (assimilation).

In collaboration with Dr. C.H. Wong from the University of Malaysia, we have examined the ultrastructure of *S. rostrata* nodules induces by *nifHDK* (20), *nifA* (39), *ntrC* (39) and *nifB* (unpublished) mutants of *A. caulinodans* by light and electronmicroscopy. Using previously described protocols (50) six week old stem- and root nodules have been examined. Most of the

cells of six week old stem nodules, induced by the *nifHDK*, *nifA*, *ntrC* and *nifB* mutant strains, appear to be uninfected, irregularly shaped (collapsing) and clear signs of early senescence (breakdown of peribacteroid membranes, bacteroids and cell cytoplasm) were evident (9). Moreover, multiple intercellular pockets of bacterial infection can be observed. Most of the cells of six week old root nodules, however, are infected and contain a clearly defined, extensive peribacteroid membrane system but hardly any (differentiated) bacteroids with these membrane pockets (9). In the infected cells of both stem and root nodules the bacteroids contain large accumulations of polyhydroxybuthyric acid crystals. Generally, the ultrastructure of the nodules induced by the different Fix⁻ mutants is similar, although the 'phenotype' of the *nifA* strain was clearly the most extreme. The difference between the morphology of Fix⁻ stem versus root nodules is quite interesting. It maybe a reflection of the different physiological conditions the infection azorhizobia and the infected plant cells of the root and the stem are exposed to. We have observed that the effect of Fix⁻ mutants on root nodule ontogeny (number, size) is more extreme than that of stem nodules in our experimental nodulation systems (green house plants; Leonard Jars; test tubes; 39).

We are presently carrying out time course experiments to establish whether the extensive peribacteroid membrane (PBM) system, containing few or no bacteroids observed in cells of the Fix⁻ root nodules, is due to bacteroid release and inclusion in PBM followed by rapid degradation of the bacteroids, or the induction of the PBM system in the absence of enclosed bacteroids. The observations support the general notion that a close scrutiny by the plant of bacterial effectiveness (nitrogen fixation) charts the course of nodule ontogeny (39) and that the particular physiological conditions of the infected plant tissue (stem versus root; different legume host), may play a major role in this process.

Structure and function of a heme biosynthesis (hemA) gene of Azorhizobium ORS571

Leghemoglobins (Lbs) are myoglobin-like proteins involved in O₂ transport, at a low oxygen concentration, in nitrogen nodules on leguminous plants (1). Apoleghemoglobin is plant-encoded, whereas the hemo prosthetic group is thought to be provided by the bacteroids (37). However, the isolation and characterization of Rhizobial mutants disturbed in heme biosynthesis has produced apparently contradictory results regarding the role of the bacteroids in providing the heme moiety for Lb synthesis. For example, *R. meliloti* mutants carrying a mutation in the structural gene for A-aminolevulinic acid (ALA) synthase (*hemA*), and therefore strictly heme deficient and ALA auxotrophs, induce inefficient (Fix⁻) nodules on *M. sativa* plants (32), in which the leghemoglobin (lb) genes are not expressed (A. Simons, P. Schreier, J. Schell and F.J. de Bruijn, in preparation). However, *hemA* mutants of *B. japonicum*, which are also strict ALA auxotrophs, induce Fix⁺, Lb containing nodules on *G. max* (22). We have extended this analysis to the *A. caulinodans* - *S. rostrata* symbiosis by cloning and mutagenizing the *A. caulinodans hemA* gene. We have used the cloned *R. meliloti hemA* gene as heterologous DNA probe to identify a recombinant plasmid of a *A. caulinodans* gene bank carrying the *hemA* locus. Using Tn5 mutagenesis and "reverse genetics" methods (5), we have constructed a *hemA::Tn5* mutant of *A. caulinodans* (ALAS122), which turns out to be a strict ALA auxotroph, but is capable of nitrogen fixation in culture (Nif⁺). Root and stem nodulation by the ALAS122 mutant strain is severely retarded and results in Fix⁻ nodules, lacking Lb proteins and *lb* MRNA entirely (K. Pawlowski, J. Schell and F.J. de Bruijn, manuscript in preparation). These observations suggest that *A. caulinodans* - *S. rostrata* symbiosis, bacteroid directed heme production is essential for nitrogen fixation, as found in the case of the *R. meliloti hemA* mutants (32).

The analysis of the *A. caulinodans hemA* locus has yielded additional interesting results. Secondary site suppressor mutations of ALAS122, capable of growth in the ab-

sence of exogenously added ALA on specific medium, have been found, which still contain the original Tn5 insertion in *hemA*. These mutants suggest the presence of a second pathway of heme biosynthesis in *A. caulinodans*, which is not expressed under normal physiological conditions but can be activated by mutation. In plant chloroplastst and some aubacteria, ALA can be generated via a pathway, proceeding from glutamate via glutamyl-tRNA; glutamate 1-semialdehyde tot ALA and this pathway is inhibited by compounds such as aminooxyacetic acid (24). We have found that growth of our ALAS122 secondary site suppressor mutants in the absence of ALA can be inhibited to a significant degree by the addition of such inhibitory compounds (K. Pawlowski, J. Schell and F.J. de Bruijn, manuscript in preparation), suggesting that *A. caulinodans* may have a second pathway for ALA synthesis analogous to the C5 pathway found in plants.

Structure and regulation of *S. rostrata* leghemoglobin (lb) genes

Stem nodules induced by *A. caulinodans* on *S. rostrata* are green and contain photosynthesizing cells immediately next to bacteroid containing, N_2 fixing cells. O_2 evolved during photosynthesis may be expected to put an additional stress on the nitrogenase enzyme complex in stem nodules. On the other hand, stem nodules may be more efficient in fixing nitrogen that root nodules due to the juxtaposition of the energy producing (photosynthesizing) and consuming (nitrogen fixing) cells. As mentioned above, leghemoglobins (Lbs) are responsible for O_2 transport in nitrogen fixing nodules to provide energy to the actively respiring bacteroids at an intercellular oxygen concentration which is low enough to prevent inactivation of the O_2-sensitive nitrogenase enzyme complex (1). It has therefore been of interest to study Lb function and regulation in the *A. caulinodans - S. rostrata* symbiosis, especially since a preliminary report indicated that a novel (unique) form of Lb may be present in stem- versus root nodules (3). Recent experiments have not been able to confirm this observation. Instead, biochemical studies have revealed that *S. rostrata* encodes seven distinct Lb

forms (LbI-VII), of which one form (LbVI) is expressed to a ~5 fold higher level in stem- versus root nodules (4). Moreover, *S. rostrata* Lbs in general, and LbII in particular, have been found to have an unusually high affinity for O_2, which allows *A. caulinodans* bacteroids to carry out nitrogen fixation at an O_2 concentration which is lower, rather than higher than usual (49). We have examined the primary structure of *S. rostrata lb* genes and their regulation in order to try to determine the reason for the high O_2 affinity of *S. rostrata* Lbs and to identify the genetic elements responsible for higher expression of the LbVI form in stem versus nodules. For this purpose we have cloned the genes encoding *S. rostrata* Lb forms VI and VII. A cDNA bank of *S. rostrata* stem nodule MRNA has been screened with heterologous *lb* gene probes from *G. max* and *Pisum sativa* (pea). One hybridizing CDNA clone has been analysed in detail and used to identify two *lb* loci (*Srglb2* and 3) in a genomic library of *S. rostrata* leaf nuclear DNA. The complete nucleotide sequence of these two *lb* loci has been determined (35, 48). The deduced aminoacid sequence of both loci reveals a 77% and 67% homology with Lbs from alfalfa and soybean, respectively (35). Twelve out of 15 amino acids, known to play a role in heme caverne formation and heme binding in the *G. max* Lba protein (38), are conserved at the analogous position in the *S. rostrata Srglb* gene product (35). Thus the structure of the *S. rostrata* Lbs, as determined by us (35), and for the *S. rostrata* LbII product by Kortt et al. (28), does not reveal significant differences with the structure of other Lbs, which could explain the observed high O_2 affinity of the *S. rostrata* Lbs. The intron-exon boundaries of the *Srglb2* and 3 loci are at analogous positions as those in the *G. max lb* genes (35). The 25 amino-terminal amino acids of the predicted *Srglb2* and 3 gene products share a 100% homology with those of *S. rostrata* Lb forms VI and VII, as determined by protein sequencing (4). Further comparison of the amino acid differences between the Srglb2 and 3 gene products and correlation of these data with the relative migration patterns of forms VI and VII during chromatography (4), suggests that *Srglb2* encodes form VI and *Srglb3* form VII (35, 48).

Leghemoglobin genes are only expressed in the nodule. Regulation and nodule specific induction of one of the *G. max lb* genes (*lbc3*) has been shown to be mediated by defined *cis*-acting DNA elements, located in the 5' upstream (promoter) region of this gene (45). This was determined by analysing the expression of chimaeric genes, consisting of distinct *lbc3* promoter elements fused to a readily assayable reporter gene (chloramphenicol transacetylase; CAT), in leaves, roots and nodules of the transgenic *Lotus corniculatus* plants (45). These elements include general regulatory motifs, such as CAAT and TATA boxes, as well as elements which play a role in nodule-specific *lb* gene induction, such as the organ specific element (45). The comparison of the nucleotide sequence of the *S. rostrata glb2* and 3 and *G. max lbc3* promoter regions reveals a very high degree of homology around the general (CAAT, TATA boxes; startpoint of transcription) regulatory elements, as well as the organ specific box (35).

The regulation of plant (as well as other eukaryotic) genes usually involves not only distinct DNA motifs in the promoter region but also the specific interaction of such *cis*-acting elements with *trans*-acting DNA binding proteins (regulatory factors; see 30). In the case of the *G. max lbc3* promoter, two distinct DNA elements have been identified, which strongly bind a nodule specific factor, which is absent in nuclear extracts from leaves and roots (26). We have found that one of these two elements is highly conserved at an analogous position in the *Srglb2* and 3 promoter regions, and that this conserved element also interacts specifically with a nodule factor (35). Moreover, we have found analogous DNA binding factors (proteins) to be present in different types of nodules (*S. rostrata* stem- and root-; *G. max* and *Medicago sativa* nodules; 35), leading to the conclusion that a highly conserved mechanism of *lb* gene induction exists in nodules of leguminous plants, involving conserved *cis*-acting elements, as well as apparently universal *trans*-acting factors. We are presently trying to purify the *trans*-acting factor(s) to study the precise interaction of these factors with their target elements. Moreover, we are trying to identify those

DNA elements (enhancers?) which may be responsible for higher expression of the *Srglb2* gene (Lb form VI) in stem versus root nodules.

S. rostrata **tissue culture, plant regeneration and transformation with Agrobacterium strains**

As mentioned above, plant gene regulation studies have greatly benefitted from the construction of chimaeric genes, consisting of distinct promoter elements fused to readily assayable 'reporter' genes, whose expression can be monitored in transgenic plants under different physiological conditions (45; for a review see 43). This approach of course requires methods to transform and regenerate the plant of interest. In addition, such methods can be beneficial for the introduction of genes conforming agriculturally useful traits into an interesting crop plant (see 43). Therefore, we have attempted to establish a system for *S. rostrata* plant regeneration from dedifferentiated cells in culture (47). We have examined different parameters of *S. rostrata* tissue culture, such as choice of explants and medium composition for callus induction/shoot and root formation, as well as direct shoot bud formation (without a callus stage). Callus formation from different explants on a variety of media is unproblematic. We have found that the use of immature embryos as starting material, yields plant regeneration frequencies (~10% shoot bud formation), which are comparable to those achieved via direct shoot induction from other mature explants (47). The phytohormone BAP (at concentrations of 0.2 - 1 mg /L) appears to be essential for shoot development and, in combination with 1 gm/L yeast extract, useful for shoot multiplication (vegetative propagation) of *S. rostrata* (47). *S. rostrata* is very sensitive to infection by *Agrobacterium tumefaciens* and *A. rhizogenes* strains. Rapid tumor (hairy root) formation can be observed, resulting often in an apparently systemic infection of the plants, due to spread of the bacteria through the vascular bundles (47). However, it has not yet been possible to use antibiotic resistance (selectable marker) genes to select from transformed *S. rostrata* tissue *and* to regenerate resistant transgenic plants which are stably transformed

(B.A. Metz, M. Vlachova and F.J. de Bruijn, unpublished). We are presently using *A. rhizogenes* strains carrying binary vectors with hygromyocin and kanamycin resistance genes as selectable markers to achieve this goal.

Conclusions

In this paper we have summarized our recent progress in trying to understand the molecular basis for the development and functioning of nitrogen fixing stem- and root nodules induced by *Azorhizobium caulinodans* on *Sesbania rostrata*. It is clear that much remains to be unlearned about the induction of nitrogen fixation genes in the bacterium and nodulin (leghemoglobin) genes in the plant partner. The studies on this symbiotic system are still in an early stage relative to those other systems. Nevertheless, it is hoped that the inclusion of the *A. caulinodans* - *S. rostrata* system in the comparative analysis, exploiting some of the unique characteristics of this symbiosis, will help advance our general understanding of both bacterial and plant aspects of the process of symbiotic nitrogen fixation. Moreover, we hope that some of the knowledge gained in our (molecular) studies will help provide tools for more 'applied' projects, such as amelioration of the *A. caulinodans* strains, genetic improvement of *S. rostrata* plants or transfer of stem-nodulation capacity to other species, the need for which has become clear from many contributions to this symposium.

Acknowledgements

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Session III

Molecular Genetics
of *Sesbania rostrata*

Rhizobium genes involved in nodulin gene expression

J.P. Nap and T. Bisseling *

The occurrence of nodulins (22) has been demonstrated in many *Rhizobium*-legume symbiose (2,14,15,23) and it has become clear that a differential expression of nodulin genes accompanies the development of a root nodule (8,9,11). Most nodulin genes are expressed simultaneously with the onset of nitrogen fixation, after the nodule structure has been formed. Hence, their gene products are most likely involved in establishing an environment within the nodule that allows nitrogen fixation and ammonia assimilation. The leghemoglobin genes are the best studies members for this class of nodulin genes, a class denoted as late nodulin genes. We have identified another class of nodulin genes, which we term early nodulin genes. These genes encode nodulins that are detectable during the formation of a nodule structure (4,6,21,24). The analysis of this second class of nodulins is an attempt to study genes and gene products most likely involved in the plant differentiation processes underlying the establishment of a nodule structure.

Vicia sativa **nodulins**

Nodulin gene expression in wild-type nodules formed on *Vicia sativa* by *R. leguminosarum* PRE was examined by analyzing RNA on Northern blots using cDNA clones as probes, and by *in vitro* translation followed by two-dimensional (2D) gel electrophoresis (16). The cDNA clones used in our studies were pENOD2 (7) and pPsLb101 (1). Clone pENOD2 was isolated from a soybean nodule cDNA library and represents a soybean early nodulin gene (7). This pENOD2 strongly cross-hybridizes to a *Vicia* early nodulin mRNA (16). The 2D pattern of *in vitro* translation products of *Vicia* root and nodule RNA revealed, after comparison, several nodu-

lins (16). Four of the major nodulins have an apparent molecular weight of 14,000. These are most probably the *Vicia* leghemoglobins. Two other major nodulins have molecular weights of 40,000 and 65,000 respectively. These nodulins are referred to as Nvs-40 and Nvs-65. Nvs-40 is an early nodulin: Nvs-65 and the Lbs belong to the class of late nodulins.

Rhizobium **nod genes and early nodulin gene expression**

To discriminate between the contribution of the *Rhizobium* chromosome on the one hand, and of the plasmid-borne nod region on the other, in the induction of nodulin gene expression, the closed nod region from *R. leguminosarum* was introduced in an *Agrobacterium* chromosomal background. This strategy of introducing the nod region in an *Agrobacterium* chromosomal background demands that the *Agrobacterium* chromosome itself does not harbour genes involved in the induction of nodulin gene expression. We examined the ability of the *Agrobacterium* genome to induce in tumors the expression of the genes identified as nodulin genes. No *Vicia* nodulins were detectable in tumors formed on the stem of *Vicia* plants after infection with *Agrobacterium* (16). Thus, it is unlikely that the *Agrobacterium* genome itself codes for signals involved in the induction of nodulin gene expression.

Nodulin gene expression was studied in nodules formed by the Ti plasmid-cured *Agrobacterium* LBA4301 (12) containing pMP104. Plasmid pMP104 contains a 12 kb nod region, carrying several nod genes from the *R. leguminosarum* sym plasmid pRL1JI (21). In the *Vicia* nodules formed by this *Agrobacterium* transconjugant the

two early nodulin genes Nvs-40 and EN-OD2 are expressed, but no late nodulin mRNAs are detectable (Nap, in preparation). This result established conclusively that the presence of only the nod region in an *Agrobacterium* background is sufficient to induce early nodulin gene expression. As the nod region is the only *Rhizobium* DNA required for the induction of early nodulin gene expression, this nod region itself must be involved in the induction of early nodulin gene expression. Plasmid pM104 contains the nod ABCDEFI and J genes. Mutations in the nodE, F, I or J genes on the total sym plasmid cause delayed root nodule formation (4). However, all nodulin genes are most probably expressed in these nodules, as they fix nitrogen. Consequently, it is not very likely that any of the nodE, F, I or J genes encode a signal essential for the induction of early nodulin gene expression. Thus, the other nod genes, nodD, A and C, are probably involved in inducing early nodulin gene expression. Of these genes, nodD has been shown to induce the expression of other nod genes on the sym plasmid, in the presence of flavones or flavanones excreted by the root (6,24,18). The nodA, B, and C genes are thus the most probable candidates for producing the signal responsible for the induction of early nodulin gene expression. It has been shown that these nod genes are absolutely essential for root hair curling (4), the infection process, and the induction of cortical cell division (5). Because the early nodulin genes we have analyzed are first expressed when the nodule primordia have been formed (7), the induction of their expression is part of a developmental stage after the induction of cortical cell division. So if the *Rhizobium* nodA, B and C genes are also involved in the induction of early nodulin gene expression, it appears that

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these genes play a role at many levels of the interaction between *Rhizobium* and its host plant.

Rhizobium **nod genes and late nodulin gene expression**

The results described above also show that the 12 kb *R. leguminosarum* nod region in an *Agrobacterium* chromosomal background is not sufficient for the induction of the expression of late nodulin genes. Introduction of pMP104 in *R. leguminosarum* 248, cured of its sym plasmid (strain 248c), and analysis of nodulin gene expression in the nodules formed by this 248c (pMP104) showed that all nodulin genes are expressed in these nodules (table 1). The same is true for strain ANU845 (pMP104), the sym plasmid-cured *R.trifolii* ANU845 harbouring pMP104. Thus, the 12 kb nod region in a *Rhizobium* chromosomal background is sufficient for induction of the expression of all nodulin genes examined. Because the engineered *Agrobacterium* is not able to induce the expression of late nodulin genes, whereas an engineered *Rhizobium* is, it seems that besides the nod region, chromosomal genes or non-sym plasmid genes are required for the induction of late nodulin genes. However, an alternative explanation could be a different reaction of the plant defense mechanism towards that *Agrobacterium* transconjugant. It is conceivable that the *Agrobacterium* transconjugant is recognized by the host plant as an "*Agrobacterium*" at a certain stage of development. Then the apparent involvment of *Rhizobium* chromosomal genes might merely reflect the requirement of a distinct surface of the bacterium to prevent an interaction with the plant defense mechanism. The importance of the outer cell surface of *Rhizobium* is illustrated by the finding that mutations resulting in an altered outer surface lead to a disturbed infection process, in which the reaction of the plant resembles a hypersensitive response (19). Because LBA4301 (pMP104) manages to "fool" the plant defense mechanism for quite a while in terms of development, the differences between the outer surfaces of LBA4301 (pMP104), 248c (pMP104) and ANU845 (pMP104) may be very minor.

Bacterial strain Lb	Chromosomal background	EARLY		LATE	
		ENOD2	Nvs-40	Nvs-65	
PRE	*R. leguminosarum*	+	+	+	+
248c (pMP104)	*R. leguminosarum*	+	+	+	+
ANU 843 (nod K11::Tn5)	*R. trifolii*	+	+	+	+
ANU845 (pMP104)	*R. trifolii*	+	+	+	+
ANU845 (pRt032) (nodE K11::Tn5)	*R. trifolii*	+	+	+	-
LBA4301 (pMP104)	*A. tumefaciens*	+	+	-	-
hypothetical (see text)	?	+	-	-	-

Table 1 : Pattern of early and late nodulin gene expression in nodules induced on Vicia sativa by the strains indicated.

Due to the limitations imposed by the use of engineered *Agrobacterium* strains, we cannot decide whether the *Agrobacterium* transconjugant has the genetic information for inducing late nodulin gene expression. If the defense mechanism counteracts nodulin gene expression before the induction of expression of these genes, it is possible that the nod region from the sym plasmid is involved in inducing late nodulin gene expression after all. As an experimental approach to this possibility, we compared nodulin gene expression in *Vicia* nodules formed by ANU845 (pMP104) and ANU845 (pRt032) (nodE K11::Tn5).

Plasmid pRt032 contains a 14 kb nod region from the sym plasmid of *R. trifolii* ANU843 (20). Also this cloned nod region contains the ABCDEFI and J genes. A Tn5 insertion in the nodE gene of pRt032 at position K11, yielding plasmid pRt032 (nodE K11::Tn5), extends the host range of the recipient ANU845 to the pea/vetch cross-inoculation group (3). In the *Vicia* nodules formed by ANU845 (pMP104) all nodulin genes identified sofar are expressed (table 1). In nodules formed by ANU845 (pRt032) (nodE K11::Tn5), the early nodulin genes ENOD2 and Nvs-40 are expressed, as well as the late nodulin gene Nvs-65. However, no Lb gene no other late nodulin transcripts are detectable. Apparently there is a marked difference between the two nodule types with respect to late nodulin gene expression. The bacterial strains used for inoculation differ only in the cloned nod region (table 1), implying that in some way the genetic

information present on pMP104 is involved in late nodulin gene expression.

Since the genetic maps of pMP104 and pRt032 (nodE K11::Tn5) are similar, the difference in the expression pattern of nodulin genes must be related to the presence of either plasmid. The major difference seems to be the presence of the Tn5 in nodE, indicating that nodE might be the cause for the observed difference. *R. trifolii* strain ANU843 (nodE K11::Tn5) (3) however, in which the nodE K11 mutation is introduced in the complete sym plasmid, forms nodules on *Vicia* in which the Lb genes are expressed (table 1). No nodE (-like) gene is present on the *Rhizobium* chromosome (Dr. M.A. Djordjevic, personal communication), hence it is very unlikely that nodE is required for the induction of expression of the Lb genes. Other distinguishing characteristics of pMP104 versus pRt032 (nodE K11::Tn5) should therefore be responsible for the observed difference in Lb gene expression. Whether this reflects a (small) difference in copy number of the two plasmids, or a difference in the presence of essential genetic information -genes on pMP104 that are not present on pRt032 (nodE K11::Tn5) - is not clear. The importance of the copy number of the nod genes has been demonstrated (13). When a high-copy number plasmid with the nodABC genes, transcribed constitutively from a vector promotor was introduced in a *R. leguminosarum* with a complete sym plasmid, nodulation ability was abolished completely. Slight differences in copy number be-

tween pMP104 and pRt032 (nodE K11::Tn5) may therefore explain the observed differences in the nodulin gene expression pattern.

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Mise en évidence de nodulines chez *Sesbania rostrata*

P. de Lajudie [1] et T. Huguet [2]

Introduction

Le développement d'un nodule et la fixation symbiotique de l'azote chez une légumineuse sont le résultat d'une série d'évènements et d'échanges continuels d'informations entre la plante et l'endophyte fixateur d'azote. Un certain nombre de gènes bactériens et végétaux, impliqués dans la reconnaissance et la différenciation des deux partenaires pendant la symbiose, ont été identifiés (pour des revues, voir référence 2 et 5). Ainsi, par des expériences de génétique classique sur plusieurs systèmes symbiotiques, il a été montré que des gènes de la plante interviennent dans le contrôle de certaines propriétés comme le délai d'apparition des nodules, leur abondance et leur taille, la multiplication des bactéries, leur libération hors des cordons d'infection, la maturation et la morphologie des bactéroïdes, l'efficacité de la fixation d'azote.

Récemment, des techniques d'immunologie et de biologie moléculaire ont permis de mettre en évidence des nodulines qui sont des protéines codées par la plante, spécifiques des nodules et qui ne sont pas retrouvées dans les racines non infectées ou les autres parties de la plante-hôte (21). Des nodulines ont été décrites chez plusieurs légumineuses à nodules racinaires comme *Glycine max* (16), *Pisum sativum* (4), *Medicago sativa* (15), et *Phaseolus vulgaris* (7).

Les fonctions de ces nodulines sont encore mal connues mais elles doivent intervenir à toutes les étapes du développement et du fonctionnement du nodule, lors de la reconnaissance entre la plante et la bactérie, au cours du processus d'infection, pour le transport des substrats vers les bactéroïdes, ainsi qu'au cours de toutes les modifications morphologiques, cytologiques et physiologiques qui interviennent dans les cellules infectées.

Quelques nodulines particulières ont été identifiées comme la leghémoglobine, cette protéine affine d'oxygène qui facilite sa diffusion vers les bactéroïdes à une tension partielle compatible avec la fixation d'azote. D'autres nodulines comme une uricase (3) ou une glutamine synthétase (7), impliquées dans l'assimilation et le transport de l'azote fixé, ou plus récemment une sucrose synthétase (20) spécifique du nodule ont également été identifiées.

Dans le cas de *Sesbania rostrata*, si la bactérie a déjà fait l'objet de nombreuses études génétiques (voir ce volume), peu d'informations sont disponibles sur la plante elle-même.

C'est ainsi que nous nous sommes intéressés à la recherche des gènes de la plante activés au cours de la symbiose dans le cas de *Sesbania rostrata*, avec les buts suivants:

▲ faire l'inventaire des nodulines et des gènes végétaux impliqués dans la symbiose,

▲ rechercher si ces gènes sont exprimés de façon identique dans les nodules de tige et de racine,

▲ étudier la cinétique de l'expression de ces gènes au cours du développement du nodule,

▲ étant donné que *Sesbania rostrata* vit en symbiose avec deux genres bactériens différents *Azorhizobium* et *Rhizobium* (9), comparer l'expression des gènes de la plante dans les deux cas,

▲ identifier les leghémoglobines grâce à un antisérum anti-leghémoglobine de *Sesbania rostrata*,

▲ rechercher si l'expression de certains gènes de la plante est en relation avec la fixation d'azote, en étudiant des nodules inefficients,

▲ les nodules de tige étant chlorophylliens, nous avons voulu voir l'incidence de la photosynthèse au niveau des nodules de tige sur l'expression des gènes végétaux en étudiant l'expression des gènes de la plante dans des nodules de tige induits par la souche sauvage ORS571 protégés ou non de la lumière. Les nodules de tige développés à l'obscurité sont de forme normale, blancs au lieu de verts à l'extérieur, roses à l'intérieur. De plus, sur certains, une petite racine émerge du nodule (14). Aucune différence dans l'activité réductrice d'acétylène n'est détectée par comparaison avec les nodules éclairés du même âge prélevés en parallèle sur les mêmes plantes.

Résultats

Analyse des protéines cytoplasmiques solubles totales

Nous avons purifié les protéines cytoplasmiques solubles totales (PCST) des racines et des tiges non infectées ainsi que des nodules de tige et de racine effectifs induits par la souche sauvage d' *A. caulidans* ORS571 et récoltés 30 jours après l'inoculation. Après séparation par PAGE-

SDS, et coloration au bleu de coomasie, environ 50 bandes correspondant à des polypeptides de masses moléculaires comprises entre 15 et 200 kDa sont révélées dans chacun des tissus (figure 1A). La plupart d'entre elles sont présentes dans tous les tissus. Cependant, certaines différences qualitatives et quantitatives apparaissent à la comparaison des différents profils.

Ainsi, nous avons pu observer une dizaine de bandes moins intenses et une dizaine d'autres plus intenses dans les nodules par rapport aux tissus non infectés. Au moins 4 bandes apparaissent spécifiques des nodules. Les profils des nodules de tige et des nodules de racine sont très voisins et seules quelques différences ont pu être décelées comme par exemple une bande de 26 kDa plus intense dans le cas des nodules de tige. Nous avons vérifié que ces différences ne sont pas dues aux protéines de bactéroïdes car les profils des protéines extraites de bactéroïdes isolés soit de nodules de tige soit de nodules de racine sont identiques (figure 1C).

Une bande majoritaire de masse moléculaire apparente inférieure à 18 kDa est spécifique des nodules de tige et de racine. Son abondance et sa taille suggèrent qu'il s'agit de la leghémoglobine, ce que nous avons vérifié par la suite par des expériences de "Western blotting" en utilisant un antisérum préparé contre les leghémoglobines purifiées de nodules de tige de *Sesbania rostrata* (fourni par D. Bogusz; figure 1B).

Analyse des produits de traduction in vitro

Ayant observé des différences dans les profils électrophorétiques des PCST des différents tissus, nous avons suivi une autre approche qui consiste à analyser l'expression des gènes végétaux au niveau transcriptionnel.

A partir des différents tissus, nous avons extrait les ARN poly A+ pour les traduire *in vitro* dans un lysat de réticulocytes de lapin en présence de Méthionine marquée au [35]Souffre, et nous avons ensuite analysé les produits de traduction par électrophorèse sur gel de polyacrylamide en une

(PAGE-SDS) ou deux dimensions (2D-PAGE) (18), suivie d'une autoradio-fluorographie.

La figure 2 présente les profils électrophorétiques en PAGE-SDS des produits de traduction obtenus à partir des ARN-poly A+ extraits des divers tissus: 50 à 60 bandes de masses moléculaires comprises entre 10 et plus de 100 kDa sont distinguables sur chaque profil. En 2D-PAGE, environ 200 à 300 polypeptides de masses moléculaires comprises entre 10 et plus de 100 kDa sont visibles sur chacun des profils obtenus (14). Dans les deux cas, la majorité des polypeptides sont communs à tous les tissus étudiés; cependant, certains apparaissent soit spécifiques d'un type de tissu (nodule ou tissu non infecté) soit plus ou moins abondants dans l'un ou l'autre tissu.

Nodules de tige et de racine induits par *A. caulidans*

En PAGE-SDS, par rapport aux tissus non infectés, les nodules efficients induits par la souche d'*Azorhizobium* ORS571 présentent environ une dizaine de bandes augmentées et une dizaine d'autres qui sont diminuées. En 2D-PAGE, nous avons dénombré au moins 16 polypeptides spécifiques des nodules, 10 qui sont fortement augmentés et une dizaine d'autres qui sont diminués dans les nodules par rapport aux tissus non infectés.

L'étude cinétique de l'expression de ces gènes spécifiques au cours du développement du nodule a montré que ces derniers sont activés de façon séquentielle. Ainsi, la majorité de ceux-ci s'expriment vers le 12ème jour après inoculation. Cependant, certains sont exprimés beaucoup plus tôt, vers les 6/7ème jour, et d'autres plus tard. Plusieurs enfin sont activés de façon transitoire: l'un d'eux est très intense au cours des premiers stades puis décroît au cours du développement: d'autres atteignent leur intensité maximale entre 12 et 16 jour, puis diminuent.

Leghémoglobine

Afin d'identifier les composants de la leghémoglobine, nous avons utilisé un antisérum dirigé contre la leghémoglobine de nodules de tige de *Sesbania rostrata* (préparé par D. Bogusz) pour immunoprécipi-

ter les produits de traduction *in vitro* et les identifier par 2D-PAGE.

Aucun produit de traduction d'ARN-Poly A+ de tiges ou de racines non infectées ne réagit avec l'antisérum; par contre, six polypeptides abondants sont immunoprécipités dans le cas des nodules de tige et de racine. Leur masse moléculaire (inférieure à 17 kDa) est en accord avec celles des leghémoglobines décrites dans d'autres systèmes (1).

Au cours du développement du nodule, dans nos conditions d'expérience, les leghémoglobines apparaissent vers le 12ème jour et leur intensité augmente ensuite au cours de notre période d'observation. De plus, leur intensité relative varie dans la même période: à 12 jours, Lb2, Lb3, Lb6 ont environ la même intensité alors que Lb5 et Lb4 sont majoritaires et que Lb1 est à peine visible; après le 16ème jour, Lb2 et Lb6 deviennent majoritaires, Lb1 reste minoritaire et les autres sont moyennement abondants.

Nodules de racine induits par *Rhizobium* sp.

Les nodules de racine induits soit par *Azorhizobium* (souche ORS571) soit par *Rhizobium* sp. (souche ORS51) présentent des profils identiques mis à part l'intensité de quelques bandes (figure 2). Cependant, en 2D-PAGE (14), deux polypeptides "racine" (rsst21 et rsst26) semblent plus abondants dans les nodules induits par ORS51 et un spot remarquable, correspondant à un polypeptide de masse moléculaire 37,5 kDA, semble spécifique.

Nodules de tige développés à l'obscurité

Le profil des produits de traduction des ARN extraits de nodules de tige développés à l'obscurité est très semblable à celui des nodules chlorophylliens et les polypeptides stimulés le sont dans les mêmes proportions (figure 2, piste 6 et 8). Cependant il est intéressant de remarquer que dans le cas de nodules à l'obscurité, l'intensité de certaines bandes est très augmentée, et ceci de façon semblable aux racines; en particulier deux bandes majeures de masses moléculaires approximatives 21 et 28,5 kDa. Ceci pourrait être en relation avec la présence de radicelles sur

Protéines solubles totales extraites de:
1.   Racines non infectées
2.   Nodules de racine Fix+ induits
     par la souche ORS571
3.   Tiges non infectées
4.   Nodules de tige Fix+ induits
     par la souche ORS571
5.   Bactéroïdes isolés de nodules de racine
6.   Bactéroïdes isolés de nodules de tige
T = Témoins de masses moléculaires

(A) PAGE-SDS/Coloration au bleu
     de Coomasie
(B) Expérience de "Western blotting"
     avec l'antisérum anti-leghémoglobines
     de Sesbania rostrata
(C) PAGE-SDS/Coloration au nitrate d'argent

●   indique les principales bandes qui sont
     plus abondantes ou spécifiques
     des nodules

◄   indique les principales bandes qui sont
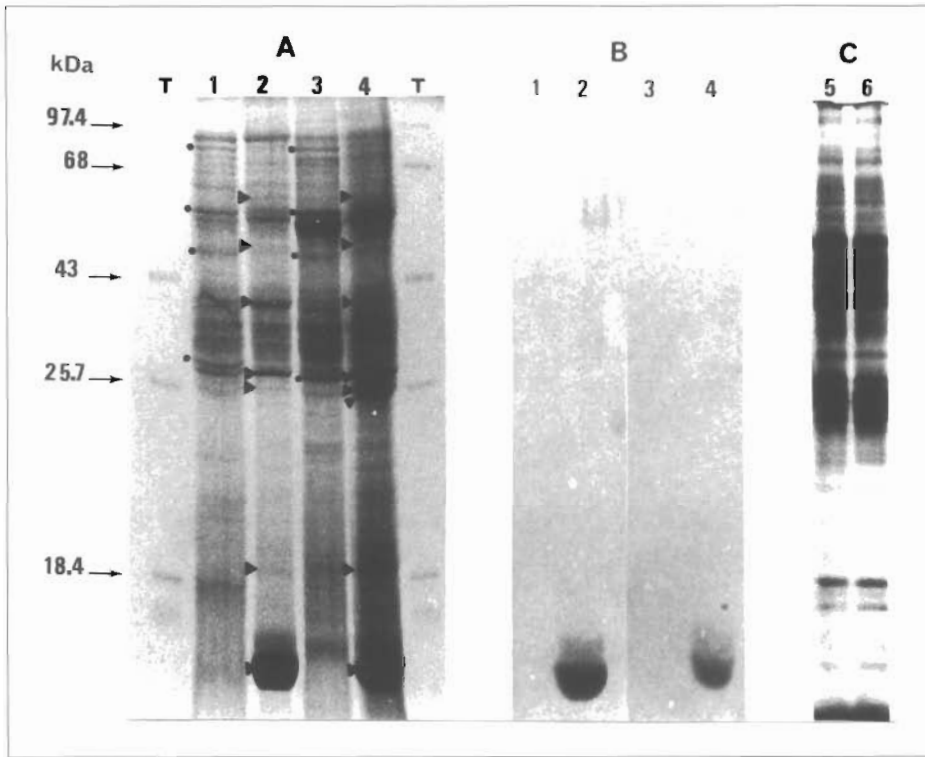   plus abondantes ou spécifiques
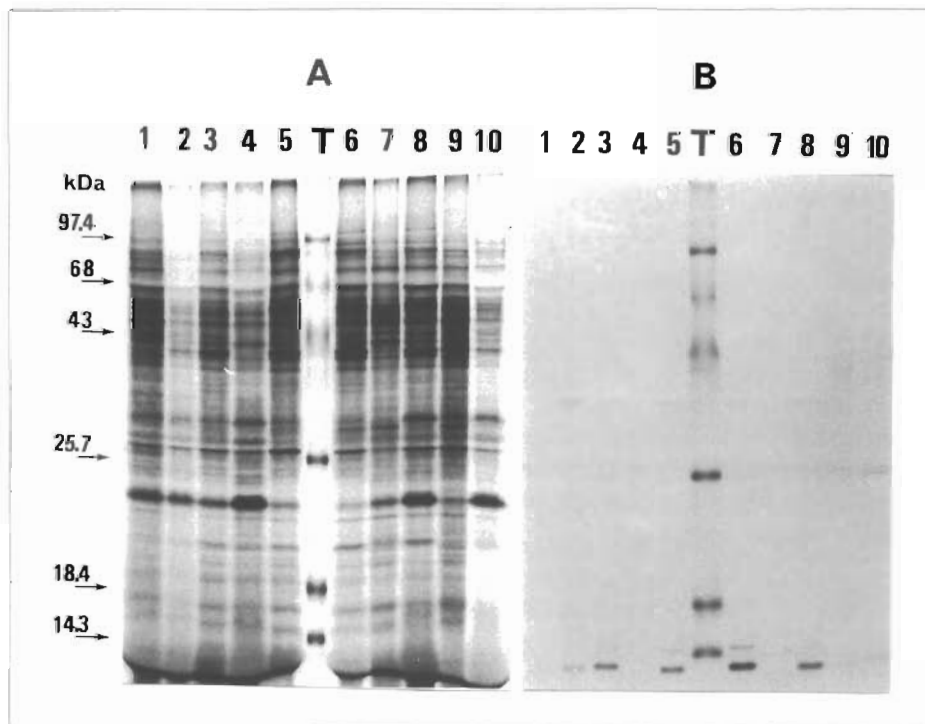   des tissus non infectés

Figure 1 : Analyse en PAGE-SDS (A et C) et "Western blotting" (B)
des protéines cytoplasmiques solubles totales de Sesbania rostrata,
de nodules et de bactéroïdes.

Protéines solubles totales extraites de:
1.   Nodules de racine Fix- induits
     par la souche ORS51
2.   Nodules de racine Fix- induits
     par la souche 5740
3.   Nodules de racine Fix+ induits
     par la souche ORS51
4.   Racines non infectées
5.   Nodules de racine Fix+ induits
     par la souche ORS571
6.   Nodules de tige Fix+ induits par
     la souche ORS571 maintenus à la lumière
7.   Tiges non infectées
8.   Nodules de tige Fix+ induits par
     la souche ORS571 maintenus à l'obscurité
9.   Nodules de tige Fix- induits
     par la souche 5740
10. Nodules de tiges Fix- induits
     par la souche 5795
T = Témoins de masses moléculaires (BRL)

(A) Produits de traduction totaux
(B) Produits de traduction après
     immunoprécipitation avec l'antisérum
     anti-leghémoglobine de Sesbania rostrata.

Figure 2 : Analyse en PAGE-SDS et autoradiofluorographie
des produits de traduction des ARNm-poly A+ purifiés de :

certains nodules développés à l'obscurité. L'analyse en 2D-PAGE a confirmé ces similitudes avec les tissus souterrains (14).

Nodules inefficients

Afin de déterminer si certains polypeptides remarquables dans le cas des nodules sont directement liés à l'activité fixatrice d'azote, nous avons analysé plusieurs types de nodules inefficients (Fix-):

▲ nodules de tige et nodules de racine induits par la souche Fix-5740, mutant ponctuel de ORS571 affecté dans le gène nif K (10).

▲ nodules de tige induits par la souche Fix-5795, mutant de régulation qui ne produit aucun des polypeptides nif normalement synthétisés par la souche sauvage (ORS571) en conditions de dérépression de la nitrogénase (8).

▲ nodules de racine induits par la souche ORS51 en conditions (température, photopériode) non permissives pour la fixation d'azote (19).

Dans tous les cas, la leghémoglobine est présente mais en quantité réduite par rapport aux nodules Fix+ du même âge. L'analyse en 2D-PAGE a confirmé ces observations et a montré que les 6 composants sont présents (14).

Ceci montre que, comme chez d'autres espèces (11, 13, 15) les gènes leghémoglobine sont activés dans des nodules inefficients mais que leur expression reste faible comparée aux nodules Fix+.

En plus des composants leghémoglobine, de nombreux polypeptides "noduline" ou dont l'intensité augmente dans les nodules fixateurs voient, dans les nodules non fixateurs, leur intensité diminuer, alors que simultanément certaines taches de type "tissus non infectés" se trouvent renforcées. Le niveau d'expression des polypeptides spécifiques du nodule est fonction du type de nodule inefficient considéré: ainsi, par rapport au nodule efficient, l'expression des gènes dans un nodule induit par la souche 5795 semble plus perturbée que dans celui induit par la souche 5740 (figure 2 et référence 14).

Conclusions

L'analyse des protéines cytoplasmiques solubles totales a fait apparaître la présence de protéines spécifiques dans les nodules.

L'analyse, par électrophorèse en une et deux dimensions, des produits de traduction des mARN-Poly A+ des nodules racinaires et caulinaires, des tiges et des racines non infectées nous a permis de suivre l'expression des gènes de la plante activés au cours de la symbiose.

Nous avons pu ainsi mettre en évidence une activité transcriptionnelle différentielle des gènes de la plante au cours de la nodulation racinaire et caulinaire de *Sesbania rostrata*.

A l'état symbiotique, l'activité d'une dizaine de gènes et diminuées, alors qu'une trentaine d'autres gènes sont stimulés, la majorité de façon identique dans les nodules de tige et de racine. Parmi eux, 16 apparaissent comme des gènes de nodulines.

Ces résultats sont comparables à ceux obtenus avec le soja et le pois, chez lesquels 20 à 30 nodulines ont été signalées (4, 16).

En ce qui concerne les nodules de racine, souche "de tige" du genre *Azorhizobium* (ORS571) et souche "de racine" du genre *Rhizobium* (ORS51) semblent induire l'activation des mêmes gènes chez la plante, mis à part un gène activé supplémentaire dans le cas de ORS51. Il serait intéressant de déterminer quel rôle pourrait jouer une telle noduline.

Une étude cinétique a montré que les gènes de la plante sont séquentiellement activés au cours du développement du nodule: certains le sont dans les premiers jours suivant l'infection, la majorité vers le 12e jour, et d'autres plus tard (vers le 18e jour). De plus, certains gènes sont activés de façon transitoire à un moment ou l'autre du développement. Une telle séquence d'évènements est également connue chez le pois (13) et le soja (12).

Au moins six différents produits de traduction *in vitro* ont été identifiés par immunoprécipitation comme étant des composants de la leghémoglobine. Leur abondance relative varie en accord avec ceux de Bogusz et coll. (6), confirmant chez *Sesbania rostrata* la présence de la plus grande famille de gènes de leghémoglobine caractérisée jusqu'à présent. Dans les deux types de nodules, ces auteurs ont séparé six composants par isoélectrofocalisation et sept par chromatographie échangeuse d'anions. Les composés apparaissent en proportions relatives différentes dans les nodules de tige et de racine. L'analyse de séquence des acides aminés amino-terminaux, ont permis à ces auteurs de conclure qu'au moins six d'entre eux sont des produits de gènes différents.

L'expression des gènes dans les nodules de tige développés à l'obscurité est comparable à celle des nodules de tige développés à la lumière; cependant, un fait remarquable est l'expression dans les nodules de tige à l'obscurité de gènes typiquement stimulés dans le cas des nodules de racine ou des racines non infectées. Ainsi, tout se passe comme si les nodules de tige à l'obscurité acquièrent certaines caractéristiques des organes souterrains.

Dans les nodules inefficients, les gènes de nodulines sont exprimés à un niveau d'expression variable en fonction du type de nodule inefficient, mais généralement réduit par rapport aux nodules fixateurs. Par ailleurs certains gènes dont l'expression est généralement renforcée dans les tissus non infectés, sont également stimulés dans le cas des nodules inefficients. Ainsi ces derniers acquièrent-ils certains caractères de type "tissu non infecté" et, comme il a été montré chez le soja (16), le pois (13) et la luzerne (17), les gènes de nodulines y sont activés indépendamment de la fixation d'azote, mais restent à faible niveau d'expression.

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Session IV

Tissue Culture
and Plant Regeneration

L'utilisation de quelques techniques de culture in vitro chez *Sesbania rostrata*

M. Sagne et R. Jonard *

Introduction

Les travaux sur la culture *in vitro* du *Sesbania rostrata* sont, à ce jour, peu nombreux (1). Cette étude aborde la multiplication *in vitro* par microbouturage, l'organogenèse et l'isolement de protoplastes à partir d'organes juvéniles tels que les cotylédons, les hypocotyles et les radicules.

La finalité des essais menés sur la culture des protoplastes étant la régénération, l'isolement de ceux-ci a été réalisé à partir d'organes juvéniles possédant souvent un bon potentiel organogène. Pour vérifier le bien-fondé de cette approche concernant le *Sesbania rostrata*, des essais préalables ont porté sur l'organogenèse de cotylédons et d'hypocotyles.

Matériel et méthodes

La multiplication in vitro

Les essais ont été réalisés à partir de graines provenant de l'ORSTOM de Dakar.

La germination des graines

Les graines de Sesbania subissent un traitement chimique, avant leur ensemencement, consistant en un trempage dans de l'acide concentré. Pour cet essai, les durées du trempage sont de 30 et 60 minutes. Les graines à macérer sont placées dans deux étuves à 35 et 24°C.

Après macération, les graines sont lavées suivant deux modes: le premier consiste en 10 lavages rapprochés et le second en 2 lavages rapprochés suivis de 8 lavages espacés de 15 minutes chacun.

Le microbouturage

La technique utilisée pour la multiplication *in vitro* est le microbouturage. Les boutures sont obtenues à partir de noeuds prélevés sur de petites plantes issues de graines cultivées *in vitro* sur le milieu suivant: solution minérale de Murashige et Skoog (2), vitamines de Morel, saccharose, 3% (w/v); agarose 0.7% (w/v). Le pH est ajusté à 5.7 avant l'autoclavage (20 mn à 110°C). L'éclairage est de 2000 lux avec une photopériode de 16 heures.

▲ a) Un seul milieu de microbouturage

- Etude de la composition en hormones
  Les hormones utilisées sont: la 6 Benzyl-aminopurine (BAP à 0.01, 0.02, 0.05, 0.1 et 0.5 mg/l) associée à l'acide naphtylacétique (ANA à 0.05, 0.1, 0.2 et 0.5 mg/l). La solution minérale est celle de Murashige et Skoog utilisée pour les plantules.

▲ b) Deux milieux de microbouturage

- Etude de la composition du milieu en sels minéraux
  Les solutions minérales suivantes ont été utilisées pour les essais concernant le débourrement des noeuds: la solution de Murashige et Skoog, cette même solution mais avec les macro-éléments divisés par deux, par quatre et par huit; soit respectivement: M.S., M.S/2, M.S/4 et Knop (3). Les autres composés du milieu et les conditions de culture sont identiques à ceux utilisés pour la culture des jeunes plantes.

- Etude de la composition du milieu en hormones
  Les deux hormones utilisées pour les milieux de débourrement et d'enracinement sont la BAP (0.1, 0.2 et 0.5 mg/l) et l'ANA (0.01, 0.05, 0.1 et 0.5 mg/l).
  Lors du repiquage des boutures dans le milieu d'enracinement, après un séjour de 10 jours sur milieu de débourrement, les explants sont soumis à un choc auxinique par trempage dans une solution d'Acide ß Indolyacétique (AIA) à 0,5 g/l durant une heure.

La culture d'apex

Les apex caulinaires, d'environ 0,5 mm de diamètre, sont prélevés stérilement à partir de bourgeons provenant de vitro plants.

Le débourrement des apex est réalisé en milieu liquide, sur pont de papier filtre, dans des tubes à hémolyse. Le milieu de base est constitué de la solution minérale de Lepoivre (4) et, pour d'élongation, de la solution minérale de M.S.. Les 5 premiers jours de culture sont conduits à l'obscurité. C'est ensuite la lumière alternée qui est utilisée. Le repiquage des explants, sur milieu d'élongation gélosé (agarose à 0,8%), est effectué au bout de 10 jours après la mise en culture des apex.

Les premiers essais réalisés avec la BAP à 0,05 mg/l dans le milieu de débourrage, ont permis de mettre en évidence un blocage ultérieur de l'élongation après le repiquage. C'est pourquoi, la concentration en BAP a été portée à 0,5 mg/l dans tous les milieux de débourrement de l'essai.

La présence d'une auxine, dans le milieu d'ensemencement des apex, entraîne une forte callogenèse qui perturbe le débourrement de l'explant. Dans cet essai, de fortes

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concentrations en gibbérelline sont utilisées pour compenser l'absence ou la très faible concentration en auxine (ANA à 0,01 mg/l) du milieu de débourrement

Dans le milieu d'élongation, la BAP est présente à 0,1 et 0,2 mg/l et l'ANA à 0,01 et 0,05 mg/l.

Les mesures, réalisées au bout de 6 semaines de culture, concernent la callogenèse, le débourrement, l'élongation et l'enracinement des explants.

L'organogenèse d'explants juvéniles

Les explants utilisés sont les cotylédons et les hypocotyles. Divers stades de développement de la plantule ont été identifiés afin de préciser le degré de juvénilité de ces explants.

Stades	Degrés de développement de la graine et de la plantule
1	Gonflement maximum de la graine
2	Cotylédons encore enfermés dans l'enveloppe. Sortie de la radicule (1 cm)
3	Cotylédons se dégagent de l'enveloppe et commencent à devenir chlorophylliens.
4	Petite plante de 10 cm de haut.

Le milieu minéral est celui de M.S.. Les essais sont réalisés avec diverses concentrations en hormones: BAP (0.1, 0.5, 1,2 et 4 mg/l) et ANA (0.05, 0.1, 0.2, 0.5, 1, 2 mg/l).

L'isolement des protoplastes

Les protoplastes sont isolés à partir de cotylédons aux stades 1 et 3 et d'hypocotyles au stade 4 issus de graines ensemencées stérilement en boîte de Pétri, sur milieu gélosé de M.S..

Avant macération, les cotylédons sont légèrement brossés sur les deux faces puis coupés en fines lamelles d'environ 1 mm de large. Les hypocotyles sont longitudinalement lacérés à l'aide d'une aiguille montée.

La solution enzymatique est composée de la solution minérale de Mabanza et Jonard

(5) contenant 0,7 M de mannitol, la cellulase Onozuka RS (1, 1.5, 2 et 2.5%) associée au mazérozyme (0.1%). Les cellulases Caylases T (0.25 et 0.5%) et 345 avec la pectinase Caylase M2 (0.1%) sont également utilisées. Le pH est ajusté à 5,6 et le milieu est stérilisé par préfiltration à 8 suivie d'une filtration à 0.22. Environ 2 g de tissus sont mis à macérer dans 20 ml de solution enzymatique, en Erlenmeyer de 100 ml. Cette macération est conduite à 25°C à l'obscurité. L'agitation est de 60 rotations par minutes. Le comptage des protoplastes libérés est réalisé à la cellule de Nageotte.

Résultats et discussion

La multiplication in vitro

La germination des graines
▲ Effet de la durée de macération
L'examen du tableau 1 montre qu'une macération de 60 minutes améliore le taux de germination par rapport à une macération de 30 minutes (95 et 76% respectivement à 35°C). Pour les lavages rapprochés, une macération de 30 minutes permet un taux de germination de 76% qui atteint 95% à 60 minutes de macération. Des germinations anormales sont observées, à un taux de 81% pour une macération d'une heure à 35°C, alors que cette macération, réduite à 30 minutes, dans les mêmes conditions de température, n'entraîne aucune germination anormale.

▲ Effet de la température d'incubation.
L'augmentation de la température d'incubation de 24 à 35°C a un effet plus important sur le taux de germination à 60 minutes d'incubation (84 et 95% respectivement) qu'à 30 minutes (71 et 76%). L'incidence de la température d'incubation sur le taux de germination anormale n'est sensible que pour 60 minutes de macération et demeure réduite (79% à 24°C et 81% à 35°C).

Le microbouturage
a) Un seul milieu de microbouturage

▲ Etude de la composition en hormones
Le taux d'enracinement le plus important (52%) est obtenu sur le milieu

M.S. sans hormones. Ce taux diminue avec l'augmentation de la concentration en BAP dans le milieu, lorsque cette hormone est utilisée seule. En association avec l'ANA (0.05 et 0.2 mg/l), la BAP à 0.5 mg/l permet un taux d'enracinement de 40%.
En présence de BAP, l'ANA réduit fortement l'élongation des pousses. En effet, celle-ci est de 4 à 5 cm au bout de 15 jours de culture pour des concentrations en BAP comprises entre 0 et 0.05 mg/l, sans ANA. La meilleure élongation obtenue n'est que de 2 cm lorsque la BAP (0.5 mg/l) et l'ANA (0.05 à 1 mg/l) sont associées dans le milieu.
La BAP favorise donc l'élongation des pousses et inhibe leur enracinement tandis que l'ANA a un effet inverse.
Si le milieu sans hormones permet d'obtenir à la fois un bon taux d'enracinement (52%) et une bonne élongation (4 cm), les pousses sont chétives et présentent un pourcentage de nécroses plus important que pour les cultures avec hormones. Les racines produites dans ces conditions sont très fines (0.5 mm) et leur élongation est bloquée à environ 1 cm.
La BAP et l'ANA ayant des effets antagonistes vis à vis de l'enracinement et de l'élongation, le milieu sans hormones ne donnant pas non plus satisfaction, le bouturage utilisant deux milieux successifs a été expérimenté. Le premier milieu pour l'élongation, le second pour l'enracinement.

b) Deux milieux de microbouturage
▲ Etude de la composition du milieu en sels minéraux

• *Le milieu de débourrement*
Les résultats obtenus, donnés dans le tableau 3, permettent d'observer que le taux d'enracinement diminue avec la dilution du milieu de culture en sels minéraux. C'est ainsi que ce taux, qui atteint 52% avec le milieu M.S., n'est plus que de 23% sur M.S./8. Cette tendance est la même pour le taux d'élongation. En effet, pour les milieux M.S. et M.S./8, celui-ci est respectivement de 35 et 27%. Le milieu M.S., caractérisé par une forte concentration en sels minéraux, offre de meilleurs résul-

tats d'enracinement et d'élonga-
tion que des milieux plus dilués. Il
sera retenu pour le débourrement
des noeuds.

- *Le milieu d'enracinement*
L'examen du tableau 4 fait appa-
raître que les milieux M.S./2 et
M.S./4 permettent d'obtenir un
taux d'enracinement de 100%. Ce
dernier n'est que de 66% pour le
milieu de Knop et de 40% pour le
milieu M.S. La meilleure élonga-
tion des racines est obtenue avec
M.S./2 (3 cm) et l'élongation des
pousses est la plus forte avec M.S.
(6.5 cm).
Le milieu d'enracinement ayant
donné les meilleurs résultats pour
l'enracinement et l'allongement
ultérieur des pousses est M.S./2
qui permet un enracinement de
100% ainsi qu'une bonne élonga-
tion des racines (3 cm) et des
pousses (5.5 cm).

▲ Etude de la composition
du milieu en hormones
- *Effet de l'ANA
du milieu d'enracinement
sur l'élongation des pousses*
Dans cet essai, représenté au ta-
bleau 5, la meilleure élongation
des pousses sur milieu d'enracine-
ment (2.4 cm) est obtenue avec
des concentrations en BAP et
ANA, dans le milieu d'élongation,
de 0.1 et 0.05 mg/l respective-
ment. Pour cette combinaison
d'hormones, la concentration en
ANA du milieu d'enracinement
(0.1 et 0.05 mg/l) n'a pas d'effet
sur l'élongation. Par contre, pour
des concentrations plus impor-
tantes en BAP, dans le milieu de
débourrement (0.2 et 0.5 mg/l),
l'élongation des pousses diminue.
Dans ces conditions, l'augmenta-
tion de la concentration en ANA
du milieu d'enracinement accen-
tue cette tendance. En effet, dans
ce milieu, l'ANA à 0.05 mg/l
n'entraîne qu'une réduction de l'é-
longation des pousses lorsque la
BAP augmente dans le milieu de
débourrement (2.2 et 1.8 cm au
lieu de 2.4 cm). Avec l'ANA à 0.1
mg/l dans le milieu d'enracine-

Milieu de débourrement	% enracinement	% élongation
MS/8	23	27
MS/	38	9
MS/2	40	30
MS	52	35

*Tableau 3 : Effet de la solution minérale du milieu de débourrement
sur l'enracinement des boutures et l'élongation des pousses*

*Le taux d'élongation est mesuré sur le milieu de débourrement et le taux d'enracinement sur le milieu
d'enracinement (solution minérale de Lepoivre) après repiquage des boutures sur ce dernier.
Le nombre d'explants par traitement est de 35 avec 3 répétitions.*

Milieu d'enracinement	% d'enracinement	Longueur moyenne des racines (cm)	Hauteur moyenne des pousses (cm)
MS	40,0	2,0	6,5
MS/2	100,0	3,0	5,5
MS/4	100,0	2,5	2,5
KNOP	66,0	1,0	2,2

*Tableau 4 : Effet de la solution minérale du milieu d'enracinement
(observation après 10 jours sur milieu d'enracinement)*

*Le milieu d'élongation: MS avec BAP 0,1 mg/l et ANA 0,01 mg/l.
Choc auxinique: AIB 0,5 g/l durant 1 heure.*

Milieu débourrement		Milieu enracinement	Allongement des pousses
BAP(mg/l)	ANA(mg/l)	ANA (mg/l)	(cm)
0,5	0,10	0,05	1,8
0,2	0,05	0,05	2,2
0,1	0,05	0,05	2,4
0,5	0,10	0,10	0,0
0,2	0,05	0,10	0,0
0,1	0,05	0,10	2,4

Tableau 5 : Effet de l'ANA du milieu d'enracinement sur l'allongement des pousses

*Le milieu de base est celui de Murashige et Skoog avec l'agar à 8 g/l, le saccharose à 30 g/l
et les vitamines du groupe B.
Les boutures ont subi un choc auxinique (AIB 0,5 g/l durant une heure) avant ensemencement
sur milieu d'enracinement.*

ment, cette augmentation de BAP
bloque complètement l'élongation.
Cet essai confirme les résultats ob-
tenus lors du bouturage à un seul
milieu, à savoir: l'ANA a un effet
inhibiteur sur l'allongement des
pousses. Si cet effet, mesuré au ni-
veau du milieu d'enracinement,
est sensible lorsque l'auxine aug-
mente dans ce dernier, il est égale-

ment mesurable pour des concen-
trations croissantes en ANA dans
le milieu d'élongation.
En définitive, l'effet inhibiteur de
l'ANA sur l'élongation, après en-
semencement des boutures sur mi-
lieu d'enracinement, se manifeste
dès le milieu de débourrement et
pas seulement au niveau du milieu
d'enracinement.

Milieu enracinement ANA (mg/l)	% enracinement	Hauteur moyenne des pousses (cm)
0	40	6,6
0,01	28	7,2
0,05	28	5,2
0,1	33	2,8
0,5	50	0

Tableau 6 : Effet de la concentration en ANA du milieu d'enracinement
(solution minérale de M.S.) sur l'élongation des pousses et sur l'enracinement des boutures.

M. de débourrement			M. d'élongation		Caulognèse	Taux de débourrement (%)	Elongation moyenne (mm)	Taux d'enra-cinnement (%)
BAP (mg/l)	ANA (mg/l)	GA3 (mg/l)	BAP (mg/l)	ANA (mg/l)				
0,5	0,01	10	0,2	0,05	++	85	8	0
0,5	0,01	10	0,1	0,01	(+)	72	17	72
0,5	0	1,5	0,1	0,01	(+)	70	0	0
0,5	0	0,1	0,1	0,01	(+)	68	4	0
0,5	0	0,1	0,2	0,05	++	65	4	0

Tableau 7 : La culture des apex caulinaires

Les solutions minérales des milieux de débourrement et d'élongation sont respectivement celles de Lepoivre et de M.S.
Chaque traitement correspond à la mise en culture de 35 apex.
Mesure de la caulogenèse: (+) = très léger développement du cal (∅ < 2 mm);
++ = ∅ d'environ 5 mm.

Il a également été constaté un effet inhibiteur de la BAP du milieu de débourrement sur l'élongation des pousses repiquées sur milieu d'enracinement.

- *Effet de l'ANA sur l'enracinement des boutures*
Le meilleur taux d'enracinement (50%) est obtenu pour la plus forte concentration en ANA de l'essai, soit 0,5 mg/l (tableau 6). Lorsque cette concentration diminue dans le milieu d'enracinement, le taux d'enracinement suit la même tendance, sauf si l'auxine est complètement absente du milieu. Ce taux est alors de 40%. L'effet inhibiteur de l'ANA sur l'élongation des pousses est de nouveau confirmé dans cet essai. En effet, l'élongation est nulle pour une concentration en ANA de 0.5 mg/l dans le milieu d'enracinement et elle est de 7.2 cm

pour une concentration plus faible (0.01 mg/l).
Pour le microbouturage, il sera donc retenu un ensemencement des boutures sur un milieu de débourrement contenant la solution minérale de M.S., avec comme hormones la BAP et l'ANA à 0.1 et 0.05 mg/l respectivement. Après une dizaine de jours sur ce milieu, les boutures subissent un choc auxinique par trempage dans une solution d'acide indolbutyrique (AIB) à 0.5 mg/l durant une heure. Le repiquage sur milieu d'enracinement est alors réalisé. Ce dernier est constitué de l solution M.S./2 sans hormones qui offre à la fois un bon enracinement (40%) et une élongation convenable des pousses (6.6 cm en 15 jours).

La culture d'apex caulinaires

Les résultats figurant au tableau 7 montrent que les apex, cultivés sur le milieu de débourrement à forte concentration en GA3 (10 mg/l), puis repiqués sur milieu d'élongation avec la BAP et l'ANA à faible concentration (0,1 et 0,01 mg/l respectivement), se développent en formant très peu de cal. Tous les explants débourrés (72%) sur ce milieu s'enracinent convenablement. La diminution de la concentration en GA3 dans le milieu de débourrement (1,5 et 0,1 mg/l) conduit à une légère baisse du taux de débourrement mais l'élongation des apex diminue fortement (0 et 4 mm au lieu de 14 et 8 mm pour le milieu à concentration élevée en GA3). Le même phénomène est observé lorsque la concentration en BAP et ANA du milieu d'élongation augmente (0,2 et 0,05 mg/l) respectivement au lieu de 0,1 et 0,01 mg/l).

Lors de l'ensemencement des apex, la prolongation de la période de culture à l'obscurité (portée à 15 jours) s'accompagne d'une diminution notable des oxydations à la base des explants. Le débourrement des apex semble également se faire dans de meilleures conditions.

La culture des apex sur milieu de débourrement est poursuivie durant 4 semaines. Après repiquage des explants sur milieu d'élongation, les vitro plants formés ne sont transférables qu'au bout de 4 semaines de culture. L'évolution de la culture des apex est donc très lente (8 semaines).

L'organogenèse d'organes juvéniles

La caulogenèse des hypocotyles

Au stade 3, le taux de caulogenèse le plus important (44%) est obtenu pour la combinaison de BAP et d'ANA de 1 et 0,1 mg/l respectivement (tableau 8).

Au stade 4, on observe une diminution de la caulogenèse pour des concentrations en BAP supérieures à 2 mg/l.

Le taux d'organogenèse des hypocotyles est très dépendant de l'âge des tissus. Prélevé à un stade très précoce (stade 3), ce type d'explant, pour exprimer son poten-

BAP (mg/l)	NAA (mg/l)	Stade de prélèvement	
		N°3	N°4
0,1	0,00	0	31
0,1	0,05	0	25
0,1	0,10	0	40
0,5	0,00	20	53
0,5	0,05	-	50
0,5	0,10	10	40
0,5	0,50	30	23
1,0	0,00	10	52
1,0	0,10	44	37
1,0	0,20	-	17
2,0	0,00	-	0
2,0	0,05	-	10
2,0	0,10	-	33
4,0	0,00	-	0
4,0	0,05	-	0
4,0	0,10	-	7
4,0	0,50	-	10
8,0	0,10	-	0

Tableau 8 : Caulogenèse des hypocotyles (résultats en % d'explants organogènes)

*Le milieu de base est celui de Murashige et Skoog avec l'agar à 8 g/l, le saccharose à 30 g/l
et les vitamines du groupe B.*
Le nombre d'explants par traitement est de 35.

Composition hormonale		Caulogenèse		Rizogenèse
		nombre de jours de culture		
BAP (mg/l)	ANA (mg/l)	10	30	10
1	0,1	0	33	0
2	0,2	0	33	25
2	0,5	10	28	22
4	0,2	0	15	9
4	0,5	0	35	0
4	1	0	12	22
4	2	0	8	5

*Tableau 9 : Organogenèse de cotylédons au stade 1
(résultats exprimés en % des explants mis en culture)*

*Le Milieu de base est celui de Murashige et Skoog avec l'agarose à 8g/l, le saccharose à 30 g/l
et les vitamines du groupe B.*
Le nombre d'explants par traitement est de 35.

tiel organogène, nécessite des concentrations en hormones supérieures à celles des hypocotyles plus âgés (stade 4).

L'organogenèse des cotylédons

Les résultats de cet essai, représenté au tableau 9 montrent que le taux le plus élevé de caulogenèse (35%) est obtenu pour la combinaison de BAP et d'ANA à 4 et 0,5 mg/l respectivement. Par contre, les premiers bourgeons se différencient au bout de 10 jours de culture, sur le milieu avec BAP à 2 mg/l et ANA à 0,5 mg/l. Ainsi, ce n'est pas ce milieu qui, au bout de 30 jours de culture, donne la meilleure caulogenèse.

Il est intéressant d'observer que les milieux ayant un rapport ANA/BAP égal à 4 (ANA 0,5 mg/l et BAP 2 mg/l ainsi que ANA 1 mg/l et BAP 4 mg/l présentent, au bout de 10 jours de culture, un même taux d'explants enracinés de 22%. Concernant la caulogenèse, c'est le milieu pour lequel ce rapport est égal à 8 (ANA 0.5 mg/l et BAP 4 mg/l) qui produit le taux le plus important d'explants avec bourgeons (35%). Viennent ensuite les milieux pour lesquels ce rapport est de 10 (ANA 0.1 mg/l et BAP 1 mg/l) ainsi que ANA 0.2 mg/l et BAP 2 mg/l), avec un taux de caulogenèse de 33%. Ces résultats vont dans le sens des observations faites par certains auteurs (6) qui faisaient remarquer qu'au delà des concentrations spécifiques de chaque hormone apportée au milieu de culture, leurs concentrations relatives sont un facteur déterminant de l'orientation de l'organogenèse, notamment le rapport cytokinines/auxine.

L'isolement de protoplastes

A partir du cotylédons au stade 3
Après avoir essayé diverses cellulases (caylases T et 345, drisélase), les meilleurs résultats ont été obtenus avec la cellulase RS Onosuka. La concentration optimale en cellulase RS est de 2% (Fig.1). Après 5 heures de macération, l'isolement est de $7.2\ 10^6$ protoplastes par gamme de tissus frais (Pr./g T.F.).

A partir de cotylédons au stade 1
Le meilleur résultat ($3.5\ 10^5$ Pr./g T.F) est obtenu avec la cellulase T à 0.5% au bout de 9 heures de macération (Fig.2). On peut observer que l'optimum forme un pic. Ceci traduit "l'agressivité" de la cellulase vis-à-vis de la membrane plasmique; celle-là conduisant à un éclatement important des protoplastes libérés. Afin de maintenir une bonne viabilité des protoplastes lors de leur ensemencement sur milieu de culture, la macération sera arrêtée une heure avant l'optimum de l'isolement; soit 8 heures après le début de la macération. L'isolement des protoplastes est alors de $2.7\ 10^5$ Pr./g T.F.

A partir de radicules
et d'hypocotyles au stade 3
L'isolement de protoplastes à partir de radicules nécessite l'utilisation d'une cellulase peu "agressive". Les essais pratiqués

ont permis de classer les cellulases suivantes par agressivité décroissante: la caylase T, la RS Onosuka, la drisélase et la caylase 345. Cette dernière à 0,5% a donné les meilleurs résultats et a permis d'isoler 4,2 10^5 Pr./g T.F.. Pour les hypocotyles, un isolement de 1,4 10^5 Pr./g T.F. a été obtenu avec la cellulase RS à 2%.

L'isolement des protoplastes à partir d'organes juvéniles (radicules, hypocotyles et cotylédons au stade 1) donne des résultats très inférieurs (de l'ordre de 10^5 Pr./g T.F.) à ceux des cotylédons matures (supérieur à 10^6 Pr./g T.F.). Pour ces derniers, les rendements sont comparables à ceux obtenus classiquement avec les mésophyles (Fig.3).

En définitive, malgré leur caractère récalcitrant à l'action des cellulases et pectinases, les organes juvéniles présentent un intérêt certain du fait de leur potentiel organogène. Ceci justifie cette étude de l'optimisation de l'isolement des protoplastes, à partir de ce type d'explants, dans une perspective de régénération.

Conclusion

Les essais sur la culture *in vitro* du *Sesbania rostrata*, qui viennent d'être exposés,

ont permis de mettre au point la technique de multiplication par microbouturage utilisant deux milieux: un milieu de débourrement des noeuds et un milieu d'enracinement permettant d'obtenir à la fois un bon taux de reprise et des petites plantes vigoureuses.

Une attention particulière doit être portée aux conditions d'éclairement (1000 à 1500 lux) et de température (25°C2 mesuré au niveau du récipient) ainsi qu'au mode de bouchage (qui doit permettre les échanges gazeux et éviter notamment l'accumulation d'éthylène). On évitera ainsi les problèmes de chlorose des tiges, les nécroses des jeunes pousses et l'apparition de cals sur les feuilles et les tiges.

Les résultats obtenus sur l'organogenèse d'organes juvéniles ont confirmé le bon potentiel organogène de ces explants. Ceux-ci seront utilisés pour la culture de protoplastes dont la finalité est la régénération.

Les essais sur les protoplastes d'organes juvéniles ont montré la difficulté d'obtenir, à partir de ce type d'explant, l'isolement d'un nombre de protoplastes comparable à celui provenant d'organes plus matures (cotylédons stade 4 ou méso-

phyles). L'étude sur l'optimisation de l'isolement des protoplastes d'explants juvéniles permettra d'aborder, dans des conditions favorables, la mise en culture qui doit conduire aux essais sur la régénération.

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Etude structurale de nodules caulinaires de *Sesbania rostrata* induits en présence d'azote combiné

A. Moudiongui [1], E. Duhoux [2] et G. Rinaudo [1]

Introduction

Il est bien établi que l'azote combiné peut inhiber les différentes étapes du processus conduisant à l'établissement d'une symbiose entre un *Rhizobium* et la légumineuse hôte. L'attachement aux poils absorbants, l'infection et la formation des nodules, sont inhibés en présence de doses élevées d'azote combiné (3, 5, 9).

Dans le cas de cultures hydroponiques de *Sesbania rostrata* la présence de concentrations élevées en azote combiné se traduit par une inhibition de la nodulation racinaire; cependant on observe des nodules caulinaires de petite taille dont l'activité fixatrice d'azote est très réduite (8).

La présente étude a eu pour objet d'analyser la structure de nodules caulinaires de *S. rostrata* induits en présence de différentes concentrations d'azote combiné, et d'évaluer leur activité fixatrice d'azote.

Matériel et méthodes

Les graines de *Sesbania rostrata* sont scarifiées et stérilisées par immersion pendant 30 mn dans l'acide sulfurique concentré. Elles sont ensuite abondamment rincées à l'eau distillée stérile, puis mises à germer sur eau gélosée (5 g/l d'Agar) en boîte de Pétri. Lorsque les plantules atteignent 1 à 2 cm de long, elles sont transférées dans des tubes de 22 x 220mm contenant environ 60 ml de milieu de culture. Le milieu utilisé est proche de celui de Betlenfalvay et Phillips (1); sa composition est la suivante (en g/l): K_2CO_3 0,23; K_3PO_4 $2H_2O$ 0,5; $MgSO_4$ $7H_2O$ 0,25; $FeCl_3$ 0,1; pH 6,8.

Les plantes sont cultivées en chambre de culture sous lumière artificielle (éclairement: 12000 lux; photopériode: 14 h d'illumination; température comprise entre 28°C et 32°C).

Les plantes sont tout d'abord maintenues pendant quatre semaines sur ce milieu additionné de 2 mM de nitrate de potassium. A compter de la cinquième semaine elles sont cultivées en présence de différentes concentrations d'azote (0, 3.0, 6.0 et 12 mM KNO_3), le milieu étant renouvelé quotidiennement; l'inoculation est effectuée au même moment par pulvérisation des tiges avec une culture d'*Azorhizobium* ORS 571.

Les mesures d'activité réductrice d'acétylène et l'étude structurale des nodules caulinaires sont effectuées 20 jours après l'inoculation.

Microscopie photonique

La fixation des nodules est effectuée par le mélange de Nawashine (7). Le matériel est ensuite rincé à l'eau courante pendant 24h, puis déshydraté durant 5 à 10 minutes dans une série de bains d'éthanol (de concentrations croissantes de 30 à 100% d'éthanol).

Après inclusion dans du Paraplast, on effectue au microtome des coupes longitudinales et transversales d'environ 10m d'épaisseur. Les coupes sont alors déparaffinées par chauffage modéré, réhydratées et colorées à l'hématoxyline de Régaud ou au Paragon (7).

Les lames sont ensuite déshydratées par traitement au toluène puis montées dans le baume du Canada.

Les coupes sont observées au photomacroscope Wild M 400 et au microscope Nikon Labophot.

Microscopie électronique

Des coupes ultrafines sont réalisées après fixation pendant 4 h dans du glutaraldéhyde en solution à 4% dans un tampon cacodylate de sodium à pH 7. Les échantillons sont ensuite fixés pendant 4 h par le tétroxyde d'osmium en solution à 1% dans un tampon cacodylate de sodium. Le matériel est inclus dans l'Epon.

Les coupes sont observées au microscope électronique Siemens Elmiskop 101 après contraste par l'acétate d'uranyle et le citrate de plomb (12).

Résultats

L'observation en microscopie photonique de coupes de nodules développés en l'absence d'azote combiné, montre que le tissu central (le méristème nodulaire) représente 40% environ du volume total du nodule (Fig. 1A). Au fort grossissement (Fig.1B) on remarque que le tissu central est constitué de grandes cellules envahies par des bactéroïdes.

En microscopie électronique (Fig-1C) on note la présence de nombreuses bactéries entourées d'une membrane péribactéroïde. L'organisation histologique de ces nodules développés en l'absence d'azote combiné, est donc conforme à la description de Duhoux (4).

Quand *Sesbania rostrata* se développe en présence de concentrations croissantes en azote combiné, on observe en premier lieu

une diminution de la taille des nodules (Fig. 2A, C et E).

A 3 mM KNO3, le tissu central ne représente plus que 25% environ du volume total du nodule (Fig. 2A). Au fort grossissement on observe que la taille des cellules du tissu central a diminué, et qu'une grande proportion de ces cellules ne contiennent pas de bactéroïdes (Fig. 2B). Ces effets sont encore plus marqués à 6 mM KNO3 (Fig. 2D) et 12 mM KNO3 (Fig. 2F). L'analyse en microscopie électronique confirme ces observations (Fig. 3A) et montre que la membrane péribactéroïde est partiellement détériorées (Fig. 3B).

En présence de fortes concentrations en azote combiné (12 mM KNO3), les cordons d'infection contiennent des bactéries dégénérescentes; les bactéries ne sont pas déversées dans le cytoplasme des cellules du tissu central (Fig. 4A, 4B).

L'activité réductrice d'acétylène des nodules est inhibée par l'azote combiné (tableau 1). En présence de 3 mM KNO3 le taux d'inhibition par rapport au témoin est de 35%. L'inhibition est totale en présence de 12 mM KNO3.

Discussion

L'azote combiné agit sur le développement et l'activité réductrice d'acétylène des nodules caulinaires de *Sesbania rostrata*. Le degré d'inhibition est d'autant plus marqué que la concentration en nitrate de potassium est plus forte (tableau 1). Lors d'une précédente étude, des résultats analogues ont été obtenus en utilisant du nitrate d'ammonium comme source d'azote (8).

On observe une bonne corrélation entre la diminution de volume du tissu central sous l'effet de concentrations croissantes en azote combiné et le degré d'inhibition de l'activité réductrice d'acétylène.

En présence de fortes concentrations en azote combiné (12 mM KNO3), on remarque la présence de cordons d'infection intracellulaires. Les premières étapes de la nodulation caulinaire de *S. rostrata* (la pénétration des bactéries dans les sites de nodulation, l'induction du méristème nodulaire et l'émission de cordons d'infection) paraissent donc insensibles à l'azote combiné. L'absence de bactéroïdes dans les cellules laisse supposer que la dernière étape du processus d'infection (la libération des bactéries du cordon d'infection dans le cytoplasme des cellules du méristème nodulaire) est bloquée par les fortes concentrations en azote combiné. Des observations analogues ont été faites par Truchet et Dazzo (13) avec des nodules de luzerne induits en absence d'azote combiné, puis développés en présence de 18 mM KNO3.

Par contre, au niveau racinaire, l'effet localisé de l'azote combiné se traduit par une inhibition des premières étapes de l'infection, notamment l'attachement des bactéries aux poils absorbants et la courbure de ces poils absorbants (2, 6, 10).

Ces résultats encore préliminaires devront être précisés par une étude en microscopie électronique plus détaillée. Cependant, en raison de sa double nodulation (caulinaire et racinaire) et la différence des modes d'infection impliqués (4,11), il apparaît que *Sesbania rostrata* constitue un modèle de choix pour étudier les effets de l'azote combiné sur la symbiose *Rhizobium*-légumineuse.

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KNO3 (mM/1)	Poids sec (mg nodules/plante)	Activité réductrice d'acétylène μmoles C2H4/plante/h
0	64,5 a	7,5 c
3	60,0 b	3,7 b
6	14,0 a	0,5 a
12	12,0 a	-

Tableau 1 : Effet de l'azote combiné sur le poids sec et l'activité d'acétylène des nodules caulinaires de Sesbania rostrata

Les valeurs suivies par une même lettre dans une colonne ne sont pas significativement différentes (test de Duncan, P = 0,05).
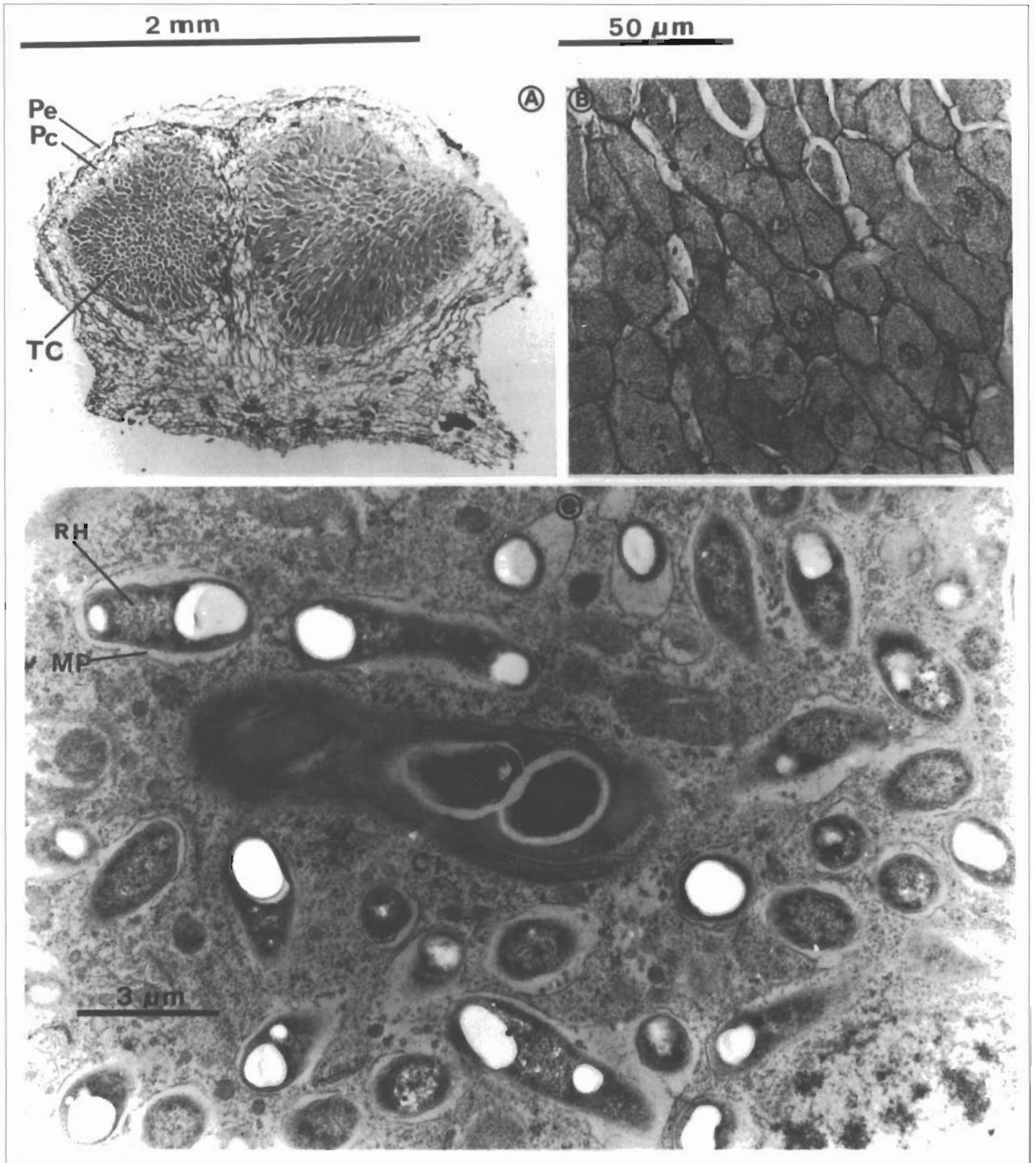
Fig 1 : Coupe d'un nodule caulinaire de Sesbania rostrata développé en l'absence d'azote combiné

A -   *Le tissu central (TC) bien développé représente environ 40% du volume total du nodule (Pe:périderme, Pc:parenchyme chlorophyllien).*
     MPH (microscopie photonique), x32.
B -   *Le cytoplasme des cellules du tissu central est totalement envahi par des bactéroïdes. MPH, x200.*
C -   *On observe des Azorhizobium (RH) entourés d'une membrane péribactéroïde (ME) (CI:cordon d'infection).*
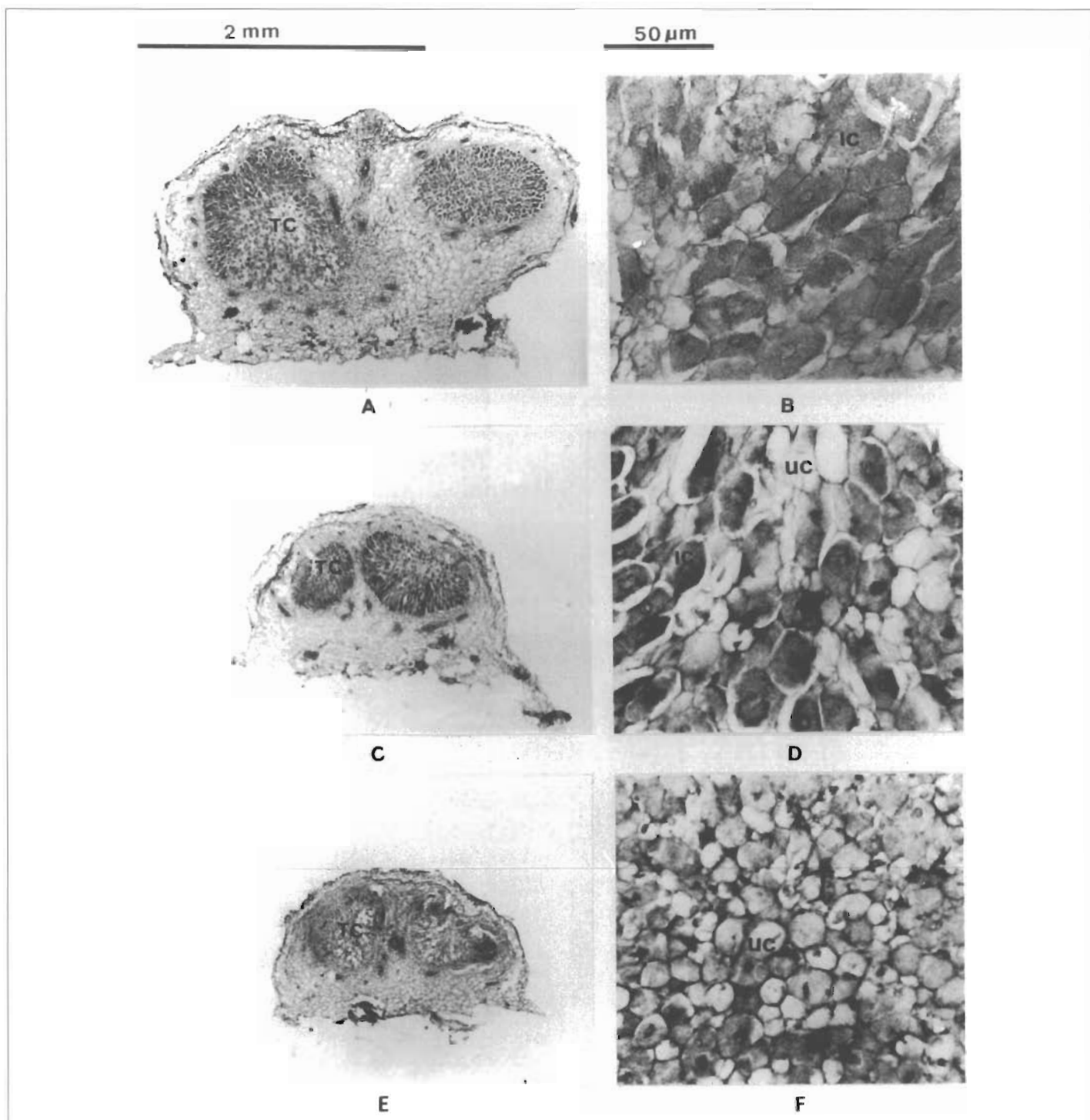   *MET(Microscopie électronique à transmission), x8000.*

Fig 2 : *Coupes de nodules caulinaires de Sesbania rostrata développés en présence de diverses concentrations d'azote combiné. La proportion de cellules envahies par Azorhizobium et leur taille, diminuent avec des concentrations croissantes en azote combiné (IC: cellules infectées, UC: cellules non infectées).*

A - *3 mM KNO₃: le tissu central est moyennement développé; il représente environ 25% du volume total du nodule MPH, x 32.*

B - *3 mM KNO₃: MPH, x 200.*

C - *6 mM KNO₃: on observe une diminution de la taille du nodule; le tissu central représente environ 20% du volume total du nodule MPH, x 32.*

D - *6 mM KNO₃: MPH, x 200.*

E - *12 mM KNO₃: on observe une diminution de la taille du nodule: le tissu central, clair par endroit, présente un contour mal défini MPH, x 32.*
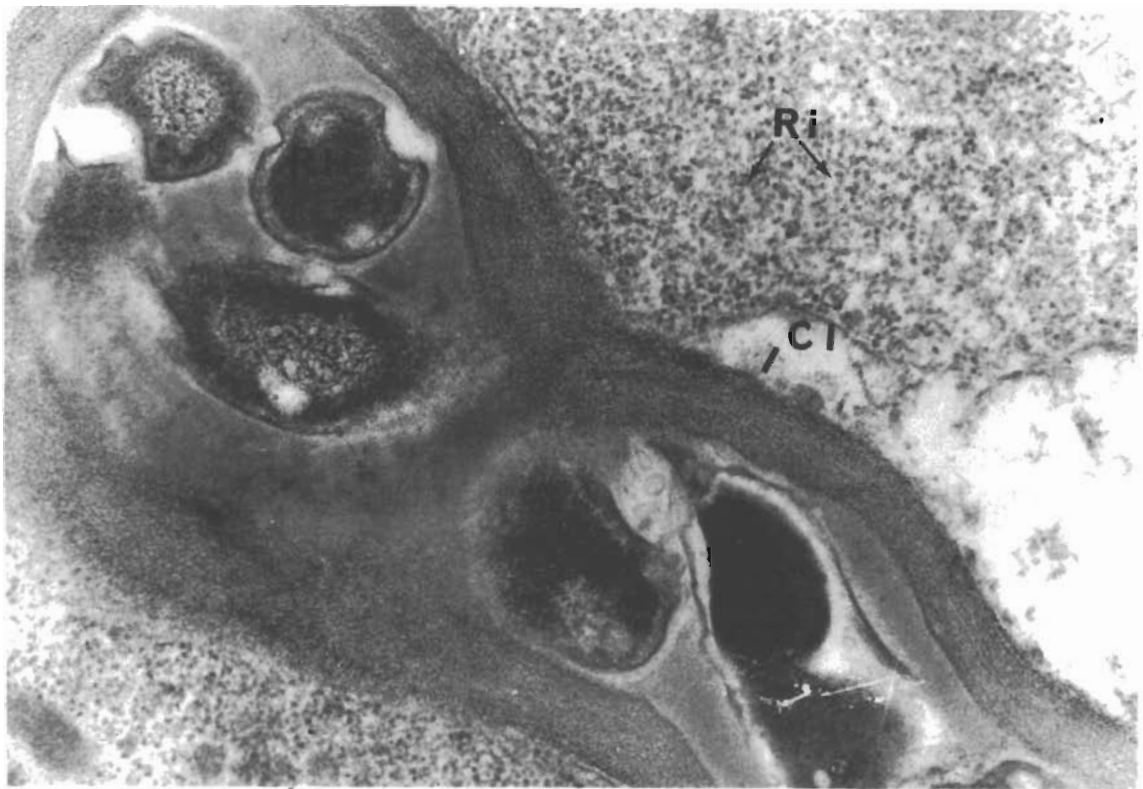
F - *12 mM KNO₃: MPH, x 200.*
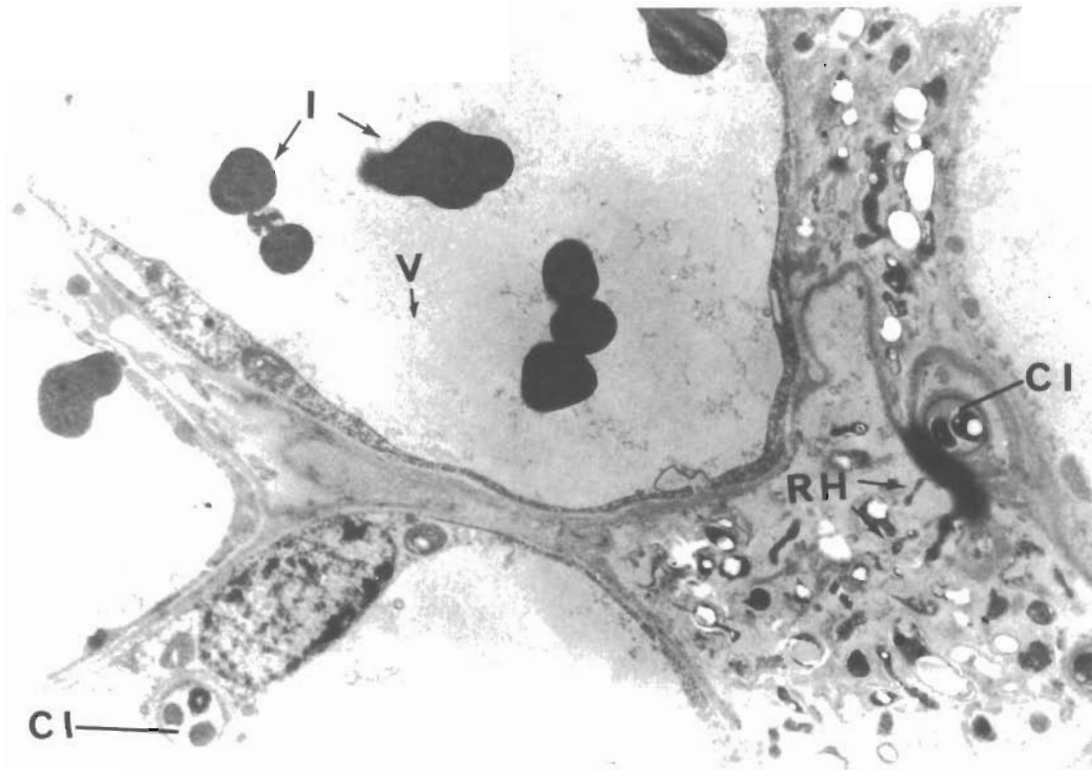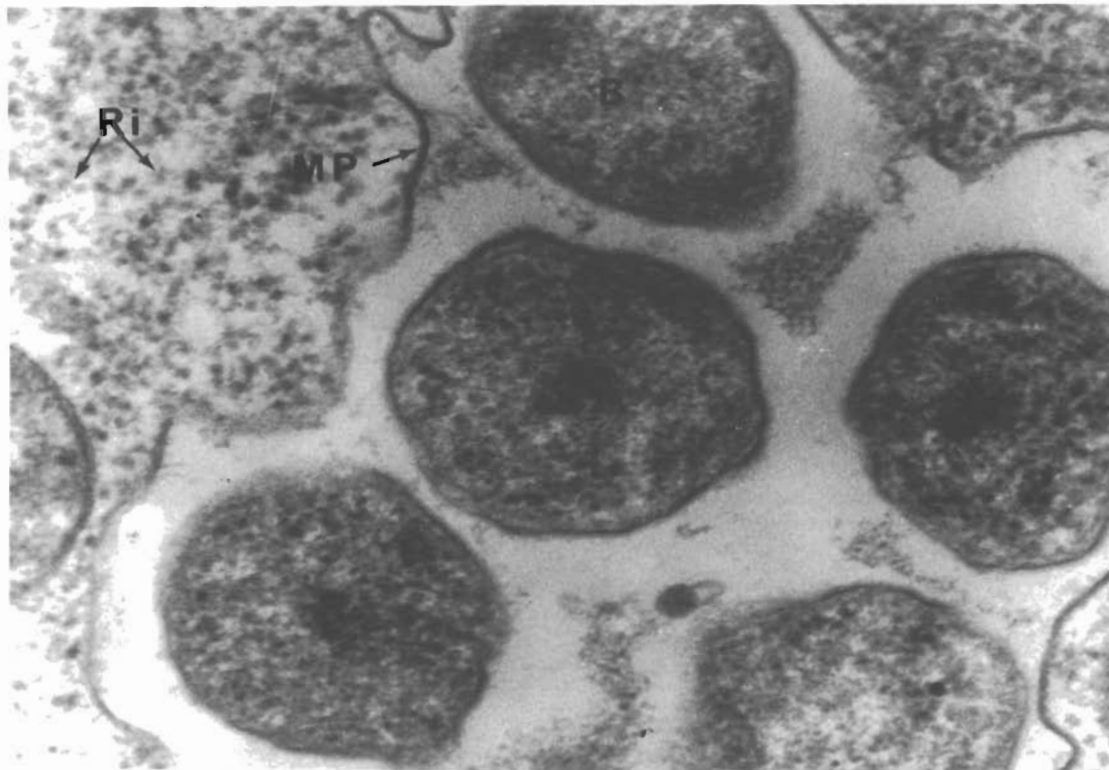
Fig 3 : Ultrastructure d'un nodule caulinaire développé en présence de 6 mM KNO₃.

A -   On observe des cellules non infectées contenant des inclusions denses aux électrons dans leur appareil vacuolaire (V). MET, x9000.
B -   Les bactéroïdes (B) sont entourés d'une membrane péribactéroïde (MP) aux contours mal définis (Ri:ribosomes). MET, x168000.
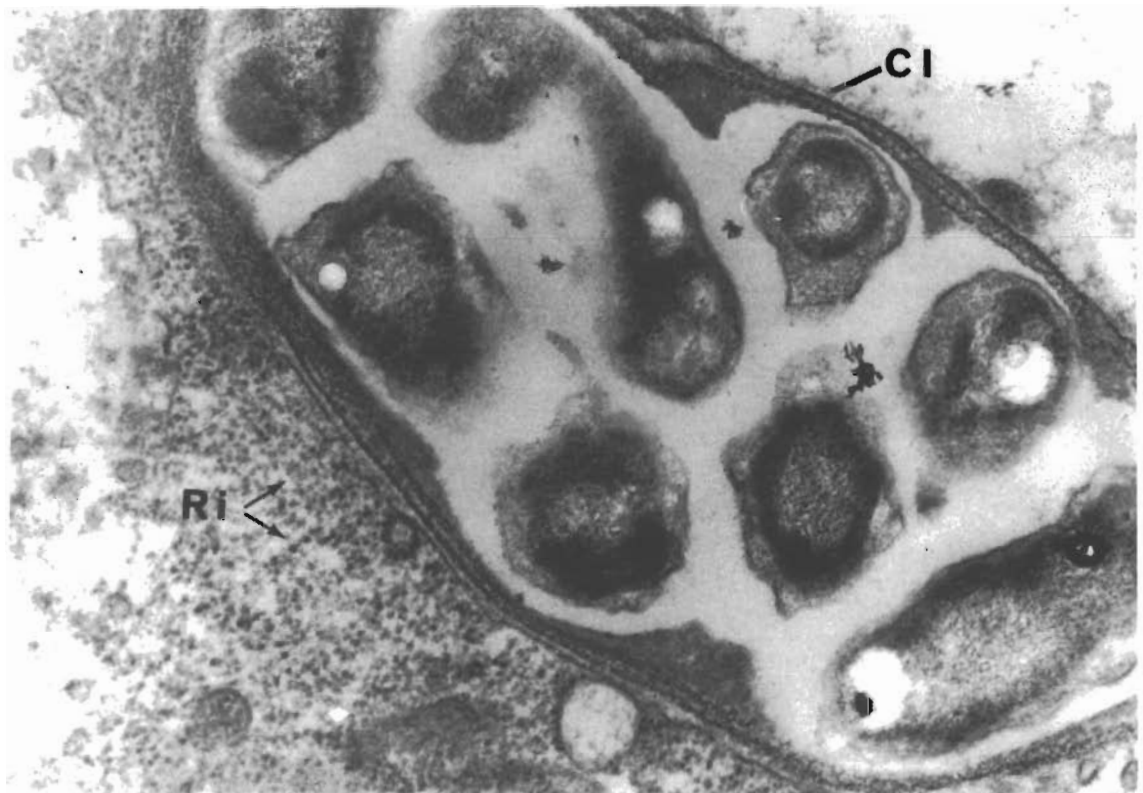
Fig 4 : *Ultrastructure de cordons d'infection de nodules caulinaires développés en présence de 12 mM KNO₃.*
On observe des bactéries dégénérescentes dans les cordons d'infection (CI). MET, x90000

Session V

Agricultural Applications

Future of stem-nodulated legumes in tropical agriculture

B.L. Dreyfus [1] and Y.R. Dommergues [2]

1. Introduction

With an expected world population of five billion by the year 2 000, the soils and other natural resources will be hard put to sustain increased yield sufficiently on land now being cultivated to meet the world's future food production needs (Brady, 1986), unless new technologies are developed to improve soil productivity.

The stem-nodulated legume technology could probably contribute to a durable nitrogen-fixing legume species produce nodules on their roots. Three genera have been reported to comprise species that form nodules on the roots and on the stems and thus are called stem-nodulated legumes (SNL viz. *Sesbania, Aeschynomene,* and *Neptunia*). So far, only one species in the *Sesbania* genus, *Sesbania rostrata,* which is native to Western Africa, has been reported to produce stem nodules. In Madagascar a *Sesbania* species similar to *Sesbania rostrata* - may be a subspecies - is known to bear stem nodules (Rakotovao, 1988). About 15 species of *Aeschynomene* are now known to have stem nodules, e.g., *A. afraspera, A. indica, A. nilotica, A. elaphroxylon* (Alazard, 1985). Several SNL (*Sesbania rostrata, Aeschynomene afraspera,* and *A. nilotica*) have an unusually high nitrogen-fixing, potential and a unique capacity to absorb combined nitrogen through their roots and, simultaneously, fix atmospheric nitrogen through their stem nodules, even when the soil is rich in mineral nitrogen (Dreyfus et al. 1984; Becker et al., 1986).

This paper focuses mainly on *Sesbania rostrata,* and to a lesser extent on *Aeschynomene afraspera* and *A. nilotica.* It considers the potential benefits of SNL, reasons for its restricted use, and improvements that could expand its field of application.

2. Nitrogen-fixing potential, biomass production and present uses

Extensive research on green manuring with *Sesbania rostrata* and, to a certain extent, with *Aeschynomene afraspera,* has been conducted in Africa and Asia. Very few studies were reported on the use of SNL in alley cropping systems and as much, compost or forage, which are probably interesting possibilities.

2.1 Nitrogen fixing potential

Between 1983 and 1987, several experiments in microplots and in 10-40 l containers were performed independently to assess the nitrogen-fixing potential, i.e. nitrogen fixed in the absence of limiting factors, of *Sesbania rostrata, Aeschynomene afraspera* and *A. nilotica.* Table 1 shows that nitrogen fixation by *Sesbania rostrata* ranged between 0.54 and 0.78 g N fixed per plant during the first 52-60 days of the plant cycle. In *Aeschynomene afraspera,* the amount of nitrogen fixed during the same period of time was similar.

Assuming a density of 140 000 plants/ha, which appears to be the optimal density, nitrogen fixation would be in the range of 70-140 kg/ha 50-60 days after planting.

In general the amount of nitrogen actually fixed in the field is much lower than the potential because of environmental constraints.

Consequently, improving nitrogen fixation by SNLs requires the reduction of the impact of limiting factors, either through managerial practices, a topic not dealt with here, or by attempting to make the symbiotic SNL system more tolerant to environmental stresses through genetic improvement.

2.2 Biomass production

The biomass produced by SNLs in the field is as variable as nitrogen fixation. Free of environmental constraints, 50 days after planting it can be as high as 0.80 g N/plant (Rinaudo and Moudiongui, 1985; N'Doye and Dreyfus, 1987). Conversely under severe environmental stress, the biomass can be as low as 0.05 g/plant (Rinaudo and Moudiongui, 1985; Saint Macary et al., 1985). In many situations, it is in the range of 0.20 - 0.30 g/plant (Saint Macary et al., 1985; Crozat and Sangchyo-Sawat, 1985). Since we know that the mean nitrogen percentage of aerial parts (stems plus leaves) of *Sesbania rostrata* is 1.8 - 2.2% (1) (Crozat et Sangchyo-Sawat 1985; Melin et al., 1987; Rinaudo et al., 1987), it is easy to calculate the biomass expressed as dry weight of the plant (40 g/plant in 50 days).

2.3 Use of SNL as green manure for rice

Field experiments have been carried out for the last 4-5 years mainly in Africa, but also in Asia (Table 2). Since the elementa-

(1) Detailed analysis of the different organs of *Sesbania rostrata* referred to in Mulongoy's paper (1986).

ry plots were usually small (1-25 m^2), the results obtained (expressed in g paddy/m^2) should not be indiscriminately extrapolated and applied to larger areas.

However, yield increases (often 100% over yields in the control plots which received non N fertilizer) in green-manured plots was substantial enough to confirm the value of green manuring with SNL. Results often appear equivalent to the application of 60-90 kg N fertilizer/ha.

Since *Sesbania rostrata* thrives in waterlogged soils (see 3.3), it may seem to excel as green manure for irrigated rice, but it appears to be exceptionally valuable as green manure for rainfed crops like rice (Vernier, 1988a) or sorghum (Moudiongui and Rinaudo, 1988).

The after effects of green manuring with *Sesbania rostrata* (and probably also with some other SNLs) may be quite substantial and enduring (Rinaudo et al., 1987), probably due to the added input of organic nitrogen, and also organic carbon into the soil, both of which contribute to the improvement of the soil's physical properties.

2.4 Other uses

Besides providing biomass for green manuring, SNL can be introduced in alley cropping systems or for mulching, composting, feeding animals and even producing fire wood (Basnet), (1988).

On a hydromorphic site at the IITA station, in southern Nigeria, *Sesbania rostrata* planted with rice in an alley cropping system proved to be a good source of nitrogen for the crop, since it was equivalent to the application of 120 kg fertilizer N/ha. Yields were 3 007; 4 477; 4 789 kg paddy/ha in control, green-manured and nitrogen fertilized plots respectively (Mulongoy, 1986; 1988).

Using *Sesbania rostrata* as a mulch on pineapple and banana plantations, and also on small farmers' crops, has been sugges-

Plant species	Experimental device	Plantage (days)	Nitrogen fixed (*)	Method (**)	Ref. (***)
S. rostrata	Microplot 1m^2	52	0.67	Bal.	(1)
S. rostrata	Container 10l	60	0.68	Differ.	(2)
S. rostrata	Container 10l	60	0.78	15N (****)	(2)
S. rostrata	Container 40l	52	0.59	Differ.	(3)
S. rostrata	Container 40l	52	0.54	^{15}N (****)	(3)
A. afraspera	Cylinder	70	1.20	Differ.	(4)
A. afraspera	Microplot 1m^2	49	0.70	Differ.	(4)
A. nilotica	Microplot 1m^2	49	0.90	Differ.	(4)

Table 1 : Estimation of nitrogen fixation by Sesbania rostrata, Aeschynomene afraspera and A. nilotica

(*)   Expressed in g N$_2$ fixed/plant
(**)   Methods of assessment of nitrogen fixation. Bal.: balance method; Differ.: difference method; ^{15}N: isotopic method.
(***)   References: (1) Rinaudo et al., 1983; (2) N'Doye and Dreyfus, 1987; (3) Rinaudo et al., 1987; (4) Alazard and Becker, 1987.
(****)   Percentage of nitrogen derived from fixation in the range of 38-43%.

Treatments					
Country	S.	Control	Green manuring	N fertilizer	Ref.
Thailand	4	340	415 (S. rostrata)	365 (80 kg N/ha)	(1)
Mali	4	415	603 (S. rostrata)	498 (100 kg N/ha)	(2)
Mali	4	401	600 (S. rostrata)	513 (100 kg N/ha)	(2)
Senegal	1	212	596 (S. rostrata)	381 (60 kg N/ha)	(3)
Senegal	1	415	1081 (S. rostrata)	784 (100 kg N/ha)	(4)
Senegal	2	5197	450 (S. rostrata)	230 (20 kg N/ha)	(5)
Senegal	?	230	450 (S. rostrata)	430 (60 kg N/ha)	(6)
Senegal	?	370	570 (S. rostrata)	670 (140 kg N/ha)	(6)
Cameroon	24	73	145 (S. rostrata)	147 (60 kg N/ha)	(7)
Senegal	1	488	881 (A. afraspera)	612 (100 kg N/ha)	(8)
Senegal	1	483	895 (A. nilotica)	612 (100 kg N/ha)	(8)

Table 2 : Effect of SNL green manuring on the rice yield expressed in g paddy/m^2 (irrigated rice fields except in Cameroon).

S.:   surface of elementary plots expressed as m^2;
(1) Crozat and Sangchyo-Sawat, 1986); (2) M. Lahbib (personal communication); (3) Rinaudo et al., 1983; (4) Rinaudo and Mondiongui, 1985; (5) Diack, 1986); (6) Camara and Diara, 1986; (7) Vernier, 1988; (8) Becker et al., 1986.
(*) Between brackets amount of N fertilizer applied.

ted by Melin et al., (1987) and Osseni et al. (1987), but has not yet been tried in agronomic experiments.

Composting SNL shoots to improve the soils is also an idea worth exploring (Ganry, 1988).

SNLs produce a nutritious forage, e.g., the average N content of the leaves of *Sesbania rostrata* is 4% (Melin et al., 1987). This resource has not yet been exploited (N'Doye, 1988).

3. Current limitations

The main drawbacks to using *Sesbania rostrata* are related to specific climate requirements, sensitivity to root-knot nematodes in well-drained soils, high water requirements, lack of competitivity against weeds and need for inoculation with the specific *Azorhizobium caulinodans* strain (Dreyfus et al., 1988).

Compared to other legumes, Crozat and Sanchyo-Sawat (1985), found that germination was not a problem, provided the seeds were treated chemically, scarified in a mortar by gently grinding them with coarse sand, or immersed into nearly boiling water and then left to cool for 10-12 hours (Dreyfus et al., 1985).

3.1. Climate requirements

At the latitude of Senegal (15N), *Sesbania rostrata* grows well during the hot rainy season (June to September); it usually behaves like an annual plant and flowers in the second or third month of its growth cycle with regular, profuse stem nodulation occurring soon (1 week) after inoculation. During the cold dry season (December to March), it grows poorly, flowers abnormally early, and nodulates with difficulty. In equatorial regions, regardless of the season, flowering is early and growth is limited. The reactions of *Sesbania rostrata* to climatic conditions may be related to sensitivity to day length, which is a typical photoperiodic response. However, other climatic parameters such as temperature, air humidity, and spectral composition of light, may also be involved. This aspect of the physiology of *Sesbania rostrata* deserves further attention.

3.2 Sensitivity to root-knot nematodes in well-drained soil

In well-drained soils, *Sesbania rostrata* roots are often severely attacked by root-knot nematodes, especially *Meloidogyne* sp., which seriously inhibit the plant growth. Infestations have been reported in several countries, e.g. Senegal, Ivory Coast (Table 3). In addition the increased pathogenic nematode populations attracted by *Sesbania rostrata* culture jeopardize the following crops.

In waterlogged soils, at least in flooded rice fields of Western Africa, where two nematode species, *Hirschmanniella oryzae* and *H. spinicauda*, are prevalent, the situation is quite different: in the presence of *S. rostrata*, the populations of these species of nematodes significantly decrease. Apparently *S. rostrata* acts like a trap-crop against *Hirschmanniella* sp. (Pariselle and Rinaudo, 1987).

3.3. High water requirements

Sesbania rostrata is reputed to be hydrophytic. This statement is based on two field observations. The first is that this legume grows best in waterlogged soils, its natural habitat in the Sahel being lowlands or temporary ponds formed during the rainy season. The second is that, in general, *S. rostrata* grows poorly in normally drained soils.

The following experiments indicate that this interpretation is incorrect, and it would be more accurate to say that *Sesbania rostrata* is much more tolerant to waterlogging than many other legumes.

In a pot experiment carried out in Thailand to demonstrate the effects of alternate flooding and dry periods on growth, nodulation and nitrogen contents of seven green manure legumes (*Desmodium tortuosum*, *Crotalaria juncea*, *Macroptilium lathyroides*, *Aeschynomene indica*, *Centrosema pubescens*, *Sesbania rostrata*, and *Vigna sinensis*) Apakupakul et al. (1986) found that in all of them, except *Sesbania rostrata* and *Macroptilium lathyroides*, and to a lesser extent *Aeschynomene indica* growth was markedly depressed by flooding.

A similar conclusion can be drawn from an experiment conducted in the Philippines by Saint-Macary et al. (1985) and more recently in Senegal by N'Doye and Dreyfus (1988) who found that *S. rostrata* was fairly tolerant to waterlogging, which only led to a 22% dry weight and 13%

		Meloidogyne			Cacopaurus	Pratylenchus
	Eggs	Larvae	Females			
Rhizosphère	220	60	2		35	1
Roots with galls	7710	940	50		4	0
Roots without galls	240	60	0		15	15

Table 3 : Number of nematodes (expressed per g fresh root) in the root system of Sesbania rostrata grown at Anguedougou Research Station, Côte d'Ivoire (Osseini et al., 1987).

Plants species	Water regime	Biomass expressed as	
		Dry w't (g/plant)	Total N (g/plant)
S. rostrata	Drained	41.3 ± 6.0	0.87 ± 0.12
	Waterlogged	32.0 ± 9.5	0.76 ± 6.21
S. sesban	Drained	29.2 ± 2.2	0.71 ± 0.07
	Waterlogged	14.2 ± 1.7	0.45 ± 0.04

Table 4 : Influence of two water regimes on the biomass of Sesbania rostrata and S. sesban (*) expressed as dry weight or total nitrogen 60 days after sowing (N'Doye and Dreyfus, 1987).

(*) Inoculated with strain ORS 571 (S. rostrata) and strain ORS 502 (S. sesban).

total N decrease in biomass loss from this treatment was 51% dry weight and 37% total nitrogen (Table 4). It is now clear that the poor growth of *Sesbania rostrata* in drained soils can be traced back to the presence of pathogenic nematodes, especially *Meloidogyne* that often proliferate in these soils.

In conclusion, when free of environmental stresses, *Sesbania rostrata* thrives in drained soils, but this in no way implies that its water requirements are low. In fact its high water requirements obviously is a problem in most of Africa, where water is always scarce.

3.4. Lack of competitivity against weeds

Weed removal is a traditional chore for the farmers but proliferating weeds may be difficult to control in plots with young *Sesbania rostrata* (Vernier, 1988; Diarra, 1988). If the use of herbicides appears to be unavoidable, *Sesbania rostrata* cultivars resistant to specific herbicides will be needed.

3.5. Need for inoculation

Because of the strict specificity of the *Sesbania rostrata-Azorhizobium caulinodans* symbiosis, stem inoculation is required in many countries, e.g., Cameroon (Vernier and Godefroy, 1987), Senegal (N'Doye and Dreyfus, 1988), West Indies (Melin, et al., 1987), Thailand (Crozat and Sanchyo-Sawat, 1985). This practice does not raise any specific difficulty since two satisfactory methods of inoculation are available: (i) spraying a liquid culture of *Azorhizobium caulinodans* on the stems, (ii) spraying a colloidal suspension of this *Azorhizobium* entrapped in alginate (Dreyfus et al., 1985). Using crushed nodules as an inoculant, which some authors suggest, should be avoided because of the danger of disseminating pests.

Infection of *Sesbania rostrata* by *Azorhizobium* is probably hindered when air humidity is too low. However, to the best of our knowledge, no systematic study has been initiated to explore this problem.

There may also be a need to inoculate stem-nodulating *Aeschynomene*, with specific strains of *Rhizobium* or *Bradyrhizobium* (Alazard, 1985).

4. Improvement of SNL symbioses

The two approaches recommended to improve nitrogen fixation in legumes and actinorhizal plant - one related to the symbiotic microorganism, the other to the host plant - should be used concomitantly. The host plant approach based on breeding for the desired traits to chance of the genetic nature of the plant might produce results rather rapidly.

4.1. Genetic improvement of the Rhizobium

In the case of SNL, nitrogen fixation is probably not limited by the nitrogen-fixing efficiency of the *rhizobium*, especially if *Azorhizobium caulinodans*, a *rhizobium* known for its exceptionally good performance, is involved.

In research on *rhizobium* improvement priority should be given to the selection or construction of strains more tolerant than wild ones to environmental constraints, especially high temperatures, drought, excessive salinity or alkalinity, pesticides.

Although classical selection techniques based on the screening of wild strains can be applied, modern molecular techniques (molecular cloning and recombination) are now available. New strains of *rhizobium* will be engineered to contain not only major genes involved in the symbiosis, but also other genes, especially genes encoding factors which enhance tolerance to environmental substances facilitating the establishment and functioning of the symbiosis, e.g., siderophores (J. Dilworth, personal communication) or indol acetic acid (Kaneshiro and Kwolek, 1985). Instead of attempting to improve the tolerance of effective strains to environmental stresses, research could focus on improving the symbiotic performances of bacteria whose genetic libraries already contain adaptation traits to prevailing environmental stresses (Roskoski, 1986).

In the case of *Sesbania rostrata*, the most desirable properties that are not inherent in wild strains are:

▲ tolerance to insecticides (whose chemicals may have to be mixed with the inoculum that is sprayed on the stems)

▲ tolerance to drought, a characteristic that would certainly improve the survival rate of the rhizobia in the soil, and possibly make it easier to infect the stems when air humidity is low.

It may be worthwhile developing *Azorhizobium* strains that can nodulate SNLs of the *Aeschynomene* group, which are host plants that are spontaneously associated with various *Rhizobium* and *Bradyrhizobium* strains (Alazard, 1985), whose efficiency may be lower than that of *Azorhizobium*.

4.2. Genetic improvement of the host plant

Three types of approaches seem appropriate:

▲ (i) The classical breeding approaches, based on the exploitation of natural plant variability or variability induced through mutation, using ionising radiations or other mutagens, such as ethylmethane sulfonate (EMS) (e.g. Gresshoff et al., 1985).

▲ (ii) The molecular engineering approach, the recombinant plant being altered to tolerate insects, diseases, or different environmental constraints, such as salt or drought (Van Montagu, 1988). However, insufficient knowledge on genes coding for specific useful traits and the absence of proper vectors limit the application of this technology.

Using vegetative propagation (Spencer Lopez Barreto, 1988) multiplication of improvement genotypes of *Sesbania rostrata* should be fairly easy.

4.2.1 Improvement of biomass production of SNL

Considering the concept that "nitrogen fixation appears to be a source rather than a sink-limited process and improved carbon nutrition is a key to increased nitrogen

input" (Hardy et al., 1974), breeding SNL for increased nitrogen fixation should primarily aim at breeding for increased foliage production.

Striking increases in *Leucaena leucocephala* biomass production have already been obtained as a result of simple improvement programs based on provenance trials (Brewbaker and Glover, 1987). A similar approach would probably also be successful in the case of SNL. More sophisticated methods, e.g., based on mutagenesis, would probably be more rewarding, but also be more time-consuming.

The molecular approach cannot be applied since progress in biochemical research does not yet allow us to identify the genes governing plant yields.

4.2.2 Breeding SNL
for adaptation to climatic conditions

To minimise the response of *Sesbania rostrata* to various climatic conditions, including day length and possibly other parameters, the best strategy may well be to exploit the natural or induced variability of the plant using approaches (i) and (ii) above.

4.2.3 Breeding *Sesbania rostrata*
for resistance to nematodes

The sensitivity to root-knot nematodes is certainly the Achilles' heel of *Sesbania rostrata*. This weak point, which seems to be common within the genus *Sesbania*, is a major defect indeed. It not only causes stunted growth of the plant, but also probably reduces nitrogen fixation by root nodules, as has been observed in many legumes, e.g. groundnuts (Germani et al., 1980), soybean (Baldwin et al., 1975). In the specific case of *Sesbania rostrata*, approach (iii) is the most promising. The transfer of genes coding for the synthesis of nematicidal compounds into the host plant genome should be feasible.

4.2.4 Reduction of water requirements

Breeding approaches (i) and (ii) are preferable to approach (iii), in solving the complex problem of reducing the plant's water needs. This situation was encountered in work on improving the yield of SNL.

4.2.5 Resistance to herbicides
Since it is now possible to engineer recombinant plants with herbicide resistance, approach (iii) can be used to develop new *Sesbania rostrata* genotypes with this trait.

4.2.6 Increased nutritional value
Approach (iii) could probably be used to construct new *Sesbania rostrata* with increased nutritional value, as already suggested by Van Montagu (1988).

5. Conclusion

To conclude, we would like to convey the following three messages:

▲ (i) The major weakness of most nitrogen-fixing, even symbiotic, systems, is that their contribution to the nitrogen pool of the agrosystems is often too low to ensure satisfactory yields of cereals or root crops which are known to "respond to levels of fertilizers far greater than those supplied by legumes" (Halliday, 1985). Fortunately SNL provide a way to increase the flux of nitrogen to the agrosystems. The input is remarkable, but SNLs can still be improved to contribute more to the nitrogen nutrition of crops and thus economically replace nitrogen fertilizers. This paper attempts to present characters of SNL which, in our opinion, should be selected or engineered to improve the nitrogen fixation in the field still further.

▲ (ii) Considering the unique properties of SNL, it seems worthwhile trying to transfer the stem nodulating ability to other legumes. This suggestion already made earlier (N'Doye and Dreyfus, 1988) would be specially valuable for three legumes, since stem nodulation would allow them to maintain their nitrogen-fixing activity in spite of the accumulation of nitrogen in the upper horizons of soils.

▲ (iii) For a strategic research topic such as the SNL symbioses, laboratory and fieldwork must be carried out with unity of purpose to avoid a paramount danger of the biotechnology age, the tendency to forget about the ecological

dimensions of phenomena under investigation (Fortin et al., 1986).

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Fixation d'azote par les légumineuses à nodules de tige : approche écologique et utilisation comme engrais vert

*G. Rinaudo et A. Moudiongui **

Introduction

L'azote est l'un des principaux facteurs susceptibles de limiter la productivité des sols. La révolution verte en riziculture est due pour l'essentiel à la sélection de variétés de riz répondant bien à la fertilisation minérale. Malheureusement, le coût des engrais, notamment des engrais azotés, limite souvent leur utilisation dans les pays en voie de développement.

La fixation biologique de l'azote peut, dans certains cas, apparaître comme une alternative intéressante aux engrais azotés minéraux (15). Cultiver une légumineuse en engrais vert avant le riz est probablement l'un des moyens les plus efficients d'améliorer la productivité des sols (7, 17, 18, 19). Toutefois, en raison du surcroît de travail qu'elle occasionne, une telle pratique ne peut être envisagée qu'à la condition d'utiliser une légumineuse à croissance rapide dont les potentialités fixatrices d'azote sont élevées et permettent d'augmenter les rendements en riz de façon significative.

C'est précisément le cas avec les légumineuses à nodules de tige *Sesbania rostrata* et *Aeschynomene afraspera*, qui fixent l'azote plus activement que la plupart des autres légumineuses et présentent en outre l'avantage d'être très tolérantes à l'engorgement du sol (14).

Des essais d'utilisation de ces légumineuses en engrais vert ont été effectués dans le cas de la culture irriguée du riz, mais aussi dans le cas d'une culture pluviale, le sorgho.

Potentialités fixatrices d'azote

La première estimation de la fixation d'azote par *Sesbania rostrata*, a consisté en un bilan d'azote effectué en 1980 à la station ORSTOM de Bel Air à Dakar (Sénégal), après une succession *S. rostrata* - riz en microparcelles de 1 m. Les résultats obtenus ont montré que *S. rostrata* avait fixé au moins 26 g N/m en 52 jours, ce qui est considérable (12). Ces premiers résultats ont été confirmés lors d'une autre expérience qui a consisté à estimer l'azote fixé au moyen de deux méthodes différentes, la méthode de dilution isotopique et la méthode par différence, les plantes étant cultivées sur sol engorgé. Par l'une et l'autre méthode la fixation d'azote par *Sesbania rostrata* a été estimée à environ 30 g d'azote/m avec des plantes âgées de 53 jours (13).

Effet de quelques facteurs de l'environnement

Facteurs climatiques

Les légumineuses à nodules de tige *Sesbania rostrata* et *Aeschynomene afraspera* se rencontrent en Afrique de l'Ouest dans des sols temporairement inondés. Il s'agit de plantes annuelles particulièrement sensibles à la photopériode et à la température, qui se développent bien en saison chaude et humide.

Les essais effectués tout au long de l'année 1983 à la station ORSTOM de Dakar, sont une bonne illustration de l'incidence des conditions climatiques sur le développement et l'activité fixatrice d'azote de *S. rostrata* (13). En particulier:

▲ la floraison intervient très rapidement (4 à 5 semaines après le semis) si la durée de l'insolation journalière est inférieure à 12 h.

▲ la fixation d'azote caulinaire est particulièrement sensible aux variations de température, la température moyenne optimale étant de 30C environ.

Voici à titre d'exemple, les résultats des mesures effectuées le 13 Septembre 1983 et le 3 Janvier 1984 sur des plantes âgées de 9 semaines:

Date	13/09/83	3/01/84
Température moyenne (°C)	29,5	25
Hauteur (cm)	213	35
Poids sec (g/plante)	94,5	3,1
Azote total (g/plante)	1,6	0,1
ARA (µmoles C_2H_4/plante/h)	171	18

Les conditions climatiques sont donc susceptibles d'avoir une incidence considérable sur le développement et l'activité fixatrice d'azote de *S. rostrata*.

Régime hydrique

Sesbania rostrata et *Aeschynomene afraspera* peuvent se développer et fixer l'azote sur sol engorgé. Nous avons remarqué que dans ces conditions, l'activité fixatrice d'azote de *Sesbania rostrata* était due essentiellement aux nodules de tige (13). La nodulation caulinaire constitue donc un

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avantage très important dans le cas d'hydromorphie excessive.

Saint Macary et al. (16) ont fait des observations analogues aux nôtres lors d'une étude concernant deux espèces de *Sesbania: S. rostrata* et *S. sesban*, cette dernière ne nodulant que sur les racines: la submersion du sol s'est avérée beaucoup plus préjudiciable à la nodulation de racines qu'à la nodulation de tige.

Azote minéral

L'azote combiné est susceptible d'inhiber la nodulation et de réduire l'activité de nodules préexistants. Ces effets sont moins marqués avec des nodules caulinaires qu'avec des nodules racinaires. Cette propriété a été notamment observée chez *Aeschynomene scabra* (5).

Les effets de l'azote minéral sur la fixation d'azote par *A. afraspera* ont été étudiés par Becker et al. (2), dans le cas de cultures hydroponiques et dans le cas de cultures en pots. Dans l'un et l'autre cas, la nodulation racinaire était fortement inhibée en présence de concentrations croissantes en azote combiné, alors que le développement et l'activité des nodules caulinaires n'étaient pratiquement pas affectés (Tableau 1).

Des résultats sensiblement différents ont été obtenus récemment dans le cas de plants de *S. rostrata* irrigués en continu au moyen d'un système de percolation automatique permettant de maintenir la concentration en azote du milieu à son niveau initial, tout au long de l'expérimentation (10).

Dans ces conditions on observe que le développement et l'activité des nodules caulinaires sont également sensibles à l'azote combiné, mais à des concentrations plus fortes que celles qui inhibent la nodulation racinaire (Tableau 2.)

Dans le cas de plantes cultivées sur sol, le comportement de *S. rostrata* vis à vis de l'azote combiné est analogue à celui d'*A. afraspera* le développement et l'activité des nodules caulinaires ne sont pratiquement pas affectés par un apport de 200 kg N/ha sous forme d'urée (9), en raison très probablement de la nutrition azotée de la

Nitrate de calcium	ARA (moles C_2H_4/plante/h)	
(mM/l)	tiges	racines
0	1,1 b	1,0 a
3	1,3 b	0,5 b
6	1,2 b	0,3 c
9	1,0 b	0,3 c
12	0,8 c	0,2 c
15	0,7 c	0,1 d

Tableau 1 : Effet de l'azote combiné sur la fixation d'azote (activité réductrice d'acétylene) d'Aeschynomene afraspera en culture hydroponique (2).

Les valeurs suivies par la même lettre dans une colonne ne sont pas significativement différentes (test de Duncan, p=0,05).
Les plantes sont été cultivées dans des tubes en verre de 125 ml et inoculées après 30 jours avec la souche Rhizobium ORS 322. La solution nutritive était renouvelée une fois par semaine jusqu'à l'inoculation, puis tous les deux jours. Les mesures ont été effectuées trois semaines après l'inoculation.

Nitrate d'ammonium	ARA (moles C_2H_4/plante/h)	
(µM/l)	tiges	racines
0	7,72 a	6,35 a
1,5	2,76 b	1,15 b
3,0	1,15 c	-
6,0	-	-

Tableau 2 : Effet de l'azote combiné sur la fixation d'azote (activité réductrice d'acétylene) de Sesbania rostrata irrigué en continu au moyen d'un système de percolation (10).

Les valeurs suivies par une même lettre dans une colonne ne sont pas significativement différentes (test de Duncan, p=0,05).
Les plantes ont été inoculées à 30 jours avec la souche Azorhizobium ORS 571. Ensuite elles ont été irriguées en continu avec un système de percolation automatique (apport de milieu nutritif à raison de 300 ml/pot/jour). Les mesures ont été effectuées trois semaines après l'inoculation.

plante, qui tend à diminuer rapidement la concentration en azote du milieu jusqu'à des valeurs compatibles avec l'activité des nodules caulinaires. On peut donc dire qu'en présence des doses d'engrais azotés

habituellement utilisées en riziculture (de l'ordre de 100 unités d'azote), il n'y a pas d'effet inhibiteur au niveau caulinaire: *S. rostrata* et *A. afraspera* assimilent à la fois l'azote de l'air et l'azote combiné.

Nématodes

En sol exondé, *S. rostrata* est particulièrement sensible à certains nématodes (*Meloïdogyne* en particulier). Leur présence éventuelle dans le sol peut constituer un facteur limitant majeur au développement de cette légumineuse.

Dans le cas de la culture irriguée du riz, par contre, *S. rostrata* peut jouer le rôle de plante piège vis à vis de nématodes appartenant au genre *Hirschmanniella, H. oryzae* et *H. spinicaudata* les nématodes phytoparasites du riz irrigué qui prévalent en Afrique de l'Ouest (6): on observe après culture de *S. rostrata* une diminution très significative du nombre de nématodes, ce qui se traduit par une augmentation des rendements en riz.

L'effet piège de *S. rostrata* vis à vis d'*Hirschmanniella oryzae* a été confirmé par Pariselle et Rinaudo (11) qui ont observé toutefois que l'augmentation des rendements en riz que l'on peut obtenir après enfouissement de *S. rostrata* est principalement due à l'effet engrais vert. Quoiqu'il en soit, l'utilisation de *S. rostrata* en riziculture est doublement bénéfique (effet engrais vert et effet nématicide).

Inoculation

Dans la nature, quand des symbiotes spécifiques sont présents dans le sol au sein de la microflore native, des nodosités apparaissent fréquemment au bas des tiges de *S. rostrata* et d'*A. afraspera*. Cette nodulation spontanée qui est probablement provoquée par des facteurs très divers (eau de submersion, pluies, insectes, vent, poussières ...) est souvent irrégulière. Il convient donc d'inoculer si l'on souhaite tirer le meilleur profit des potentialités fixatrices d'azote de ces légumineuses.

L'inoculation consiste en une pulvérisation des tiges avec une suspension du symbiote spécifique (10^8 bactéries/ml). Cette suspension peut être: (i) une culture diluée du symbiote, (ii) une suspension colloïdale obtenue en mélangeant de l'inoculum en

poudre (bactéries incluses dans un polymère d'alginate) à du tampon phosphate (0,06 M, pH 6,8), (iii) une suspension obtenue à partir de nodules écrasés dans de l'eau après passage à travers un filtre.

Il convient également de choisir judicieusement la date d'inoculation. Il est généralement conseillé d'inoculer 4 semaines avant la coupe de la légumineuse (14). La coupe est effectuée quand les plantes sont âgées de 8 à 9 semaines. L'inoculation doit donc avoir lieu 4 à 5 semaines après le semis. La taille des plantes est alors comprise entre 50 et 70 cm, et on obtient ainsi un très grand nombre de nodosités.

Utilisation des légumineuses à nodules de tige comme engrais vert

Riziculture irriguée

Les essais effectués à la station ORSTOM de Dakar en microparcelles de 1 m, ont mis en évidence l'intérêt des légumineuses à nodules de tige utilisées comme engrais vert avant une culture de riz au début de la saison des pluies (1, 12, 13).

Nous citerons, à titre d'exemple, les résultats d'expériences réalisées d'une part avec *S. rostrata* cultivé sur un sol Dior de texture sableuse (sol de Bel Air, Dakar). Dans l'un et l'autre cas, les rendements obtenus sur les parcelles témoins; ils sont également significativement plus élevés que les rendements obtenus avec 100 unités d'azote/ha apportées sous forme d'urée (Tableau 3).

Des collaborations ont été développées avec l'Institut Sénégalais de Recherches Agricoles (ISRA) et l'Association pour le Développement de la Riziculture en Afrique de l'Ouest (ADRAO), afin de préciser les possibilités d'utilisation des légumineuses à nodules de tige en riziculture irriguée, dans le cadre d'essais réalisés à plus grande échelle.

Les résultats obtenus à la station ISRA de Djibelor en Casamance, au sud du Sénégal, ont montré que l'engrais vert *S. rostrata* permettait de doubler les rendements

Traitement	Grains (g/m^2)	Azote total (g/m^2)	Talles (nb/m^2)	Augmentation des rendements en grain (%)
Expérience I (S. rostrata sur sol de Tiléne)				
Engrais vert	1080 a	17,24 a	402 a	160
Fumure minérale (100 N = urée)	784 b	11,99 b	347 b	89
Témoin	415 c	5,37 c	213 c	-
Expérience II (A. afraspera sur sol de Bel Air)				
Engrais vert	881 a	23,9 a	193 a	82
Fumure minérale (100 N-urée)	612 b	11,4 b	145 b	27
Témoin	483 c	9,0 c	123 b	-

Tableau 3 : Utilisation des légumineuses à nodules caulinaires Sesbania rostrata et Aeschynomene afraspera comme engrais vert pour le riz.
Essais en microparcelles de 1 m.

Traitement	Grains (t/ha)	Pailles (t/ha)	Talles (nb/m^2)	Augmentation des rendements en grain (%)
Engrais vert	4,50 a	12,36 a	465 a	96
Matière organique *	2,92 b	7,68 b	318 b	27
Témoin	2,30 b	5,93 b	301 b	-

Tableau 4 : Utilisation de Sesbania rostrata comme engrais vert pour la culture irriguée du riz en Casamance, Station ISRA de Djibelor, parcelles de 25 m (4).

Les valeurs suivies par une même lettre dans une colonne ne sont pas significativement différentes (test de Duncan, p=0,05).

* *Matière organique: mélange fumier + compost.*

Traitement				
Urée (kg N/ha)	Engrais vert	Grains (t/ha)	Pailles (t/ha)	Talles (nb/m^2)
0	-	2,3 a	2,5 a	238 a
60	-	4,3 b	5,5 b	310 b
120	-	6,2 bc	7,6 b	363 b
0	+	4,5 b	5,7 b	325 b
30	+	5,9 bc	7,6 b	405 b
60	+	6,8 c	7,7 b	400 b

Tableau 5 : Utilisation de Sesbania rostrata comme engrais vert pour la culture irriguée du riz dans la Région du fleuve Sénégal. Station ADRAO de Fanaye, parcelles de 25 m (3).

Les valeurs suivies par les mêmes lettre dans une colonne ne sont pas significativements différentes (test de Duncan, p = 0,05).

Traitement	Grains (g/m^2)	Pailles (g/m^2)	Augmentation des rendements en grain (%)
Engrais vert	525 a	1610 a	79
Fumure minérale (150 N = urée)	522 b	1685 a	87
Témoin	260 b	900 b	-

Tableau 6 : Utilisation de Sesbania rostrata comme engrais vert pour la culture du sorgho. Essai en parcelles de 4 m (9).

Les valeurs suivies par une même lettre dans une colonne ne sont pas significativement différentes (test de Duncan, p = 0,05).

en riz (par rapport au traitement témoin sans azote) et avait un effet plus marqué que l'apport d'un mélange "compost + fumier" (Tableau 4) (4).

De même, l'essai effectué à la station ADRAO de Fanaye, au nord du Sénégal, a montré que l'on pouvait économiser plus de 50% des quantités d'engrais azotés préconisées pour la culture irriguée du riz dans la vallée du fleuve Sénégal, en associant fertilisation azotée minérale et engrais vert (Tableau 5) (3).

Culture du sorgho

L'essai réalisé à la station ORSTOM de Dakar en parcelles de 4 m a montré (Tableau 6) que *S. rostrata* peut également constituer un excellent engrais vert dans le cas d'une culture exondée, la culture du sorgho: les rendements obtenus après enfouissement de 15 kg de matière verte par parcelle (37,5 t/ha), sont équivalents à ceux que l'on obtient avec un apport de 150 unités d'azote sous forme d'urée: les rendements observés sont environ deux fois supérieurs à ceux des parcelles témoin (9).

Les résultats de cet essai préliminaire ont été confirmés dans le cadre d'un essai mis en place à Balandine, village de Casamance au sud du Sénégal, avec la collaboration du groupement des jeunes paysans du village: l'engrais vert *S. rostrata* a provoqué des augmentations de rendements significativement plus fortes qu'un apport de 100 unités d'azote sous forme d'urée (9).

Conclusions

Des résultats extêmement encourageant ont été obtenus en Afrique de l'Ouest avec les légumineuses à nodules de tige *S. rostrata* et *A. afraspera* utilisées comme engrais vert en riziculture au début de la saison des pluies. Elles peuvent produire environ 10 t/ha de matière sèche en 8 à 9 semaines, ce qui correspond à plus de 200 kg N/ha. Si l'on considère que près de 50% de cet azote provient de la fixation biologique de l'azote, c'est donc environ 100 kg N/ha que ces légumineuses peuvent fournir à une culture de riz. Des résultats analogues aux nôtres ont été obtenus aux Philippines à l'IRRI (International Rice Research Institute) au cours de ces dernières années (8).

Toutefois, la mise en évidence des potentialités de ces légumineuses en tant que source d'azote, aussi exceptionnelle soient-elles, ne peut interesser la riziculture que si l'on précise au préalable leurs limites. Avant de les utiliser dans une région rizicole donnée, il conviendra en particulier de déterminer l'effet des principaux facteurs de l'environnement sur leur développement et de définir un calendrier cultural qui tienne compte des exigences particulières de la légumineuse et du riz ainsi que des disponibilités en eau (pluviométrie et/ou irrigation).

Les expériences réalisées avec le sorgho, ont montré que les légumineuses à nodules de tige peuvent également constituer un excellent engrais vert dans le cas de plantes de culture pluviale. Il est apparu également que des groupements villageois sont susceptibles d'adopter cette pratique

de l'engrais vert à base de légumineuse, pour accroître la productivité du sorgho ou de tout autre culture céréalière pluviale.

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Utilisation de *Sesbania rostrata* comme engrais vert et fourrage

I. N'Doye *

Introduction

L'eau et l'azote constituent deux des facteurs majeurs régissant la production agricole. Les exigences en azote des plantes cultivées sont considérables. C'est ainsi que pour le riz, aliment de base de près de la moitié de la population mondiale, il a fallu accroître la consommation des engrais azotés et phosphatés pour faire face à une demande de plus en plus forte. Pour produire 100 kg de grains, le riz "consomme" de 1,8 à 2 kg d'azote quelque soit le type de sol ou la date de plantation (Patnaik et Rao, 1979). Cependant, l'emploi des engrais azotés est souvent limité par leurs prix élevés, sans cesse croissants, incompatibles avec les ressources financières de la majorité des paysans des pays en voie de développement. La fixation biologique de l'azote constitue une alternative attrayante et de moindre coût. L'enfouissement dans le sol, des parties aériennes d'une plante fixatrice d'azote et les résidus végétaux en résultant jouent alors le rôle d'engrais azoté organique susceptible de se substituer aux engrais minéraux. Le nom d'engrais vert est donné à cette pratique.

L'utilisation des légumineuses comme engrais vert en vue d'améliorer les rendements des rizières est une pratique ancienne, en particulier en Asie du Sud-Est et en Inde où de nombreuses légumineuses sont utilisées, *Aeschynomene americana, Aeschynomene indica, Astragalus sinicus, Crotalaria juncea, Crotalaria stricta, Indigofera tinctoria, Lablab purpureux, Medicago hispida, Medicago officinalis, Vicia officinalis, V. cracca* et plusieurs espèces du genre *Sesbania* qui ne portent pas de nodules sur la tige, *Sesbania aculeata, S. cannabina, S. sesban, S. paludosa* (Vachhani et Murty, 1964; Patnaik et Rao, 1979; Watanabe et App., 1979; Allen et Allen, 1981; Singh, 1984; Roger et Watanabe, 1986; Brewbaker et Glover, 1988).

Des progrès ont été enregistrés dans le domaine des engrais verts avec la découverte des légumineuses comme *S. rostrata*, dont la double nodulation conduit à l'obtention de nodules 5 à 10 fois plus nombreux que chez la plupart des autres légumineuses (Dreyfus et Dommergues, 1981). Cela se traduit par une capacité de fixation d'azote très supérieure à celle par exemple, d'autres espèces asiatiques de *Sesbania* utilisées jusqu'alors comme engrais vert. *S. rostrata* ayant l'aptitude à pousser dans de sols submergés pouvait donc être utilisée comme engrais vert en riziculture.

Testée comme engrais vert, en parcelles de 1 m^2 ou 25 m^2, *S. rostrata* a permis de doubler le rendement en riz par rapport à la manière traditionnelle et permet l'obtention de riz dont la teneur en protéines est accrue de 37 à 50% par rapport aux teneurs habituelles (Rinaudo et al., 1982; Dreyfus et al., 1985; Rinaudo et Moudiongui, 1985; Diack, 1987).

Des expériences conduites en 1985-1986 aux Philippines à l'IRRI (International Rice Research Institute) ont montré que *S. rostrata* était la légumineuse la plus performante parmi les espèces testées comme engrais vert. Son incorporation au sol de la rizière peut apporter l'équivalent de 120 kg N par hectare (Ladha et al., 1988).

Parallèlement à ses propriétés d'engrais vert, *Sesbania rostrata* est très utilisée comme fourrage vert donné aux animaux et en particulier aux bovins. L'étude de la valeur fourragère de *S. rostrata* n'ayant jusqu'à présent jamais été abordée, il nous a paru utile d'envisager son utilisation comme fourrage en introduisant sa production parmi celle d'autres plantes fourragères dans la nouvelle zone économique du fleuve Sénégal, zone naturelle d'élevage. De telles productions fourragères devraient donc conduire à une amélioration de la nutrition animale et par voie de conséquence à une augmentation de la production de viande et de protéines animales. Il en résulterait une augmentation de la consommation par tête d'habitant, actuellement faible au Sénégal.

Dans un premier temps, l'utilisation de *Sesbania rostrata* comme engrais vert est envisagée avec pour objectifs la réduction des lourdes importations de riz par le Sénégal et l'amélioration des rendements de rizières permettant l'autonomie alimentaire du pays.

Des essais en milieu paysan ont eu pour but de tester la validité de la démarche, d'identifier les éventuelles contraintes liées à l'introduction de cette biotechnologie dans les habitudes et pratiques culturales des agriculteurs, et enfin éventuellement de trouver des solutions aux problèmes liés à l'emploi des engrais verts. Un projet utilisation de *Sesbania rostrata* comme engrais vert a été mené en Casamance (Sud du Sénégal), région de riziculture traditionnelle où les rizières présentent de grave pénuries en azote. Les expériences réalisées ont été conduites en liaison avec l'ORSTOM (Institut Français de Recherche Scientifique pour le Développement en Coopération), l'ISRA (Institut Sénégalais de Recherches Agricoles) et l'Université de Gand en Belgique.

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Dans un deuxième temps, l'utilisation de *Sesbania rostrata* comme plante fourragère pour les animaux a été envisagée. En effet, la connaissance de la valeur alimentaire des fourrages revêt une importance particulière au Sénégal où ils entrent dans la composition de différentes rations. Les fanes en général, particulièrement celles d'arachide et de niébé sont très recherchées. Récoltées avec soin, elles entrent, en partie ou en totalité, dans la composition de rations d'embouche ovine ou bovine (Roberges et al., 1984).

L'utilisation de *Sesbania rostrata* comme plante fourragère est menée depuis 1985, en étroite liaison avec le L.N.E.R.V. (Laboratoire National de l'Elevage et des Recherches Vétérinaires).

Résultats

1. Influence de *S.rostrata* utilisée comme engrais vert sur le rendement en riz

1.1 Essais en parcelles expérimentales

Les résultats obtenus en 1985 montrent une très bonne croissance des plantes de *S. rostrata* ainsi qu'une nodulation de tige importante. Au bout de 6 semaines, *S. rostrata* peut ainsi atteindre une hauteur moyenne de deux mètres et produire une biomasse de 37 t/ha. La croissance de *S. rostrata* illustrée sur la Figure 1 peut, dans la meilleure période c'est à dire après 3 semaines de croissance, atteindre 10 cm par jour.

L'apport azoté de l'engrais vert durant 6 semaines de croissance est équivalent à environ 150 kg/ha (Tableau 1). L'inoculation de *A. afraspera*, légumineuse tropicale à nodulation caulinaire n'a pas donné satisfaction en comparaison de la masse de matière organique enfouie 27 t/ha.

Le rendement du riz en grains, exprimé par hectare a été évalué respectivement à 2,0 t dans la parcelle témoin, à 2,9 t dans les parcelles ayant reçu de l'engrais azoté et à 4,9 t dans le cas ou *S. rostrata* est utilisée comme engrais vert (Tableau 1). Le rendement dans les parcelles traitées avec *S. rostrata*, 2,4 t/ha, correspond à un gain de

rendement de sulement 20% par rapport au témoin.

1.2 Essais menés par les paysans

Les dispositifs utilisés, plus simples, se résument à des parcelles avec l'engrais vert *S. rostrata* et des parcelles témoins sans engrais vert. Ces expériences se sont étendues sur 4 années, 1986, 1987, 1988 et 1989. Les *Sesbania* ont été enfouies 5 semaines après le semis en 1986 et 1987 et seulement après 4 semaines en 1988.
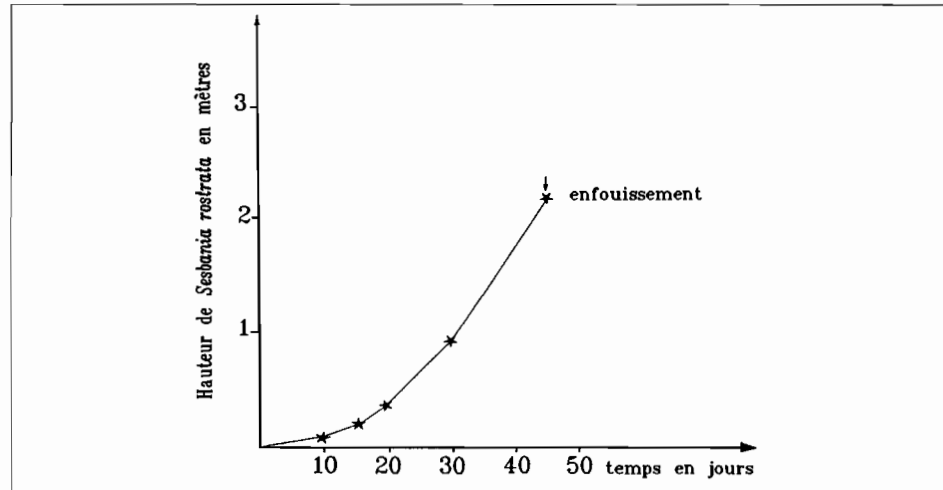

Figure 1. Croissance de Sesbania rostrata avant enfouissement.

Traitements	Biomasse	Equivalent azote total	Rendements	
	t/ha	kg/ha	kg/40m²	t/ha
Sesbania rostrata (engrais vert)	37	153	19,83 b	4,9 b
Aeschynomene afraspera (engrais vert)	27	-	9,51 a	2,4 a
Fumure azotée (60 kg N/ha)			11,55 a	2,9 a
Témoin (sans engrais vert)			8,21 a	2,0 a

Tableau 1 : Influence de Sesbania rostrata comme engrais vert sur le rendement du riz (grains): Essais sur parcelles expérimentales (1985).

Les chiffres (moyenne de 8 répétitions) affectés des mêmes lettres ne sont pas significativement différents au seuil de 5% (test de Newman et Keuls).

Traitements	Rendement t/ha			
	Fanghote		Bayottes	
	1986 (1)	1987	1988	1987
Sesbania rostrata (engrais vert)	3,2	4,3	3,4	2,5
Témoin (sans engrais vert)	1,7	2,2	2,1	1,3

Tableau 2 : Influence de S. rostrata comme engrais vert sur le rendement du riz: Essais Paysans.

(1) La Biomasse de l'engrais vert est de 25 t/ha et l'équivalent en azote enfoui de 110 kg/ha.

▲ En 1986, les essais ont été effectués sur une surface de 3000 m. Le rendement du riz est passé de 1,7 t/ha dans la parcelle témoin à 3,2 t/ha dans la parcelle ayant reçu l'engrais vert, soit un gain de rendement de 85% (Tableau 2). La biomasse produite et enfouie est d'environ 25 t/ha et correspond à plus de 100 kg N/ha.

▲ En 1987, à Fanghote, les essais ont été effectués sur une surface beaucoup plus grande de 10.000 m (1 hectare). A cours de cette année, la nodulation spontanée des tiges de *S. rostrata* étant limitée à la base de la tige, l'inoculation de la souche ORS 571 a été réalisée afin que les plantes puissent exprimer totalement leurs capacités de nodulation et de fixation. Dans ces conditions le rendement en riz s'est avéré de 2,2 t/ha dans la parcelle témoin à 4,3 t/ha dans la parcelle témoin à 2,5 t/ha dans la parcelle avec engrais vert.

▲ En 1988, à Fanghote la surface de la parcelle a été de 1000 m. Cette année là, aucune inoculation n'a été jugée nécessaire du fait de la précense d'*Azorhizobium* dans le sol. Le rendement de 2,1 t/ha dans la parcelle témoin, à 3,4 t/ha dans la parcelle avec engrais vert, a montré un gain de 62% (Tableau 2).

2. Utilisation de *S. rostrata* comme fourrage

2.1 Productivité

Afin de mesurer la productivité de *S. rostrata*, des essais de rendement ont été réalises en saison des pluies en sol exondé argilo sableux à Sangalkam dans la région de Dakar. Les résultats obtenus montrent que *S. rostrata* a une bonne productivité,

Traitements	Biomasse t/ha
S. rostrata	25 a
S. sesban	12 b

Tableau 3 : Productivité de Sesbania rostrata comme fourrage après 45 jours de croissance à Sangalkam.

Les chiffres (moyenne de 6 répétitions) affectés des mêmes lettres ne diffèrent pas au seuil de 5% (test de Newman et Keuls).

Constituants	Composition (g/kg MS)			Digestibilité des constituants de la plante entière
	Foin	Tiges	Feuilles	
Cendres	87	63	158	
Matière organique	913	937	842	54,7
Matières azotées totales	163	115	305	75,3
Cellulose brut	424	528	114	36,6
Extrait éth éré	17	9	42	-
E.N.A.	309	285	381	62,6
N D F	610	751	186	38,8
A D F	500	615	155	39,8
Lignine	98	118	38	0

Tableau 4 : Teneur en cendres, composition de la matière organique et digestibilité des constituants (analyses faites à l'IEMVT, Maisons-Alfort, responsable H. Geurin).

Matière sèche du foin distribué: 879 g/kg brut.
Digestibilité de la matière sèche: 52,2.
Consommation de matière sèche/jour: 38,1 g/kg $P^{0,75}$ soit 426 g pour un mouton de 25 kg.

	Plante entière	Tiges	Feuilles
Cendres totales (g/kg MS)	87,0	63,0	158,0
Insoluble chlorhydrique "	12,5	3,0	40,5
Ca (gr/kg MS)	8,30	3,55	13,7
P (gr/kg MS)	3,15	3,05	3,45
K (gr/kg MS)	18,20	17,60	19,80
Mg (gr/kg MS)	1,87	1,35	3,40
Na (ppm/kg MS)	2712,0	2861,0	2264,0
Cu (ppm/kg MS)	7,54	6,75	9,9
Zn (ppm/kg MS)	35,3	29,5	52,9
Mn (ppm/kg MS)	61,8	24,3	174,2
Fe (ppm/kg MS)	537	140	1730
Co (ppm/kg MS)	0,25	0,13	0,63

Tableau 5 : Composition minérale du Sesbania rostrata.

25 t de matière verte par ha pour une période de 45 jours en comparaison de *S. sesban* qui ne produit que 12 t (Tableau 3). En outre *S. rostrata* est assez prolifique du point de vue grainier. Coupé à 30 cm du sol, *S. rostrata* fournit en effet des rejets permettant une deuxième coupe. Les rendements obtenus sur deux coupes sont de 3,5 à 4 t de matière sèche alors que le niébé fourrager donne des rendements de 4,5 t et les fanes d'arachide, 1,3 t (Roberges et Faye, 1983).

2.2 Valeur alimentaire de *S. rostrata*

Les études de digestibilité, d'ingestibilité et des analyses bromatologiques ont été réalisées sur du foin de *S. rostrata*. Les résultats sont représentés sur les Tableaux

4 et 5. La Tableau 4 montre ainsi une teneur de la matière sèche du fourrage distribué de 879 g/kg brut, une digestibilité de 51-53% et une ingestibilité de 38 g de MS/jour et par kg de poids métabolique ou 1,7 kg pour 100 kg de poids vif. Par comparaison, les fanes d'arachide vulgarisées offrent une digestibilité de la matière sèche de 56% et une ingestibilité de 75-100 g par kg de poids métabolique équivalent à plus de 3 kg de MS/jour pour 100 kg de poids vif (Roberges et Perrot, 1987); la consommation moyenne normale étant de 2,5 kg de MS/jour (Chenost, 1972). Pour le niébé fourrager cette consommation est d'environ 76 g/kg de poids métabolique (Milford et Minson, 1965).

Besoins des animaux			Fanes d'arachides
Ca	2	g/kg MS	8
P	1,2	g/kg MS	1,2
K	10	g/kg MS	13
Mg	2	g/kg MS	4,8
Na	0,8	(mg/kg MS)	
Cu	10	(mg/kg MS)	
Co	0,1	(mg/kg MS)	
Mn	40	(mg/kg MS)	
Fe	8	(mg/kg MS)	
Zn	50	(mg/kg MS)	

Tableau 6 : Besoins des animaux en éléments minéraux des plantes consommées
(Quittet, 1969) et compositions minérales de fanes d'arachide (Rivière, 1978).

On notera chez *S. rostrata*, une digestibilité moyenne de la matière organique et une bonne digestibilité des matières azotées totales conformes à la teneur élevée en MAT de la plante entière (Tableau 4).

Le Tableau 5 donne la comparaison de la teneur en éléments minéraux de *S. rostrata*. Comparé aux besoins des animaux (Quittet, 1969) et à la teneur des fanes d'arachides (Rivière, 1978) (Tableau 6), l'apport de *S. rostrata* en éléments minéraux apparaît donc satisfaisant.

Discussion et conclusion

Les résultats obtenus au cours des 4 dernières années montrent que *S. rostrata* constitue un engrais vert tout à fait exceptionnel dans les sols inondés et en particulier dans les rizières. Dans tous les cas l'apport d'engrais vert a été supérieur à 100 kg d'azote à l'hectare. Les rendements du riz ont doublé dans les conditions de culture en milieu paysan. Ces résultats ont, depuis, été confirmés dans de nombreux pays et en particulier en Asie du Sud Est, aux Philippines, et en Inde où l'apport en azote de *S. rostrata* est du même ordre de grandeur (Ladha et al., 1988). L'apport d'engrais doublé d'un apport de matière organique et probablement d'une diminution de la population de nématodes phytoparasitaires, a donc permis de doubler les rendements du riz sans avoir recours aux engrais chimiques dont le coût élevé est incompatible avec les revenus modestes des paysans de ces pays.

Au Sénégal, *Sesbania rostrata* dont l'effet engrais vert est désormais reconnu, a toutefois du mal à être intégrée dans les pratiques culturales paysannes et être utilisée à grande échelle en riziculture. Plusieurs problèmes demeurent, contrairement aux paysans asiatiques qui, traditionnellement utilisent les engrais verts dans la riziculture, les paysans africains, en général et les sénégalais en particulier n'ont aucune pratique des engrais verts. L'introduction de *Sesbania rostrata* demande donc un effort multidirectionnel, allant de la modification des pratiques culturales à la prise en compte des aspects socio-économiques. En Casamance, les premières pluies débutant vers les mois de juin-juillet et le repiquage du riz s'effectuant généralement vers fin août et début septembre, la période intermédiaire comprise entre le premier juillet et le 15 août devrait toutefois permettre la culture de *Sesbania rostrata* en rizière sans perturber le calendrier traditionnel de la riziculture.

L'enfouissement de l'engrais vert représente indéniablement la partie nécessitant le travail le plus intense. En Casamance, les paysans ayant l'habitude d'enfouir de grandes quantités d'herbe dans leur rizière, une différence de travail minime entre l'enfouissement des mauvaises herbes ou l'enfouissement de l'engrais vert serait nécessaire. Cet effort est fait par les paysans qui utilisent, à ce titre leur outillage traditionnel efficace pour traiter de petites surfaces. Le traitement de surfaces plus étendues demande en revanche la mise en place de moyens de mécanisation tractée

ou attelée adaptée à l'enfouissement rapide de l'engrais vert dans les rizières.

Les résultats présentés dans ce mémoire montrent par ailleurs que les *Azorhizobium* une fois introduits sont capables de coloniser les rizières même dans le cas de sols acides comme ceux de Fanghote. Cette propriété observée également aux Philippines et en Inde (Watanabe et Ladha, communication personnelle), permet donc aux paysans d'éviter toute pratique expérimentale liée à l'inoculation.

Un autre facteur limitant de l'emploi de *Sesbania rostrata* est la production de semences. Les paysans devront garder un certain nombre de plantes pour récolter des graines.

Notons avec intérêt qu'à la suite des expériences menées depuis 1986, les paysans de Casamance ont bien maîtrisé la technologie de l'engrais vert *S. rostrata* qu'ils jugent supérieure, en rendements en riz à l'utilisation d'engrais chimiques azotés.

Dans le futur, l'utilisation à grande échelle de *Sesbania rostrata* au Sénégal dépendra d'une part, d'une collaboration accrue entre les différents Instituts de recherche, ISRA, ORSTOM, ADRAO (Association pour le Développement de la Riziculture en Afrique de l'Ouest) et les organismes socio-éducatifs et de développement, et d'autre part, d'une réelle volonté politique du gouvernement. Il est urgent que l'engouement porté au *Sesbania rostrata* en Asie, le soit aussi au Sénégal. Il serait en revanche incompréhensible que les excellents résultats obtenus par l'utilisation de cette plante tant au plan fondamental qu'appliqué, soient exploités dans les pays asiatiques et non au Sénégal où la plante a été découverte.

Les résultats relatifs à l'utilisation de *S. rostrata* comme fourrage sec (foin) sont moins prometteurs. Un certain nombre de problèmes se posent:

▲ (1) la sensibilité de *S. rostrata* aux insectes nécessitant des traitements chimiques constitue un inconvénient majeur pour sa vulgarisation.

▲ (2) *S. rostrata* perd une très grande partie de ses feuilles qui se séparent

des tiges en séchant et tombent sur le sol. La nécessité de peser les tiges et les feuilles pour reconstituer un fourrage complet distribué aux animaux, paraît inconcevable.

▲ (3) Le dernier problème est la faible ingestibilité du fourrage. En effet, la consommation volontaire du fourrage de *S. rostrata* est faible par rapport aux légumineuses tropicales, qui en général, ont une ingestibilité assez élevée surtout en début de floraison (Roberges, communication personnelle). Cette faible consommation est particulièrement due à la teneur élevée en constituants pariétaux des tiges (forte teneur en cellulose et en lignine) qui ne sont pas digestibles. C'est ainsi que sur des tiges hachées et distribuées, les taux de refus élevés atteignent environ 48%. Par rapport au foin brut, le poids des tiges représente 75% du poids total.

Il est clair qu'actuellement *S. rostrata* ne peut comme fourrage sec, concurrencer les niébés, et fanes d'arachides vulgarisés au Sénégal. Toutefois, les résultats ne sont pas exhaustifs et on pourrait se poser les questions suivantes: pour une légumineuse à forte croissance comme le *S. rostrata*, ne faudrait-il pas l'exploiter en vert plutôt qu'en foin?

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Synthèse des essais avec *Sesbania conduits* au CNRA de Bambey : *production et compostage*

F. Ganry et F. Gueye *

Dans le but d'étudier la valorisation des bas-fonds et d'accroître la production de la biomasse en zone soudano-sahélienne, nous avons réalisé à Bambey deux années de culture pluviale en 1984 et 1985 avec *Sesbania rostrata*. Sous l'isohyète 400 mm, seuls les bas-fonds, inondés ou partiellement inondés, peuvent permettre la culture de cette plante.

Première année de culture

L'objectif de la première année de culture était de voir le comportement de *Sesbania rostrata* et de nous procurer de la semence et des nodules pour l'année suivante. La pluviométrie a été de 460 mm.

Les techniques culturales ont été réduites au minimum: labour avant semis; binage avant et après semis; démariage. Les graines, semées à la volée, ont germé au bout d'une vingtaine de jours; les herbes apparues avant la levée des plantules de *Sesbania* ont été détruites par un binage. Les tiges de *Sesbania* ont été inoculées en deux fois par la souche de *Rhizobium* ORS 571: trois semaines et cinq semaines après la levée. Aucune fertilisation chimique ni organique n'a été appliquée: la richesse en matière organique des bas-fonds et la fixation symbiotique du *Sesbania* devant contribuer *à priori* suffisamment à une nutrition correcte de la plante. Notons à cet égard que l'apport de phosphates naturels au sol serait vraisemblablement bénéfique; le *Sesbania* produit serait alors enrichi en N par la fixation de N et en P par le phosphate naturel).

A la maturité, nous avons obtenu sur 200 m de surface, 980 g de nodules à 5%

d'humidité et 9,8 kg de graines, lesquelles ont été traitées au granox pour la conservation. Les nodules on été séchés au soleil et conservés dans un flacon à la température ambiante du laboratoire.

Deuxième année de culture

L'objectif de la deuxième année de culture était de produire assez de matériel végétal pour être composté en fosse *in situ*. La pluviométrie a été de 390 mm.

La semence produite la première année a permis de réaliser la deuxième année de culture. Les plantes ont été inoculées par les nodules récoltés la première année selon le mode opératoire suivant: les nodules secs sont broyés et mis dans l'eau; une partie de la parcelle de *Sesbania* est inoculée; 15 jours après, les nodules frais produits sont récoltés (inoculées avec les nodules secs, les tiges ne sont nodulées qu'à 20 à 30% de leur surface et écrasées au mortier; le jus est récupéré, dilué dans l'eau; l'inoculum est alors prêt (20 g de nodules frais donnent environ 6 litres d'inoculum qui peuvent inoculer 40 m de surface cultivée. Cette façon de fabriquer l'inoculum à partir des nodules s'avère plus accessible, moins onéreuse et autant efficace que la fabrication en fermenteur de l'inoculum à partir d'une souche bactérienne. Cette technique présente cependant le risque de transmettre et induire le développement de germes pathogènes.

Le matériel végétal récolté à 2 mois, soit 310 kg M.S. sur 600 m 52 g M.S./ha), nous a permis de mettre en route un essai compostage avec un ou sans phosphate tricalcique incorporé, essai dont le but était

de produire un compost enrichi en azote et en phosphore. La réussite de ce compost a été compromise d'une part par le fait que le *Sesbania* est ligneux et d'autre part par le manque d'eau (le compost n'étant pas suffisamment dense en raison de sa structure ligneuse, l'eau s'évapore rapidement). Pour être composté, le *Sesbania* gagnerait donc à être mélangé à d'autres matières végétales ou fumier, ou servir de litière. La valorisation la plus sûre consisterait en une utilisation fourragère par des bovins en stabulation, les résidus non consommés passant dans la litière.

Conclusion

Nous pensons qu'en zone soudano-sahélienne l'avenir du *Sesbania* réside dans une utilisation fourragère et comme apport de matière végétale pour la litière des étables, en vue d'accroître la production de fumier. Pour appréhender l'importance de cette production en biomasse des bas-fonds, il importe d'évaluer les superficies inondées, ou partiellement inondées, pour une région donnée, à partir de photo-interprétation ce qui est en cours pour la région de Bambey ou à partir de la télédétection qui pourrait sans doute fournir des résultats supérieurs. Nos premiers essais et ceux de l'ORSTOM montrent que la production de semences et d'inoculum est réalisable dans l'exploitation sans avoir recours à un appui extérieur. On peut penser que la survie du *Rhizobium* permettra après quelques inoculations d'arrêter celle-ci, ce qu'atteste l'existence rare cependant, de certains bas-fonds peuplés spontanément de *Sesbania* nodulés.

(*) ISRA, B.P. 51, Bambey, Sénégal.

Sesbania rostrata comme source d'azote en rizière irriguée dans le delta et la vallée du fleuve Sénégal

*H.F. Diara et I. Camara **

Introduction

De 1975 à 1985 la quantité totale de riz consommée au Sénégal a augmenté de 90% (plus de 100% pour l'ensemble des pays Ouest-africains). Dans le même temps la production rizicole a augmenté de 13% et les importations de 240% (40 et 580% pour l'ensemble de l'Afrique de l'Ouest) (2). Ce déséquilibre considérable se traduit par une ponction énorme sur les ressources en devises du pays.

Devant une telle situation la recherche rizicole se doit de proposer des technologies propres à augmenter la production certes, mais aussi adaptées aux possibilités réelles des utilisateurs, notamment par la valorisation des ressources locales.

Sesbania rostrata est une légumineuse arbustive sauvage très commune dans le delta et la vallée du fleuve Sénégal où les paysans la considèrent comme une mauvaise herbe. Sa particularité remarquable est de porter des nodules à la fois sur ses racines et sur ses tiges (4). De 1979 à 1982 diverses études, menées principalement à l'ORSTOM de Dakar, ont mis en évidence sa capacité élevée à fixer l'azote atmosphérique (4)(5) et ses effets bénéfiques sur le développement et le rendement du riz (5)(6).

Des expérimentations ayant pour but d'évaluer la valeur agronomique de *Sesbania rostrata* ont été réalisées de 1982 à 1987 à la station régionale de recherche sur le riz irrigué de l'Association pour le Développement de la Riziculture en Afrique de l'Ouest (ADRAO).

Résultats obtenus de 1982 à 1987

Pour l'essentiel les expérimentations ont été conduites dans la station expérimentale de Fanaye, située dans la moyenne vallée du fleuve Sénégal, sur des sols argileux du même type que ceux rencontrés dans les casiers rizicoles de la région du fleuve. Un essai de culture en grandes parcelles a été réalisé hors-station, dans le delta.

Les résultats de ces expérimentations (1)(3) ont montré que:

▲ L'enfouissement de *Sesbania rostrata* permet de doubler le rendement en grain du riz (Tableau 1);

▲ Un rendement optimum peut être obtenu par l'application complémentaire de 30 à 60 kg N/ha d'engrais minéral azoté;

▲ Le meilleur effet est obtenu quand cet engrais minéral complémentaire est apporté soit en totalité 20 ou 40 jours après repiquage du riz soit en deux fractions égales au repiquage et à l'initiation paniculaire du riz;

▲ Compte tenu des limitations liées aux caractéristiques climatiques locales qui commandent le calendrier cultural du riz d'hivernage, la date de semis de *Sesbania rostrata* la plus appropriée se situe en début juin (Tableau 3).

▲ Pour éviter les effets nocifs de certaines substances libérées par la décomposition de la matière organique on a avantage à ne pas repiquer le riz moins d'une semaine après enfouissement de *Sesbania rostrata*; mais au delà de deux semaines le rendement du riz baisse (Tableau 2).

▲ La perte en biomasse et en azote organique est négligeable quand on cultive *Sesbania rostrata* hors rizière;

▲ *Sesbania rostrata* montre une tolérance remarquable au sel (Figure 1).

Traitement	Rendement en grain (1)	Gain par rapport au témoin (%)
N_0	2,3 d	-
N_{60}	4,3 c	87
N_{120}	6,2 a	170
N_0 + *Sesbania*	4,5 bc	96
N_{30} + *Sesbania*	5,9 ab	150
N_{60} + *Sesbania*	6,8 a	196
N_{90} + *Sesbania*	6,8 a	196
N_{120} + *Sesbania*	6,8 a	196
Test F (traitement)		9,8 **
C.V.	16,7 %	
E.S.	0,5 t/ha	

Tableau 1 : Effet de l'enfouissement de Sesbania rostrata sur la réponse de l'azote minéral du riz irrigué d'hivernage cultivé dans la vallée du fleuve Sénégal.

(1) Test de Duncan: les moyennes suivies de la même lettre ne sont pas significativement différentes au niveau 5%.

(*) Station Régionale Riz Irrigué, Saint-Louis, Sénégal.

Traitement (enfouissement	Rendement en grain (2) (t/ha)	Grain (t/ha) par rapport à	
		N_0	N_{120}
0 jour A.R. (1)	5,0 b	+ 2,9	-
1 semaine A.R.	6,4 ab	+ 4,3	0,4
2 semaines A.R.	6,5 a	+ 4,5	+ 1,0
3 semaines A.R.	6,0 ab	+ 3,9	+ 1,2
4 semaines A.R.	6,1 ab	+ 4,0	+ 0,6
N_0	2,1 c	-	+ 0,7
N_{120}	5,4 ab	+ 3,3	- 3,3
Test F.	24,4 **		
C.V.	11,8 %		
E.S.	0,3 t/ha		

*Tableau 2 : Rendement du riz irrigué en fonction de la longueur de la période
entre l'enfouissement de Sesbania rostrata et le repiquage du riz.*

Les moyennes suivies de la même lettre ne sont pas différentes significativement au niveau 5%.

Date de semis	N_{30} (urée)	N_{30} (urée) + *Sesbania*
10-04-1986	2,7	3,5
08-05-1986	4,5	5,9
05-06-1986	5,4	7,8
03-07-1986	5,5	6,0
Test F D/semis	(a)	23,5 **
Trait. *Sesbania*	(b)	125,9 **
D/semis x tr. *Sesbania*		13,3 **
E.S.	(a)	0,4 t/ha
E.S.	(b)	0,2 t/ha
C.V.	(a)	16,8 %
C.V.	(b)	6,3 %

*Tableau 3 : Influence de la date de semis de Sesbania rostrata
sur le rendement en grain (t/ha) du riz irrigué (Fanaye, 1986).*
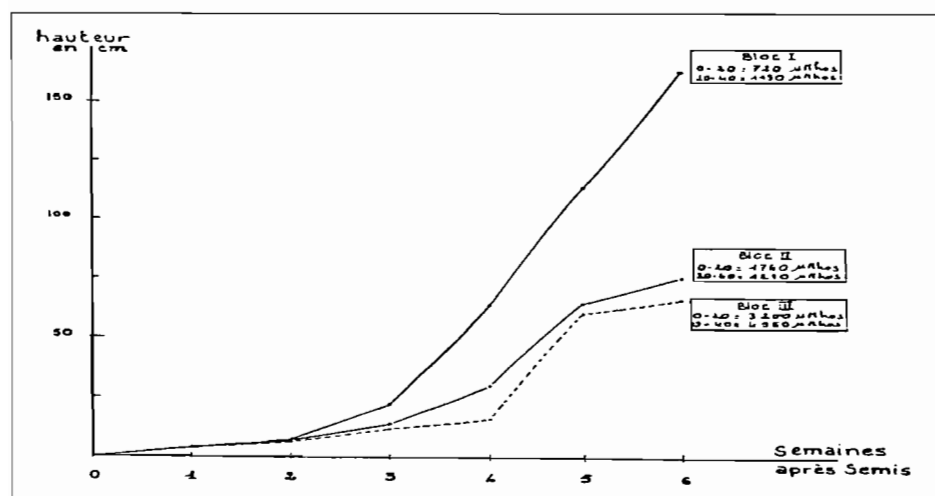
*Figure 1. Courbe de croissance de Sesbania rostrata en fonction du degré de concentration
en sel dans le sol (conductivité électrique en uMhos aux horizons 0-20 et 0-40)
(Ross-Bethio, Delta du fleuve Sénégal).*

Conclusion

Sesbania rostrata s'est avéré être un très bon engrais vert pour le riz. Son utilisation pourrait permettre d'économiser au moins 50% de l'engrais minéral actuellement utilisé pour obtenir de hauts rendements. Dans la région du fleuve Sénégal cette légumineuse se trouve dans son habitat naturel; elle est donc bien adaptée aux conditions pédo-climatiques qui y prévalent et elle est bien connue des paysans, même s'ils en ont une perception plutôt négative actuellement. Avec de tels atouts son adoption par les riziculivateurs du nord Sénégal peut s'envisager avec une dose raisonnable d'optimisme, si tant est qu'un certain nombre de contraintes majeures auront été levées; parmi lesquelles le temps d'occupation de la parcelle, le régime d'irrigation, la production massive d'inoculum et de semences, la période et la méthode d'enfouissement, la susceptibilité aux attaques de certains insectes.

Toutes opérations qui devront s'insérer harmonieusement dans le calendrier agricole déjà existant.

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Use of *Sesbania rostrata* in alley cropping systems

K.Mulongoy and N.V. Nguu

Introduction

The high green manuring value of *Sesbania rostrata* for wetland rice is known. The green manure is usually generated and ploughed under before transplanting rice. Since growing Sesbania in rotation with rice or in a cut-and-carry system requires additional land or labour essentially for land preparation and weed control, we tested *S. rostrata* in the alley cropping system.

Alley cropping is the intercropping of (food) crops with fast-growing trees or shrubs. The hedgerow trees or shrubs are pruned periodically to provide nutrients, mulch and browse materials (4). *S. rostrata* presents a number of characteristics desirable for hedgerow trees or shrubs in alley cropping systems. It is a fast-growing legume, easy to establish, regenerating rapidly after pruning, and unique for its profuse stem nodulation. It nodulates freely on both Alfisols and Ultisols in southern Nigeria (5). A population of 400 000 plant/ha can symbiotically fix more than 200 kg N/ha in 50 days (7).

Before intercropping *S. rostrata* with rice and using its prunings as source of N effectively, it is necessary to determine the population of the shrub that will provide enough biomass without reducing rice growth and yield, and to assess the benefit of sesbania prunings applied after rice has been transplanted. We conducted at the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria, a number of pot and field experiments. We report a few of them to illustrate the suitability of *S. rostrata* in alley cropping system with rice.

Materials and methods

Experiment I

This field experiment was carried out in a paddy field to evaluate the performance of rice intercropping with *S. rostrata*. It consisted of 7 treatments arranged in a randomized complete block design with 4 replications: (1) rice only; (2) rice intercropped with *S. rostrata* planted at a 0.2x3 m spacing and harvested at 4 weeks after planting (WAP); (3) like (2) but with *S. rostrata* harvested at 8 WAP; (4) like (2) but with *S. rostrata* planted at a 0.3x3 m spacing; (5) like (4) but with *S. rostrata* harvested at 8 WAP; (6) like (2) but with *S. rostrata* planted at a 0.4x3 m spacing; and (7) like (6) but with *S. rostrata* harvested at 8 WAP.

The rice variety used was ITA 306, an improved lowland variety with a growth duration of 130 days. It was established from 21-day-old rice seedlings and was planted 2 per hill at spacings of 15x30 cm. *S. rostrata* from 21-day-old seedling was then planted in some plots as defined above. The general pattern of intercropping *S. rostrata* and rice is shown in Figure 1. The treatment plot size was 6x6 m. Some properties of the soil in the experimental plot are shown in Table 1. We applied fertilizers (40-30-50 kg N-P-K/ha) before planting and kept the field weed-free with frequent hand weeding. The plot was flooded with 3 to 5 cm of water above the soil surface throughout the experiment.

At harvest dates, we cut *S. rostrata* at ground level, and determined its dry weight and N content as described by Mulongoy (5). These prunings were not applied. Rice yield was estimated in each plot from the harvest of a 5.265 m centre area (Figure 1).

Experiment II

The objective of this experiment was to study the effect of post-planting application of *S. rostrata* on rice. Eight combinations of N rates, amounts of *S. rostrata* prunings, and application times were arranged in a randomized complete block design with 4 replications. The treatments
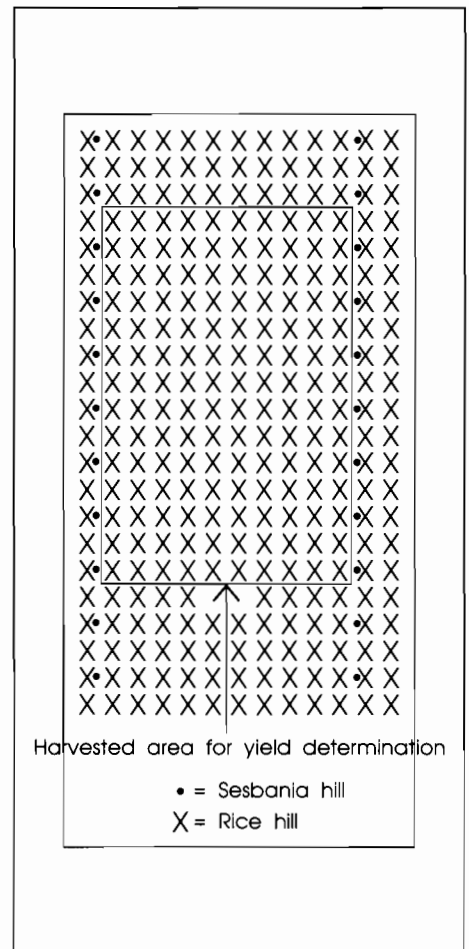

Harvested area for yield determination

• = Sesbania hill
X = Rice hill

Figure 1. Schematic representation of the planting pattern used in the rice-Sesbania intercropping system.

were: (1) a control without N and sesbania prunings; (2) *S. rostrata* prunings applied at 3 tons/ha at 4 WAP; (3) like (2) but with prunings applied at 8 WAP; (4) like (2) but with 6 tons of *S. rostrata* prunings; (5) like (4) but with prunings applied at 8 WAP; (6) with 40 kg N/ha broadcast before transplanting rice; (7) like (6) but with additional 3 tons/ha of *S. rostrata* prunings applied at 4 WAP; (8) like (6) but with additional 6 tons/ha of *S. rostrata* prunings applied at 4 WAP.

Single superphosphate at 30 kg P/ha and muriate of potash at 50 kg K/ha were broadcast prior to planting. Fresh prunings of *S. rostrata* containing 2.56% N on a dry weight basis were carried from 56-day-old (on the application dates) nurseries. The were cut into small pieces, distributed evenly between rice rows, and incorporated into the soil by stepping on them. Rice variety ITA 306 was planted at 20x20 cm from 21-day-old seedlings (2/hill). Weed and water were controlled as in Experiment I. The treatment plot size was the same as in Experiment I. Rice yield was estimated in a 5.265 m area.

Experiment III

This experiment was set out to assess N contribution of *S. rostrata* to rice in an alley cropping system. It was carried out in a hydromorphic soil. Some characteristics of the soil is the experimental plot are presented in Table 1. The treatments consisted of a factorial combination of 3 rates of Sesbania prunings (0, 3 and 4 tons of dry matter per hectare) and 2 rates of urea (0 and 120 kg N/ha), arranged in a randomized complete block design with 4 replications. The treatment plot size was 4x6 m.

At planting, seeds of both rice and *S. rostrata* were drilled in rows. The seedlings were thinned to the desired spacing at one week after emergence. Rice cultivar ITA 117 was planted the first year, and ITA 212 the following year, both at the spacing of 25x25 cm. *S. rostrata* was planted at 0.1x2 m (low population) and 0.1x1.5 m (high population) spacings, and was pruned at 8 and 12 WAP by cutting plant materials above the rice canopy. The two prunings of low and high population of Sesbania provided 3 and 4 tons/ha of dry matter, respectively. Urea was applied in two splits of 60 kg N/ha each at the time when *S. rostrata* was pruned.

Nitrogen contributed by Sesbania prunings and urea to rice was calculated as the difference between rice N contents in unfertilized plots that received no prunings and rice N contents in plots where Sesbania prunings or urea were applied.

A decomposition study was conducted to monitor the release of N from *S. rostrata* in relation to rice N uptake. Fresh prunings of *S. rostrata* (200 g) were secured in 30x40 cm nylon bags of 1 mm mesh size. The were laid at 8 WAP in unfertilized plots where Sesbania was applied at 4 tons/ha. The lay-out was a complete randomized design with three replications.

We prepared enough bags to allow sampling at 1, 2, 4, 6, 8 and 10 weeks after application. At sampling times, the content of the bags was dried in an oven at 60C, weighed and ground for N content determinations. In the second decomposition study, we compared the rate and extend of decay and N release from all parts

of mature plants of *S. rostrata*. The procedure was the same as for the first decomposition study except that stem nodules were placed in 10x10 cm nylon bags, and bags containing roots were buried at 10 cm from the soil surface. We fitted to the decomposition curves the double exponential function of the remaining organic matter (z):

$$z = Le^{-kt} + Re^{-ht} \quad (3)$$

with the simplification that L, the labile component of applied organic matter decomposing at a rapid rate (k) was 100%, whilst R, the resistant component decomposing at a slow rate, was nil. This simplification can only be justified if one considers that decomposition of low C:N ratio plant materials from legumes is rapid and almost complete over a cropping season, in the humid tropics. Thus N or dry matter losses (y) were calculated as

$$y = 100 - Le^{-kt}$$

Results and discussion

Experiment 1

The biomass and N yields of *S. rostrata* harvested at 4 and 8 weeks after transplanting are presented in Table 2.

Dry matter produced by intercropped *S. rostrata* increased rapidly from the first toe the second harvest. At the same intercropping pattern, an average of 5.7 fold increases was recorded at 8 WAP as compared to the biomass yield at 4 WAP. The same trend was observed for N content with an average of 5.2 fold increases. *S. rostrata* height (data not presented) also increased rapidly from 25 cm at transplanting to about 80 cm at 4 WAP and 2.5 m at 8 WAP. Grain yield of rice was not affected by *S. rostrata* planted at different populations or harvested at different times (Table 2).

These data indicate that intercropping of *S. rostrata* with rice can generate green manure in-situ without negative effects on grain yield of the associated rice. The population of *S. rostrata* in this experiment was however too low to supply the 3 and 6 tons of green manure applied per hectare

Soil parameters	Paddy soil[a]	Hydromorphic soil[b]
pH (1:1)	6.5	6.3
Total N, %	0.03	0.14
Organic C, %	0.44	0.80
Extractable P (Bray 1), ppm	2.9	32.2
CEC, meq/100 g	1.4	4.2
Sand, %	91	85
Silt, %	6	12
Clay, %	3	3

Table 1 : Some characteristic of the soil at the experimental sites.

[a] *Soil used in Experiments I and II*
[b] *Soil used in Experiment III*

Sesbania	Rice grain		Sesbania		
Spacing (cm)	yield[b]	Harvest time (WAP)	dry weight	N content	
			kg/ha		
300 x 20		4	240 c	6.80 c	3920 a
		8	1250 a	32.50 a	3920 a
300 x 30		4	132 c	3.82 c	4000 a
		8	845 b	21.97 b	3920 a
300 x 40		4	145 c	4.20 c	3970 a
		8	810 b	21.05 b	3920 a

Table 2 : Nitrogen content and biomass production of Sesbania rostrata planted at different spacing and harvested[a] at 4 and 8 weeks after planting (WAP), and its influence on grain yield of the intercropped rice variety ITA 306 (Nguu, unpublished data).

N.B.   *Means in the same column followed by different letters are significantly different at 0.05 probability level.*
[a]   *Harvested Sesbania was not applied to intercropping rice*
[b]   *Grain yield of rice grown alone was 3970 kg/ha.*

in Experiment II. Thus, we monitored in other experiment (not all reported here) the influence of higher populations of Sesbania on rice yield. For instance, in Experiment III, higher population of *S. rostrata* established from seeds did not affect rice growth significantly.

Experiment II

Table 3 shows the response of rice to fertilizer N and to different rates of *S. rostrata* prunings collected from an adjacent plot and applied at 4 and 8 weeks after transplanting rice.

Application of *S. rostrata* and fertilizer N increased rice grain yield by at least 15%. The highest yield increases were obtained with 6 tons/ha of *S. rostrata* prunings applied at 4 WAP, and with fertilizer N applied at 40 kg N/ha. No further increase in rice yield was obtained when urea (40 kg N/ha) was supplemented with 3 or 6 tons/ha Sesbania prunings. For the same amount of sesbania prunings, application at 4 WAP tended to be more effective in increasing rice yield than at 8 WAP. Efficiency of pruning N utilization is high when there is a synchrony between N release from decomposing prunings and the food crop N requirement. Similarly, we observed that N contribution of hedgerow trees alley-cropped maize is higher when the prunings are close to the planting of the food crop then when they are applied

too long before or after the planting of maize (2).

When the efficiency of applied *S. rostrata* is defined as the ratio of rice yield increase to de quantity of applied *S. rostrata*, the efficiency obtained in this experiment is comparable to that of pre-plant incorporation of *S. rostrata* reported by Furoc *et al.* (1). They obtained a 37.5% rice yield increase due to the pre-plant application of 7.7 tons/ha of *S. rostrata*.

Experiment III

Table 4 presents yield increases due to application of sesbania pruning and fertilizer N for rice variety ITA 212. Similar significant increases were obtained the previous year for rice variety ITA 117. There was little or no difference between the effect of sesbania prunings on rice yield and that of 120 kg N/ha applied alone or in association with the prunings. But this is not to say that N contribution of *S. rostrata* was equivalent to application of 120 kg N/ha of urea since we did not use intermediate N rates between 0 and 120 kg N/ha.

Nitrogen contribution of *S. rostrata* to rice variety ITA 212 was 38.0 and 43.2 kg N/ha (Table 4) representing 89 and 75% of the total N released during decomposition of sesbania prunings at low and high sesbania populations respectively. The first prunings applied at 8 WAP released, until the time rice was harvested at 16 WAP, 64% of the total N content of the prunings (Figure 2). The second prunings applied at 12 WAP released, until the harvest time, 40% of the total N of the prunings, assuming that decomposition rates were the same for the first and the second prunings. The latter assumption is supported by the fact that sprinkler irrigation was provided to compensate for low rainfall and to average a rainfall of 8 mm/day. The total amounts of N released from the two prunings of *S. rostrata* were 56 and 75 kg/ha when 3 and 4 tons of prunings were applied, respectively.

Fertilizer H (kg/ha)	Sesbania prunings[b]		Rice grain yield[a] (kg/ha)
	Application time (WAP)	Rate (tons/ha)	
0	4	3	3180 bc
		6	3680 ab
	8	3	2896 bc
		6	3249 bc
40	4	3	4113 a
		6	4269 a

Table 3. Influence of fertilizer N and different rates of Sesbania prunings[b] applied at 4 or 8 weeks after planting (WAP) on grain yield of rice variety ITA 306 (Nguu, unpublished data).

[a]   *Values followed by the same letters are not significantly different at 0.05 probability level. Grain yield of rice grown alone and without fertilizer N was 2520 kg/ha, not significantly different from the lowest yields in the Table.*
[b]   *Sesbania prunings were brought from an adjacent plot.*

Figure 3 shows that stems, roots and pods of *S. rostrata* decompose slowly. Assuming that 54% (on a dry weight basis) of young plants of *S. rostrata* i.e. before podding, consists of stems (0.85% N), 21% of roots and root nodules (1.50% N), 21% of

Sesbania prunings Nitrogen (tons/ha) contribution	Rice		
	N content	yield[b]	
	kg/ha		
Without fertilizer N	51.1	3010	NA[b]
0	89.1	4480(49)	38.0
3	94.3	4670(55)	43.2
4			
With 120 kg N/ha	91.5	4790(59)	40.4
0	101.2	4710(56)	50.1
3	107.0	4420(47)	55.9
4	23.7	670	ND[c]
LSD (0.05)			

Table 4. Nitrogen contribution and effect of Sesbania rostrata prunings and urea on rice yield (adapted from Mulongoy, 1986).

[a] *Sum of two prunings applied during the cropping season: 3 tons/ha in plots with 10x200 cm plant spacing and 4 tons/ha in plots with 10x150 cm spacing.*

[b] *Values between brackets represent the percentage of yield increases due to application of Sesbania pruning and fertilizer N.*

[c] *NA = not applicable; ND = not determined.*

leaves (3.80% N), and 4% of stem nodules (4.40% N) (5), and taking into consideration the extent of N release over a decomposition period of 8 weeks (Figure 3), 1 ton of Sesbania plants materials including roots will release 6 kg N from the leaves, and in average 2 kg N from the stems, from the stem nodules or from the roots. Nitrogen contribution of stems and roots to the intercropped rice is therefore relatively small. But their benefit may be a long term one as they can enrich the more stable fraction of soil organic matter.

Pruning N utilization was more efficient in this alley cropping experiment (Table 4) than is other trials where maize was cropped in alleys of Leucaena (6). Efficiency of nitrogen utilization by maize is usually low. Better N utilization in this experiment can be explained by the fact that rice population was higher than maize population in the other trials. Also Sesbania pruning ap-
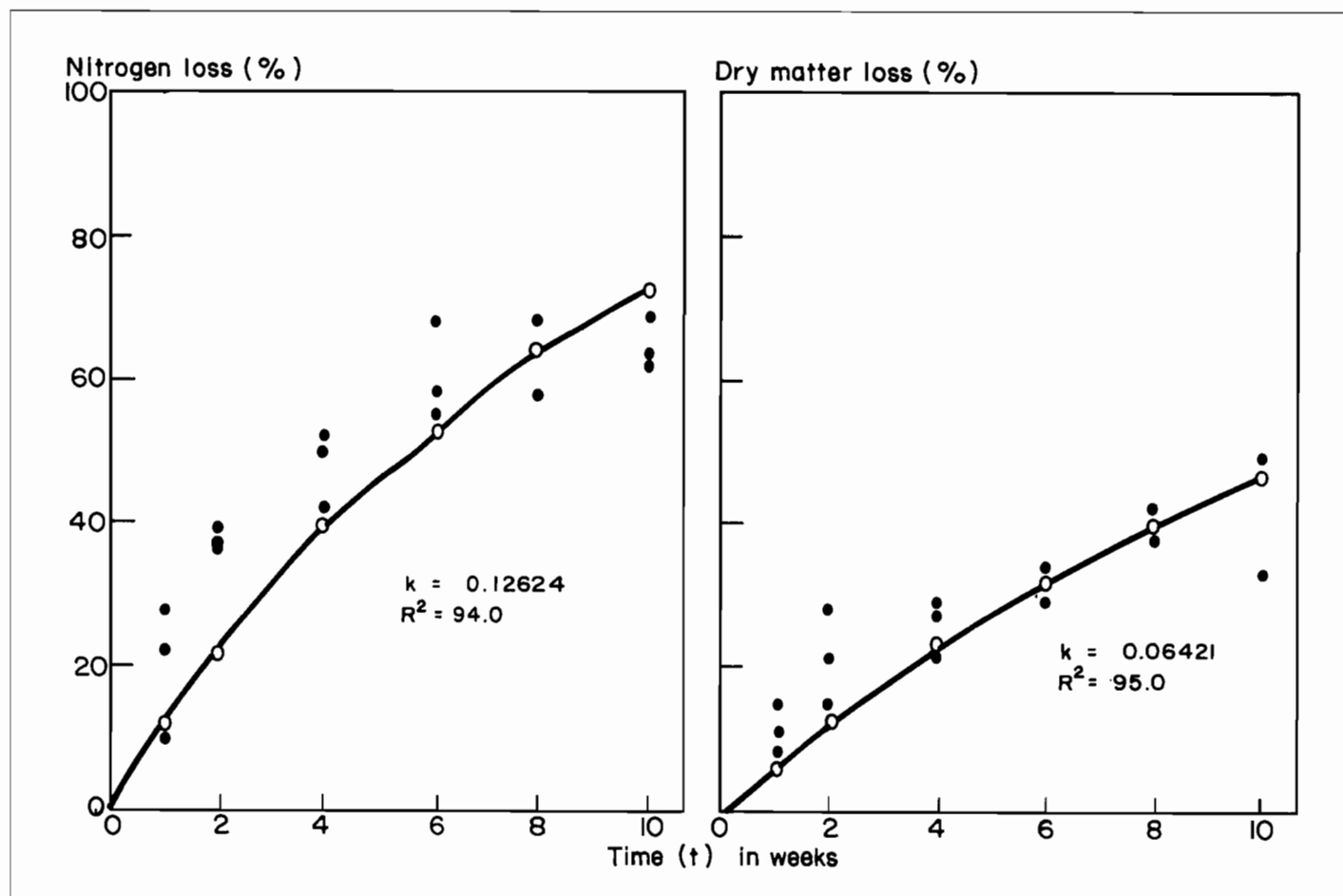
Figure 2. Nitrogen and dry matter losses during decomposition of sesbanian prunings, expressed as percentage of the initial values (losses y = 100 - Le^{-kt}, assuming L = 100%; - • - observed values, - o - fitted values) (Mulongoy, unpublished data).
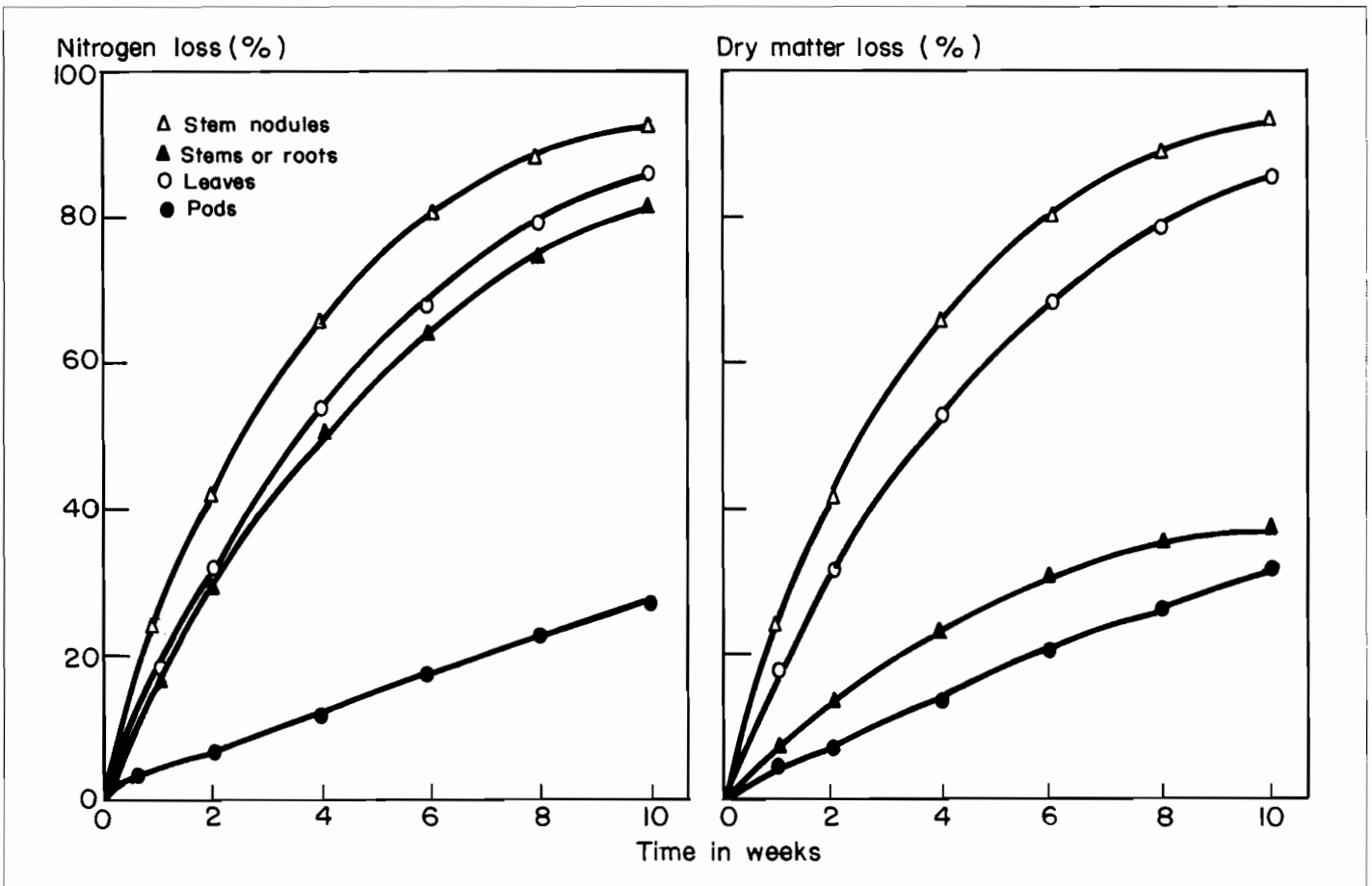
Figure 3. Nitrogen and dry matter losses during decomposition of different parts of S. rostrata, expressed as percentage of the initial values (Mulongoy, unpublished data).

plication coincided with weeding times. Hoe-weeding following application of prunings buried them to some extent. This probably resulted in reduced volatilization losses.

Since pruning application is efficient in terms of N contribution when it is done close to the planting of rice, alley cropping rice with *S. rostrata* may be much successful only when long duration varieties of rice like ITA 212 and ITA 306 are used. The small biomass of *S. rostrata* produced at 8 WAP (960-1280 kg/ha) accounts for only 38% of the total N released from the prunings (131 kg N/ha) during the cropping season. And N released (81 kg N/ha) from the second prunings (2040-2720 kg/ha) applied a 12 WAP may take place when rice does not need is much, if we use short duration rice varieties.

Results of experiments conducted at IITA demonstrated the potential of intercropping *S. rostrata* with rice in alley cropping

systems. The major advantage of the system is that the green manure is produced *in situ* in the growing rice field. The effectiveness of the system is supported by (1) the absence of (or little) competition between S. rostrata and rice (2) and by the significant yield increase due to post-planting application of *S. rostrata* to rice.

Evaluation of *S. rostrata* as source of N in comparison with other potential hedgerow trees in farmers' field as well as economic analyses of the system are needed before the system can be recommended to extension bodies. Present data, however, show that the system is technically feasible.

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Identification des paramètres limitant le développement de *Sesbania rostrata* au sud de la Côte d'Ivoire

B. Osseni

Introduction

L'agriculture ivoirienne, à l'image de celle des pays en voie de développement des régions tropicales, est caractérisée par une faible productivité. Les fortes productions végétales de ces deux dernières décennies ont été obtenues essentiellement par l'extension des surfaces cultivées, alors que les rendements en milieu réel, pour la plupart des cultures à caractère villageois, notamment le café, le caco, le palmier à huile, l'ananas ..., les cultures vivrières (le maïs, le riz, le manioc, l'igname, l'arachide...) sont demeurés stationnaires, voire en régression dans certaines régions.

En effet, dans les régions à croissance démographique élevée et à forte pression foncière (surface cultivée/surface cultivable), les jachères qui constituent l'unique forme traditionnelle de restauration de la fertilité du sol, s'étendent sur des périodes de moins en moins longues. Ainsi, au sud de la Côte d'Ivoire où autrefois, la durée de jachère était de 10 à 12 ans, elle est actuellement de 3 à 4 ans (Osseni, 1983; Biarnes et Colin, 1985).

Pour obtenir des rendements élevés dans de telles conditions, l'utilisation des fumures minérales en quantité importante dans les sols généralement pauvres en éléments fertilisants, devient une nécessité. Cependant, une mauvaise conduite des engrais encadrés, peut aboutir à des effets néfastes au niveau du sol (épuisement en éléments fertilisants, acidification, ...). En outre le coût élevé des engrais (souvent importés) rend leur accès problématique pour la plupart des paysans aux ressources financières limitées.

De ce fait, tout en intensifiant l'agriculture pour améliorer de façon régulière sa productivité, les pays tropicaux en voie de développement doivent veiller à la conservation et à l'amélioration de la fertilité (physique, chimique et biologique) des sols. Ces objectifs peuvent être atteints non seulement par l'application de certaines techniques comme le bon travail du sol, l'utilisation des variétés de plantes adaptées, la meilleure conduite des engrais, le choix judicieux des associations et des rotations de cultures... mais aussi par l'introduction des légumineuses fixatrices d'azote dans les systèmes de culture.

Parmi ces légumineuses, une attention particulière a été portée sur *Sesbania rostrata* par l'IRFA (Institut de Recherche sur les Fruits et Agrumes) suite aux performances de cette plante au Sénégal (travaux des chercheurs de l'ORSTOM: G. Rinaudo et al., 1983; B. Dreyfus et al., 1985) et au Nigéria (travaux réalisés à l'IITA en 1983).

La première étape des études entreprises au sud de la Côte d'Ivoire, a consisté à suivre le comportement de *Sesbania rostrata* (Osseni et al., 1987).

Principales caractéristiques de la région

Situation

Le sud de la Côte d'Ivoire, concerné par les études de *Sesbania rostrata* est compris entre 5 et 6 de latitude Nord. Située en bordure du Golfe de Guinée, la région a pour domaine initial la forêt en reconstition, entrecoupées par des emplacements de cultures vivrières et de cultures de rente pérennes ou semi-pérennes.

Climat

Le climat est caractérisé par:

▲ une pluviométrie contrastée en quatre saisons dont deux saisons pluvieuses (de mai à mi-juillet et de mi-septembre à mi-novembre) et deux saisons sèches (de mi-juillet à mi-septembre et de mi-novembre à avril). La hauteur des pluies annuelles avoisinait 2000 mm (1959 à 1982), mais actuellement, elle est de l'ordre de 1500 mm (1983 à 1986). Les pluies sont mal réparties avec un mois de juin où il tombe le plus souvent près du quart du total;

▲ une température moyenne de 26C avec de variations mensuelles de faible amplitude (2 à 4 C);

▲ une humidité relative mensuelle de 90% en moyenne pendant plus de 12 h par jour;

▲ une durée d'insolation moyenne de 5 h par jour mais variant de 3 à 7 h selon les mois.

Sol

Le sol est du type ferrallitique désaturé (classification française CPCS) ou orthic ferralsol (classification FAO), développé sur des sables argilo-ferrugineux d'origine tertiaire. La fraction sableuse est toujours élevée (supérieure à 70%). La teneur en argile est généralement inférieure à 25% et la fraction limoneuse est toujours faible (moins de 5%). Les propriétés chimiques de ce type de sol, d'après J. Godefroy (1975), se caractérisent par des teneurs en cations échangeables très faibles (moins de 1 meq/100 g de terre), un coefficient de

saturation faible (moins de 20%) et un pH à réaction fortement acide (inférieur à 5,0).

Matériels et méthodes

Conduite des essais

Tous les essais ont été réalisés à la station fruitière de l'Anguededou, situé à 25 km à l'Ouest d'Abidjan. Les coordonnées géographiques sont: latitude: 525' N; longitude: 408' O; altitude: 30 mètres.

La surface d'une parcelle élémentaire est de 3 m (2,0 x 1,5 m) et comporte 3 lignes de *Sesbania* (20 plants par ligne). Le nombre de plants par hectare est de 200.000 (écartements: 0,10 x 0,50 cm)

Les semis sont réalisés en poquets à raison de deux graines par poquet. Une semaine après la levée, un démarrage a été fait en condition humide du sol et en gardant la plantule la mieux développée.

Le premier site des essais (site 1), avait porté plusieurs cycles d'ananas depuis plus de dix ans. La croissance très hétérogène de *Sesbania rostrata* sur ce site, a conduit à choisir un autre du même sol qui n'a jamais été planté en ananas mais ayant pour précédent du gazon, *Chrysopognon aciculatus* (site 2).

Les dates prévisionnelles de semis, à trois mois d'intervalle, conformément au protocole expérimental de l'ORSTOM de Dakar, devraient être: octobre 1984, janvier 1985, avril 1985 et juillet 1985.

Mais, afin de faire coïncider certains semis avec les deux saisons pluvieuses, les deux derniers semis ont été réalisés en mai et septembre 1985.

En fumure de fond, il a été appliqué juste avant les semis:

▲ 30 g/m de CaO et 20 g de MgO sous forme de dolomie;

▲ 3,5 g/m de P_2O_5 sous forme de phospal;

▲ 7,5 g/m de K_2O sous forme de sulfate de potassium.

En période de déficit hydrique, des arrosages fréquents ont été effectués de façon à apporter environ 150 l d'eau (pluie et arrosages) par mois. Les semis de mai réalisés en début de la grande saison pluvieuse, sont les seuls qui n'ont pas été arrosés.

Deux désherbages ont été effectués par sarclage manuel.

Traitement de graines avant les semis

Les graines de *Sesbania*, avant leur mise en terre, ont été immergées dans de l'acide sulfurique à 50% pendant trente minutes afin de provoquer une germination rapide et homogène. Elles ont été ensuite lavées immédiatement et abondamment trois fois à l'eau.

Inoculation des plantes

L'inoculation des plantes a été réalisée par pulvérisation des parties aériennes par une culture du rhizobium ORS 571, à quatre et six semaines après chaque semis. La composition d'un litre de solution tamponnée à pH 6,8 est:

▲ 1 g de poudre sèche de la souche ORS 571 incluse dans un polymère type alginate;

▲ 4,3 g de KH_2PO_4;

▲ 4,9 g de K_2HPO_4.

L'inoculum ainsi préparé renferme environ 10^8 bactéries/ml. La solution a été diluée 20 fois (11 dans 19 l d'eau) avant d'être pulvérisée sur les plantes de chaque parcelle.

Résultats

Données climatiques

Les données climatiques durant toutee la période des essais (octobre 1984 à décembre 1985), sont représentées par les figures suivantes:

▲ Figure 1: durée du jour (a) et durée d'insolation journalière (b)

▲ Figure 2: températures maximales, minimales et moyennes mensuelles

▲ Figure 3: pluviométrie mensuelle.

Caractéristiques physico-chimiques des sites d'étude

Avant le début de la mise en place des essais, il a été prélevé par site deux échantillons de terre de 0 à 20 cm det de 20 à 40 cm de profondeur (mélange de 15 "carottes" par niveau de prélèvement) pour la détermination des caractères physico-chimiques. Les résultats des analyses sont présentés dans le Tableau 1.

Croissance de la plante

Elle a été mesurée sur l'ensemble des plantes de chaque parcelle (60 plantes) toutes les deux semaines. La hauteur moyenne de la plante estimée durant le cy-
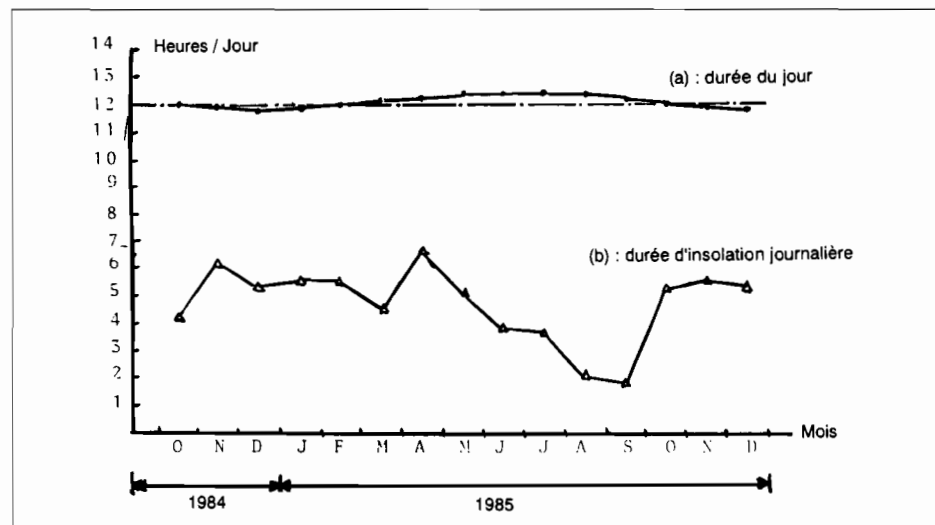

Figure 1. Durée du jour et durée d'insolation journalière selon les mois durant la période des essais.
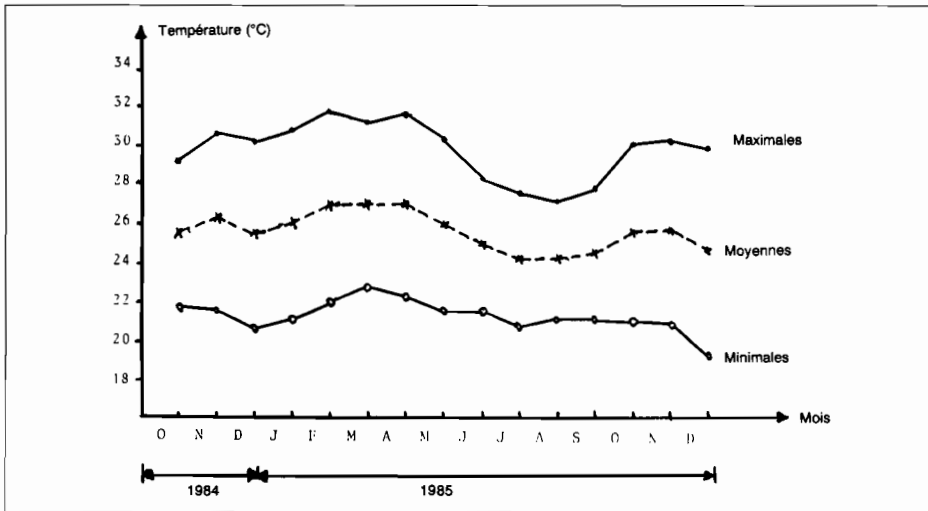
Figure 2. Evolution des températures maximales, minimales et moyennes pendant la période des essais.
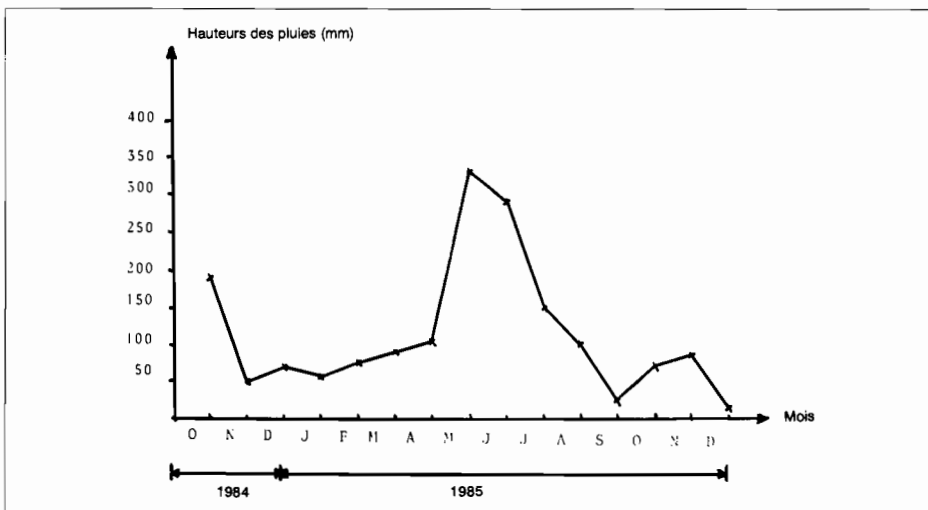

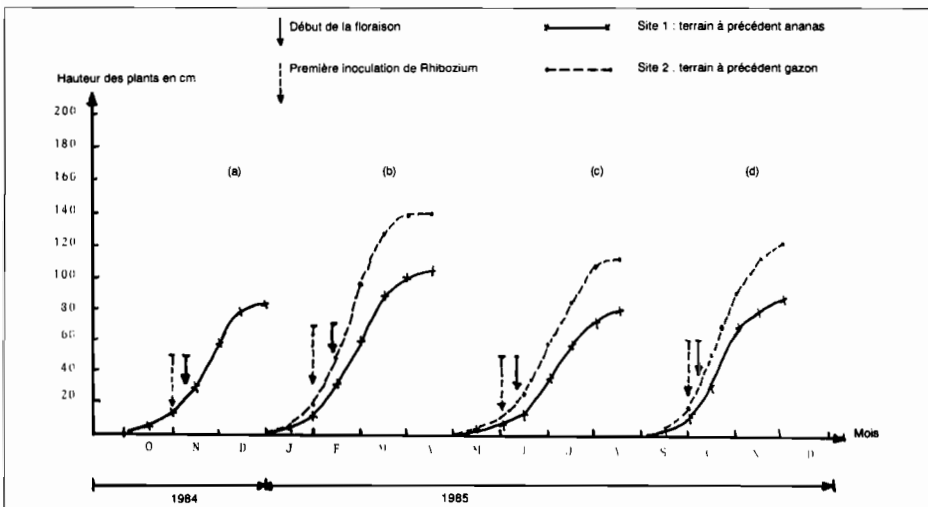*Figure 3. Evolution des sommes mensuelles des pluies durant la période des essais.*

Figure 4. Croissance de Sesbania rostrata sur un même sol à précédent cultural différent.

cle et aux différentes dates de semis est représentée par la Figure 4.

Production de biomasse et teneurs en éléments minéraux de Sesbania rostrata

La production de biomasse a été estimée à deux stades: floraison et maturité des graines. A chaque stade, 12 plantes tirées au hasard à raison de 4 par ligne ont été arrachées au ras du sol. Le poids moyen de matière fraîche des organes aériens (feuille, tiges + rameeaux) a été enregistré (Tableau 2).

Un aliquot de 200 g de chaque organe a été prélevé et mis à sécher à 80C à l'étuve jusqu'à l'obtention de poids constant de matière sèche. Les teneurs en N, P. K, Ca et Mg de chaque organe de la plante aux différentes dates de semis ont été déterminées (Tableau 3).

Attaque des racines par les nématodes

Le dénombrement des populations de nématodes a été réalisé sur le site 2 lors du semis de janvier 1985. Les genres de nématodes identifiés ainsi que leur nombre sont consignés dans le Tableau 4.

Discussion des résultats

Influence des facteurs climatiques sur la croissance de Sesbania rostrata

Durant toute la période des essais, on note que:

▲ la longueur du jour est relativement constante (12 heures) et les amplitudes sont très faibles, le maximum se situant à 36 mn (Figure 1a);

▲ la durée moyenne d'insolation journalière est de 5 heures et les faibles valeurs (environ 2 heures par jour) sont enregistrées en août et septembre (Figure 1b);

▲ les amplitudes de températures mensuelles se situent à 4,5 C en moyenne (Figure 2).

Site	1		2	
Précédent cultural	**Ananas**		**Gazon**	
Horizon (cm)	**0-20**	**20-40**	**0-20**	**20-40**
Granulométrie (p. 100)				
Argile	20	20	16	18
Limon fin	3	3	3	3
Limon grossier	2	2	2	1
Sable fin	16	22	24	20
Sable grossier	59	53	55	58
Matière organique (p. 1000)				
Matière organique	15,2	15,2	-	-
C organique	8,8	8,8	9,8	10,8
N total	0,9	0,8	0,8	0,7
C/N	9	11	13	15
Phosphore (Pppm)				
Total	385	189	522	491
Assimilable (Dyer)	6	6	14	9
Complexe absorbant (1) (mé/100 g)				
Calcium	0,3	0,2	1,7	0,8
Magnésium	0,1	0,1	0,6	0,4
Potassium	0,1	0,1	0,2	0,1
Somme des cations échang.	1,3	0,5	0,4	2,5
CEC	4,8	4,7	5,9	5,8
E cations/CEC (p. 100)	10	9	42	22
pH (pâte saturée)	4,1	4,1	5,4	5,0
Al^2	0,7	0,7	0,04	0,3

Tableau 1 : Caractéristiques physico-chimiques du sol des deux sites d'étude.

(1) extraction à l'acétate d'ammonium N à ph 7,0;
(2) extraction au chlorure de cobaltihexamine non tamponné.

L'observation de l'évolution des courbes de croissance durant cette période (Figure 4) montre que pour chaque site, aux différentes dates de semis, la croissance de *Sesbania rostrata* est équivalente (80 à 90 cm pour les plants du site 1 et 110 à 120 cm pour ceux du site 2), exception faite des semis de janvier où on note une nette amélioration de la croissance (105 cm en moyenne pour les plants du site 1 et 140 pour ceux du site 2).

Dans les conditions très favorables du Sénégal, la plante de *Sesbania rostrata* peut atteindre 3 à 4 mètre de hauteur à 4 mois (Dreyfus et al., 1985). Les faibles valeurs de l'ensoleillement journalier pourraient être l'un des facteurs limitant la croissance de *Sesbania rostrata*, du moins dans les régions maritimes de Côte d'Ivoire où l'ensoleillement est généralement considéré insuffisant pour la croissance de certaines plantes.

Le début de la floraison pour toutes les dates de semis a lieu environ 1,5 mois après le semis; cette floraison apparaît plus précoce alors qu'elle se situe à 2-3 mois dans la région de Dakar (Dreyfus et al., 1985) et à 2,5 mois au centre de la Martinique (Melin et al., 1987). Les deux régions sont respectivement localisées à 1434' et 1440' de latitude Nord.

La meilleure croissance de *Sesbania rostrata* au sud de la Côte d'Ivoire a été notée sur les plantes issues des semis de janvier. Cette période correspond à la grande saison sèche, donc très chaude comme l'indique la Figure 2. Du fait des déficits

hydriques importants à cette période, dus aux niveaux très faibles des pluies (Figure 3), des apports fréquents d'eau ont été réalisés et c'est cette parcelle qui a été la mieux irriguée (625 mm contre 435 à 460 mm pour les autres parcelles. En période de la grande saison pluvieuse (mai à mi-juillet) où aucun arrosage n'a été effectué, la hauteur de pluies était de 435,7 mm durant le cycle de *Sesbania rostrata*. La croissance (85 cm sur le site 1 et 110 cm sur le site 2) aurait pu être meilleure s'il n'y avait pas eu une interruption des pluies durant douze jours consécutifs, six semaines après le semis. *Sesbania rostrata* est donc une plante très sensible au déficit hydrique et le manque de pluie en période pluvieuse sur une période de jours consécutifs, relativement courte, peut être préjudiciable au développement de la plante.

Il est alors fort probable que les apports d'eau réguliers durant la période très chaude aient permis de maintenir le sol à un taux d'humidité suffisant ayant entraîné une amélioration très sensible de la croissance par rapport aux autres périodes.

Ces résultats confirment la préférence de *Sesbania rostrata* pour les zones hydromorphes comme cela a été montré par Dreyfus et al. (1985) et Dommergues et al. (1985).

Influence des facteurs liés au sol

L'analyse du Tableau 1 montre qu'il existe des différences entre les caractéristiques chimiques du sol du site 1 et du site 2. Ces différences, de 0 à 20 cm de profondeur sont:

▲ le pH du site 1 est plus acide que celui du site 2 (4,1 contre 5,4);

▲ la somme des bases échangeables du site 1 est plus faible que celle du site 2 (0,5 contre 2,5 meq/100 g de terre);

▲ la teneur en Al est, corrélativement au pH, plus élevée dans le site 1 que dans le site 2 (63 ppm contre 4 ppm);

▲ la teneur en phosphore total et assimilable du sol du site 1 est plus faible (respectivement 385 et 6 contre 522 et 14 ppm).

Les écarts de croissance des plantes de *Sesbania rostrata* issues d'une part du site

Précédent cultural	Date de semis/ stade de la plante	Organes	Matière fraîche (MF) g/plante	Matière fraîche (MS) g/plante	MS/MF %
Ananas	19 octobre 1984 maturité des graines (7 janvier 1985)	feuilles (17,6%) tige + rameaux (82,4%) plante entière	6 23 29	1,2 5,8 7,0	20 25 24
Gazon	28 janvier 1985 floraison (21 mars 1985)	feuilles (47,5%) tiges + rameaux (52,5%)	24 31 55	4,3 4,7 9,0	18 15 16
	maturité des graines (30 avril 1985)	feuilles (32,0%) tiges + rameaux (55,3%) racines (12,7%) plante entière	29 93 10 132	7,3 12,6 2,9 22,8	25 14 29 17
Gazon	23 mai 1985 floraison (23 juillet 1985)	feuilles (47,6%) tiges + rameaux (52,4%) plante entière	25 32 57	4,3 4,7 9,0	17 15 16
	Maturité des graines (14 septembre 1985)	feuilles tiges + rameaux plante entière	- - -	- - 12,9	- - -
Ananas	28 septembre 1985 maturité des graines (12 décembre 1985)	feuilles (43,0%) tiges + rameaux (57,0%) plante entière	- - -	3,7 4,9 8,6	- - -
Gazon	Maturité des graines (12 décembre 1985)	feuilles (43,7%) tiges + rameaux (56,3%) plante entière	- - -	3,5 5,1 9,0	- - -

Tableau 2 : Poids de matière fraîche et sèche de Sesbania rostrata aux différentes dates de semis.

1 et d'autre part du site 2, peuvent s'expliquer par les différences entre les caractéristiques chimiques.

Le pH très acide des sols ferralitiques désaturés du sud de la Côte d'Ivoire peut être considéré comme l'un des facteurs limitant la croissance de *Sesbania rostrata*.

Influence d'autres facteurs

Le système racinaire de *Sesbania* en condition de croissance plus favorable a été infecté par des nématodes (Tableau 4). Parmi ces nématodes, le genre *Méloïdogyne* se trouve en proportion plus élevé dans les racines. D'autres genres, comme *Cacopaurus* et *Pratylenchus* ont été identifiés mais leur nombre est très faible par rapport à celle de *Méloïdogyne*.

La plante de *Sesbania rostrata* apparaît donc très sensible au *Méloïdogyne* et les dégâts causés par ce nématode sur les racines auraient pu influencer négativement la croissance de la plante.

En outre l'intensification de la culture d'ananas exige l'utilisation importante de pesticides notamment de désherbants (Bromacyl et Diuron). Le mode d'action et surtout la longue rémanence de ces deux formes d'herbicide, deux à quatre ans selon PY et al., (1984) pourraient être l'une des causes de la croissance très lente et très hétérogène de *Sesbania rostrata* dans le sol du site 1 à monoculture d'ananas depuis plusieurs années.

Bilan de la biomasse et de l'azote

La production de la matière végétale est très sensible aux dates de semis et selon le site (Tableau 2). Sur le site 2, dans le cas le plus favorable (semis de janvier), la production de matière sèche par plante entière (feuilles, tiges, rameaux) est d'environ 20 g. Cette production est très faible par rapport à celle enregistrée dans le centre de la Martinique (latitude 1440' N), dans le cas le plus favorable (semis de novembre), qui est de l'ordre de 51 g de matière sèche par plante entière (feuilles, tiges, non aoûtées). Au sud de la Côte d'Ivoire, la production de matière sèche par m (densité: 20 plants/m) est de 400 g alors qu'elle est de

Précédent cultural	Date de semis et stade de la plante	Organe	N	P	K	Ca	Mg
Ananas	19 octobre 1984 maturité des graines	feuilles	3,18	0,3	2,5	1,8	0,3
		tige + rameaux	0,78	0,1	1,8	0,4	0,1
		plante entière	1,2	0,2	1,9	0,6	0,2
Gazon	28 janvier 1985 floraison	feuilles	5,22	0,4	3,1	1,3	0,2
		tiges + rameaux	1,29	0,2	3,7	0,6	0,1
		plante entière	3,2	0,3	3,4	0,9	0,2
	maturité des graines	feuilles	4,19	0,3	2,4	1,4	0,2
		tiges + rameaux	0,94	0,1	1,2	0,3	0,1
		racines	0,99	0,1	1,3	0,3	0,1
		plante entière	2,0	0,2	1,6	0,7	0,2
Gazon	23 mai 1985 floraison	feuilles	5,56	0,3	2,8	1,6	0,2
		tiges + rameaux	1,47	0,2	3,1	0,5	0,2
		plante entière	3,4	0,2	3,0	1,0	0,2
Ananas	28 septembre 1985 maturité des graines	feuilles	4,66	0,2	2,3	1,7	0,3
		tiges + rameaux	1,23	0,1	1,6	0,5	0,2
		plante entière	2,7	0,2	1,9	1,0	0,2
Gazon	Maturité des graines	feuilles	4,70	0,2	1,9	1,3	0,4
		tiges + rameaux	1,24	0,1	1,0	0,3	0,2
		plante entière	2,8	0,1	1,4	0,8	0,3

Tableau 3. Teneurs en quelques éléments minéraux majeurs des différents organes de la plante de Sesbania rostrata exprimées en pour cent de matière sèche.

Genre	Meloidogyne			Cacopauruus	Pratylenchus
	Oeufs	**Larves**	**Femelles**		
Rhizosphère	220	60	2	35	1
Racines + galles	7710	940	50	4	0
Racines sans galles	240	60	0	15	15

Tableau 4 : Population de nématodes dans 1 g de matière fraîche.

700 g à la densitée réelle de 14 plants/m au centre de la Martinique.

La composition minérale de la plante est indiquée au Tableau 3. L'organe le plus riche en azote et potassium au stade "maturité" des graines constitue les feuilles. Pour ces deux éléments, il a été dosé 3,2 à 4,7 de N et 2,0 à 2,5 de K (en p. 100 de matière sèche). Ces teneurs sont variables selon les dates de semis. Au stade "floraison", la teneur en azote des feuilles est de 5.2 p. 100 de matière sèche (semis de janvier) et de 5,6 p. 100 de matière sèche (semis de juillet). Des résultats similaires ont été obtenus en Martinique: les feuilles sonts les plus riches en azote et en potassium au stade "floraison" qu'au stade "fructification".

Dans les conditions optimales de croissance, l'immobilisation de l'azote est de 0,46 g/plante entière, fruits exclus (feuilles, tiges, rameaux, racines) dont 94 p. 100 dans les parties aériennes. Cette quantité d'azote qui représente 9 g/m ou 92 kg de N/ha dans la localité des essais (525' de latitude Nord) paraît très faible.

En effet, les immobilisations d'azote enregistrées à Dakar (latitude 1434' N) sont de 1,6 g/plante (Rinaudo et Moudiongui, 1985) et au centre de la Martinique (latitude 1440' N) sont de 2,0 g/plante (Melin et al., 1987). On rapporte que les légumineuses du genre *Sesbania* dans d'autres pays comme le Népal, le Sri Lanka, Costa Rica pourraient fixer jusqu'à 500 kg N/ha (Mathieu, 1984).

Conclusion

Les études de *Sesbania rostrata* entreprises au sud de la Côte d'Ivoire situé entre 5 et 6 de latitude Nord, montrent que les performances de cette plante fixatrice d'azote sont médiocres dans un sol bien drainé et exondé ayant porté ou non plusieurs

cycles d'ananas. Les performances médiocres dans de telles conditions peuvent s'expliquer par:

▲ le pH très acide (pH 5,0) du sol à monoculture d'ananas avec une teneur en aluminium échangeable élevée;

▲ la très longue rémanence de désherbants (Bromacyl et Diuron) utilisés en culture d'ananas;

▲ la grande sensibilité de la plante au déficit hydrique et au nématode *Méloïdogyne*.

Dans les sols jamais cultivés en ananas, le développement de *Sesbania rostrata* pourra se révéler intéressant à condition que l'eau ne soit pas le facteur limitant. Il apparaît, dans ce cas, que l'utilisation de la plante en culture d'ananas qui exige un sol bien drainé semble limitée au sud de la Côte d'Ivoire. Par contre, *Sesbania rostrata* pourrait se révéler d'un grand intérêt dans les terrains situés le long des marigots et souvent gorgés d'eau ou dans les bas fonds hydromorphes inondés en saisons pluvieuses.

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The influence
of sub-humid tropical stress factors
on legume production with special reference
to *Sesbania-Azorhizobium* symbiosis

K. Olufemi Awonaike

Introduction

In addition to forage legumes serving as rich sources of protein for animals, a lot of interest is also shown in the use of legume-based forage in cropping systems for maintaining soil fertility and controlling erosion. There potentials have to a large extent not been harnessed in most parts of the sub-humid tropics as compared to the temperate regions.

It is a recognized fact that to adequately feed the world's ever increasing population, it is essential that food production be increased in the sub-humid tropics where it will be mostly needed. The use of fertilizers is one out of many means of increasing crop production and nitrogenous fertilizers are by far the most widely used among these. With specific regards to the tropics, the package of improvement practice must of essence include an increased input of biologically fixed nitrogen to complement the use of chemical nitrogen fertilizer if it is available. It should be borne in mind though that contrary to popular opinion, the growing of a legume crop does not necessarily result in a nitrogen gain for a farming system, except when an affectively nodulated forage legume is ploughed under as a green manure.

It is important to note also that improving nitrogen fixation per se is not necessarily the main priority in increasing production, other of equal or even in some cases of higher significance include judicious ferti-

lize use, improved soil and water management, an effective insect and disease control and planting super cultivars. With these priorities taken care of under the most suitable environmental condition, an adaptive high yielding legume plant genotype and an effective *Rhizobium* strain in a symbiosis will perform optimally either in terms of production of vegetative matter or grains or both depending on the purpose for which the legume is grown.

In the sub-humid tropics however, an ideal or suitable environmental condition is rarely obtained throughout the growth cycle of the legume crop as it is subjected to various environmental stresses - which invariably limit its production.

This paper therefor aims at highlighting the influence of the main sub-humid tropical stress factor on legume production with special reference to *Sesbania-Azorhizobium* symbiosis. Reviews by Gibson (2), Franco (3, 4) and Eagleshan and Ayanaba (5) are highly recommended to complement this report.

Amounts of nitrogen fixed by various legumes in the field and scope for improvement

An FAO handbook publication (6) estimated that leguminous plants fix 80 million tons of nitrogen annual from the vast supply in the air. Of this, crop legumes ac-

count for around 35 million while other in meadows, grasslands and forests accounts for the remaining 45 million tons.

Desmodium spp. and *Sesbania* spp. are some of the highest forage legume nitrogen fixers while *Calopogonium* spp. and *Cajanus* spp. are some of the highest grain legume nitrogen fixers (Table 1).

It is apparent also from Table 1 that tropical legumes are not inferior nitrogen fixers when compared with temperature legumes. Earlier reports (till the 1970s) of poor nitrogen fixing abilities of tropical legumes (8) may be due to such studies being conducted under simulated tropical conditions. This does not in any way suggest that food and forage legumes perform optimally in the farmers fields in the sub-humid tropics. A host of factors is responsible for this, the most prominent being environmental stresses.

A meeting of consultants on Mutation Breeding for Improved Nitrogen Fixation by 10 per cent in the tropics is a realistic goal. This increase would be roughly equivalent to the uptake from 20 kg N/ha fertilizer at a basic cost of approximately $ (US) 10/ha without taking account of transportation and application costs. This would represent an annual saving of at least $ US 600 million in India or $ US 200 million in Brazil (7).

Efforts aimed at increasing biological nitrogen fixation in the tropics in general should therefore be intensified.

Plant	Nitrogen fixed kg N/ha/year
(a) Food legumes	
Calapo *Calopogonium* spp.	370-450
Horse bean *Vicia faba*	45-552
Pigeon pea *Cajanus cajan*	168-280
Cowpea *Vigna unguiculata*	73-354
Mung bean *Vigna mungo*	63-342
Soybean *Glycine max*	60-168
Groundnut *Arachis hypogea*	72-124
Pea *Pisum sativum*	52-77
Bean *Phaseolus vulgaris*	40-70
(b) Forage legumes	
Tick clover *Desmodium intortum*	897
Sesbania *Sesbania cannabina*	542
Leucanea *Leucaena leucocephala*	74-584
Centro *Centrosema pubescens*	126-398
Alfalfa *Medicago sativa*	229-290
White clover *Trifolium repens*	128
Subclover *Trifolium subterraneum*	207

Table 1 : Estimated amount of nitrogen fixed
by various under field conditions (FAO publication (6)).

Brief climatic consideration of the sub-humid tropics

The sub-humid tropics cannot be defined on a simple latitudinal basis for the discussion of plant growth, Coaldrake (9) however categorized the sub-humid tropics of eastern Australia with relation to plant growth as the region where plants may be subjected to water stress or surplus in any month of the year and where water rather than energy is likely to limit plant growth.

The sub-humid tropical climate prevails in four of the five continents of the world. These are usually areas of relative mild high temperatures with rainfall from equatorial and tropical cyclonic systems in summer, from high altitudes in winter and from local thunderstorms.

In some years. an area in this zone may receive from all of these systems, in other years from only one or two. The result is the great variability in rainfall characteristics of the sub-tropics (10 and 11). The rainfall pattern inevitably produces droughts and floods.

With regards to temperature in the sub-tropics, the annual range is moderate while the diurnal range in generally wide - with the range of temperatures increasing away from the coast.

Mean monthly relative humidity at 15.00 hours is in the range of 50 to over 60 per cent all through the year, though in some inland areas the figure could be as low as 30 per cent.

The sub-tropics are areas of comparatively high incoming solar radiation. From the estimates of Ransom (12), the amount of incoming solar radiation of the sub-tropics of north-eastern Australia was surpassed only by those of the arid zones o Africa, Australia and Central South America.

Sub-humid tropical stress constraints on legume production

An essential requirement in pasture legumes is an efficient *Rhizobium* symbiosis associated with a high yield of dry matter and protein, and with the ability to add enough nitrogen to the soil/plant/animal system to maintain productivity in the persistence under grazing in a mixed pasture and that mineral nutrition with elements other than nitrogen is adequate.

As expected, significant interactions between host and strain are increasingly common and favourable responses to inoculation with effective strains are more frequently observed. However, the popular consensus remain that tropical legumes do not respond to inoculation because they lack specific *Rhizobium* requirements and nodulate effectively with a wide range of strains indigenous to tropical soil. This no doubt reflects historical experience with most of the generally grown including *Arachis, Centrosema, Vigna, Desmodium, Glycine, Stylosanthes,* and *Trifolium* (African) (13, 14, 15).

The amount of nitrogen which is fixed will depend on many factors, not least among which is the nitrogen status of the soil. Nevertheless, annual amount of 500 kg N/ha for a forage legume and 250 kg N/ha for a grain legume crop are reasonable targets (16). Although reports of higher amounts fixed are by no means exceptional in literature, the range of estimates differs widely and is to great to be accounted for by difference in methods of measurement (17). Much of this variation stems from a number of environmental stresses which may prevail at one or more stages in crop duration (18, 19).

The main environmental stress factors in the sub-humid tropics are moisture excess and deficiency, the extremes of temperature and relative humidity. Soil acidity, salinity and alkalinity are the other lesser and more localized stress factors.

(a) Moisture excess

Soil waterlogging commonly occurs in the sub-humid tropics during and after prolonged and intense rainfall during the growing season. Since rhizobia are aerobic organisms, the anoxic condition of waterlogged soils would be expected to have a detrimental effect on their survival. This has been found to be true for populations of *Cicer, Rhizobia* and *R. japonicum* after paddy rice in india and Thailand (20, 21) and prior flooding adversely affected nodulation of *Centrosema* and soybean in Brazil (22). A comparison of a strain of *R. trifolii* and *R. japonicum* indicated that the former was the more flooding-sensitive (23). Some rhizobia (especially among the slow growers) were found not only to sur-

vive in anaerobic conditions, but also to increase in numbers (24). The reason being that they can utilize nitrate as electron acceptor under anaerobiosis (24, 25).

Oxygen deficiency which accompanies waterlogging of soil generally inhibits nodulation and nodule development (26, 27). In contrast, nodulation of submerged stems of Aeschynomene and Sesbania was not adversely affected by waterlogging (28, 29) probably because of their air-conducting lacunae (29). Severely restricted root growth when submerged undoubtedly contributes to decrease legume root nodulation, although in microaerobic condition, the former may be more adversely affected than the latter (26).

Ethylene, which has been found to be produced in waterlogged organic soil (30) has also been found to be powerful inhibitor of nodulation (31). The waterlogging of nodulated roots of soybean plants resulted in acetylene reduction activity declining to zero within a day of flooding (32). Again, in contrast, Aeschynomene root nodules retained the potential to reduce nitrogen even after long term flooding, as shown by acetylene reduction activity in air (33).

Flooding tolerance in plants has been linked to the ability to utilize nitrate as a terminal electron acceptor (24, 25, 34) and to the ability to accumulate malate rather than ethanol in their roots when anoxic (35).

Manganese toxicity has also been implicated as a further constraint to plant growth when soil are inundated (36).

(b) Moisture deficiency

The distribution of the rains during the wet season is the sub-humid tropics is usually uneven resulting in the surface layers of the soil becoming desiccated during crop growth.

Chen and Alexander (37) reported a significant reduction in bacterial populations during prolonged soil desiccation, with the slow-growing bacteria being more tolerant to desiccation than the fast-growing bacteria (38). The greater susceptibility to desiccation of the fast-growing bacteria was explained not in terms of differences in internal solute concentrations in water permeability, but rather that the fast growers had a greater affinity for water because of higher surface energy (39).

There are very few reports on the effects of low moisture on the nodulation process. Worral an Roughley (40) however reported that a reduction of soil moisture by only 2 percent had severe effects on root hair infection of *Trifolium subterraneum*. They found that nodulation failure at the moisture level was not due to death of rhizobia, but rather the root structure which appeared to be immune to rhizobial penetration, but which became amenable to infection after watering.

Pot grown Siratro and *Desmondium intortum* subjected to weekly cycles of water stress suffered more in terms of shoot and root growth than in nodule mass or acetylene reduction activity (although the latter was measured under non-stressed conditions (41).

Nodules on water-stressed clover plants resumed meristic activity and quickly increased in fresh weight and nitrogen fixing activity after rewatering (42), whereas those of soybean (without localized meristem) similarly treated were shed (43).

The superior drought tolerance of Lotus corniculatus in comparison with *Medicago lupulina* and *Trifolium repens* was correlated with its ability to retain rather than shed its root nodules under dry conditions (44).

(c) High temperature

In the sub-humid tropics, air temperatures exceeding 38C are rare (45) but when the bare surface of a cultivated soil is exposed to direct sunlight, its temperature can greatly exceed the air temperature. In seasonal rainfall areas, crops are usually sown at the end of the hot dry period (i.e. at the start of the rains). After planting there may be dry hot days when germinating seeds and the roots of developing seedlings are exposed to elevated temperatures. Temperatures in excess of 40C have been accorded at planting depth (46, 47, 48). The available data indicate that rhizobial survival and/or early nodulation are adversely affected by tropical soil temperatures (46, 47, 49).

Legumes species show varying tolerance to high temperature in their nodulation abilities. While chickpea failed to nodulate with any of five rhizobial strains at a constant temperature of 33C (50), cowpea nodulated well with all ten rhizobial strains at a constant temperature of 35C (51).

Rhizobial strain to strain variation exists in nodulation potential under temperature stress conditions, this has been demonstrated with several host species including cowpea (51, 52 and lotus 53).

(d) Other

Other environmental stress factors in the sub-humid tropics include soil acidity, soil alkalinity and salinity. These are felt in more localized areas due to the microclimates prevailing in the areas.

▲ (i) Soil acidity
In an area where rainfall exceeds evapotranspiration for much of the year, this results in soils becoming thoroughly leached of calcium and magnesium, leaving the soils markedly acid. Such soils are characterized by high concentrations of hydrogen ions and free aluminium, and low concentrations of calcium and available phosphate. Some acid contain manganese at phytotoxic levels and in some molybdenum is unavailable. Legume dependent on the root nodule symbiosis for nitrogen showed a range of responses to low pH, but in general, nodulation was reduced or eliminated at pH values below 5 (54, 55). The inhibition of nodulation appears to result from a combination of low calcium and low pH since it was alleviated by increasing either of the two (56).
The presence of available aluminium is acid soils inhibits nodulation directly (57) and indirectly by stunting root growth, and also tends to compound the effects of low levels of calcium by inhibiting its uptake (58).
Depending on the degree of tolerance to acid conditions, some crop species respond more strongly than others to lime application (59).

Legumes in acid soils dependent on nodule fixed nitrogen generally derive greater benefit from liming than when there is sufficient nitrogen available (54). High levels of lime application where pH is raised to between 0 and 7 have been found to have deleterious effects on plant growth (60, 61).

▲ (ii) Soil salinity and alkalinity
Saline soils are common in regions where transport of soluble salts to the ocean does not occur because of low rainfall, while alkaline soils usually develop from saline soils with ion calcium reserves (45). These soils are characterized by the presence of high levels of neutral salts in the surface layers resulting from the capillary rise of water when evaporation exceeds precipitation. The predominant salt and usually sulphate and chlorides of sodium, calcium and sometimes magnesium, and small quantities of carbonates and bicarbonates are often present.

Rhizobia exhibit a large range of sensitivities to salinity. *R. trifolii* is reported to be a saline sensitive species (62, 63) while rhizobial strains from *Sesbania cannabina, Crotolaria juncea* and soybean are saline tolerant (64). Indigenous rhizobia which effectively nodulate some legume species including *Sesbania* and which can survive extreme saline-alkaline conditions for long periods have been reported (65).

Suggested strategies to increase yield of legumes in the sub-humid tropics

An essential aspect of the strategy to improve the yields of sub-humid tropical legumes in stressed environments must involve combining stress-tolerant cultivars with stress tolerant rhizobia. The latter may exist already in the soil or be introduced as seed or soil-applied inoculants.

In soil waterlogged areas, in addition to screening for flooding tolerance, a combination of superior lines with rhizobia possessing dissimilatory nitrate reductase may also lead to significantly increased yields in hydromorphic soils.

Although there are several reports on the adverse effect of dry soils on legume symbiosis and of supra-optimal soil temperatures on survival of ameliorating these effects. Surface mulches may conserve soil moisture, cool the top soil, control weeds and reduce available nitrogen concentration which otherwise may delay nodulation of crops especially sensitive to soil nitrogen status (16).

Further studies should therefore be done to evaluate the responses of nodulated legumes in the field and of the effects of different management practice on nitrogen fixation. With this done, nitrogen fixation by sub-humid tropical legumes can be exploited fully to enhance agricultural productivity.

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Agricultural use of root and stem nodulated legumes and their potential in Egypt

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The local cultivation of forage crops to support the rapid increasing interest and importance given to animal production in Egypt can not be over emphasized.

Since a very long time "berseem" *Trifolium alexandrinum* was the only legume crop in Egypt and used as green manure and green animal fodder. At present time more than half million hectares are cultivated with berseem in winter.

The high yield produced is not yet enough to cover all the needs of green fodder and this caused a complete stop for its use as a green manure. Shortage of forage crops is very clear in summer where limited area are cultivated with sorghum and sordan, and importation of animal fodder is needed to cover all shortage of animal fodder in that time.

In Egypt, the soils are characterized by a very low organic matter content which not exceed more than 2% in the fertile soils, and 1% in the sandy and newly reclaimed soils. This is due to the continuous and intensive cultivation. All efforts should be done to cover the continuous loss of that indispensable material for soil fertility and productivity.

It is well known that the "Green Revolution" of the last five decades is based, in particular, on the wide production and application of mineral fertilizes for growing crops. However, the practice of soil fertilization with mineral fertilizer is not without adverse, long term environmental consequences with regard to increasing the food supply, there is growing concern about the deterioration of soil fertility and environmental quality as a result of using vast amounts of chemicals and inorganic fertilizer to produce more food. Fertilization by organic materials and green manure is, therefore, an alternative approach for restoring environmental resources while minimizing the reliance of fertilization by inorganic chemicals and maintaining soil productivity.

This emphasize the need for seeking new organic manures specially in the newly reclaimed sandy soil.

Cultivation of Sesbania plants may be the solution of these problems.

It is known as a common wild leguminous in Nile Valley in Egypt, growing in summer surroundings cotton fields as wind break. According to their high dry biomass production is a very rapid growth period, ability of nitrogen fixation, they may be used as a green manure, and also as a very good summer forage crop.

Sesbania plants are tall shrubs or sometimes annuals, with long virgate branches. Leaves are paripinnate with numerous leaflets.

S. sericea and *S. sesban* are recorded in Egypt and cultivated as shade plants surroundings cotton fields. *S. rostrata* has not yet been recorded (Täckholm,1974).

In Egypt, recently, several investigators start to study the growth of *S. sesban* and the effect of different growth factors vitamins on their growth, nodulation and Nitrogen fixation. (El-Gamal, 1985; El-Gamal and Alaa - El-Din, 1984; Elwan *et al.*, 1983a).

Elwan et al, (1983b) studied the root nodule bacteria *Rhizobium sesban* and the role of vitamins, indole compounds and gibberelic acid int its nutrition.

Haroun and El-Gamel (1986) studied the sensitivity or *R. sesbani* to some antibiotic and its efficiency on symbiotic nitrogen fixation.

Sesbania as Green Manure

Two of experiments were carried out at the experimental farm of the Faculty of Agriculture, Cairo University at Fayoum, Egypt.

▲ 1. Container Experiments: - *Sesbania sesban* and *S. sericea* were grown in drained conditions (1 plant container) in plastic containers with 20 kg of deficient payment sandy soil supplemented with 1 g K_2HPO_4.

▲ 2. Plot Experiment: - The two types of Sesbania plants were grown in sandy soil (350 plants per plot). Latin square of 5x5 ^{m} (1/400h) plots designed were used. N_2 fixation was assessed using the "N" balance in the plot experiment. Super phosphate was added at the rate of 60 k/h$_2$ of P$_2$O.

Generally, the results showed that plants of *S. sesban* were taller and heavier in their fresh and dry weights of leaves and stem/plant, total plant and consequently fresh and dry yield/unit area than those of *S. Sericea*. The amount of biomass incorporate after seven weeks of cultivation was 22 t. and 20 t. fresh weight for *S. sesban* and *S. sericea* respectively.

Nitrogen fixation was 0.16 g and 0.12 g by *S. sesban* and *S. sericea* respectively. That means 56 g N per plot and 22.5 kg N per ha for *S. sericea* during the same period of seven weeks. N'doye and Dreyfus, 1987 found that *S. sesban* gave higher yield of biomass and fixed nitrogen, specially under waterlogged conditions.

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However, the fixed nitrogen by the two tested Sesbania plants was very low comparing with of nitrogen fixed by *S. rostrata*.

From these primary studies is can be concluded :

▲ 1. Since *S. rostrata*, the root and stem noduled legumes has not been yet detected in Egypt, efforts should be done to import and introduce recommended samples of seeds from Senegal and adapt them and test their possible use as green manure and green fodder.

▲ 2. It is of great value to try transferring the stem-nodulating character to non-stem nodulating legumes, Which can be chosen among those known to be the best adapted species to a given climate and the most interesting for agriculture e.g. *S. sesban*.

▲ 3. About one million hectares are cultivated with rice in Egypt with an average of (8 tons/ha). The irrigation water is the limiting factor controlling the expansion of rice cultivation.
Growing Sesbania as a green manure before rice is controlled by crop notation, availability of irrigated water and using fast growing types of rice.

▲ 4. Intercropping of *S. rostrata* in the rice fields may be of a great value for producing green manure and green fodder in the same time during rice cultivation.

▲ 5. Cultivars of *S. rostrata* resistant to shortage of water, should be grown as green manure, in the newly reclaimed and sandy soils irrigated by sprinkle or dripping system. Rinaudo *et al.* (1983) showed that *S. rostrata* used as green manure have marked aftereffect and that can be attributed to the improvement of soil properties, such as a more stable structure and a higher content in organic matter.

▲ 6. Studying the best time of cutting the Sesbania plants and testing their nutritional value as green fodder with higher yield and protein content.

▲ 7. Finding the best method of composting Sesbania alone or with other organic materials.

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Etat d'une recherche pluridisciplinaire sur *Sesbania rostrata* et d'autres légumineuses à nodules caulinaires de Madagascar

L.H. Rakotovao

Depuis 1984, un programme sur la "fixation biologique de l'azote" a été initié à Madagascar en vue de l'amélioration de la production rizicole. Des missions d'identification ont été effectuées par Messieurs M. Toure et B. Dreyfus dans le cadre d'un programme de coopération technique entre le Gouvernement Malgache et la FAO. Une équipe pluridisciplinaire mise en place et animée par le Ministère de la Recherche Scientifique et Technologique pour le Développement (MRSTD), actuellement opérationnelle, rassemble des chercheurs de diverses spécialités (botanique, microbiologie, écologie, physiologie végétale, biochimie, agronomie) ainsi que des vulgarisateurs agricoles.

Bien qu'un programme existe sur l'utilisation comme engrais verts de l'association *Azolla-Anabaena*, la présente communication ne concerne que les légumineuses à nodosités caulinaires qui fixent l'azote atmosphérique, existant à Madagascar.

Deux genres de légumineuses poussant à l'état sauvage ont retenu particulièrement notre attention en attendant de pouvoir effectuer un inventaire exhaustif d'autres légumineuses à nodulation de tige. Les genres *Sesbania* et *Aeschynomene* ont pour caractéristiques communes, la nécessité de la levée de la dormance des graines pour obtenir la germination, la sensibilité à la photopériode, le fait de supporter une immersion prolongée, ce qui permet leur utilisation en riziculture irriguée.

Une espèce du genre Sesbania très proche, semble-t-il, de Sesbania rostrata décrit au Sénégal, a été localisée dans les milieux humides et exondés de la côte Ouest (Sud-

Ouest et Nord-Ouest) ou le climat chaud et sec ne comporte que 4 à 5 mois de pluie par an. Cette espèce cohabite avec une autre sans nodosités caulinaires.

Trois espèces d'*Aeschynomene* ont été observées sur les Hauts-Plateaux du centre de Madagascar; il s'agirait d'*A. uniflora, A. schimperi* et *A. sensitiva* dont la nodulation se trouve à la base des tiges et semble être tributaire de la présence d'une lame d'eau.

Afin de pouvoir utiliser ces espèces de légumineuses comme engrais verts en riziculture, le **programme scientifique** concerne plusieurs volets:

▲ la prospection botanique des espèces locales, répartition géographique, étude systématique;

▲ des études microbiologiques des souches de *Rhizobium* isolées de *Sesbania* et d'*Aeschynomene* de Madagascar;

▲ des études physiologiques: levée de dormance, essais de germination, essais de comportement en fonction de la photopériode, induction de la nodulation, caractérisation des sites de prélèvement;

▲ des essais agronomiques au champ dont l'effet de l'enfouissement;

▲ des analyses en vue de leur utilisation possible comme plantes fourragères;

▲ des études de vulgarisation en milieu paysan sont également en cours.

L'état d'avancement des travaux tel que nous allons les présenter est en partie tiré

de notre exposé au colloque de l'Académie Malgache de septembre 1987 sur l'"Amélioration de la riziculture par des méthodes biologiques".

1. Etudes systématiques

Sesbania rostrata de Madagascar a été déterminé vers l'année 1900 par Perrier de la Bathie. La plante est répandue sur la Côte Ouest de l'Ile de Majunga (Nord-Ouest) à Fort-Dauphin (Sud-Ouest).

Les études systématiques ont été entreprises afin de discerner les ressemblances et les différences de l'espèce malgache avec *Sesbania rostrata* du Sénégal.

A. Au point de vue botanique, les critères suivants ont été considérés:

▲ morphologie de la graine;

▲ hauteur de la plante;

▲ nombre de nodosités par plante;

▲ morphologie des graines de pollen;

▲ cytologie;

▲ étude chimiotaxonomique des graines;

▲ morphologie de la graine.

A.1.

Les résultats ont confirmé que l'espèce malgache possède des graines deux fois plus petites que celles de l'espèce du Sénégal tant au point de vue de la dimension que du poids. Il en est de même pour le nombre de graines par fruit et la dimension des folioles.

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Direction du Ministère de la Recherche Scientifique et Technologique pour le Développement, Madagascar.

	Sesbania rostrata **du Sénégal**	**Sesbania rostrata de Morondava**
Poids	$\bar{x}$ = 0,135 g	$\bar{x}$ = 0,04 g
Dimension	$\bar{x} = \dfrac{3,26 \text{ mm.}}{2,15}$ $\dfrac{L}{1}$	$\bar{x} = \dfrac{2,4 \text{ mm.}}{1,86}$
Gousse/longueur	$\bar{x}$ = 23,7 cm.	$\bar{x}$ = 8,7 cm.
Nombre de graines par gousse	$\bar{x}$ = 38,7	$\bar{x}$ = 23,4
Nombre de folioles par feuille	$\bar{x}$ = 52,5	$\bar{x}$ = 20,3
Dimension des folioles	3.13/.68 cm	.9/.39 cm

Tableau 1 : Comparaison concernant Sesbania rostrata de Sénégal et Sesbania rostrata de Morondava.

Date d'observation	Hauteur moyenne (cm)	Nombre de nodules	Nombre de feuilles	Remarques
01-20/12/85	-	-	-	Germination
04/01/86	58,6	100	-	
21/01/86	111,8	378	131	
27/01/86	183,4	1.328	317	
03/02/86	212,5	2.041	450	Apparition des premières inflorescences
27/02/86	297,5	4.174	1.444	

Tableau 2. Observation de la croissance sur terrain du Sesbania rostrata de Morondava.

A.2. Hauteur de la plante

Dans son habitat naturel, *Sesbania rostrata* de Madagascar peut atteindre jusqu'à 4 m de haut.

A.3. Nombre de nodules par plante

Des observations sur le terrain ont montré que les nodosités caulinaires peuvent apparaître jusqu'à une hauteur de 3 m. Le nombre moyen de nodosités par plante peut atteindre 4.174.

A.4. Analyse de la morphologie des grains de pollen

L'étude comparée des grains de pollen acétolysés a montré que les 2 taxons sont proches l'un de l'autre. Les grains de pollen sont légèrement hétéropolaires, tricolorés. La seule différence réside dans la taille plus petite des grains de pollen de l'espèce malgache.

A.5. Cytologie

Utilisant la solution de Carney modifiée (Carney's modified solution), le nombre de chromosomes a été compté afin de dé-terminer si la différence de taille des 2 taxons est basée sur une différence de ploi-die. Le nombre n = 6 II (pairs) pour *Sesbania rostrata* du Sénégal a été retrouvé confirmant des comptages effectués par d'autres chercheurs. Cependant le nombre de chromosomes de l'espèce malgache n'a pu être encore obtenu; ce travail sera certainement fait dans l'avenir.

A.6. Etude chimiotaxonomique des graines

Les graines des 2 taxons ont été analysées et comparées avec 20 autres espèces de *Sesbania* au laboratoire de Kew Royal Botanic Gardens par Docteur R. Nash. Les 2 taxons ont un profil identique qui est différent de celui des 20 autres *Sesbania* analysés. Aucune trace d'alcaloïdes n'a été détectée. Il est envisagé également l'étude des flavonoïdes des graines.

A.7. Anatomie des folioles

Une observation de l'architecture des folioles a montré que les 2 taxons possèdent la même architecture des folioles. Cependant on a noté que le *Sesbania* malgache possède certains caractères primitifs, no-tamment l'angle de divergence des nervures secondaires - 40-68 contre 48-52 pour *Sesbania rostrata* du Sénégal.

Ces résultats sont encore préliminaires. Beaucoup reste à faire sur la systématique des *Sesbania* à nodosité caulinaires malgaches ainsi que celles des *Aeschynomene*.

II. Etudes microbiologiques

Cf. communication de Fetiarison et al. CENRADERU-FOFIFA.

III. Etudes physiologiques

Du point de vue physiologique, les recherches actuelles concernent la comparaison des deux espèces de *Sesbania* et les trois espèces d'*Aeschynomene* sur certains points dont la germination, le comportement vis-à-vis de la photopériode, l'influence du niveau d'eau sur la nodulation ainsi que la fixation d'azote par activité réductrice d'acétylène.

A. Levée de dormance et germination

Quatre (4) méthodes ont été utilisées pour la levée de la dormance des différentes graines suivant les espèces:

▲ traitement par l'acide sulfurique concentré;

▲ scarification au sable;

▲ immersion dans l'eau chaude;

▲ méthode mixte scarification-immersion dans l'eau chaude (à 50 et 70, 1 à 15 minutes).

La vitesse et le pourcentage de germination sont différents pour chaque espèce.

▲ *A. uniflora* a présenté un pourcentage de germination de 84 après traitement de 90 minutes à l'acide sulfurique. La scarification seule ou combinée au traitement à l'eau chaude a permis d'obtenir 70 à 75% de germination de cette espèce.

▲ *A. schimperi* est l'espèce la plus rapide à germer (3 cm après 2 jours au lieu de 5 à 6 jours). Le pourcentage de germination est compris entre 80 et 85.

▲ Pour les espèces de *Sesbania* de Morondava, le taux de germination est plus élevé par la méthode mixte scarification-eau chaude. *Sesbania* de Fort-Dauphin n'a pu présenter qu'un taux de germination supérieur à 60% dans les conditions des expériences.

B. Etudes de comportement en vases de végétation: Essais d'étude de l'effet de la photopériode

Six (6) semis ont été effectués de novembre 1986 à mars 1987; ceux-ci ont permis d'observer des différences réelles de comportement entre les 7 espéces étudiées. Une plus grande production végétale est obtenue au cours des premiers semis.

▲ Le cycle végétatif est plus court pour *A. schimperi* (127 jours au lieu de 145 jours ou plus pour les autres espèces).

▲ La floraison se produit plut tôt pour *S. rostrata* que pour *Aeschynomene* dans les mêm conditions. Il en est de même de la fructification. Puis viennent *A. schimperi, A. uniflora, A. sensitiva* et *Sesbania* sp. Une précocité de la floraison a été observée pour les premiers semis.

▲ La résistance aux insectes (pucerons) est différente; elle se présente comme suit: *A. schimperi, A. uniflora, Sesbania* sp. *S. rostrata, A. sensitiva.*

On observe une prolifération des insectes aux 5ème et 6ème semis.

Les expériences relatives à l'effet de la hauteur d'eau sur la nodulation caulinaire ainsi qu'à la mesure de l'activité réductrice d'acétylène (ARA) n'ont pas donné de résultats probants en vases de végétation; l'insuffisance d'immersion a limité la nodulation. Le problème d'induction de la nodulation reste encore à résoudre.

Les divers résultats obtenus ont fait l'objet d'analyses statistiques en composantes principales (ACP) pour ce qui concerne les corrélations entre divers paramètres, les corrélations entre espèces étudiées et les divers paramètres, le comportement des espèces suivants le traitement.

IV. Essais agronomiques

Les essais d'application agronomique ont été entrepris par la FOFIFA[*] en procédant à des tests de comportement et d'enfouissement en différents endroits pour déterminer les conditions optimales d'utlisation des engrais verts dans différentes zones rizicoles malgaches ([*] FOFIFA ou CENRADERU = Centre Nationale de Recherche Appliqué au Développement Rural).

A. Les essais de comportement des divers engrais verts ont été effectués en rizières dans différentes régions:

▲ A Tanandava (Côte Ouest, chaud et sec, 0 m d'altitude)
Une forte croissance et une bonne nodulation caulinaire ont été observées pour *Sesbania rostrata* (de Madagascar et du Sénégal).

 • Hauteur maximale: 3-4 m au bout de 4 mois de végétation;

 • Production de matière sèche abondante: 6 tonnes/ha au bout de 8 semaines après le semis;

 • Date de semis optimale: mois de novembre où la photopériode et la pluviosité sont favorables à une croissance rapide;

 • Inoculation des souches bactériennes non indispensable.

 • Scarification des graines soit par friction sur sable silicieux, soit à l'acide sulfurique (80% de levée).

▲ Sur les Hauts-Plateaux (1.400 m d'altitude)
Deux essais sur le comportement de *Sesbania* et d'*Aeschynomene* ont été menés à Betafo et Manjakandiene. Les *Sesbania* germent très mal et leur croissance est faible (30 cm de haut en 4 mois et uniquement 300 kg de matière sèche/ha).

B. Les essais d'enfouissement ont été entrepris sur la Côte Ouest à titre démonstratif en parcelle de 10 m x 10 m pour voir l'effet sensible sur le rendement rizicole; les résultats après extrapolation sont:

▲ Parcelle 1 traitée avec 60 kg d'urée, 5,2 tonnes de riz/ha et 9 tonnes de paille/ha.

▲ Parcelle 2 traitée avec *S. rostrata* de Madagascar, 5,4 tonnes de riz/ha et 11 tonnes de paille/ha.

▲ Parcelle 3 traitée avec *S. rostrata*, 5,8 tonnes de riz/ha et 14 tonnes/ha paille.

(le rendement moyen du paysan étant 2 tonnes de riz/ha).

Compte tenu du calendrier rizicole et des taux de croissance des plantes, l'usage des *Sesbania* dans la zone Sud-Ouest en tant qu'engrais vert semble tout à fait concevable (en 50 jours de végétation). Par contre, bien que *Aeschynomene uniflora* présente une bonne indication de matière sèche produite (6,3 t/ha en 121 jours), seules *A. uniflora* et *A. sensitiva* semblent convenir à l'apport d'azote au profit des rizières des Hauts-Plateaux. Cependant, leur période de croissance maximale coïncide avec le calendrier rizicole de la région, ce qui exclut leur culture avant la culture de riz.

V. Conclusions et perspectives

Compte tenu des résultats préliminaires particulièrement prometteurs obtenus sur *Sesbania* et d'autres légumineuses à nodosités caulinaires de Madagascar, et ce, malgré des moyens modestes, de nombreuses perspectives s'ouvrent à l'équipe pluridisciplinaire qui s'est constituée à notre niveau. Peu de recherches ont été effectuées sur le plan fondamental, mais les applications agronomiques s'avèrent nombreuses.

Si *Sesbania rostrata* de la Côte Ouest peut être inséré avant le repiquage du riz, des problèmes restent encore à résoudre: durée de culture, mode d'incorporation, étude de l'arrière effet, détermination du seuil de rentabilité.

Aeschynomene uniflora, espèce qui a survécu à la saison sèche après sortie de serre, pourrait être mise en culture entre deux saisons pour être enfouie avant le repiquage du riz. Toutefois, une méthode de culture intégrant les engrais verts dans la rizière en même temps que le riz est à rechercher: culture intercalaire (alley cropping), en bordure de rizière, etc... .

En guise de conclusion, nous reprendrons à notre compte les recommandations remises lors du Colloque International de l'Académie Malgache sur l'"Amélioration de la riziculture par des méthodes biologiques" (Antananarivo, Fianarantsoa, 7-15 septembre 1987). Il s'agit de:

▲ l'intensification et la continuation des recherches sur l'inventaire et la systématique des espèces fixatrices d'azote par des études fines de cytogénique et de phytohistologie, sur des études écophysiologiques en laboratoire et sur le terrain, et sur des études comparatives avec d'autres espèces africaines;

▲ la sélection, la conservation et la multiplication des souches de microorganismes fixateurs d'azote en vue de l'amélioration des légumineuses et des cultures vivrières;

▲ les études sur la valeur alimentaire et la valeur fourragère des parties aériennes de *Sesbania*;

▲ la conduite d'essais d'enfouissement sur différents types de sols (notamment les sols pauvres) ainsi que l'évolution des matières organiques et des éléments nutritifs libérés dans ces sols par des méthodes de marquage isotopique (^{14}C et ^{15}N);

▲ le développement simultané de la recherche fondamentale et de la recherche appliquée en vue d'une meilleure utilisation des légumineuses à nodosités caulinaires en riziculture;

▲ une collaboration étroite avec d'autres organismes de recherche et de développement nationaux, pour la poursuite des études pluridisciplinaires, et internationaux (ORSTOM, IRRI, IRAT par exemple) ainsi qu'avec d'autres pays travaillant sur le même sujet (Sénégal, Philippines).

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Possibilité d'utilisation des légumineuses à nodules caulinaires dans la riziculture malgache

R. Fetiarison, D. Montange, ET C. Samson

Les légumineuses à nodulation caulinaire comme le *Sesbania* ou l'*Aeschynomene* présentent la particularité de porter un nombre de nodosités bien supérieur à celui des légumineuses comme le soja ou le haricot, ce qui entraîne une potentialité fixatrice beaucoup plus importante. Ce sont des arbustes vivant dans des milieux inondés et ce fait les désigne comme susceptibles d'être cultivés en tant qu'engrais vert pour la riziculture irriguée. En cette première année d'essais, nous avons d'abord voulu déterminer si ces plantes croissaient bien dans quelques régions rizicoles malgaches et si l'enfouissement de la matière végétale issue de cette culture avait un effet sur la récolte suivante.

Etude comparative des deux espèces de *Sesbania*: *S. de Madagascar* et *S. rostrata* au point de vue microbiologique

Des études microbiologiques concernant les deux espèces de Sesbania: *Sesbania de Madagascar* et *Sesbania rostrata* du Sénégal ont été effectuées.

Etude du spectre d'hôte des rhizobium isolés

Des souches de bactériennes isolées à partir des nodules de tiges du *Sesbania de Madagascar* ont été comparées à la souche de *Azorhizobium* ORS 571 du *Sesbania rostrata* du Sénégal.

Les souches isolées du *Sesbania de Madagascar* et la souche ORS 571 ont été inoculées d'une part à *Sesbania rostrata* du Sénégal et d'autre part à *Sesbania de Madagascar* sur les racines et sur les tiges.

Ces souches nodulent et fixent l'azote sur les racines et sur les tiges de ces deux espèces de *Sesbania* montrant ainsi une grande similitude au niveau de la spécificité de nodulation des deux espèces malgré leur origine géographique très éloignée.

Etude préliminaire sur la physiologie des souches de Rhizobium isolées

Les souches de Madagascar sont apparues très proches de la souche ORS 571 sur plusieurs points:

▲ les types de colonies et la vitesse de croissance

▲ l'utilisation préférentielle du lactate comme source de carbone et une très faible croissance sur les sucres tels que glucose, arabinose, ribose, fructose et saccharose

▲ ces souches sont capables comme ORS 571 de fixer l'azote en culture libre, propriété unique parmi les *Rhizobium*.

Etude de croissance du *S. de Madagascar* et du *Sesbania rostrata* du Sénégal

Une différence de croissance apparaît rapidement entre les deux espèces. *Sesbania de Madagascar* présente un plagiotropisme net et une tendance à la ramification plus développée que *Sesbania rostrata*.

On peut conclure que *Sesbania de Madagascar* est bien différent de *Sesbania rostrata* du Sénégal. Sur le plan de la spécificité de nodulation, il semble qu'ils font partie de la même espèce. Peut-être s'agit-il d'une sous espèce du *Sesbania rostrata*.

D'autre part, nos résultats confirment que le groupe de *Rhizobium* des tiges est un groupe très homogène puisque nous avons trouvé des caractères identiques entre les souches isolées à Madagascar et la souche de référence ORS 571.

Aussi les résultats des "essais de comportement - date de semis" et les "essais préliminaires d'effet d'enfouissement" confirment notre conclusion sur cette partie.

Des tests de comportement de légumineuses à nodosités caulinaires ont donc été mis en place en plusieurs points de Madagascar, sur les Hauts-Plateaux à Betafo près d'Antsirabe, à Manjakandriana, au Lac Alaotra et sur la Côte Ouest à Tanandava dans la région de Tuléar.

Les résultats de deux points seulement seront analysés ici, ceux de Batafo (1400 m) et ceux de Tanandava (au niveau de la mer), les autres essais ayant connu des fortunes diverses.

Sur les Hauts-Plateaux, les essais consistaient d'une part à comparer la croissance de 5 légumineuses à nodulation caulinaire en fonction de 3 dates de semis afin de déterminer les espèces les mieux adaptées à leur utilisation en tant qu'engrais vert.

Les 5 arbustes testés sont:

▲ *Sesbania de Madagascar*

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▲ *Sesbania rostrata* du Sénégal

▲ *Aeschynomene uniflora*

▲ *Aeschynomene sensitiva*

▲ *Aeschynomene schimperi.*

Sur la Côte Ouest, seuls les deux *Sesbania* ont été comparés.

D'autre part, un essai d'enfouissement a été mis en place pour déterminer l'effet de la matière organique apportée au sol sur le rendement de la culture de riz suivante. A Betafo, *Aeschynomene uniflora* et *Sesbania de Madagascar* ont été comparés, alors qu'à Tanandava, ce sont les deux *Sesbania* qui l'ont été.

1. Croissance des 5 légumineuses à Betafo

Des courbes (Figures I, II, III) représentent la croissance moyenne de toutes les plantes de l'essai pour 3 dates de semis. Il est à remarquer qu'il n'y a pas d'analyse statistique car le nombre des plantes mesurées est très variable d'une parcelle à l'autre, ceci étant dû tant à des hétérogéneités dans la levée qu'à des attaques d'insectes. De plus, ce test ne comportait que 2 répétitions.

La première remarque concerne l'absence de *Sesbania de Madagascar* dans ces courbes. Ce fait provient de la très mauvaise levée des semences (inférieure à 5%), malgré la scarification effectuée par abrasion à l'aide de sable. Les graines scarifiées avec de l'acide sulfurique concentré germaient un peu mais les plantules ont été détruites par des attaques d'insectes. Sur 100 graines semées, 3 plants seulement sont arrivés jusqu'à la floraison.

Quelle que soit la date de semis, une plante semble donner les meilleurs résultats pour ce qui est de la taille, *Aeschynomene uniflora*. En ce qui concerne *Aeschynomene sensitiva*, la taille n'est pas en rapport avec le poids de matière végétale de la plante qui a un port très étalé. Pour la première, à 133 jours, une plante de 185 cm pèse 32 g en moyenne, alors que la seconde, au même âge, ne mesure que 64 cm pour un poids moyen de 27 g. Si l'on veut donc continuer à comparer ces 2 espèces, il sera

nécessaire de trouver un critère non destructif autre que la taille.

Il semble ne pas y avoir une forte influence des dates de semis retenues sur la croissance de ces légumineuses à nodulation caulinaire. Mais il est nécessaire de les semer avant la fin du mois de novembre, car les semis effectués dans le courant du mois de décembre n'ont donné qu'un développement très réduit. Seule *Aeschynomene schimperi* subit une diminution de sa croissance en rapport avec une date de semis tardive, mais ceci n'est pas étayé par une étude statistique.

Il est à remarquer que les croissances reflètent assez bien la courbe de température, les plantes testées poussent beaucoup mieux quand les températures sont plus clémentes (janvier, février).

Ceci aura son importance lors de l'emploi en tant qu'engrais vert. Pour *Aeschynomene uniflora*, les plantes issues des 2 premières dates de semis ont une taille moyenne identique à la fin de leur cycle de développement.

Par contre, on observe chez cette même plante, une diminution avec l'âge, de la proportion de feuilles par rapport à la masse totale de matière végétale: le 9 mars, soit 119 jours après le semis (2ième date), les feuilles représentent 55% de la masse de la plante; le même jour, soit 133 jours après le semis (1ère date), les feuilles ne représentent plus que 39% du total. Ce fait peut avoir de l'influence sur l'utilisation en tant qu'engrais vert, car on peut penser que les feuilles se dégradent plus facilement dans le sol que les tiges lignifiées.

En ce qui concerne la nodulation, seules les graines de *Sesbania* ont été inoculées avec la souche de *Azorhizobium* ORS 571 du laboratoire de Microbiologie de l'ORSTOM à Dakar (Sénégal). Lors des prélèvements nous avons noté que les *Aeschynomene* étaient très nodulées sur les racines, au niveau de collet et sur les branches touchant le sol. Par contre, les *Sesbania* ne portaient pas de nodosités, ni sur leur système racinaire, ni sur les tiges malgré une inoculation de celles-ci par pulvérisation.

2. Croissance de *Sesbania* à Tanandava

A Tanandava, le *Sesbania rostrata* du Sénégal a un développement végétatif beaucoup plus important que celui de Madagascar. 140 jours après le semis, les premiers mesurent en moyenne 4 mètres alors que les seconds arrivent à peine à la moitié. A 8 semaines, date prévue pour l'enfouissement, les *Sesbania rostrata* du Sénégal mesurent 1,20 m alors que ceux de Madagascar ne font que 0,40 m. Mais le pourcentage de feuilles est le même dans les deux cas: 37% de la masse totale (Figures IV, V, VI, VII, VIII).

Les semis effectués après le début du mois de décembre semblent donner des croissances moins importantes que les semis plus précoces. Il faudra en tenir compte dans l'établissement du calendrier cultural de ces engrais verts.

3. Enfouissement à Betafo

Ce test, sans répétition, compare *Sesbania de Madagascar* et *Aeschynomene uniflora*. Les plantes des 2 parcelles devaient être enfouies 8 semaines après le semis et l'effet de la matière végétale apportée au sol comparé à l'action de 60 unités d'azote sous forme d'urée.

A 8 semaines, les plantes ne mesuraient que quelques centimètres et la matière végétale qui aurait été ainsi enfouie n'aurait repésenté qu'une dizaine de kilogrammes par hectare. Nous avons préféré laisser pousser ces plantes jusqu'à une taille suffisante et, en tout état de cause, l'enfouissement devait avoir lieu avant la fructification.

Afin d'éviter les problèmes liés à la mauvaise levée des *Sesbania*, nous avons semé des graines déjà germées, ce qui a permis d'avoir une densité convenable (250.000 pieds/ha). Malgré cela les *Sesbania* n'ont eu qu'une croissance très médiocre. A 121 jours, la masse végétale sèche n'était que de 300 kg/ha (extrapolation à partir d'une parcelle de 50 m), alors qu'au même moment, la productivité d'*Aeschynomene* était de 6,3 tonnes/ha. Seules les *Aeschynomene* ont donc été enfouies, 17 semaines après le semis.

L'enfouissement a eu lieu le 16 mars 1987, mais il n'était alors plus possible de repiquer du riz sur ces parcelles, la saison étant déjà trop avancée. Le riz sera repiqué dès le début de cette saison, la matière végétale ayant ainsi séjourné 6 mois dans le sol durant la saison sèche.

On peut imaginer d'autres moyens d'enfouissement, comme le piétinement de zébus par exemple. L'incorporation de ces cultures d'engrais vert entraîne un surcroit de travail pour le cultivateur ce qui ne facilitera sans doute pas l'adoption de ce procédé dans le cas où l'augmentation de rendement n'est pas importante. Des moyens mécaniques de fauchage et de hachage économiseraient beaucoup de main d'oeuvre.

4. Enfouissement à Tanandava

Les 2 espèces de *Sesbania* sont ici comparées pour leur valeur fertilisante, avec un témoin recevant 60 unités d'azote sous forme d'urée avant le repiquage. Le test a lieu sur des parcelles de 100 m sans répétition.

Les 2 espèces ont été enfouies 9 semaines après le semis. Les masses de matière sèche diffèrent beaucoup: le *Sesbania rostrata* du Sénégal a produit 10 tonnen/ha

alors que le *Sesbania de Madagascar* a produit 10 fois moins environ. Ces chiffres ne donnent qu'un ordre de grandeur car ils sont extrapolés à partir d'une ligne de 2 mètres linéaires.

Dix jours après l'enfouissement, les plants du riz ont été repiqués. La végétation de la parcelle ayant reçu le *Sesbania rostrata* du Sénégal était plus dense et plus haute, avec un certain moutonnement dû sans doute à une mauvaise répartition des émondes.

La parcelle ayant recu le *Sesbania rostrata* du Sénégal a donné 14,6 t/ha de paille et 5,8 t/ha de paddy, alors que celle avec urée portait 9,3 t/ha de paille et 5,4 t/ha de paddy.

Traitement	Paille T/ha	Paddy T/ha
1 *Sesbania de Madagascar*	11,9	5,65
2 *Sesbania rostrata* Sénégal	14,6	5,80
Tém. 60 U. azote	9,45	5,44

Des différences existent, surtout au niveau des pailles, mais ceci demande confirma-

tion avec une étude comportant des répétitions. De même, il serait intéressant de suivre la fertilité de ces parcelles lors de la prochaine saison de culture afin de voir s'il existe un arrière effet de l'incorporation au sol de cette matière organique (fertilité, portentiel hydrique).

Nous pouvons déjà entrevoir ce que pourrait devenir l'emploi des légumineuses à nodulation caulinaire en tant qu'engrais vert dans la riziculture malgache.

Dans les zones tropicales, il est possible d'insérer avant le repiquage du riz une culture d'engrais vert. Des problèmes restent à éclaircir: la durée de cette culture, le mode d'incorporation, sans oublier bien entendu la détermination du seuil de rentabilité (rapport du temps de travail supplémentaire sur l'augmentation de rendement des cultures qui suivent cette incorporation; différence de coût avec l'apport d'urée équivalent).

Sur les hauts-Plateaux, il faut rajouter à cela le mode de culture de cet engrais vert en rizière. En tout état de cause, il faudra choisir une légumineuse n'ayant pas une trop forte dormance tégumentaire, ce qui semble exclure *Sesbania de Madagascar*.
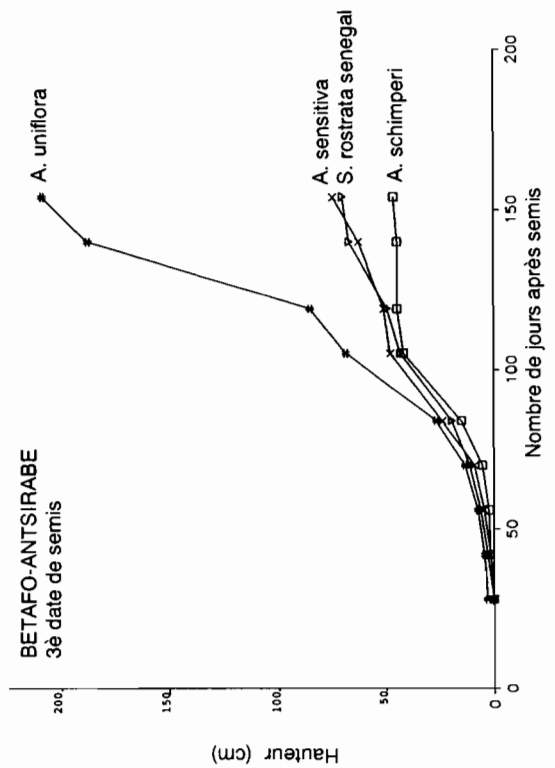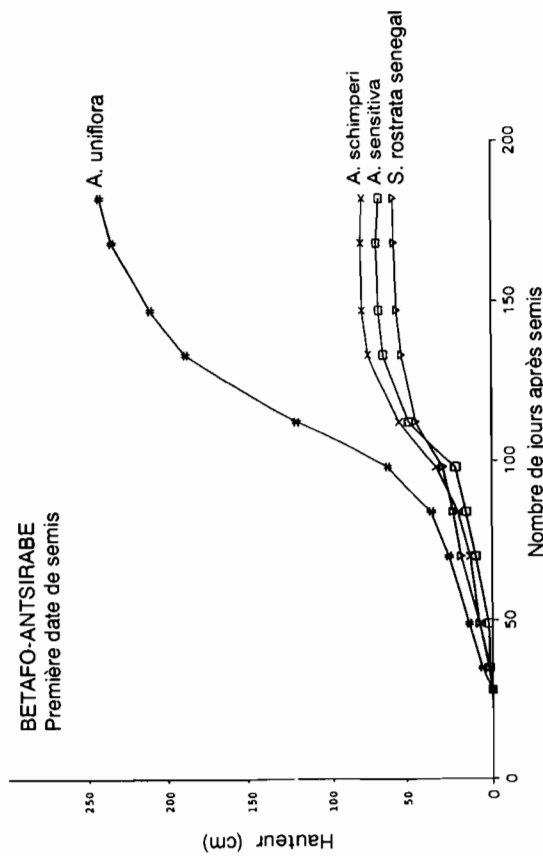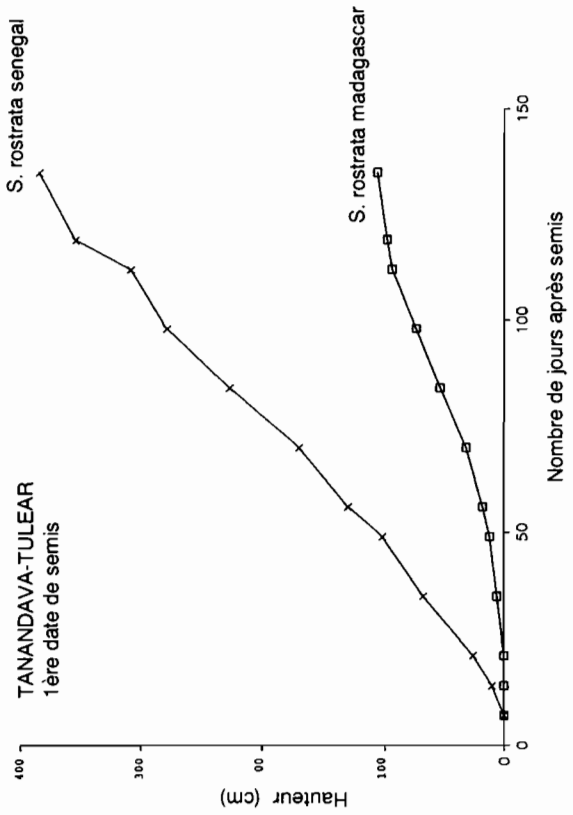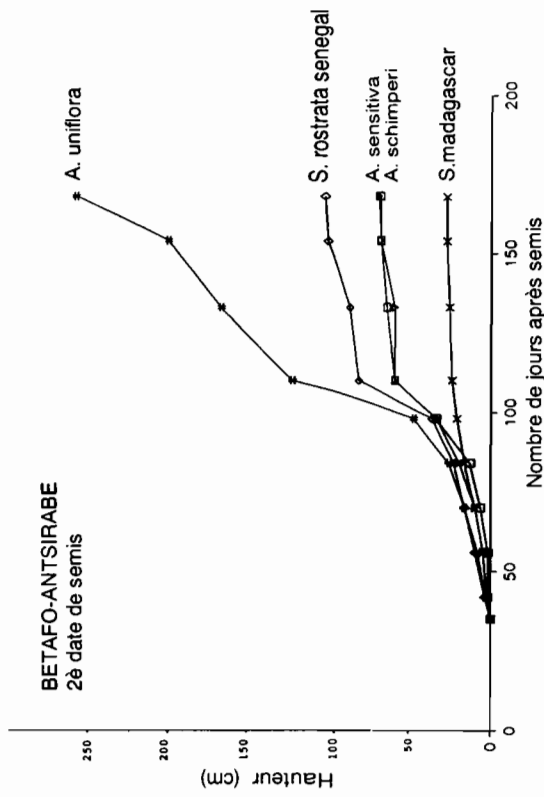
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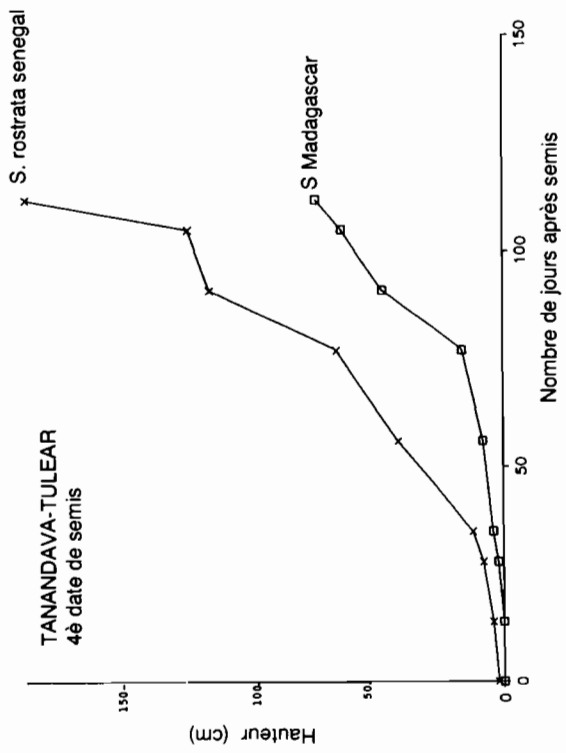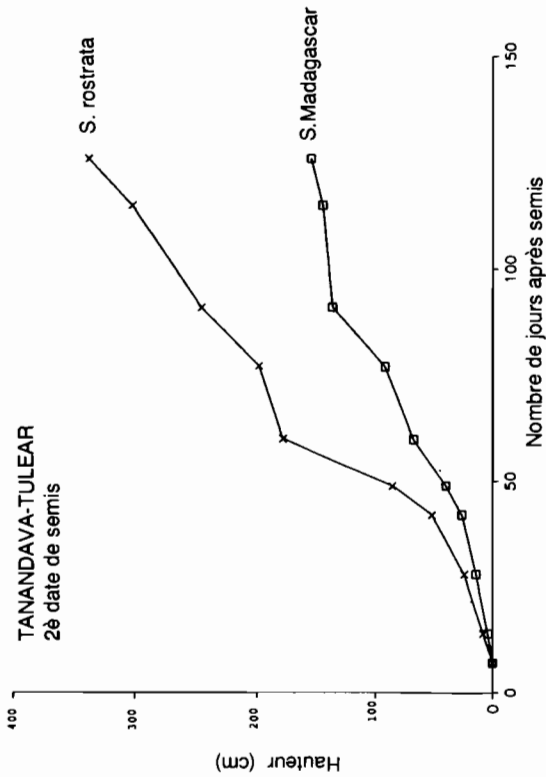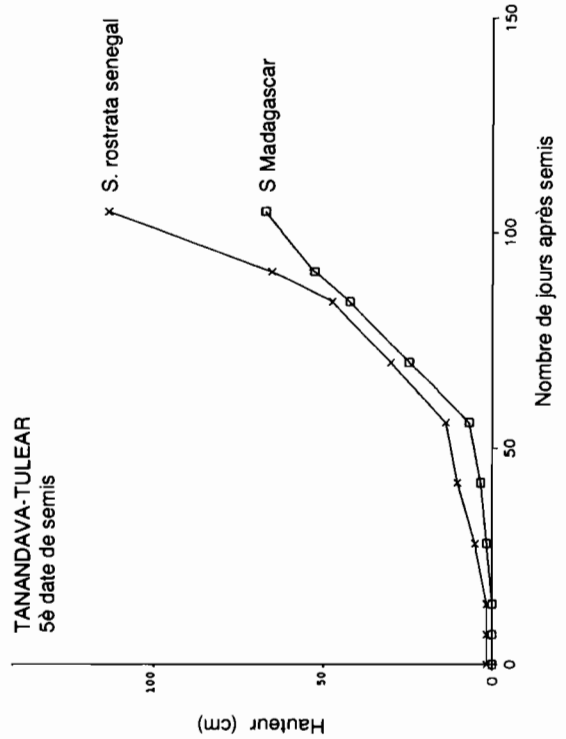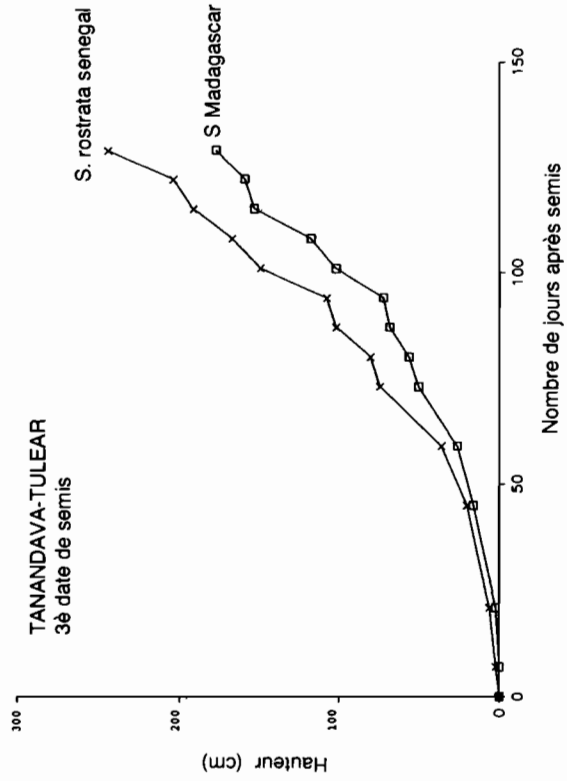
Graphique 2

Recherche de solutions aux problèmes limitant la vulgarisation de *Sesbania* sp. en riziculture paysannale malgache

R. Rabezandrina

Introduction

Nous sommes particulièrement convaincus de l'intérêt que présente l'utilisation de *Sesbania* comme engrais vert dans les rizières.

Cette plante, rappelons-le, peut se développer en sols inondés et atteindre plus de 2 mètres de haut en 2 mois quand les conditions qu'elle exige, notamment celles concernant la température et la durée du jour, sont remplies. Ses nodules caulinaires sont installés sur des sites prédéterminés, alors que ses racines se trouvent dans des conditions de submersion permanente, si bien que ses performances dans les rizières pour apporter non seulement de la matière organique mais aussi de l'azote, devraient être exceptionnelles, après enfouissement.

En outre, elle a la possibilité de "piéger" certains nématodes du riz et d'accroître la résistance de ce dernier vis-à-vis de ces parasites (Alazard et al., 1987).

Il ne fait aucun doute que l'utilisation raisonnée de *Sesbania* comme engrais vert fera augmenter le rendement des rizières malgaches dont la plupart souffre du manque d'azote et/ou d'une mauvaise structure du sol.

Toutefois, cette utilisation se heurte encore à un certain nombre de problèmes dont il convient de chercher ensemble les solutions.

Problèmes posés par Sesbania en riziculture

Sesbania utilisée comme engrais vert en riziculture pose les problèmes suivants:

▲ 1. Elle est sensible, d'après les résultats des travaux effectués jusqu'à maintenant, à la photopériode et ne présente un développement végétatif intéressant qu'en période de jour long, de décembre à février à Madagascar.
Or cette période, également chaude et pluvieuse, est aussi la période de culture du riz dans toute l'île. Elle est même la seule possible sur les Hauts-Plateaux à saison sèche et fraîche marquée.
Sesbania ne pouvant être cultivée en période de contre-saison devrait donc occuper une campagne rizicole entière. Cette éventualité n'est pas envisageable pour une rizière.

▲ 2. Les semences de *Sesbania* comme celles de beaucoup de légumineuses, sont dures et nécessitent, pour obtenir une germination homogène de la plante, une scarification mécanique ou chimique (Dreyfus, 1982), difficile à faire exécuter par les paysans.

▲ 3. Les semences de *Sesbania* germent en sol humide mais pas submergé. Cette propriété limite la période de semis, mais peut être intéressante en évitant que les semences dures à germination tardive ne deviennent des mauvaises herbes pour le riz quand celui-ci pousse dans l'eau.

▲ 4. *Sesbania* ne libère l'azote qu'elle a fixée qu'après son incorporation au sol, c'est-à-dire après hachage et enfouissement, deux opérations relativement pénibles si elles doivent être faites à la main et que le paysan malgache n'est pas volontiers disposé à exécuter sans être bien convaincu de leur intérêt ou de leur rentabilité. La pratique de l'engrais vert ne peut être vulgarisée et adoptée sans une technique paysannale d'incorporation au sol appropriée ou sans la disponibilité de moyens mécaniques convenables (Rabezandrina, 1986).

▲ 5. Enfin, en plus de ces quatre problèmes, il y en a un autre à résoudre de nature psychologique, et qui n'est pas le moindre: le paysan malgache, estimant qu'il n'a pas suffisamment à récolter pour avoir de quoi se nourrir, se demande pourquoi labourer le sol, et entretenir une plante uniquement pour tout enfouir ensuite.

Recherche de solutions

On peut avoir deux attitude vis-à-vis de ces problèmes. Il est possible de:

▲ soit continuer les recherches fondamentales et appliquées pour trouver des *Sesbania* adaptées aux conditions de culture du riz;

▲ soit adapter les modes traditionnels de culture du riz aux exigences de *Sesbania*.

Dans le premier cas, il faut continuer la recherche systématique de légumineuses à nodules caulinaires peu sensibles à la photopériode par toutes les voies possibles: prospections dans la nature, traitements

mutagènes ..., et étudier ensuite le comportement des variétés sélectionnées ou obtenues.

C'est un travail de longue haleine mais qui doit être poursuivi.

Dans le deuxième cas, on peut amener, par persuasion, les paysans de la Côte Est de Madagascar par exemple, où les conditions de pluviométrie et de température sont favorables à la riziculture toute l'année, (1) à ne pas faire une double riziculture, (2) à réserver à *Sesbania* la campagne rizicole qui a lieu en période de jour long.

C'est possible, d'autant plus que dans cette zone, la double riziculture n'est pas systématique et que la terre ne manque pas.

Toutefois, dans les deux cas, les problèmes psychologiques et ceux posés par le hachage et l'enfouissement de *Sesbania* restent entiers et demeurent sans solutions.

Aussi, nous sommes-nous orientés vers une autre voie qui consiste à étudier systématiquement les différents modes de riziculture malgaches.

Nous nous sommes dits que dans cette mosaïque de types de riziculture qui couvrent Madagascar, il y a des chances qu'il y en ait un qui puisse convenir à *Sesbania* et nous pensons justement en avoir trouvé un à Marovoay.

Insertion de *Sesbania* sp. comme engrais vert à Marovoay

Présentation de la région

Marovoay est située au Nord Ouest de Madagascar, dans une zone où *Sesbania rostrata* a été trouvé à l'état spontané (Perrier de la Bathie).

C'est le deuxième "grenier à riz" de Madagascar après Alaotra avec ses 17.000 ha de rizières et son surplus de production de 35.000 tonnes de paddy commercialisé par an.

La culture du riz à Marovoay

Il y a 3 saisons de culture du riz dans cette zone:

▲ a) la saison Asara, de novembre à avril, pendant laquelle on cultive le riz sur les bordures non inondées de la plaine. Elle représente environ 15% de la production de la région.

▲ b) la saison Jeby, période de jour court, d'avril à octobre (80%). Elle est celle qui nous intéresse et qui concerne la plaine proprement dite de Marovoay.

Cette plaine, en saison pluvieuse de novembre à mars, risque d'être plus ou moins inondée suivant l'importance des crues du fleuve Betsiboka qui la traverse. On ne la cultive donc pas pendant cette saison et la végétation spontanée s'y installe abondamment, favorisée par la chaleur et l'humidité.

En avril, dès le début des retraits des eaux, on travaille le sol soit par piétinage animal, une technique traditionnelle employée dans la zone bien avant ce siècle, soit par piétinage mécanique, assuré depuis 1960, par un organisme de développement (FI.FA.BE.) qui utilise des tracteurs dont on a changé les roues arrières contre de grandes roues-cages métalliques. Le tracteur ainsi modifié, en faisant des va-et-vient sur la rizière submergée, piétine celle-ci à la façon d'un troupeau de zébus.

Dans les deux cas, le travail obtenu par malaxage intime de la végétation spontanée et du sol, est excellent. Cette végétation est un véritable engrais vert qui permet d'obtenir 3 à 4 tonnes/ha de paddy sans aucun autre apport de fumure.

Concordance heureuse entre la campagne rizicole "Jeby" de Marovoay et l'utilisation de Sesbania comme engrais vert

▲ a. Marovoay est situé dans la zone où l'on trouve *Sesbania* à l'état spontané. Il doit donc y avoir une concordance positive entre les exigences écologiques de *Sesbania* et les conditions climatiques et édaphiques de la région.

▲ b. La campagne rizicole de la saison Jeby, en période de jour court et relativement fraîche, ne coïncide pas avec la période favorable au développement de *Sesbania*, en saison de jour long et chaude.

▲ c. Si on considère *Sesbania*, non comme un engrais vert planté spécialement pour cet objectif, mais comme une plante semée sans préparation particulière avant la submersion de la rizière, et se mélangeant avec la végétation spontanée ensuite, le problème de scarification ne se pose pas, surtout si on utilise une quantité de semences suffisante. Les semences dures qui ne germent pas la première année, se développeront l'année suivante après assèchement de la plaine une fois le riz récolté. Elles constitueront les engrais verts de la campagne suivante.

▲ d. Ces semences à germination tardive ne seront pas des mauvaises herbes pour le riz, car le jour court et la submersion des rizières les inhiberont jusqu'à la fin de la campagne rizicole.

▲ e. Avec le système traditionnel de préparation des sols de la région, par piétinage animal ou mécanique, le problème de hachage et d'enfouissement de *Sesbania* est résolu.

▲ f. Le problème psychologique ne se posera pas non plus: *Sesbania* sera considérée comme une végétation spontanée qu'il faut enfouir comme les autres.

▲ g. La zone de Marovoay, deuxième région rizicole de Madagascar, rappelons-le, est suffisamment importante pour que l'impact de l'**action *Sesbania*** effectuée dans cette zone, soit perceptible au niveau national.

Conclusion

L'insertion de *Sesbania* sp. comme engrais vert spontané sur des parcelles paysannales réelles cultivées en saison Jeby à Marovoay, mérite d'être réalisée.

Nous allons justement essayer de le faire en tenant compte des acquis actuels sur les connaissances de *Sesbania* et sur les techniques culturales adoptées depuis des années dans la région.

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Sesbania rostrata as green manure for amelioration of salt-affected soils

H.Q. Yao, J.Q. Cao, and B.Y. Chen.

Introduction

Sesbania has been cultivated as one kind of green manure and fodder crop in China for a long time. Although it was originally grown tropical and subtropical regions, many new varieties obtained by breeding or selection were adaptable to be grown in different localities. There are 7 species of *Sesbania* cultivated in our country; they are: *Sesbania cannabina* pers, *Sesbania aegyptiaca* pers, *Sesbania aculeata* pers, *Sesbania paludosa* prain, *Sesbania grandiflora* pers, *Sesbania speciosa* and *Sesbania sesban*, 1986). The total acreage Sesbania planted in China has amounted to about 0.8 million ha constituting 7.8% of the total area of green manure crops. *Sesbania* was introduced into China in 1984 (Chen Li, 1986) and attracted much attention because of its high activity of N-fixation. However, it can only be cultivated in the region south to 25N, because it can not pod under long-day length and low temperature. This paper deals with its response to illumination and both the effect of N fertilization and soil salinity on growth and activity of N-fixation of *S. rostrata*.

Materials and methods

▲ 1. Pot experiment: 0.5 g ammonium phosphate per kg soil was added to the soil used. In short-day length treatment, the exposure time was 12.0, 11.50, 11.0, 10.0 hrs/day and each was lasted for 7, 15, 30, 45 and 60 days respectively. Each treatment was triplicated.

▲ 2. Solution culture experiment: Added ^{15}N labelled $(NH_4)_2SO_4$ (10.27 N% abundance) in 4 levels of N (2.5, 5.0, 10.0, 20.0 ppm) to Withman solution without N, and it was used in solution culture to investigate the effect of fertilizer-N on growth and N-fixation of *S. rostrata*. The final pH of this solution was adjusted to pH 5.5 with HCl. The culture solution was changed every two weeks during the experiment and distilled water was added to supplement the water loss due to evaporation. All the treatments were replicated four times.

▲ 3. Pot experiment: 0.2%, 0.3%, 0.4%, 0.48%, 0.6% of sodium chloride were added respectively to a calcareous soil for studying the salt tolerance of *Sesbania rostrata*. Each treatment was replicated four times. Three plants were cultivated in each pot and harvested 55 days after sowing.

Results and discussion

Sesbania rostrata is a short day plant; the optimum temperature for its growth is 17-38 C, and the most suitable day length is 11-13 hrs (Rinaudo et al., 1987). Because of its sensitivity to temperature and day length, cultivation of this species is limited in the region south to 25N in China. In the region north to 25N, because of the vegetative over-growth in late spring and summer, its reproductive stage is delayed resulting in the plant being not able to seed and mature in time. Based on the observation on photoperiodic phenomenon of *S. rostrata*, experiment of day length shortened by artificial shading was carried out to stimulate its development for early blooming and seeding. It is indicated in Table 1 that days required from sowing to blooming were decreased with the shortening of day length. The period from sowing to blooming was 52 days, 46 days, 37 days and 35 days at day length of 12, 11.5, 11, and 10 hrs/day respectively. The period from sowing to blooming was shortened by 60, 66, 75 and 77 days as compared with that of the treatment under natural length of day (14.4 hrs). After the blooming of the plants under shading treat-

Treatment		Days from seeding to	Vegetative period shortened	Grain yield g/plant	1000 grains wt
Day length hrs/day	Duration days				
14.5	-	112	-	0	0
12.0	45	-	-	0	0
	60	52	60	6.5	15.3
11.5	45	-	-	0	0
	60	46	66	12.7	16.9
11.0	45	37	75	6.2	n.d.
	60	37	75	17.6	18.3
10.0	45	35	77	7.0	n.d.
	60	35	77	14.8	21.6

Table 1 : Physiological effect of day length treatment on Sesbania rostrata.

Mean of triple determinations.
n.d. = not determined.

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ment, it must be shaded again for 15-20 days. Otherwise the flowers bloomed would fade. According to our experiment the optimal conditions for growing *Sesbania rostrata* to maturity in Nanjing are: sowing in early June, after its sowing, the plant should be put under the day length of 11.0 hrs/day by artificial shading for 60 days.

By this way *Sesbania rostrata* blooms 38 days after sowing and it can mature and seed in September. The whole growth cycle of *Sesbania rostrata* is completed in four months. Thus our experiment has shown that it is possible to plant *Sesbania rostrata* in the region north to 25N.

N-free sand culture experiment showed that the height, dry weight and nitrogen content of *Sesbania rostrata* were much greater than those of *Sesbania aegyptiaca*, indicating that the activity of N-fixation of the former was much higher than that of the later (Table 2).

It is well known that symbiotic N-fixation is decreased with the increase of mineral nitrogen applied. Bernard et al (1981) reported that the growth of stem nodules of *Sesbania rostrata* was not affected by fertilizer-N. Our experiment showed that the biomass and total N content of *Sesbania rostrata* were increased with the increase of fertilizer-N, but the amount of N biologically fixed remained unaffected in the range of concentrations of 0-10 ppm N. In treatment of 20 ppm N, 66.7% of the total N of plant was biological fixed, while in the rest of the treatments the total N biologically fixed ranged from 83.4% to 94.6% of the total N of plant (Table 3).

In treatment of 20 ppm N, amount of N fixed by both stem and root nodules decreased significantly, especially that of root nodules. These results indicate that N-fixation of *Sesbania rostrata* by stem and by root nodules are influenced by fertilizer-N. However, at concentration of 0-10 ppm N, the amount of N fixed by root nodules was increased and then decreased with the increase of fertilizer-N, while that fixed by stem nodules was decreases and then increased with the increase of fertilizer-N (Table 4). It is evident that the activity if N-fixation of stem nodules is affected by that of root nodules, and it is less affected by fertilizer-N than that of root nodules.

Species	Height	Dry matter g/plant	N %
S. rostrata	60 a	0.68 a	1.71 a
S. aegyptiaca	17 b	0.05 b	0.60 b

Table 2 : Biomass yield and amount of N accumulated of Sesbania rostrata and Sesbania aegyptiaca in sand culture.

* *Means in each column followed by different letter are significantly different at the 1% level.*

N ppm	Biomass yield g/plant	Total N accumulated g/plant	N dfa	
			mg N/plant	%
0	6.09	150.3 a	150.3 a	100
25	7.05	152.4	144.2	94.6
5.0	7.25	160.7 ab	144.2 a	89.7
10.0	8.05	171.5 b	142.9 a	83.4
20.0	8.53	173.1 b	115.5 b	66.7

Table 3 : Effect of fertilizer-N on the growth and biological N-fixation of S. rostrata.

* *Means in each column followed by different letter are significantly different at the 5% level.*

N ppm	N dfa, mg/3 plants		
	Total	Stem nodule	Root nodule
0	450.9 a	218.8 a	232.2 c
2.5	432.6 a	137.8 c	294.8 a
5.0	432.7 a	160.8 b	269.6 b
10.0	428.6 a	204.9 a	223.7 c
20.0	346.4 b	176.2 b	170.2 d

Table 4 : Effect of concentration of (NH4)2SO4-N on the biological N fixation by stem and root nodules of S. rostrata.

* *Means in each column followed by the same letter are significantly different at the 5% level.*

NaCl %	Height cm	Dry matter g/3 plants	Total N mg/3 plant
0.04 (ck)	122.9	25.4 a	753.1 a
0.20	125.0	25.7 a	745.6 a
0.30	122.2	20.0 b	610.1 b
0.40	107.4	17.2 b	487.5 c
0.48	107.5	13.6 c	372.6 c
0.6	21.7	0.4 d	12.9 d

Table 5 : Effect of salt content of soil on the growth of S. rostrata, pot culture.

* *Means in each column followed by the same letter are not significantly different at the 5%.*

Performance of growth	NaCl (%)	
	S. rostrata	S. cannabina
Normal	0.2	0.15 - 0.26
Retarded	0.3 - 0.48	0.29 - 0.31
Seriously retarded	0.5 - 0.6	0.38 - 0.44

Table 6 : Salt tolerance of S. rostrata and S. cannabina.

Sesbania rostrata is a species with strong salt tolerance.

Experimental results showed that it grew normally on soils containing salt of less than 2%, while on soils with total salt content from 0.3 to 0.48%, the dry matter produced and total N content of plant were decreased along with the increase in salt concentration. Its growth was severely when salt content in soil was higher than 0.48%, and even died when it amounted up to 0.6% (Table 5).

Results also showed that the length and biomass of roots of *Sesbania rostrata* were decreased with the increase of salt content. It is concluded that the salt-tolerance of *Sesbania rostrata* is comparable to that of *Sesbania cannabina* (Table 6).

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Green manuring *Sesbania* in different cropping systems

O.P. Meelu

Introduction

The vast majority of soils in south and south-east Asia are deficient in nitrogen and organic matter. application of fertilizers and manures is, therefore, necessary. Role of inorganic fertilizers in agriculture productions cannot be over emphasised. There is need and lot of scope to increase fertilizer consumption to meet increasing food requirements. However, the fertilizer production is energy input intensive. It is estimated that fertilizer production accounted for about 45% of energy used in agriculture worldwide. Of this 73% is used in the manufacture of nitrogen fertilizer (Mc Cune, 1984). However, except for energy conservation measures, little attention has been given to devising management methods which maintain soil productivity while reducing dependence on non-renewable resources. This calls for the attention to mobilize all the local manurial resources and use of green manures as substitute for inorganic fertilizer and maintenance of soil fertility.

During the last few decades, organic manures were abandoned in favour of inorganic fertilizers that cam the modern varieties or rice, maize and wheat. But in the face of high fertilizer input cost and for maintaining soil fertility organic manures and biofertilizers are once again gaining popularity.

This paper present the results of integrated management of green manure as substitute for fertilizer N and effect of soil productivity.

Green manure

N-accumulation

Biomass production and N accumulation of green manures vary widely in response to soil, weather and crop management. The range in N incorporated by green manures from a few experiments are shown in Table 1. From a comparison of the *Sesbania aculeata*, sunhemp (*Crotalaria juncea*) and guara (*Cyamopsis tetragonaloba*), Uppal (1955) reported that the former was the best green manure for rice in alkali soils. Even in normal soils. *Sesbania* is often superior to other green manures because of its ability to withstand prolonged moist conditions.

Time of incorporation

Traditionally green manures were grown in fallow fields on rain water and incorporated about 2 to 4 weeks before sowing of the following dry season crop. However, this practice in not feasible in the context of intensive agriculture where there is a fallow period of only 40 to 60 days before transplanting rice. Berri and Meelu (1981) in a study on the effect of incorporation of green manure incorporated immediately before transplanting was delayed 1 to 2 weeks (Fig. 1). Bhardwaj et al. (981) reported similar results.

Effect of P application

Beri and Meelu (1980) found that on a soil testing low in available P in Ludhiana, India, application of P to a green manure crop was beneficial to green manure production and N accumulation (Table 2) and gave better rice yield than P application to rice (Fig. 2). On a soil testing high in available P, in the Philippines, however, Meelu et al. (1986) obtained mean N

accumulation of 199 kg/ha from *Sesbania aculeata* without P application. This need to be further investigated.

Fertilizer N substitution in rice

A wide range in N accumulation is possible (Table 1) and the quantity of green manure N incorporated is likely to determine yield responses to green manuring. Beri and Meelu (1981) reported a substitution of 60 kg N/ha by a green manuring with *Sesbania rostrata* in rice (Fig. 3). Substitution of 45 to 120 kg N/ha with green manuring was reported by Williams et al. (1972), Tiwari et al. (1980), Bhardwaj and Dev (1985), and Morris et al. (1986). Differences in N accumulated by the green manures, and in the recovery and utilization of the incorporated N owing to differences in crop management, cultivars, soils and weather, may explain the wide substitution range. Factors contributing to the range are not clear form the reports.

N substitution in maize

Singh et al. (1981) reported that incorporation of 60 days cowpea green manure about a week before sowing of maize gave N substitution of 75 kg N/ha in maize grain yield (Table 3).

N substitution in oilseeds

Dhillon et al. (1984) studied the effect of application of cut and chopped 20 t/ha guara (*Cyamopsis tetragonaloba*) adding 132 kg N/ha incorporated about 10 days before sowing raya (*Brassica juncea* L.) on its yield. The results showed that 50 kg N/ha in combination with green manure gave more grain yield of raya than 150 kg/ha fertilizer N application indicating a substitution of 100 kg N/ha with green manuring.

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Green manuring under rainfed conditions

Crops in rainfed areas area not only thirsty but hungry also. In situations where wet season rainfall is not enough to raise maize grain crop, legume green manure incorporation may help to improve soil fertility and increase yield of following winter season crop. Singh et al. (1986) obtained considerable increase in wheat yield by incorporation 50-60 days sunhemp green manure grown in wet season in about mid of August and allowing sufficient time for decomposition before sowing wheat in last week of October to first week of November (Table 4).

Mungbean for gain and green manuring

With the introduction of high yielding cereal varieties, cereal hectareage has increased at the cost of pulses. One effective way to bring a pulse into rotation is to adopt wheat-summer mung or cowpea-rice sequence. This is possible with short duration mungbean and cowpea varieties which mature in 60 to 70 days. Meelu and Rekhi (1983) found that in a wheat-summer mung-rice rotation mungbean produced 0.86 t/ha grain and incorporation of straw gave a substitution of 60 kg N/ha in rice (Table 5; Fig. 4).

Green manure and micronutrient availabilities

From a two year field experiment on a coarse textured soil at Ludhiana, Takkar and Nayyar (1986) reported that Fe deficiency in rice was more effectively corrected by *Sesbania* green manuring alone or in combination with soil applied Fe than by soil applied Fe alone (Fig. 5). Gopala Rao (1956) reported that green manuring with Glyricidia maculata in a pot experiment increased iron and manganese availability and shoot weight of rice. The results indicate the *Sesbania* green manuring maintained higher N, increased availability of Fe^{++}, and Mn^{++} in soil and gave higher rice grain yield.

Residual effect of organic manures

Sethi et al. (1952) reviewed green manuring research in India and concluded that the residual value of green manure applied to rice was low. Singh (9181) also did not obtain residual effect from green manure incorporated before rice in a rice-wheat rotation. However, Tiwari et al. (1980) and Singh (1971) reported a residual effect from green manuring rice on a succeeding wheat crop.

Effects of green manuring have been observed on soil fertility parameters. Sahu and Nayak (1971) studied the effect of fertilizers alone and in combination with FYM and green manure. They observed that FYM and green manure improved soil fertility relative to control and fertilizers treatments (Table 6). Green manuring increased total soil N and maintained organic carbon and available P. However, available K declined slightly. Bhardwaj and Dev (1985) obtained soil organic carbon and total N increases from incorporation of *Sesbania* in an experiment in a subtropical humid climate.

Greenmanure						
Age days	Sesbania Cannabina	Crotolaria juncea	Cowpea N kg/h	Ipomea	Pillipasara	References
45	79	-	32	-	-	Rajbhandari, 1984
56	57	78	-	28	-	Bhardway et al., 1981
57	102*	-	-	-	-	Ghai et al, 1985
60	105*	-	-	-	-	Beri & Meelu, 1981
65	165	-	-	-	-	Bhardwaj & Dev, 1985
60	133	134	74	-	102	Sanyasi Raju, 1952

Table 1 : N accumulation in green manures.

* *S. aculeata.*

Treatment	Green weight t/ha	Dry weight t/ha	N-added kg/ha
G.M. without P	17.9	4.0	88.2
G.M. without P	20.8	4.2	-

Table 2 : Effect of application on green matter and nitrogen addition in soil.

Nitrogen applied to maize (kg/ha)	Grain yield (t/ha)	
	Fallow	Cowpea
50	2.9	3.3
75	2.9	3.7
100	3.2	4.0
125	3.4	4.2
Mean	3.1	3.8

Table 3 : Effect of cowpea green manure and nitrogen rates on maize yield (3 years average).

Crop	Average of year	No. of trials	Grain yield (t/ha)			
			Control	Fertilizer	Control + Sunhemp	Fertilizer + Sunhemp
Wheat	Two	8	0.9	1.7	1.4	2.5

Table 4 : Effect of fallow-green manuring on succeeding crop.

Particulars	1980	1981	1982	Mean of 3 years
Grain yield of mung, t/ha	0.84	0.84	0.89	0.86
N content (%)	3.84	3.92	3.64	3.80
N removal by grain, kg/ha	32.30	33.40	32.40	32.70
Fresh weight of mung straw, t/ha		7.60	7.60	7.60
Dry weight of mung straw, t/ha		4.60	4.60	4.60
N content of mung straw, (%)	2.16	2.21	2.26	2.21
C:N ratio	16.10	16.10	15.10	16.10
N added through straw, kg/ha	99.40	101.70	101.70	101.70

Table 5 : Grain and straw yield of mung and N addition in soil.

Treatment	Total N %	O.C. %	Available P_2O_5 (ppm)	Available K_2O (ppm)
At start of trial, 1956	0.66	0.029	30	150
At the end of tenth year, 1965 No manure (control)	0.58	0.021	20	103
Organic manures 45 kg/ha N FYM	0.78	0.058	34	166
Green manure	0.67	0.054	33	130
Ammonium sulphate				
N 22.5 kg/ha	0.60	0.032	24	130
45.0 kg/ha	0.61	0.037	30	123
67.5 kg/ha	0.62	0.045	26	103
90.0 kg/ha	0.65	0.065	24	103

Table 6 : Effect of organic and inorganic fertilizers on nutrient status of soil.

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Sesbania rostrata : a source of nitrogen farming in Nepal

Bholoa Man Singh Basnet Kayo Devi Ysmi

Introduction

Nepal lies in between 804' and 8812' East longitude and 2622' and 3027' North latitude. The total cultivated area is 2,653,300 hectares with 338,672 hectares (up to 1984/85) under irrigation. Nepal is located in between China and India.

About 93 percent of Nepal's population are farmers. Indicating a vigorous dependence of the Nepalese economy on agriculture. The farming system is still traditional having limited improved facilities and practices. Among the different inputs to boost the agriculture production, fertilizer and the manure play an important role. The use of synthetic fertilizers in Nepal started since early sixties. Prior to the introduction of chemical fertilizers organic manure were basically the only source of nutrients for crops. The introduction of fertilizers reduced the use of organic manure to a great extent. However, the availability of chemical fertilizers in the country are still scarce specially in the hilly region. It is estimated that only 5 percent of the fertilizers imported is being used in mountains which represent 64 percent of the land (ADB 1982). So His Majesty's Government of Nepal given priorities in promotion and use of locally available resources as green manure to sustain the agriculture productions by maintaining soil fertility too.

Recent trends have indicated that one of the major factors responsible for the downward trend in agricultural productivity is the decline in soil fertility. For maintaining a uniform policy and insuring timely availability of fertilizers throughout the country. HMG/N has been providing transportation and other subsidies amounting to approximately 63 percent of the total cost of the fertilizer.

But experience has indicated however that majority of those that benefit from such subsidies have been those farmers that are already applying high quantities of fertilizer in their fields like the farmers in Kathmandu Valley and getting higher crop yields, and not farmers in the most of the hilly areas farming soils with inherently low fertility and marginal agricultural lands, where the fertilizer is most needed to raise productivity.

In spite of all these efforts, the unaccessibility of the hilly and mountainous terrains (which comprises most part of the country), soil erosion and degradation, the high cost of transportation, international price rise of fertilizers, timely unavailability of right kinds of fertilizers and distribution have become major constrains in increasing crops productivity. On the other hand, heavy and unbalanced use of chemical fertilizers in some parts of the country especially in Kathmandu Valley is also causing various soil problems.

The use of locally available different plant materials as nutrient source is a traditional practice of hill farmers in Nepal. However, dwindling supplies of these plant materials preclude their continued use as a source of green manure.

The use of dhaincha (*Sesbania cannabina*) as a green manure crop is common in the teras and inner teras regions of Nepal. Experiments conducted at several research farms and station showed that green manuring with dhaincha in rice increased the grain yield by about 20-25 percent. In the eastern terai, dhaincha (*Sesbania cannabina*) alone or in combination with fertilizer N significantly increased rice yield and was economically profitable. Many reports suggest that incorporating green manuring plants such as dhaincha has increased the paddy yield by 21 percent (Maskay et al., 1978). It was also reported

that the grains yield produced by the application of dhaincha as green manure plus 25 kg N/ha fertilizer is equal to the grains yield produced by 75 kg/ha fertilizer N alone. These results indicate that about 50 kg/ha fertilizer nitrogen can be saved by using dhaincha as green manure.

There is an urgent need to search for alternative sources of plant nutrients and utilize them more efficiently in order to raise crops productivity on sustained basis.

Among the various sources of locally available plant nutrients, dhaincha (Sesbania species) has the potential for supplementing plant nutrients and increasing crop yields.

Nepal is totally dependent on imports for its chemical fertilizer supply, much of it financed by donor agencies and the rest purchased by the government. Having no proven reserves of fertilizer raw materials, no production capability and a limited market, it is highly doubtful that this situation will change in the foreseeable future (Hill, 1982).

As the use of synthetic fertilizers alone eventually leads to productivity decline and organic matter depletion in the soil. Through exploitation of green manuring technology, farmers can possibly grow their own nitrogen and reduce dependence on expensive fertilizers, especially in remote areas. Fertilizer use in Nepal at present is about 18 kg/ha of nutrient, among the lowest in the world.

No doubt, the enthusiasm in green manuring have been renewed with the identification of stem nodulating species like *Sesbania rostrata* and other related plants which can tolerate both drought and temporary flooding and are able to accumulate substantial quantities of nitrogen and green biomass. Thus, the species such as

Sesbania rostrata offer a unique opportunity for increasing crops productivity by reducing the cost of chemical fertilizers plus enhancing the soil fertility build-up too.

The plants are grown a hedges, the wood is used for making gun-powder charcoal or fire wood, the fibre made into ropes, the foliage used as cattle feed and its ashes work as cleaning powder for the utensils.

Dhaincha is an annual plant and acts as a green manure or green leaf manuring crop or biofertilizer. Biofertilizers which are finding large-scale application as supplements to chemical fertilizers in the modern-day crop cultivation practices.

Sesbania is a good candidate for wetland rice cultivation.

Dhaincha can be used for shade loving plants like tea, ginger, turmeric etc.

Fisheries, livestocks, horticulture, tea, sugar cane programs are also interested in Sesbania program.

There is no fertilizer factory in Nepal and we have to import from foreign countries. Thus source, timely availability and kinds of fertilizers are not guaranteed. If we do not get chemical fertilizers then what will be the fate of our soils? Maximum exploitation of the locally available material have to be done very carefully.

Long term soil fertility trial

These results of the replicated long term soil fertility trials (10 years) at Hardinath Agriculture Farm are also hinting out that the use of nitrogen, phosphorus, potash plus compost are must to get higher productivity of rice and wheat on sustained basis.

Seed germination and production

The problem of poor seed germination have been experienced on *Sesbania rostrata* in Nepal even up to the extent of 89 percent hard seededness after 72 hours of soaking in water (Rajbhandary and Shakya, 1980). It has been observed that the seed coat is more thick and hard in comparison to *Sesbania cannabina*. The special treatment is required to promote seeds germination in rostrata.

On the basis of preliminary study, it is possible to make some suggestions for immediate use and further research needs. Seed scarification by concentrated H_2SO_4 proved to be a potential technique and soaking in it for 30-40 minutes will be as appropriate time to overcome 85 percent hard seededness of *S. rostrata*. Although it might not be feasible at farmers' level, in term of both availability and safety risk, but is can be practised at Agricultural Farms/Stations. The safe post scarification storage effect up to 30 days will be an advantage even to the farmers as they can store pretreated seeds stock upto that period without any appreciable loss in germination.

Immersion in water at boiling and near boiling point would have been a low-cost practical technique for farmers provided it were effective as the acid and sand paper scarification techniques. More studies should be conducted with repeated immersion in water at near boiling point and immersion at boiling point for less than 3 seconds to determine maximum possible germination of the vailable seeds without injury.

Seeds scarification by sand paper No. 60, although little bit time consuming but proves to be equally effective as acid scarification by concentrated H_2SO_4. Fifty seeds at a time scarified between sand papers for continuous 2 minutes gave maximum germination percent (87%). This will be practical technique of farmers' level when both safety risk and costs are considered. However, further studies need to be carried out to determine the optimum size of sand papers as well as the number of seeds and time to obtain maximum possible germination. Studies should also be conducted to develop simple low-cost manual sand paper or mechanical scarifier for use at the farmers' level. Similarly, studies should be carried out to determine how many times a particular sand paper can repeatedly be used without any appreciable loss in germination of the scarified seeds in each scarification. Post scarification storage effects for various periods also need to be studied.

It is advisable to encourage individual farmers in achieving seed self-sufficiency. This approach will assure on-farm seeds availability at a least cost. The self grown seeds will be seen by small farmers as a non-cash input, the use of which should facilitate adoption. For seeds production it takes about 5-6 month in teras situation.

There was no any organized seed production in the past for *Sesbania*. Some quanties are produced by stations and farms plus farmers. Agriculture Inputs Corporation (AIC), the main seed out-fit has recently interested to deal with sesbania seeds otherwise there were no private agency, except perhaps on occasional across-the-border seed trade. It remains to be seen if new opportunities will arise form the adoption of *Sesbania rostrata* on a large scale, both in the hill and terai, heading to planned seed production in Nepal.

Production of biomass

We can incorporate about 25 metric tons of green biomass of sesbania within 2 month of emergence.

Obstacles on *Sesbania* extension

▲ 1. Seeds unavailability,

▲ 2. Incorporation problem,

▲ 3. Irrigation problem,

▲ 4. Detail informations are lacking,

▲ 5. Farmers are unaware about dhaincha,

▲ 6. Conflict with other crops.

Selection of *rostrata* as a potential green manure

All the legume crops contain nodules on their roots with Rhizobia bacteria for fixing atmospheric nitrogen. But this wri-

ter brought 10 grams seeds of new dhaincha (*Sesbania rostrata*) from Philippines in the Fiscal Year 1984-85. This produced 500 grams in FY 1985-86, 100 kilograms in FY 1986-87 and ultimately kilograms in FY 1987-88 at Hardinath Agriculture Farm, Baniniya Dhanusha, Janakpur, Nepal alone. I am expecting 3-4 metric tons seeds of *Sesbania rostrata* from Nepal in 1987/88. This introduction of *Sesbania rostrata* in 1984/85 is the first case in Nepal.

Sesbania rostrata, a leguminous plant that traces its origin to the Senegal Valley in Africa, was introduced in the Philippines in 1982 by the French government via the Nitrogen Fixation by Legumes and Soil Fertility Project.

According to the proponents of the Biofertilizer Production and Utilization Programme, *Sesbania rostrata* possess a high capability of fixing atmospheric nitrogen. Hence, they are able to accumulate high percentage of nitrogen in their biomass. The nitrogen that they accumulate in their tissues could be made available to other agricultural crops through green manuring.

Green manuring as an alternative source of nitrogen for agricultural crops has been accepted as a farm practice for several decades in countries like India and the United States. Hardinath Agriculture Farm in Nepal is also practising dhaincha as legume green manure crops from eight to ten years back and getting higher and sustained productivity of rice by maintaining soil fertility too.

Sesbania rostrata, no doubt a miracle, the best legume tested thus far, forms nitrogen-fixation nodules on its roots, stems, branches too and has 5 to 10 times or even more nodules than most legumes. The stem nodules allow *Sesbania rostrata* to fix nitrogen in flooded paddies.

The newly introduced species, *Sesbania rostrata* has exciting possibilities with virtually whole plant nodulation and potential for 4-5 times more biological nitrogen fixation than most legumes. Thus a new impetus is in sight for green manuring.

In Senegal, it has been estimated that *Sesbania rostrata* contributes 267 kilograms nitrogen per hectare (nearly equivalent to 6 quintals urea application per hectare) of which one-third is transferred to the rice crop and the remainder is left behind in the soil for subsequent utilization.

IRRI has also reported that *Sesbania rostrata* can fix more than 200 kg N/ha.

In Kathmandu Valley 6 percent nitrogen content of stem nodules of *Sesbania rostrata* was recorded on dry weight basis.

Time and areas to be grown

Sesbania as green manure is taken in between harvesting of winter crop like wheat and transplanting of paddy and can be grown on bunds, as intercrop or can be composted too.

Dhaincha in grown in teras, inner teras and the regions upto 1,000 meter above mean sea level. However, the possibility of exploring various altitudes must be tried.

Program in Nepal

Eighty kilograms seeds of *Sesbania rostrata* were distributed from Hardinath Agriculture Farm to the various agricultural stations, farms, centres, projects, extension offices and the farmers (more than 150 agricultural units) throughout Nepal in the Fiscal Year 1986-87 for the feasibility studies cum seeds production.

By now, the *Sesbania rostrata* is also included in the field experiments under various farms of Nepal. The Sesbania (Dhaincha) is already included in the basic needs program of Nepal.

This writer also did follow-up in 16 sites and found that *Sesbania rostrata* were growing well with good stem nodulation even without inoculation at Hardinath Agriculture Farm, Horticulture Station (Nawalpur), Rapti Commercial Fishery Development Centre (Bhandara), National Wheat Development Program (Bhairahawa), Horticulture Station (Panchkhal), Fis-

hery Development Centre (Trisuli) etc. In some sites, the plants grew well but there were no nodulation. It is also heard that *Sesbania rostrata* is coming up well even at the extreme eastern and western parts of Nepal.

I hope to get the well filled performa about *Sesbania rostrata* from all the sites where the seeds were distributed in near future.

Future prospect of improving *Sesbania* green manure by biological nitrogen fixation

The local species which an indigenous to Nepal is *S. cannabina*. It has root nodules rather than stem nodules. Since 1985 many field trials of *S. cannabina* have been performed at different agricultural farm stations of Nepal for seed production and green manure. But no attention has yet been given to the *Rhizobium* symbiont of the plant. As *Rhizobium* is very much the integral part of the Biological Nitrogen Fixation in the legume plant, this should not be ignored.

Dreyfus, Rinaudo and Dommergues (1984) have reported that there are two typed of rhizobial strains in *Sesbania rostrata*, stem *Rhizobium strain* capable of nodulating both stem and roots, and root *Rhizobium* strain capable of nodulating roots only.

Sesbania rostrata is a newly introduced species in Nepal. At present many trials of *S. rostrata* have been carried out at different farm stations of Nepal mainly for seeds production. These trials showed that root nodulation is very sparse where as stem nodulation is not only confined to the main steam but also to all lateral branches of the stem. If best root strains of Rhizobium for *Sesbania rostrata* could be isolated and used for inoculation the dual nodulation of stem as well as root will tremendously increase the biomass yield of the plant. At one site, Rampur Agricultural Station neither stem nor root nodulation occurred as a result of which seed yield is very very poor. In such conditions the

yield could be increased by selecting best Rhizobium partner of the plant.

Hence, the future trend in the case of Sesbania green manure in Nepal will be the isolation and selection of best strains of Rhizobium from the local species *S. cannabina* as well as from the imported species *S. rostrata*. The biofertilizer project which has been carried out by the Royal Nepal Academy of Science and Technology in collaboration with the Department of Agriculture has this as one of its project activities.

Sesbania rostrata : seed production under on-farm conditions Thailand

S. Arunin [1], B. Hamman [2], and A. Yuvaniyama [1]

Introduction

The Northeast's 18 million inhabitants live on 17 million hectares of land: both population and land area represent about one third of the country. According to Prapertchob's estimates: over 80% of this population is engaged in agricultural production, with an average farm size of 4.3 hectares supporting a family of six. The per capita income in 1985 was 8,124 Bath. The low per capita income is due to poor agricultural output. The Northeast plants 46% of the country's total paddy rice, and yet, contributes only 35% to the national harvest.

The problem with most Northeast soils is infertility; a deficit not redressed by simply adding chemical fertilizers. The root-zone limitation to crop growth is due to the near absence of clay in the top soils of all except a few soils adjoining the rivers. The consequence of little inactive clay is low cation exchange capacities, which are inadequate for retention and effective delivery of plant nutrients for good yields (Ragland and Arunin, 1987).

In addition to this agronomic limitation to the use of chemical fertilizers, there is the economic restraint: chemical fertilizers are too expensive for the Northeast farmer because of the large amounts needed to improve yields versus the cash value of the rice yields (House of Agriculture Magazine, 1987). The low fertility and low CEC could be countered by adding organic matter to the soil.

The potential utility of *Sesbania rostrata* for green manure is well established. *Sesbania* can be used as a green manure to improve soil fertility and increase the amount of available nutrients for the subsequent rice crop. It will increase the soil's capacity to adsorb nutrients, improve soil structure and microbial activity. Soil chemical properties will also be improved; with a greater buffering capacity (Evans and Rotar, 1986; Dreyfus et al., 1983; Singhabutra et al., 1987; Ragland, 1986). In addition, *S. rostrata* has stem nodulation; an asset in flooded conditions. The profusion of stem nodules will be incorporated into the soil and decompose liberating nitrogen for the rice crop (Dreyfus et al., 1983). In this regard, 2/3 of the nitrogen fixed is released to the soil (Rinaudo et al., 1983). The high nitrogen fixing capability of *S. rostrata* was also reported by Gines et al., 1986).

Preliminary trials on farms and in experimental plots indicate that *S. rostrata* is well adapted to the Northeast conditions. The ussefulness of *S. rostrata* for soil improvements supported by other researchers: Kumazawa, 1984; Jefferies et al., 1981; App and Eaglesman, 1981. Much literature is available on the benefit of *S. rostrata* for green manure, but little work has addressed the problem of seed production.

The purpose of this paper is to document the experience of *S. rostrata* seed production under on-farm conditions and non-saline soils in the Northeast of Thailand.

Methodology

Three rainfed locations in Khon Kaen province were selected for seed production trials: Don Mong (non-saline), Pra Yeun (saline ECe; Electrical Conductivity of saturation extract, 8.0), and Nong Bua Dimi (ECe = 5.0). The soils of all three locations are Roi-Et sandy loam both on upland and lowland conditions. The first two locations were on-farm's ability to sacrifice land for green manure seed production. The last location was set aside green manure seed production. The last location the seed yields for experimental trials and experimental plot conditions.

At Don Mong there are nine plots ranging in size from 152-1679 m. Six kg of P_2O_5 and 3 kg of K_2O were applied per hectare, while the control had none. A starter level of phosphorous and potassium is needed to boost the vegetative growth of *S. rostrata* (Ragland and Arunin, 1987). The spacing of the Sesbania was 50 by 75 m. The number of plants per hill varied from 2-6 resulting in differing plant densities from 1.5 to 5.83 plants/m. All plots were inoculated with ORS 571 at 25 days. Two of the nine plots were under lowland conditions: one was Sesbania intercropped with rice, where both Sesbania seed and rice were harvested; and the other was Sesbania grown as green manure incorporated after 53 days then rice was transplanted the same day (Beri and Meelu, 1981).

At Pra Yeun, a 530 m lowland plot had a 10 kg/ha seed broadcast (Garrity and Flinn, 1987). No fertilizer was used. The plants were inoculated 25 days after planting.

At Nong Bua Dimi, the objective was to observe the vegetative growth and seed production under lowland and upland conditions in order to compare with the on-farm conditions. The experiment was carried out with three replications of *S. rostrata*. The treatments were: no inoculation, inoculation with a native strain, and inoculation with ORS 571 for a total of 18

plots of 25 m each. The plant spacing was 100 by 50 cm, which represents a density of 2 plants per m.

Results and discussion

Under on-farm conditions:

▲ 1. Seed yield under various plant densities are presented in Table 1. Under fertilized conditions, the highest yield has 910 kg/ha at 4.29 plants/m2, and the lowest yield was 560 kg/ha at 2.45 plants/m^2; it was observed that seed yield was related to plant density. The variation in yield depends upon plant density. It is simply concluded that under the farmer's conditions a density between 4-5/m^2 will give a higher yield when compared to the plant density at 2.45 m^2.

Plot	Yield (kg/ha)	Plant density (cm^2)
Ban Don Mong: non-saline ECe = 0.3 MS/cm		
A	660	2.45
B	560	2.45
C	910	4.29
D	860	5.54
E	700	5.33
F	840	4.72
G	380	5.83
H	107	1.50
1	none	1.50
Ban Pra Yeun : saline ECe = 8.0 MS/cm		
S	340	8.74

Table 1 : Ban Don Mong: non-saline ECe = 0.3 MS/cm

1) Intercrop with rice (rice yield = 894)
2) Green manure (rice yield = 828.9).

The vegetative growth of the unfertilized field was much lower than the fertilized fields; whereas the average height 3.5 m of the fertilized plants compared to 2.0 m of the unfertilized plants.

It is remarkable that although the saline site was unfertilized its vegetative growth was higher (at 2.5 m) than the upland unfertilized non-saline site.

▲ 2. The salinity as high as 8.0 mS/cm does not affect seed production (this finding agrees with Anuluxtipun, 1988).

▲ 3. Under lowland conditions at the same plant density of 1.5 plants/m rice yields were about the same; either growing *Sesbania* for seed production or for green manure, but *Sesbania* grain yield under these conditions is low (107 kg/ha).

Experimental conditions

▲ 1. The seed production of *S. rostrata* under lowland conditions is much higher than upland conditions under all treatments (Table 2).

▲ 2. The yield of non-inoculated *Sesbania* on both upland and lowland conditions is higher than *Sesbania* ioculated with the native strain, however, there is not much difference between treatments under lowland conditions.

▲ 3. Biomass (dry weight) of plants under non-inoculated conditions are much higher than the plants inoculated with native or ORS 571 rhizobium. The nitrogen content, however, in the non-inoculated plants was 1.77% compared to 2.04% for the plants inoculated with ORS 571. This finding is in accord with Anuluxtipun, 1988. The plant is losing energy due to rhizobium activity, which slows plant vegetative growth (Bach et al., 1958). It should be noted that under flooded conditions non-inoculated *S. rostrata* developed stem nodulation in the latter stages of the season, the rhizobium can migrate through a water media. This nodula-

tion was also observed under upland conditions but at a lower magnitude; this indicates that rhizobium are being carried by air or insects (Rinaudo et al., 1983).

▲ 4. Insects (stem and pod borers) will attack the upland inoculated plots while they not attack the flooded plots.

▲ 5. Seed production under the experimental conditions with a density of 2.0 plants/m gives a higher yield then the on-conditions due to more intensive management.

Conclusion

▲ 1. Low plant densities give a lower grain yield.

▲ 2. Under on-farm conditions seed production is lower than under experimental conditions.

▲ 3. Inoculation may not be necessary provided that the plots have been previously inoculated.

▲ 4. *S. rostrata* seed production can be achieved under both lowland and upland conditions.

▲ 5. The areal extent necessary for seed production is nearly the same as for non-saline sites.

▲ 6. Inoculation may reduce vegetative vigour, but it does induce improved grain yields, especially if pests are not a factor.

Performance	Non inocu-lated	Native strain	ORS 571
Upland height (cm)	177	152	188
Dry weight (kg/ha)	5800	2056	2813
Grain(kg/ha)	948	23*	163*
Lowland height (cm)	206	215	240
Dry weight (kg/ha)	6076	3852	3531
Grain (kg/ha)	1440	1066	1139

Table 2 : Plant height, dry weight and grain yield of Sesbania rostrata with native strain and ORS 571.

*   *insect damage.*

Planning for seed production under on-farm conditions

The long term goal is to promote the practice of green manuring to the farmers of the Northeast. The Royal Thai Department of Land Development recognizes the need for an on-going seed multiplication programme; therefore, it will provide interested farmers with a loan of seed. The expectation is that the farmer will grow enough for his next year's requirements plus the amount he borrowed, which he would return to the Department.

The average land holding is 4.0 hectares (Prapertchop, 1987) and the broadcast seed rate for *S. rostrata* green manure would be 20 kg/ha (Garrity and Flinn (1987) refer to a range of 10-50 kg/ha); a farmer will need to borrow 80 kg of seed to begin green manuring practices. In the first year, the farmer will have to produce 160 kg (80 kg for his subsequent year's green manure effort and 80 kg to give back to the Department).

In the second year, the area needed for seed production can be halved because there will be no pay back required.

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First Year	Average for years' yield kg/ha	Area needed to achieve 160 kg/ha	Proportion of area needed for seed production
Non-saline			
Non-fertilized	380	0.42 (~0.50)	1:8
Fertilized	755	0.21 (~0.25)	1:16
Intercropped	107	1.49 (~1.50)	1:2.67
Saline			
Non-fertilized	340	0.47 (~0.50)	1:8

Growth and nitrogen fixation in *Sesbania rostrata* under flooded and non flooded conditions

S.A. Kulasooriya and I.M. Samarakoon

Introduction

Sesbania rostrata (Brem.) is an annual leguminous shrub that belongs to the sub-family Papilionatae. It produces nitrogen fixing nodules profusely on its stems and roots. These nodules are produced by a rhizobium genus now named as *Azorhizobium* (4). The nodulation sites are predetermined and remain sensitive to infection throughout the life cycle. Using the acetylene reduction technique, the rate of nitrogenase activity of this plant has been reported to be 600 moles of ethylene per hour (2) and the stem nodule activity has been calculated to exceed 250 kg N/ha/52 days (3). Besides its high nitrogen fixing activity, the possession of a large number of stem nodules endows upon this plant a special advantage over root nodulated plants. Since the stem nodules are away from the soil, their nitrogen fixation is not affected by combined nitrogen in the soil It has been demonstrated that nitrogenase activity is not inhibited by the addition of 200 kg N/ha (3).

Unlike most other legumes, *S. rostrata* is capable of growth under flooded conditions. This enables these plants to be grown under wetland conditions for use as a green manure in rice cultivation. It has been reported that *S. rostrata* is sensitive to certain environmental factors such as photo-period, temperature and humidity (3). It was therefore felt necessary to conduct preliminary studies on the year-round growth of this plant and to compare it with other flood tolerant legumes, prior to its introduction as a green manure for rice in Sri Lanka.

Materials and methods

Year round growth of *S. rostrata*

The lay-out of the experiment was essentially similar to that suggested by Dreyfus et al. (2). Two sets of main plots (2x6 m) were divided into four sub-plots (2x1.5 m) each. 20 kg/ha of concentrated super phosphate and 12 kg/ha of muriate of potash were applied as a basal dressing to all the plots. Each sub-plot was planted at three month intervals, four times during the year (Dec 23, Mar 23, Jun 23, and Sep 23). Prior to planting, seeds were surface sterilised by immersing in concentrated sulphuric acid for 30 min and washed with ten changes of sterilised, distilled water. *Azorhizobium* (ORS 571) was cultured in sodium lactate medium for 6 days prior to the inoculation of seeds. The seeds were immersed in this culture (10^8 cells/ml), for 1 to 1.5 hours. (The seeds and the specific rhizobia were kindly provided by Professor Y.R. Dommergues of ORSTOM, Senegal). Two seeds each were placed in premarked holes at 10 cm intervals, along three rows 50 cm apart, in each sub-plot. Seedlings were thinned to 20 plants per row and gaps filled in 15 days. When the plants were 30 cm high, one set of plots was flooded, while the other was kept moist. The floodwater level was maintained between 5-7 cm throughout the experiment. Triplicate plots per treatment were maintained. Stem inoculation was done when the plants were about 30 cm high, by spraying with a culture of ORS 571 (10^8 cells/ml), using a hand sprayer.

The following data were collected.

▲ (1) Plant height from base to apex, measured every fortnight from 30th day until harvest.

▲ (2) Dry weights of the plants harvested from the 1st row, 50 days after sowing (DAS) and from the 3rd row, 100 DAS, after drying to a constant weight at 60C.

▲ (3) The number and dry weight of nodules of plants harvested from rows 1 and 3, 50 and 100 DAS, respectively.

▲ (4) Nitrogenase activity of different parts of plants harvested 100 DAS, estimated by the acetylene reduction technique. Plant parts were placed in 2L plastic bottles, with injection ports. After incubating for 30 min with 10% acetylene in air under room light and temperature, the ethylene produced was estimated using a Perkin-Elmer Sigma-4 gas chromatograph fitted with a 2 m column, packed with Poropak T. Detection of gases was done at 80C on a H_2 flame-ionization-detector, with N_2 at 30 ml/min as carrier gas.

Comparison of the growth and nitrogen fixation of *S. rostrata* with that of *S. aculeata* and *S. sesban*

The same site used for the previous experiment was ploughed and reused for this experiment. The three different *Sesbania* species were grown in separate plots, each 2x1.5 m and 4 replicate plots arranged on a randomized-complete-block design, were used for each species. A basal dressing of P and K fertilizers was applied to all the plots as before. *S. rostrata* seeds from the previous experiment together

with ORS 571 was used. Seeds of *S. aculeata* and the corresponding rhizobia were kindly supplied by Dr. D. Morris of the International Rice Research Institute, Philippines. *S. sesban* seeds were collected from the Agricultural Research Centre at Maha Illuppallema, in North Central Sri Lanka and were used without inoculation. Seed inoculation prior to planting was done as before by immersing in a culture suspension of the corresponding rhizobia, for 1 to 2 h. Seeds were initially sown along 3 rows, 50 cm apart and the seedlings were thinned to 20 plants per row and gaps filled, one week later. All the plots were watered regularly, but not flooded during early seedling growth and they were flooded 30 DAS and the flood water level was maintained around 5 cm until the end of the experiment. Commencing from the 2nd week after seeding, the height from base to apex of five randomly selected, pre-marked plants from each plot was recorded at weekly intervals. Two randomly selected plants from each plot, were harvested every two weeks and their nitrogenase activity was estimated by the acetylene reduction technique. The harvested plants were dried to a constant weight at 60C and their dry weights recorded. Dates of flowering in the different plant species were observed and recorded. The experiment was terminated in 14 weeks, when all the three species appeared to have reached their stationary phase of growth, in terms of plant height.

Results

The growth curves of *S. rostrata* during the four periods (Dec-Apr, Mar-Jul, Jun-Oct, and Sep-Jan) are given in Figure 1. It is seen from this figure that the overall growth during Dec-Apr and Mar-Jul are better than that during the other two periods, and that growth under flooding and non-flooding was similar during these two periods. This is also reflected in the plant biomass and nodule production during these periods. Thus, the dry weights of plants (Table 1), their nodule number and dry weights (Table 2), with and without flooding, during Dec-Apr and Mar-Jul are not different from each other whereas during the other two periods, flooding has

clearly had a positive effect on both these parameters.

Nitrogenase activity of the different parts of 100-day old *S. rostrata* plants (Table 3) shows that the apex without stem nodules has no activity. While nitrogenase activity expressed as total or as per length of stem is higher towards the base, highest specific activity (M C_2H_4/mg nodule dry weight/h) was associated with stem parts having young nodules.

A most noticeable effect of flooding was the production of massive roots, extensively covered with aerenchymatous tissues (Fig. 2). The production of such roots appeared to be an adaptation that enabled these plants to tolerate flooding. A simple experiment conducted to examine the effect of flooding on the root growth of *Glycine max*, *Vigna unguiculata* and *Vigna mungo*, showed that all three species could not withstand inundation for more than 3 weeks. However, all of them showed an initial formation of aerenchymatous tissue around their stem bases and surface roots under flooding, but this could not be sustained, and the plants died. The comparative growth of the three species *S. rostrata*, *S. aculeata* and *S. sesban* are shown in Figure 3, from which it is seen that although the growth of *S. sesban* was slow, it eventually grew taller than the others. *S. rostrata* plants reached a maximum height similar to that in the previous experiment, but *S. aculeata* showed poor growth. Biomass production has followed similar trends (Table 4). Comparison of the nitrogenase activities (Table 5) clearly show that the stem nodulated *S. rostrata* has a much higher rate of nitrogen fixation than the other two plant species. Nevertheless, the activities of the other species are not negligible, being about 1/2 and 1/6th of the former.

In both *S. rostrata* and *S. aculeata*, initiation of flowering was observed within 5 weeks and by 6 to 8 weeks they were in full bloom. On the other hand, *S. sesban* started to produce flowers by about 8 weeks indicating its insensitivity to the photoperiod in Sri Lanka.

Discussion

The lack of enhancement of *S. rostrata* growth by flooding during periods favourable to its growth (Fig. 1 and Table 1), indicates that inundation with water is not a specific requirement for its rapid growth as long as the soil is saturated with water, and other environmental factors favour its growth. This makes the potential use of the plant greater, as it may grow well during the wet seasons, even if standing flood water cannot be maintained throughout its growth. Extrapolation of biomass production by *S. rostrata* in 50 days under flooding (Table 1), give values less than those reported (5). The comparative study *S. rostrata* with *S. aculeata* and *S. sesban*, showed that although the initial growth of *S. sesban* is slower, it eventually increased its rate of growth and finally produced the highest biomass. Both *S. rostrata* and *S. aculeata* commenced flowering earlier than *S. sesban*, confirming the limitation tropical photoperiodism could impose on these plants (1). The higher growth recorded by *S. sesban* under flooding, is not in agreement with an earlier report (5), but confirm our observations that this plant grows luxuriantly in flooded rice fields in the dry zones of Sri Lanka (personal communication, Mr. W.L. Weerakone, Agricultural Research Centre, Maha Illuppallema). Although nitrogenase activity of *S. rostrata* is much higher than those of the other two species tested, the activity of the latter is not negligible. Both biomass production and nitrogen fixation in *S. rostrata* has been much higher than in *S. sesban*, during the first 6 weeks (Tables 4 and 5). This is important because the fallow period between two rice crops may not provide a much longer time for the growth of a green manure crop.

It has been suggested that genetic studies be directed to produce photoperiod insensitive varieties of *S. rostrata* (2) and to transfer stem nodulation ability to other Sesbania species used as green manure (5). An additional desirable objective would be to transfer and stabilise the ability to produce aerenchymatous tissue by grain legumes as a response to flooding so as to make them flood tolerant, as most farmers would be tempted to grow flood

tolerant cash crops than pure green manure species.

It may be concluded that these preliminary studies have shown *S. rostrata* to be flood tolerant legume capable of fixing nitrogen at much higher rates than the other two species tested. However, its sensitivity to the photoperiod in Sri Lanka would be a limitation to its use as a green manure in rice production.

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Period	non-flooded		flooded	
	50 days	100 days	50 days	100 days
Dec 83 - Apr 84	5.4 ± 1.5	33.8 ± 5.2	6.8 ± 2.1	35.7 ± 4.3
Mar 84 - Jul 84	4.8 ± 1.6	49.3 ± 6.2	6.5 ± 2.3	48.1 ± 5.8
Jun 84 - Oct 84	1.8 ± 0.62	10.3 ± 2.4	2.4 ± 1.6	34.6 ± 4.7
Sep 84 - Jan 85	2.2 ± 0.81	11.4 ± 3.2	2.1 ± 0.43	25.2 ± 3.6

Table 1 : Biomass production in S. rostrata, during the four periods in g.dwt/plant *.

Period	non-flooded				flooded			
	50 days nodule		100 days nodule		50 days nodule		100 days nodule	
	No.	dwt mg	No.	dwt mg	No.	dwt mg	No.	dwt mg
Dec-Apr 83 84	68 ± 12	105 ± 24	208 ± 22	425 ± 43	74 ± 14	146 ± 28	215 ± 32	487 ± 52
Mar-Jul 84 84	91 ± 18	226 ± 34	456 ± 69	1338± 96	98 ± 16	237 ± 40	428 ± 74	1218± 92
Jun-Oct 84 84	33 ± 11	81 ± 6	83 ± 12	246 ± 28	58 ± 10	138 ± 21	218 ± 22	457 ± 61
Sep-Jan 84 85	36 ± 9	88 ± 11	92 ± 12	308 ± 42	52 ± 6	166 ± 28	162 ± 18	402 ± 57

Table 2 : Nodule production per plant in S. rostrata during the four periods.

*   *Mean value of ten replications.*

Height from apex (cm)	total ARA μM h^{-2}	ARA per length of stem μM cm^{-1} h^{-1}	ARA per nodule biomass μM mg dwt^{-1} h^{-1}
0-10	-	-	-
10-045	34	0.96	3.8
45-120	109	1.44	1.7
120-240	884	6.30	1.0

Table 3 : Acetylene reducing activity (ARA) of the different parts of 100-days old S. rostrata plants.

Age of plant week	mean dry weight * (g/plant)		
	S. rostrata	S. aculeata	S. sesban
2	0.4 ± 0.8	1.2 ± 0.6	1.6 ± 0.9
4	2.6 ± 0.7	2.8 ± 0.8	2.5 ± 11
6	12.9 ± 2.0	10.3 ± 1.8	6.9 ± 1.1
8	30.3 ± 4.8	21.9 ± 3.0	20.4 ± 3.2
10	48.7 ± 5.1	30.7 ± 4.5	38.2 ± 4.1
12	56.8 ± 5.7	33.8 ± 4.6	62.1 ± 5.7
14	58.4 ± 6.1	36.4 ± 5.0	68.7 ± 7.2

Table 4 : Biomass produciton in the three Sesbania species.

*   *Mean value of eight plants.*

Age of plant weeks	mean acetylene reducing activity plant (*) (μM C_2H_4 g dwt^{-1} h^{-1})		
	S. rostrata	S. aculeata	S. sesban
2	0.17 ± .02	0.04 ± .01	0.02 ± .01
4	0.94 ± .04	0.13 ± .06	0.09 ± .05
6	3.63 ± .53	0.98 ± .32	0.13 ± .09
8	5.87 ± 1.1	1.62 ± .78	0.92 ± .34
10	6.83 ± 1.8	2.01 ± 1.05	1.04 ± .81
12	7.21 ± 2.1	3.68 ± 1.3	1.56 ± .92
14	7.78 ± 2.6	4.02 ± 2.0	1.63 ± 1.2

Table 5 : Nitrogenase activity of the three Sesbania species.

(*) Mean value of 8 replicates
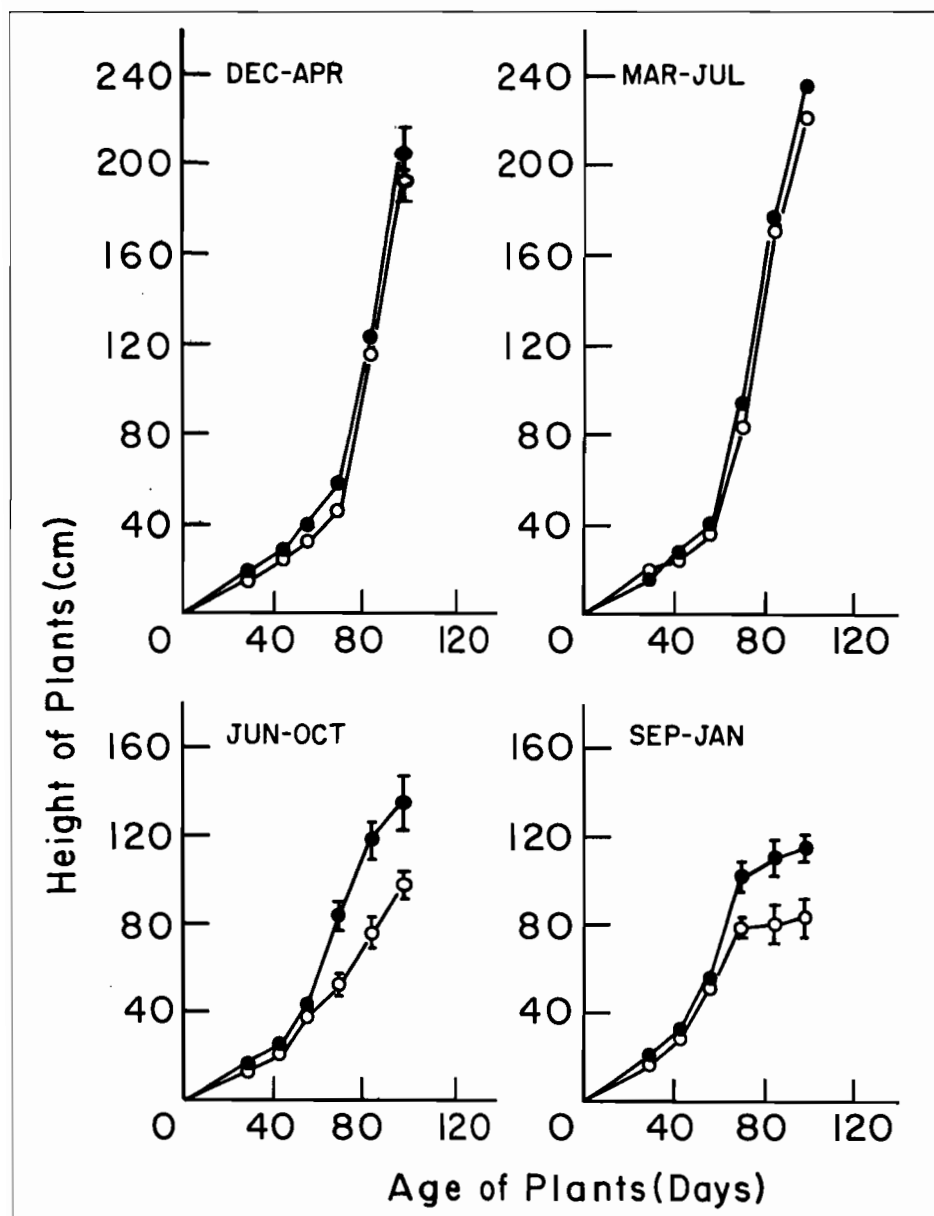
*Figure 1. Growth of Sesbania rostrata under flooded (●)
and non-flooded (O) conditions, during four periods of the year.*

*Figure 2. Effect of flooding on the growth of roots
of Sesbania rostrata during the dry season.*
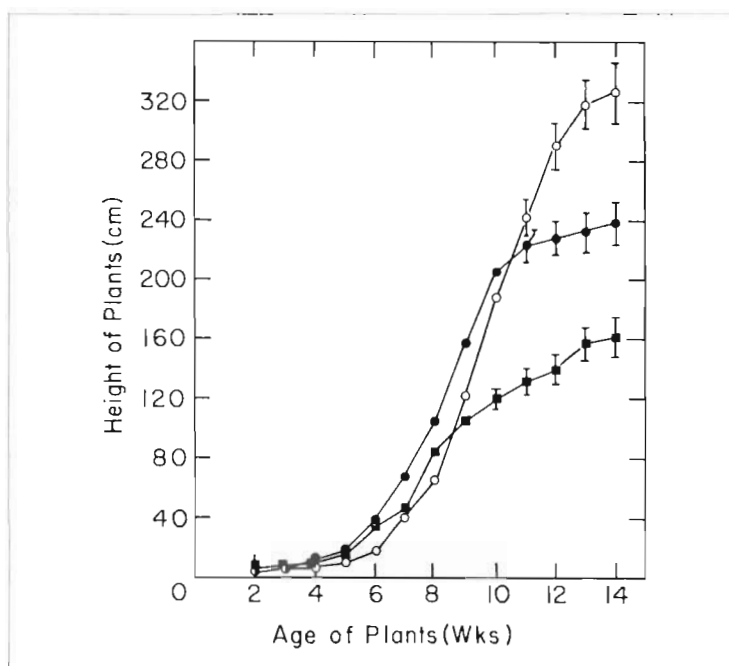
*Figure 3. Growth of Sesbania rostrata (●), S. aculeata (■),
and Sesbania sesban (O), under flooded conditions.*

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(Synopsis: R.A. Ludwig)

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Agricultural applications