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African
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AABN

A C T E S

MAXIMISER LA FIXATION BIOLOGIQUE DE L'AZOTE POUR LA PRODUCTION AGRICOLE ET FORESTIÈRE EN AFRIQUE

IIIème Conférence de l'AABNF
7-12 Novembre 1988, Dakar, Sénégal

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SOMMAIRE

		Pages
Les perspectives de la fixation 'biologique de l'azote en Afrique	GUEYE, M.	1
FIXATION BIOLOGIQUE DE L'AZOTE CHEZ LES LEGUMINEUSES A GRAINES		
Inoculation du soja (Glycine max (L.) Merr:) et du niébé (Vigna unguiculata (L.) Walp.) sur sol ferrallitique fortement désaturé en basse Côte d'Ivoire	KIMOU, A. et ZENGBE, M.	7
Influence of rhizobia inoculation on soybean (Glycine max L.) performance in Nigeria.	AWONAIKE, K.O., OLUFAJO, O.O. and ADU, J.K.	15
Nodulahon and growth of bambara groundnut (*Vigna subterranea*) in tree soils.	MULONGOY, K. and GOLI, A.E.	27
Effet de l'inoculation avec des souches de *Rhizobium* et de la fertilisation azotée sur le rendement en grains du Voandzou (*Vigna subterranea* (L.) Thouars) au Sénégal.	GUEYE, M.	34
FIXATION BIOLOGIQUE DE L'AZOTE CHEZ LES LEGUMINEUSES FOURRAGERES		
Nitrogen fixation of the forage legumes and their residual effects on wheat growth and yield.	HAQUE, I	40
FIXATION BIOLOGIQUE DE L'AZOTE CHEZ LES ARBRES		
Effect of the addition of phosphorus and *Rhizobium* inoculum on thenodulation and the growth of two species of gum arabic tree *Acacia senegal* L. Willd and *Acacia laeta* R. Br. ex Benth.	BADJI, S ; THOEN, D. ; DUCOUSSO, M. and COLONNA, J.P.	54
Nodulation survey of leguminous and non leguminous trees growing at ICRAF's field station.	ODUOL, P.A. ; AKUNDA, E.M. and WAMBUGU, P.N.	63
Effect of inoculation withe *Rhizobium,* P application and liming on early growth of leucaena (*Leucaena leucocephala* Lam. de wit).	GICHURU, M. and MULONGOY, K.	72

A preliminary study on the compatibility between indigenous rhizobia and some tree legumes in Kenya	ODEE, D.W.	81

FIXATION D'AZOTE ASSOCIATIVE

Effect of inoculation on the growth and yield of three maize cultivars.	MWAURA, F.B. and WIDDOWSON, D.	92
Population of nitrogen fixing bacteria in sweet potato fibrous roots.	HORTENSE, W.D. ; WALTER, A.H. ; MULONGOY, K. ; ADEYEYE, S.O. and HAHN, S.K.	99

ASSOCIATION AZOLLA / ANABAENA

La recherche sur *Azolla* et ses applications : évolution et tendances actuelles.	VAN HOVE, C. et DIARA, H.F.	106
Phosphorus needs and accumulation potential of various *Azolla* species and strains.	DESMADRYL, D. ; GODARD, P. ; WAUTHELET, M. and VAN HOVE, C.	118
Sensitivity to Aluminium of various *Azolla* species and strains.	WAUTHELET, M. ; GODARD, P. ; DESMADRYL, D. and VAN HOVE, C.	126

INOCULATION : PRODUCTION D'INOCULUM
MATERIALS D'INOCULATION

Production and uses of rhizobium inoculants.	BORDELEAU, L.	132
Caractérisation et choix de supports pour inoculum.	BEUNARD, P. et SAINT MACARY, H.	144
Progrès récents dans la technologie des inoculums utilises en agriculture et en foresterie.	DIEM, H.G. ; BEN KHALIFA, K. ; NEYRA, M. and DOMMERGUES, Y.R.	153

ECONOMIE DE L'AZOTE DANS LES SYTEMES DE CULTURE EN ASSOCIATION ET EN AGROFORESTERIE

Biological nitrogen fixation and nitrogen transfer in multiple cropping in tropical Africa.	MULONGOY, K. and EHUI, S.K.	174

AMELIORATION DE LA FIXATION SYMBIOTIQUE DE L'AZOTE PAR LA SELECTION DES PLANTES

Importance de la plante-hôte et amélioration génétique de la fixation biologique de N_2 chez les arbres tropicaux. — DUHOUX, E. — 193

Analysis of the plant genes involved in the nitrogen fixing symbiosis. *Sesbania rostrata as* a model. — De LAJUDIE, P. — 207

Sesbania rostrata : un modèle pour la biologie moléculaire de la nodulation — TOMEKPE, K. ; HOLSTERS, M. ; GOETHALS, K. ; VAN DEN EEDE, G. ; De LAJUDIE, P. ; TRAN, P. and DREYFUS, B. — 213

AMELIORATION DE LA FIXATION SYMBIOTIQUE DE L'AZOTE PAR LES PRATIQUES CULTURALES

Amélioration de la Fixation Biologique de l'Azote (N_2) par les pratiques culturales. — GANRY, F. — 221

Response of *Phaseolus vulgaris* L. to inoculation with *Rhizobium* and fertilization with nitrogen and phosphorus in Northem Tanzania. — AMIJEE, F. ; EDJE, O.T. ; KOINANGE, E.K. ; BITANYL, HF. ; BRODRICK, S.J. and GILLER, K.E. — 228

Variation in acid Al tolerance of *Bradyrhizobium japonicum.* — ASANUMA, S. and AYANABA, A. — 239

The influence of some parameters of soil fertility on early growth of *Leucaena leucocephala* and *Cassia siamea* — LIYA, S.M. ; MULONGOY, K. ; ODU, C.T.I. and AGBOOLA, A.A. — 250

MESURE DE LA FIXATION D'AZOTE

Evaluation of biological nitrogen fixation in plants. — DANSO, S.K.A. — 258

Influence du déficit hydrique sur la fixation symbiotique de l'azote atmosphérique chez le soja — SALL, K. ; DREVON, J.J. et OBATON, M. — 272

Effect of ^{15}N-labelled mineral-N and strains of *Bradyrhizobiumon* biological nitrogen and N-partitioning in cowpea (*Vigna unguiculata*) (L.) Walp.	LUYINDULA, N. and WEAVER, R.W.	288
Quantification of the contribution of BNF to field grown plants : the use of the ^{15}N isotope dilution technique - problems and some solutions.	BODDEY, R.M. and URQUIAGA, S.	298
Utilisation de la méthode ^{15}N pour estimer la fixation symbiotique de l'azote chez les plantes herbacées et ligneuses.	DOMENACH, A.M. ; KURDALI, F. et BARDIN, R.	317
Nitrogen fixation in tropical trees : estimations based on ^{15}N techniques.	SAGINGA, N. ; ZAPATA and DANSO, S.K.A.	337

CONCLUSIONS ET RECOMMANDATIONS

Les recherches sur la fixation biologique de N_2 et leur impact présent et futur sur la production végétale et forestière en Afrique.	DOMMERGUES, Y. ; BORDELEAU, L.M. et GUEYE, M.	352

PREFACE

Après les réunions à Nairobi en 1984 et au Caire en 1986, l'Institut Sénégalais de Recherches Agricoles (ISRA) a organisé la troisième conférence de l'Association Africaine pour la Fixation Biologique de l'Azote (AABNF). Le thème général de cette troisième conférence était « Maximiser la Fixation Biologique de l'Azote pour la production agricole et forestière en Afrique ».

Maximiser la « Fixation Biologique de l'Azote » pour la production agricole et forestière est sans nul doute une des voies que les pays en voie de développement, particulièrement ceux d'Afrique, doivent adopter pour relancer leur production agricole dont la tendance est à la décroissance et sauvegarder leur patrimoine forestier exposé dangereusement à la dégradation.

En effet, dans un environnement à forte propension aux bouleversements écologiques et aux déséquilibres d'écosystèmes naturellement fragiles, les pays africains ont beaucoup de défis à relever parmi lesquels :

- la sécurisation de la production agricole et la couverture de la demande alimentaire,
- la sauvegarde du milieu naturel.

Il s'agit là d'enjeux de toute importance exigeant la mise en œuvre d'actions correctrices permanentes liées aux opérations agricoles. Deux actions paraissent prioritaires à l'heure actuelle pour la sauvegarde des agrosystèmes : le maintien et l'amélioration de la fertilité des sols (pour la plupart potentiellement pauvres et fragiles) et la lutte contre la désertification par un effort soutenu de reboisement.

Dans ce cadre, la biotechnologie en général et la fixation biologique de l'azote en particulier offrent d'excellentes perspectives pour les pays africains. La mise en œuvre effective de cette technologie nécessite :

- l'isolement et la culture en quantités industrielles de souches de microorganismes fixateurs d'azote;
- la mise au point de méthodes pratiques et efficaces d'inoculation à la portée du développement;
- [illegible] végétatives [illegible] en fonction de leur aptitude à la nodulation et à la fixation de l'azote;
- l'isolement et l'identification de souches de champignons micorhiziens et la mise en œuvre de méthodes pratiques d'inoculation facilement utilisables en pépinière.

L'Association Africaine pour la Fixation Biologique de l'Azote devra aider les pays africains à atteindre ces objectifs pour l'accroissement du niveau de vie des populations rurales par la promotion d'opérations agricoles intégrant l'utilisation des microorganismes fixateurs d'azote.

Je remercie toutes les institutions nationales et internationales ainsi que la communauté scientifique mondiale qui ont aimablement assisté l'ISRA dans l'organisation de cette conférence de l'AABNF.

Mouhamudou El Habib LY
Directeur Général de l'I.S.R.A.

AVANT PROPOS

La troisième conférence de l'Association Africaine pour la Fixation Biologique de l'Azote (AABNF) a été organisée à Dakar, Sénégal, par l'Institut Sénégalais de Recherches Agricoles (ISRA) du 7 au 12 Novembre 1988.

L'ISRA, établissement public chargé de concevoir, organiser et mener à bien toutes les recherches relatives au secteur rural au Sénégal, bénéficie de la collaboration très étroite de l'Université Cheickh Anta Diop de Dakar(UCAD) et de celle de l'Institut Français de Recherche Scientifique pour le Développement en Coopération (ORSTOM) au Sénégal.

Plusieurs agences internationales ont contribué financièrement à l'organisation de la 3ème conférence AABNF :

- l'Organisation des Nations Unies pour l'Education, la Science et la Culture (UNESCO) à Paris par ses divisions : SER, ABN, ROSTA et MAB. L'UNESCO centralise le réseau mondial des MIRCENs ;
- le Programme des Nations Unies pour l'Environnement à Nairobi ;
- l'Organisation des Nations Unies pour l'Alimentation et l'Agriculture (FAO) à Rome ;
- le Programme des Nations pour le Développement (UNDP) à Dakar ;
- le Centre Technique de Coopération Agricole (CTA), installé depuis 1983 à Ede/Wageningen au titre de la convention de Lomé entre les états du groupe ACP. Le CTA est à la disposition des états ACP pour leur permettre un meilleur accés à l'information, à la recherche, à la formation ainsi qu'aux innovations dans les secteurs de développement agricole et rural et de la vulgarisation ;
- le Centre de Recherche pour le Développement International (CRDI) à Ottawa ;
- la Fondation Internationale pour la Science (FIS) à Stockholm ;
- l'Union Internationale des Sociétés de Microbiologie (IUMS).

La troisième conférence AABNF a réuni 80 participants venant de : Angleterre, Angola, Autriche, Belgique, Brésil, Burkina Faso, Canada, Congo, Côte d'Ivoire, Egypte, Etats Unis d'Amérique, Ethiopie, France, Ghana, Haïti, Italie, Kenya, Liberia, Mali, Maroc, Nigéria, Ouganda, Pays Bas, Rwanda, Sénégal, Sierra Leone, Tanzanie, Tunisie, Zaïre et Zimbabwé.

Comité d'organisation

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Les perspectives de la fixation biologique de l'azote en Afrique

GUEYE, M.
MIRCEN/CNRA BP. 53 Bambey, Sénégal

La satisfaction des besoins nutritifs et nutritionnels des populations africaines dépend en premier, lieu et en très grande partie des progrès de l'agriculture, qui sont eux-mêmes handicapés par la pauvreté des sols peu fertiles, carencés en éléments majeurs (N, P, K) et en oligoéléments (Zn, Cu). Pour obtenir des rendements agricoles suffisants dans de tels sols, il est nécessaire de recourir à un emploi massif d'engrais chimiques. Or, les cultivateurs africains, dans leur grande majorité. n'utilisent pas ces engrais parce qu'ils sont importés, et donc trop coûteux. Ainsi, pour améliorer la production agricole en Afrique, il faudra obligatoirement faire appel à de nouvelles technologies à la fois efficaces et moins onéreuses. Parmi ces technologies, l'introduction systématique des plantes fixatrices d'azote dans les systèmes agricoles devrait permettre le maintien et l'amélioration de la fertilité des sols. L'agriculture itinérante en est un exemple. Les plantes fixatrices d'azote jouent un rôle prépondérant dans le cadre des systèmes destinés à recycler ou à valoriser la matière organique, dans les rotations culturales et dans les cultures en -association ou en couloir.

Importance des plantes fixatrices d'azote dans l'agriculture et la foresterie en Afrique

Les légumineuses à graines ont fait l'objet de nombreuses études. Elles occupent une place prépondérante dans l'agriculture et y jouent un rôle très important aussi bien dans la satisfaction des besoins alimentaires des hommes et des animaux que dans le maintien de la fertilité des sols. En effet, grâce à leur capacité de fixer l'azote atmosphérique en association avec les *Rhizobium*, les légumineuses à graines permettent d'économiser l'azote du sol. L'utilisation des variétés à haut potentiel fixateur d'azote dans les systèmes culturaux accroit la teneur en azote des sols et en assure la fertilité. D'autre part, dans le cadre d'une rotation de culture légumineuse-céréale, au cours des processus de décomposition, l'azote des racines et des nodules peut être transféré à la céréale suivante. En Afrique, une dizaine de légumineuses à graines sont cultivées dans les différentes zones écologiques : ce sont notamment le soja (*Glycine max*), le haricot (*Phaseolus vulgaris*), le niébé (*Vigna unguiculata*), l'arachide (*Arachis hypogaea*), le pois chiche (*Cicer arietinum*) et le pois bambara ou voandzou (*Vigna subterranea*).

Les espèces ligneuses fixatrices d'azote sont appelées elles aussi à jouer un rôle de plus en plus important en Afrique pour plusieurs raisons. D'abord, elles peuvent pousser sur des sols très pauvres ou totalement dépourvus d'azote fournissant ainsi du bois ou du fourrage pour l'alimentation du bétail : c'est le cas de *Acacia albida* et d'autres acacias introduits en Afrique sahélienne ainsi que de *Medicago arborea* et *Acacia cyanophylla* en Afrique du Nord. D'autre part, beaucoup d'espèces ligneuses contribuent à la régénération des sols en reconstituant le stock de matière organique dans le cadre des cultures intercallaires en systèmes agroforestier (*Leucaena leucocephala, Gliricidia sepium*) ou dans le *cas* de la riziculture (*Sesbania rostrata*). Les espèces ligneuses contribuent également à *la* fixation des dunes : c'est le *cas* du filao (*Casuarina equisetifolia*).

Il convient donc, à l'étape des recherches actuelles de tirer le maximum de profit des connaissances acquises pour, maximiser au mieux la fixation biologique de l'azote dans le but d'améliorer la production agricole et forestière en Afrique.

Amélioration de la fixation biologique de l'azote par action sur les micro-organismes fixateurs d'azote

La première démarche pour augmenter la fixation biologique de l'azote par action sur les microorganismes consiste à sélectionner judicieusement pour chaque espèce de légumineuse, voire pour chaque variété de légumineuse fixatrice d'azote, une ou des souches de *Rhizobium* capable de fixer très effectivement l'azote atmosphérique à la suite de leur installation sur la plante-hôte considérée. Le schéma de sélection a été très bien décrit par beaucoup d'auteurs (Vincent, 1970). Il repose sûr 'la connaissance de critères bien précis : (ij infect&&& et effectivité dans la fixation biologique de l'azote ; (ii) compétitivité pour la formation des nodules et (iii) adaptation aux conditions de l'environnement : température, pH, concentration en sel, type de sol, taux d'azote et/ou de matière organique du sol, antagonistes (certains actinomycètes...).

L'inoculation des plantes fixatrices d'azote avec des souches sélectionnées de microorganismes symbiotiques a généralement donné de bons résultats dans le cas des légumineuses suivantes : soja (Burton, 1976). haricot (Keya *et al.*, 1982), luzerne (*Medicago sativa*), (Bordeleau *et al.*, 1977). stylosanthes (*Stylosanthes guineansis*) leucaena (Skerman, 1982)

et filao (Diem *et al.*, 1983). Cependant, cette démarche n'a pas toujours été couronné de succès. C'est le cas de certaines légumineuses du groupe cowpea très répandues en Afrique et cultivées à grande échelle telle que l'arachide, le niébé pour lesquelles l'inoculation est sans effet en raison de la présence dans le sol de souches de *Rhizobium* natives compétitives et ineffectives sur les variétés utilisées. Toutefois, des résultats positifs peuvent être obtenus dans une zone déterminée en réintroduisant massivement dans le sol une souche de *Rhizobium* effective isolée de ce sol, à partir d'une légumineuse qui s'y est adaptée. Par cette méthode, l'inoculation du voandzou avec la souche de *Rhizobium* MAO 113 (Gueye et Bordeleau, 1988) a augmenté le rendement en grain de 51% en 1986 et de 52 % en 1988 (voir page 34 pour plus de précision).

Actuellement, on commence à faire appel aux techniques de génie génétique pour créer des souches plus performantes par les processus de transformation, de traduction et de conjugaison bactérienne. On sait actuellement que l'information génétique qui détermine la fixation biologique de l'azote dans les bactéries est contenue dans une séquence de gènes dénommés Nif dont la structure et le fonctionnement sont décrits avec précision (Sasson, 1984). Les *gènes Nif* de *Klebsiella pneumoniae* ont été transférés avec succès par conjugaison chez *Escherichia coli* K12 (Postgate, 1987). Depuis lors, de nombreuses expériences ont été effectuées et ont abouti à la création de nouvelles bactéries fixatrices d'azote. Les hybrides de *E. coli* ont été les premières nouvelles bactéries à être développées par manipulation génétique. Ensuite, plusieurs transferts de gènes Nif ont été effectués : de *Rhizobium trifolii* à *Klebsiella aerogenes*, de *Bradyrhizobium japonicum* à *Azotobacter vinelandii* (Postgate, 1987).

Ainsi, il est permis d'espérer que l'on pourra agir encore plus puissamment sur la fixation biologique de l'azote en manipulant génétiquement les microorganismes fixateurs d'azote. C'est ainsi qu'on peut envisager le transfert du potentiel fixateur d'une souche de *Rhizobium* effective et non compétitive à une souche de *Rhizobium* native compétitive, très peu effective et modulant une légumineuse adaptée à une zone déterminée.

Amélioration de la fixation biologique de l'azote par la sélection des plantes

Rappelons que la symbiose est par définition une association entre deux partenaires pour un bénéfice réciproque. Il est donc logique de chercher à maximiser la fixation biologique de l'azote en agissant non seulement sur le partenaire bactérien, mais aussi en agissant sur la plante-hôte elle-même.

De nos jours, il apparait de plus en plus évident que l'approche génétique végétale doit être nécessairement combinée à l'approche microbiologique si l'on veut atteindre rapidement l'objectif visé qui est d'augmenter les protéines végétales et/ou d'obtenir des variétés rustiques adaptées aux conditions sévères de l'agriculture en zone tropicale. L'aptitude à mieux fixer l'azote doit être nécessairement prise en compte dans les schémas de sélection.

A la fin de la décennie 1980, une nouvelle technologie s'est développée : il s'agit de la multiplication végétative in vitro qui permet une exploitation raisonnée de la variabilité intraspécifique chez les plantes. Dans le cas des espèces fixatrices d'azote, cette technologie permet d'accroître substantiellement la quantité d'azote fixé en permettant la multiplication des clones identifiés comme les plus performants.

Cette démarche peut facilement s'appliquer aux arbres fixateurs d'azote car ils présentent une variabilité très importante et leur densité à l'hectare est comprise entre 2000

et 5000 plants. En revanche, on ne peut envisager de recourir à cette méthodologie dans le cas des plantes fixatrices d'azote annuelles dont la densité est de l'ordre de 100.000 plants à l'hectare (Dommergues, 1987).

Les centres de ressources microbiologiques (MIRCENs)

L'amélioration de la fixation biologique de l'azote étant une priorité pour les pays africains, puisqu'elle contribue à l'autosuffisance alimentaire avec un minimum d'engrais azotes, il y a lieu de collecter et de préserver les microorganismes fixateurs d'azote capables de s'associer de façon effective avec les plantes améliorées. C'est la un des rôles majeurs des MIRCENs. Le rôle de ces MIRCENs est de :

- fournir l'infrastructure d'un réseau mondial de laboratoires travaillant en collaboration au niveau régional et interrégional à la distribution et à l'utilisation des pools de gènes des microorganismes ;
- renforcer les efforts relatifs à la préservation des microorganismes d'intérêt économique ;
- promouvoir l'application de la microbiologie à l'agriculture ;
- servir de centre de formation pour la diifusion des connaissances sur la microbiologie.

Actuellement, il y a trois MIRCENs en Afrique. Le MIRCEN de Nairobi avec une collection de *500* souches de *Rhizobium,* produit de l'inoculum sur un support de boue filtrée pour la culture du haricot. Le MIRCEN du Caire renferme une collection de *200* souches de *Rhizobium* et conduit plusieurs expériences sur l'inoculation de la *fève* (*Vicia faba*). La'collection du MIRCEN de Bambey renferme *300* souches de *Rhiwbium* pour les expériences sur la fixation biologique de l'azote chez **l'arachide, le niébé, le voandzou, le soja et les acacias.**

L'association africaine pour la fixation biologique de l'azote (AABNF), les institutions internationales

Dans la plupart des pays africains, existe une institution nationale, privée ou semi-privée, travaillant sur un aspect de la fixation biologique de l'azote. Cependant, ces institutions nationales ont des moyens très limites qu'il conviendrait de rassembler sous la forme d'un réseau. C'est encore là un des objectifs des MIRCENs et par delà un objectif de l'AABNF. Il n'est donc pas étonnant que les MIRCENs d'Afrique aient soutenu et supporté les premières activités de l'AABNF. A côté des institutions nationales, il y a les institutions internationales.

L'impact dè l'International Institut of Tropical Agriculture (IITA) de Ibadan, Nigeria dans les programmes de recherches sous régionaux n'est plus à démontrer : depuis les recherches sur le niébé en particulier jusqu'aux récents travaux sur l'exploitation des arbres fixateurs d'azote dans les systèmes de culture en couloir. L'International Council of Research in AgroForestry (ICRAF) a démontre, dans les régions au Sud du Sahara, l'importance des arbres fixateurs d'azote et leur intégration dans les systèmes agroforestiers.

Il y a tgalemnt les nombreuses agences internationales oeuvrant pour le développement de l'agriculture en Afrique à travers des programmes axes sur la fixation biologique de l'azote : CRDI, CTA, FAO, FIS, NAS-BOSTID, UNDP, UNEP, UNESCO.

Il apparait donc indispensable d'évaluer dès à présent, le potentiel humain dont dispose actuellement l'Afrique pour mener à bien les recherches envisagées. C'est pourquoi, l'AABNF, par l'intermédiaire de l'IITA à Ibadan, dresse un annuaire des microbiologistes en Afrique travaillant dans le domaine de la fixation biologique de l'azote. Il faut maintenant raisonnablement mesurer ce qui reste à faire pour que tous les pays africains puissent utiliser avec le maximum de profit toute la technologie de la fixation biologique de l'azote. Cet objectif requiert au préalable la formation d'un personnel qualifié et une plus large diffusion des connaissances acquises sur la fixation biologique de l'azote en Afrique.

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FIXATION BIOLOGIQUE DE L'AZOTE CHEZ LES LEGUMINEUSES A GRAINES

Inoculation du Soja *Glycine max* (L.) Merr.) et du niébé *Vigna unguiculata* (L.) Walp.) sur sol ferrallitique fortement désaturé en basse Côte d'Ivoire.

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Résumé

Des essais d'inoculation du soja (Glycine max) et du niébé *(Vigna unguiculata)* ont été conduits en serre et en plein champ sur un sol ferrallitique fortement désaturé en Mai, Juin et Juillet 1987. Les résultats obtenus indiquent d'une part qu'en sol très acide (pH 4,8), il est possible d'avoir une bonne nodulation avec certaines associations symbiotiques (Légumineuses-Rhizobium) bien adaptées aux conditions extrêmes de culture. D'autre part, l'inoculation du soja et du niébé peut être bénéfique en Côte d'Ivoire, du moins dans la région où l'expérience a été conduite.

La nutrition azotée des légumineuses est un important facteur limitant du rendement en graines du soja (*Glycine max*) et du niébé (*Vigna unguiculata*) et de leur rôle en tant que précédent cultural. or, cette nutrition azotée est assurée par deux voies : l'assimilation de l'azote combiné du sol ou des engrais azotes et la fixation biologique de l'azote atmosphérique. Ces deux voies sont influencées différemment par les facteurs de l'environnement si bien qu'elles peuvent être concurrentes ou complémentaires suivant les stades physiologiques de la plante ou les conditions du milieu (Decau *et al.*, 1975 ; Obaton et Kimou, 1985).

La modification de l'équilibre entre les contributions de ces deux sources peut avoir d'importantes implications agronomiques et économiques. En effet, des gains substentiels de fixation et de rendement sont possibles chez certaines espèces de légumineuses par inoculation avec des souches très efficientes. Mais l'efficacité de cette inoculation peut être compromise par des conditions pédoclimatiques inadéquates. Dans le présent travail, nous avons cherché à estimer l'influence des modalités de fourniture d'azote par le sol ou par la fumure sur la participation relative de l'une ou l'autre source et à en évaluer les conséquences agronomiques en sol ferrallitique fortement désaturé très typique des zones forestières de la basse Côte d'Ivoire.

Matériels et méthodes

Le niébé cv. V_1 et le soja cv. Tropical ont été cultivés parallèlement en champ et en serre en présence de 0 et 90 kg N/ha. L'azote est appporté sous forme d'urée. Pour chaque type de légumineuses, les graines ont été stérilisées par trempage pendant 30 mn dans une solution d'hypochlorite de calcium (66 g/l) puis abondamment rincées à l'eau distillée stérile et ensuite réparties en deux lots. Un lot est inoculé avec une suspension de *Rhizobium japonicum G49* (INRA-France) pour le soja et de *Rhizobium tropical* N7 (ENSA-Côte d'Ivoire) pour le niébé. L'inoculation a été réalisée à une dose forte (10^9 *Rhizobium* par graine) juste avant le semis; L'autre lot de graines est semé sans inoculation.

L'expérience au champ a été conduite sur la parcelle expérimentale de l'Ecole Nationale Agronomique d'Abidjan en une seule saison culturale de Mai à Juillet 1987. Cette période correspond à la grande saison des pluies, saison de culture en Côte d'Ivoire. Les sols sont du type sablo-argileux contenant en moyenne 2,84 % de matière organique et 1080 ppm d'azote total ; 17,5 ppm de phosphore assimilable ; complexe absorbant 25,11 % ; Mg : 0,23 meq/100 g ; Ca : 0,84 meq/100 g ; K : 0,05 meq/100 g et pH eau 4,8. Le site chosi pour l'expérience est relativement homogène et n'a jamais porté de culture de soja et de niébé.

En serre, les plantes ont été cultivées en pots de 5 1 sur des échantillons de sol provenant de la parcelle d'expérience au champ. La serre est un abri avec un toit en verre transparent qui n'arrête que les eaus de pluies. I-es murs sont constitués de grillage de 0,5 cm de maille. Ainsi les plantes en serre bénéficient des mêmes conditions d'éclairement naturel (durée d'ensoleillement variant entre 10 et 12 heures), de température (température diurne : 28° à 30°C ; température nocturne : 24 à 26°C) et d'humidité (humidité relative pendant la période de cullture 95 à 100 %) que celles en plein champ. Les plantes sont arrosées au fur et à mesure des -besoins, en solution non coulante à l'eau distillée.

En pleine floraison, 45 jours après le semis pour le niébé et 60 jours après le semis pour le soja, quelques plantes sont déterrées pour le dénombrement des nodosités formées. La partie aérienne de chaque plante est sectionnée au noeud cotylédonnaire et mise à sécher à l'étuve à 75 °C pendant 48 h. Le poids de matière sèche de la partie aérienne est déterminé par pesée. Les rendements en graines sont mesurés après la récolte à la maturité et après séchage au soleil jusqu'à 15 % d'humidité.

Le dispositif expérimental utilisé en champ et en serre est en blocs de Fisher entièrement randomisés. Il comporte 4 traitements (témoin absolu, témoin avec inoculation, 90 kg N/ha et 90 kg N/ha avec inoculation.

Résultats et discussion

Les résultats de l'inoculation sont indiqués dans les Tableaux 1 et 2 respectivement pour le soja et pour le Niébé.

Inoculation du soja - Les effets de l'inoculation du soja, cv. Tropical avec la souche G49 sont très nets. Au champ, en l'absence de fumure azotée, l'inoculation permet de passer de 0,78 t/ha (témoin absolu) à 2.62 t/ha soit un accroissement du rendement en graines de 23 % par rapport au témoin non fumé et non inoculé (Tableau 1A). En l'absence d'inoculation, l'apport de 90 kg N/ha augmente le rendement en graines, d'environ

67 % mais ne provoque aucune différence significative sur la production de matière séche des parties aériennes. En présence d'inoculation, l'apport de 90 kg N/ha augmente significativement la production de matière sèche des parties aériennes de 13.9 % et provoquent dans le même temps une diminution de 21 % du rendement en. graines. Cette baisse du rendement est due à un avortement élevé des fleurs suite à un développement végétatif excessif des plantes suivi de leur verse. Ces différentes observations faites au champ sont confirmées par les résultats en serre (Tableau 1B).

Tableau 1A : Essai d'inoculation de Glycine max (cv. Tropical) avec l'inoculum G49 (INRA-France) : résultats au champ.

Traitements	Nombre de nodosités par plante	Poids de matiére sèche t/ha	Rendement en graines t/ha
Témoin non inoculé			
0 kg N/ha	0 b	5,69 c	0,78 c
90 kg N/ha	0 b	5,92 c	1.30 b
Inoculé			
0 kg/ha	72 a	7,97 b	2,62 a
90 kg/ha	69 a	9,08 a	2,16 a

Dans chaque colonne les valeurs suivies de la même lettre ne diffèrent pas au seuil de 5 % (méthode Newman-Keuls)

Tableau 1B : Essai d'inoculation de Glycine max (cv. Tropical) avec l'inoculum G49 (INRA-France) : résultats en serre

Traitements	Nombre de nodosités par plante	Poids de matière sèche g/plante	Rendement en graines g/plante
Témoin non inoculé			
0 kg/ha	0 b	18,28 d	3,57 d
90 kg/ha	0 b	24,32 c	8,90 c
Inoculé			
0 kg/ha	50 a	31,14 b	15,04 a
90 kg/ha	56 a	41,68 a	11,83 b

- 90 kg N/ha correspond à 3 mg N/100 g du sol utilisé en serre
- Chaque valeur est une moyenne sur 16 plantes (4 plantes x 4 répétitions)
- Dans chaque colonne, les valeurs suivies de la même lettre

Inoculation du niébé - Les observations faites sur l'inoculation du soja, cv. Tropical sont identiques à celles faites sur l'inoculation du niébé cv. V,. Cependant, l'effet de cette inoculation semble moins spectaculaire sur le niébé comparé aux résultats obtenus sur le soja. Au champ, en l'absence de fumure azotée, l'inoculation augmente le rendement en graines de 32,75 % par rapport au témoin non inoculé (Tableau 2A). Pour les autres traitements, aucune différence significative n'est observée. La différence de résultats observée entre le soja et le niébé pourrait être justifiée par le fait que le niébé non inoculé porte des nodosités qui possèdent une certaine aptitude à fixer l'azote atmosphére (section rouge des nodosités). Un traitement témoin non nodulé aurait été intéressant pour infirmer ou confirmer cette hypothèse.

Tableau 2A : Essai d'inoculation de Vigna unguiculata (V_1) avec l'inoculum local N 7 (ENSA-Côte-d'Ivoire) : résultats au champ.

Traitements	-Nombre de nodosités par plante	Poids de matière sèche t/ha	Rendement en graines t/ha
Témoin non inoculé			
Ø kg N/ha	8 b	6,00 b	0,76 b
90 kg N/ha	7 b	6,65 ab	0,85 ab
Inoculé			
Ø kg N/ha	20 a	5,86 b	1,001a
90 kg N/ha	15 ab	7,08 a	0,86 ab

Dans chaque colonne les valeurs suivies de la même lettre ne diffèrent pas au seuil de 5 4 (Méthode Newman-Keuls).

Tableau 2B : Essai d'inoculation de Vigna unguiculata (V1) avec l'inoculum local N7 (ENSA-Côte-d'Ivoire) : Résultats en serre

Traitements	Nombre de nodosités par plante	Poids de matière g/par plante	Rendement en graines g/plante
Témoin non inoculé			
Ø kg N/ha	8 b	23,08 b	3,75 c
9Ø kg N/ha	8 b	23,30 ab	3,95 c
Inoculé			
0 kg N/ha	24 a	21,43 a	4,85 b
9Ø kg N/ha	27 a	24,90 a	4,99 b

- 9Ø N/ha correspond â 3 mg N/100 g du sol utilisé en serre
- Chaque valeur est une moyenne sur 16 plantes (4 plantes x 4 répétitions.
- Dans chaque colonne, les valeurs suivies de la mëme lettre ne diffèrent pas au seuil de 5 % (Méthode Newman-Keuls).

Conclusion

De ce travail deux points essentiels semblent retenir particulièrement notre attention :

- en sol très acide, il est possible d'avoir une bonne nodulation avec certaines associations symbiotiques *«légumineuse-Rhizobium»* bien adaptées aux conditions extrêmes.
- l'expérience conduite sur le domaine expérimental de l'Ecole Nationale Supérieure Agronomique d'Abidjan montre que l'inoculation du Soja cv. Tropical et du Niébé cv. V, peut être bénéfique. Bien que l'inoculation du niébé soit rarement pratiquée dans le monde, cette augmentation de rendement d'environ 33 % dans le sol présentant de telles caractéristiques nécessiterait d'effectuer des expérimentations analogues en d'autres points du pays. La confirmation de tel effet bénéfique pourrait conduire à une application pratique (inoculation du soja et du niébé) en milieu paysan.

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Maximiser la FBA pour la Production Agricole et Forestière en Afrique

Influence of rhizobia inoculation on soybean (*Glycine max* L.) performance in Nigeria

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Abstract

The response of soybean (*Glycine* max L.) to inoculation with exotic and indigenous rhizobia (no inoculation) and inorganic nitrogen fertilization was studied at Ilorin and Samaru, Nigeria, during the 1985 cropping season In the two locations. nodulation was enhanced by all inoculants, while inorganic nitrogen fertilization significantly enhanced vegetative dry matter production. Location effect was observed for the response of soybean to inoculation with regard to seed yield. While in Ilorin, grain yield was not enhanced by inoculation, in Samaru, only strain IRj 2133 significantly influenced the seed yield of some soybean cultivars.

A generalization of the need or otherwise to inoculate soybean for enhanced performance in Nigeria is not possible from this study. However, a more justifiable conclusion may be reached if other parameters, such as amount of nitrogen fixed and the proportion of it which is available to a subsequent grown crop, are assayed.

Introduction

The potential contribution of soybean to the improved nutrition of most Nigerians that can ill-afford animal proteins cannot be over-emphasized. Although it has been cultivated for sometime in traditionally growing areas of Nigeria, its wide national acceptance and cultivation is relatively recent.

Soybeans derive N directly from the soil as inorganic residuals or as mineral&d organic soil fractions and indirectly by the symbiotic nodule relationship where the bacterium *Bradyrhizobium* juponicwn fix N present in the atmosphere. Harper (1974). confirmed that both symbiotically fixed and inorganic N are required for highest yields of soybean, while estimates of the fixed N to the total N range from 25 to 60% (Vest et al., 1973). Thus, yield increases from N fertilization would be expected to be relatively common. Yet only a few reports of measured yield increases from N fertilization are available in the literature (Bhangoo and Albritton, 1972 ; Johnson and Hume, 1972) and these increases were often very small (Sorrensen and Penas, 1978). Somme other investigations however found no effect of N fertilization on soybean field (Wagher, 1962, Beard and Hoover, 1971).

Ayanaba (1977) reported that the tropical soils are devoid of indigenous *B. japonicum*, although the cowpea type *Rhizobium* strain (which is prevalent in these soils) readily form effective nodules on the roots of soybean varieties of Asian origin (Nangju, 1980). It is generally believed that when soybean is cultivated in a field with no previous history of soybean cultivation, artificial inoculation with *B.japonicum is essential to* ensure high seed and protein production (Graham, 1985). Although several investigators have reported beneficial effect of inoculation on soybean (Kang, 1975 ; Pal and Saxena, 1975 ; Chowdhury, 1977 ; Singh and Tilak, 1977), others have reported inoculation failures (Salema and Chowdhury, 1980 ; Awai, 1981 ; Awonaike, 1988).

Addition of combined nitrogen and inoculation have been shown to respectively enhance vegetative matter yield and nodulation in some grain legumes (Ayanaba and Nangju, 1973). Their combined effect on seed yield of some tropical grain legumes under field conditions is inconclusive (Ofori, 1973 ; Luse *et* al., 1975 ; Pal et al., 1985).

The objectives of this study were therefore to ascertain the need or otherwise to inoculate soybean for enhanced performance in Nigeria and if necessary, to identify strains of ***B. juponicum*** that are adapted to the environmental conditions.

Materials and methods

Field experiments were conducted on newly cleared land (without a history of soybean cultivation) in 1985 at the Ballah substation of the Institute of Agricultural Research and Training in Ilorin (8°30"N, 4°35"E) and on the Institute for Agricultural Research's experimental farm at Samaru, Nigeria (11°11"N, 7°38"E). The soil at Ilorin belongs to the Apomu series and that of Samaru is a well-drained, leached ferrogenous tropical sandy loam.

(i) : Treatments of the Ilorin trial . Eleven soybean cultivars were subjected to three nitrogen nutrition regimes (viz., N_1 : uninoculated control treatment without nitrogen fertilization ; N_2 : 150 kg urea per ha provided in three split applications at 25 days interval commencing at planting ; N_3 : inoculation with a mixture of ***B. japonicum*** strains IRj 2114 and IRj 2133). A randomized complete block design incorporating four replicates

of the 33 treatments was used. A basal application of 400 kg single superphosphate per hectare was applied before planting because of the low P status of the soil (3.2 ppm P).

(ii) : Treatments of the Samaru trial - Treatments consisted of five soybean cultivars grown with four single IRj strain peat inoculants (2114, 2123, 2133 and 2144) and a mixture containing equal proportions of the four strains. An uninoculated control treatment without nitrogen fertilizer and another uninoculated treatment with 100 kg N/ha applied as calcium ammonium nitrate (CAN) (26 % N) were also included. A randomized complete block design incorporating three replicates of each of the 35 treatments was used.

Each plot size in the both trials was 6 m by 2.25 m made up of four rows plants, 75 cm between the rows and '5 cm within the row.

Symbiotic effectiveness was evaluated at 9 weeks after planting (WAP) by assessing fresh weight of nodules produced and dry weight of the shoots (Ilorin trial) and the nodules number and dry weight (Samaru trial) of five competitive plants per plot, The remaining plants were left to mature when the seeds were harvested.

All the data collected were subjected to statistical analysis of variance.

Results

Nodulation and vegetative matter production. The nodulation data as expressed by the fresh weight of nodules per plant (Ilorin trial) and number and dry weight of nodules per plant (Samaru trial) at 9 WAP arepresented in Tables 1.2 and 3, respectively. Averaged over cultivars, all inoculants significantly enhanced nodulation when compared to the uninoculated control in both locations. Also, nitrogen fertilization decreased nodulation, but not significantly. Among the inoculants at Samaru, strain IRj 2133 gave the higest nodules number and weight while strain IRj 2123 performed poorest. The cultivars also differed significantly in their ability to nodulate. As expected at Ilorin, Bossier cultivar nodulated very poorly with the indigenous rhizobia but nodulated highly with the mixture of inoculant strains, while cultivars TGx 539-5E, Samsoy 2 and M-90 nodulated well with both the indigenous rhizobia and the inoculant mixture. At Samaru however, cultivar TGx 814-26D produced the highest number and weight of nodules while the Samsoy cultivars produced the least number and weight of nodules. Significant interaction between cultivar and inoculant strain was observed at Samaru in that while cultivar TGx 888-49C responded to all inoculants except strain IRj 2144, cultivars TGx 536-02D, Samsoy 1 and Samsoy 2 responded to all inoculants except strain IRj 2123. TGx 814-26D responded to all inoculants except the mixture of strains.

At Ilorin (Table 4) and Samaru (result not shown) inorganic nitrogen fertilization significantly enhanced vegetative matter production. There was however no significant difference in the mean vegetative matter production of the inoculated and uninoculated control plants. Varietal differences were also observed.

Seed yield. The seed yield data are presented in Tables 5 and 6.

Despite the significant effects of source of nitrogen on nodulation and vegetative matter production at Ilorin, these were not reflected in the seed yield (Table 5). Significant varietal effects were however observed. TGx 814-26D and TGx 539-5F significantly out yielded the rest of cultivars. The very poor yield of TGx 573-209D was due to poor

Table 1. Effect of inoculation with B. japonicum strains on nodulation of soybean cultivars (Ilorin location).

Cultivars	Fresh weight nodules (ng per plant)			
	Uninoculated (indigenous rhizobia)	Uninoculated + 150 kg N/ha	Mixture of strains IRj 2114 and 2133	Mean
Samsoy 1	0.06	0.05	0.24	0.12
TGx 297-10F	0.05	0.03	0.14	0.08
TGx 539-5E	0.10	0.03	0.38	0.17
M-90	0.05	0.03	0.11	0.06
TGx 814-26D	0.04	0.04	0.16	0.08
TGx 136-02D	0.02	0.01	0.15	0.06
TGx 813-6D	0.01	0.05	0.08	0.05
Samsoy 2	0.08,	0.01	0.16	0.08
TGx 573-209D	0.02	0.02	0.13	0.05
TGx 888-49C	0.05	0.05	0.19	0.10
Bossier	0.01	0.01	0.46	0.16
Mean	0.04	0.03	0.20	

C.V. % 32.5

LSD (P = 0.01) N = 0.01

LSD (P = 0.01) V = 0.02

LSD (P = 0.05) N x V = 0.07

Table 2 : Effect of inoculation with *B. japonicum* strains on the nodulation of soybean cultivars (Samaru location),

Inoculant	Number of nodules per plant					
	TGx 88-49C	TGx 536-02D	Samsoy 1	Samsoy 2	TGx 814-26D	Mean
IRj 2114	70.2 (b-h)	43.5 (c-i)	67.9 (b-h)	14.5(hi)	98.9 (a-c)	59.0 (tu)
IRj 2123	51.3 (b-i)	22.3 (f-i)	29.1 (e-i)	16.7 (g-i)	93.0 (a-d)	42.5 (uv)
IRj 2133	79.7 (b c)	87.3 (b-d)	72.5 (b-g)	88.4 (a-d)	140.3 (a)	93.6 (s)
IRj 2144	23.7 (f-l)	102.7 (a b)	69.7 (b-h)	75.0 (b-f)	104.1 iab)	75.1 (st)
Mixture	59.8 (b-h)	63.7 (b-h)	43.3 (c-i)	59.2 (b-i)	82.3 (b-e)	62.3 (tu)
Uninoculated	23.3 (f-i)	6.8 (i)	17.3 (g-i)	23.1 (f-i)	41.2 (d-i)	22.3 (vw)
Uninoculated+ 100 kg N/ha	9.4 (i)	7.3 (i)	20.2 (f-i)	18.3 (g-i)	49.1 (b-i)	20.9 (w)
Mean	45.4	48.2	45.7	42.2	87.0	

SE inoculant means = 7.26; SE cultivar means = 6.13 ; SE inoculant x cultivar mean = 16.23. Mean followed by the same letter or letters are not significant at the 5 per cent level, according to Duncan's multiple range test. Means followed by 2 letters connected with a hyphen are included *in* all groupings between the two letters in the alphabet.

Table 3 : Effect of inoculation with B japonicum strains on the nod ulation of soybean cultivars (Samaru location).

Inoculant	Nodule dry weight (mg per plant) TGx 88-49C	TGx 536-02D	Samsoy 1	Samsoy 2	TGx 814-26D	Mean
IRj 2114	382.9	222.4	353.9	55.6	254.7	253.9 (bc)
IRj 2123	267.6	100.5	99.7	83.3	344.1	179.0 (cd)
IRj 2133	429.8	472.7	304.7	524.5	486.5	443.6 (a)
IRj 2144	139.2	364.0	327.3	336.3	460.3	325.4 (b)
Mixture	472.4	315.5	159.4	320.3	299.1	313.3 (b)
Uninoculated	153.4	43.8	64.4	88.1	173.7	104.7 (d)
Uninoculated+ 100 kg N/ha	52.2	45.1	91.3	68.8	218.9	94.7 (d)
Mean	271.1	223.4	200.1	210.5	319.6	

S.E.D. of inoculant means = 44.4 ; S.E.D. of variety means = 37.5 ; S.E.D. of inoculant x variety means = 99.25.

Values in column followed by the same letter are not significantly different at the 5 percent level, according to Duncan's multiple range test.

establishment of the plants. At Samaru, the relatively earlier maturing cultivars TGx 888-49C and TGx 536-02D yielded significantly higher than the other three cultivars which did not differ from each other. Also, the interactive effect of cultivar and strain on seed yield was significant ($P = 0.05$) at Samaru. Inoculants did not increase the yields of cultivars TGx 888-49C, TGx 536-02D, Samsoy 1 and TGx 814-26D, while the yield of Samsoy 2 was improved by strain IRj 2133.

Discussion

Nangju (1980) postulated that the local cultivars can form effective nodules with indigenous rhizobia and therefore can be grown without seed inoculation with *R. japonicum*. All the cultivars used in this study (except cultivar Bossier) were selected under the local conditions and were found to respond to inoculation. The fact that the strains differed in their ability to form effective nodules with the different cultivars indicated that the most important factor is the *use of* efficient strains of *R. japonicum* that can compete with the indigenous rhizobia. The comparative advantage of higher number of organisms in the inoculants than in the soil may also account for the response. Other researchers have reported differential performance of soybean inoculants (Kang, 1975 ; Chowdhury, 1977 ; Chowdhury *et* al., 1983).

The positive responses of all the cultivars to inorganic N fertilization (with regard to vegetative matter production) and to inoculation (with regard to nodulation) suggest that the indigenous rhizobia do not adequately supply the N needs of the plants. It is however not clear why these (increased matter production and nodulation) do not enhance seed yield. Further studies on the distribution of absorbed and fixed N to the parts of an actively fixing soybean plant especially at its reproductive phase should help to explain the situation.

Until this is done however, the inoculant requirement situation in Nigeria for enhanced soybean production is uncertain.

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Table 4 : Effect of inoculation with B. japonicum strains on vegetative dry matter yield (mg per plant) of soybean cultivars (Ilorin location).

Cultivars	Uninoculated (indigenous)	Uninoculated+ 150 kg N/ha	Mixture of strains IRj 2114 and 2133	Mean
Samsoy 1	10.5	13.2	9.3	11.0
TGx 297-10F	12.3	14.1	11.4	12.6
TGx 539-5E	10.6	15.8	7.6	11.4
M - 90	8.5	12.8	7.0	9.4
TGx 814-26D	10.8	15.9	7.9	11.5
TGx 536-02D	12.6	16.9	9.6	13.0
TGx 813-6D	13.6	14.9	11.1.	13.0
Samsoy 2	9.5	18.2	10.7	12.8
TGx 573-208D	12.2	15.6	11.0	13.0
TGx 888-49C	10.7	16.8	11.5	13.0
Bossier	7.2	22.4	9.2	12.9
Mean	10.8	16.1	9.7	

C.V. (%) = 19.5
LSD (P = 0-01) N = 3.6
LSD (P = 0.05) V = 1.0

Table 5 : Effect of inoculation with *B. japonicum* strains on the seed yield (kg per ha) of soybean cultivars (Ilorin location).

Cultivars	Uninoculated (indigenous rhizobia)	Uninoculated+ + 150 kg N/ha	Mixture of strains IRj 2114 and 2133	Mean Mean
Samsoy 1	1941	2499	2335	2285.3
TGx 297-10F	2260	1665	2173	2032.7
TGx 539-5E	3648	3203	3242	3364.7
M - 9 0	2508	2320	1930	2286.0
TGx 814-26D	3608	3143	3170	3307.0
TGx 536-02D	1578	2008	2903	2163.0
Samsoy 2	1917	1685	1814	
TGx 573-209D	860	218	337	471.7
TGx 888-49C	2812	2588	2773	2724.3
Bossier	1615	2092	1908	1871.7
Mean	2208.1	2118.0	2201.8	

C.V. (%) = 15.8
LSO (p = 0.01) v = 268.3

Table 6 : Effect of inoculation with B. japonicum strains on the seed yield (kg per ha) of soybean cultivars (Samaru location).

Inoculant	TGx 88-49C	TGx 536-02D	Samsoy 1	Samsoy 2	TGx 814-26D	Mean
IRj 2114	1792 (a-f)	2137 (a)	1694 (a-h)	1388 (e-j)	1081 (i-k)	1618 (st)
IRj 2123	1637 (a-h)	1625 (a-i)	1184 (g-k)	1436 (c-j)	1238 (a-j)	1422 (tu)
IRj 2133	2028 (ab)	1923 (a-d)	1131 (h-k)	2026 (ab)	1287 (e-k)	1679 (s)
IRj 2144	1588 (e-i)	1726 (a-g)	1307 (e-j)	1447 (c-j)	1035 (jk	1421 (tu)
Mixture	1972 (a-c)	1676 (a-h)	1168 (g-k)	1211 (g-k)	931 (k)	1352 (u)
Uninoculated	1525 (b-j)	1665 (a-h)	1305 (e-j)	1354 (e-k)	1142 (b-k	1398 (tu)
Uninoculated + 100 kg N/ha	1823 (a-c)	1713 (a-g)	1524 (b-j)	1461 (i-j)	1348 (a-e)	1674 (s)
Mean	1766	1781	1330	1474	1193	

SE inoculant means = 72.5 ; SE cultivar means = 61.3 ; SE inoculant cultivar means=162.2
Means followed by the same letter or letters are not significant at the 5 per cent level, according to Duncan's multiple range test. Means followed by 2 letters connected with a hyphen are included in all groupings between the two letters in the alphabet.

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Nodulation and growth of bambara groundnut (*Vigna subterranea*) in three soils

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Abstract

Nodulation and dry matter production of ten cultivars of bambara groundnut differing in growth habit, maturity period and yield potential were assessed in potted soils from three locations in southern Nigeria. Nodulation occurred in all three soils with the least number of nodules plant-' in the acid soil (Ultisol) from Onne, near Port Harcourt.

Urea did not affect nodulation in general. Differences were observed between cultivars on the basis of nodule number but not of nodule' dry weight. Relative symbiotic effectiveness values based on plant dry weight averaged 92 % for all cultivars grown in the three soils. This indicated that bambara groundnut establishes effective symbioses with indigenous rhizobia in these soils. Only cultivar TVsu 1231 responded to urea N significantly (at P = 0.05) in soils from the International Institute of Tropical Agriculture (IITA) and Fashola (Alfiiols). There was no correlation between growth habit or yield potential and plant dry matter production.

Introduction

A few years back, bambara groundnut *(Vigna subterranea* (L.) *Verdcourt)* was one of the most widely cultivated leguminous crops in Africa (Doku and Karikari, 1971). Nowadays, with the expansion of groundnut production for oil. bambara groundnut is only sparsely grown in Africa (Goli, 1987) in spite of its pest and disease tolerance both in fields and during storage, its nutritional value and its adaptation to a wide range of soils (Doku and Karikari, 1971).

Few studies have been conducted to document the ability of bambara groundnut to fix nitrogen and grow on low-N soils. Duke et *al.* (1977) reported in their review that high soil N promotes vegetative growth of bambara groundnut at the expense of seed production. Stanton (1968) found that inoculation of new fields of bambara groundnut with soil from plots where the crop grew well improved crop yields. But Johnson (1966) and Denarit *et al.* (1968) showed that inoculation with rhizobia did not improve yields significantly. More inoculation studies are being carried in parts of Senegal where bambara groundnut does not nodulate freely (Gueye, M. pers. comm.).

The germplasm unit of the International Institute of Tropical Agriculture @TA), Ibadan, Nigeria, possesses an important collection of bambara groundnut with 2027 accessions. In view of the potential of this legume in tropical agriculture, we assessed its growth and nitrogen-fixing ability in three low-N soils comprising an Ultisol from the humid region, and two Alfisols from the subhumid forest-savanna transition zone. Cultivars used in this study represented a range of growth habits, maturity periods and yield potentials of bambara groundnut

Materials and methods

Ten cultivars of bambara groundnut were compared on the basis of their nodulation, dry matter production and response to urea to assess their nitrogen-fixing ability. The experiment was conducted in pots at IITA. Growth characteristics and yield potential of the cultivars used are presented in Table 1. The completely randomized design was used with five replications and a factorial combination of 10 bambara groundnut cultivars, three soils low in N from IITA, Fashola (at about 70 km North of Ibadan) and Onne near Port Harcourt, all in Nigeria, and two rates of urea applied at 0 and 100 mg N kg^{-1} soil. Some properties of the experimental soils are presented in Table 2. Urea was applied as a water,, solution at one and two weeks after plant emergence. All pots received a basal application of single superphosphate (25 mg P kg^{-1} soil) and muriate of potash (15 mg K kg' soil).

Five seeds of each cultivar were sown in each pot containing 3 kg of soil previously passed through 'a 10 mm sieve to remove stones and large plant parts. Seedlings were thinned to three at one week after emergence. Plants were harvested at 8 weeks after planting for nodule count and mass, and plant dry weight.

Results and Discussion

Nodulation occurred in all the soils with an average of 30 nodules plant-' in Fashola soil (Table 3). Nodule number was 21 % and 44 % less in IITA and Onne soils respectively ; but plant nodule dry weight was the same in the three soils indicating that nodules

Table 1. Growth characteristics and yield potential of cultivars of Bambara groundnut studied.

Cultivars (TVsu)	'Growth habit'	Days to maturity	Yield potential (g plant-')
5	spread	123	4
9a	bunch	113	27
9b	spread	115	15
132	spread	131	18
409	intermediate	105	5
465	bunch	105	15
790	bunch	127	0,4
1 055	bunch	150	5
1 061	intermediate	157	5
1 231	intermediate	123	87

Table 2. Some properties of the experimental soils collected from IITA, Fashola and Onne.

Parameter	IITA	Fashola	Onne
PH	5.8	6.2	4.6
Organic C, %	Ø.53	Ø.36	1.01
Total N, %	Ø.11	Ø.Ø4	Ø.13
Extractable P (Bray-1), mg kg^{-1}	21.Ø	7.8	54.8
CEC[a], meq $100g^{-1}$	1.89	1.21	2.32
Total acidity (Al + H), meq $100g^{-1}$	Ø.Ø2	Ø.Ø1	1.72

[a]Cation exchange capacity

were relatively smaller in IITA and Fashola soils. Thompson and Dennis (1977) reported numbers ranging between 92 and 318 nodules plant-' in Ghana. Free modulation was also reported in Madagascar (Denarié et *al.*, 1968) and in Zaire (Rassel, 1960).

Nodulation in this experiment was not affected by fertilization with urea except in Fashola soil (results not presented) where nodule number of cultivars TVsu 9b and TVsu 409 decreased and that of cultivar TVsu 9a increased in the N treatment These differences were also observed when the average nodule number for all three soils was calculated (Table 3). In general, there were in both N treatments differences between cultivars of bambara groundnut on the basis of nodule number but not of nodule dry weight (Table 4). Correlations between nodule. numbers or weights and shoot, root or plant weights were poor at P = 0.05 ($r < 0.253$) in the no-fertilizer N treatement. For instance, plant dry matter production was highest in Onne soil where nodulation was the poorest (Table 3). Also, there was apparently no relationship between growth habit, maturity period or yield potential and nodulation, relative effectiveness or dry matter production.

Table 3. Nodulation and growth of Bambara groundnut in three different soils (1)

Soil	Plant dry weight (g plant-')	Nodule number plant^{-1}	Nodule dry weight (mg plant-')	Single nodule dry weight (mg)
Ibadan	4.2	23	87	3.8
Fashola	3.2	41	100	2.4
Onne	5.3	16	80	5.0
LSD (0.05)	0.89	4.3	NS	1.6

(1) Means of 10 cultivars in the no-fertilizer N treatment.

Table 4. Response of ten cultivars of bambara groundnut to fertilizer N_1 and their relative effectiveness (R.E.)[2] in nitrogen fixation based on plant dry weight.

Cultivar	Plant dry weight		Nodule number		Nodule dry weight		Single nodule dry weight		R.E.
(TVSu)	N_0	N_1	N_0	N_1	N_0	N_1	N_0	N_1	
	g plant^{-1}		No. plant^{-1}		mg plant^{-1}		------mg------		%
5	4.3	5.3	30	27	168	98	5.6	3.6	82
9a	3.5	4.0	18	31	54	71	3.0	2.3	88
9b	3.7	4.4	22	16	80	126	3.6	7.9	84
132	5.3	4.0	23	27	88	59	3.8	2.2	133
409	3.3	4.0	34	21	111	146	3.3	7.0	a3
465	5.1	4.7	28	32	92	97	3.3	3.0	109
790	5.5	6.3	25	32	83	73	3.3	2.3	87
1055	4.5	3.8	22	33	88	132	4.0	4.0	118
1061	5.7	6.0	20	13	69	56	3.5	4.3	95
1231	4.3	6.7	11	16	60	71	5.5	4.4	64
LSD(5%)	1.48		11.1		96		4.32		ND[3]

1N_0 = without fertilizer N; N_1 = with 100 mg urea N kg-1 soil

2R.E. : Relative effectiveness = Plant dry weight at N0 * 100/Plant dry weight at N_1

3ND : not determined as R.E. values were obtained from computed values.

Dry matter production was only slightly increased by N fertilization. Except for cultivar TVsu 1231. relative effectiveness based on plant dry weight ranged between 82 and 132 %. This was an indication of effective symbiosis between bambara groundnut and indigenous rhizobia present in the three soils studied. Cultivar TVsu 1231 had relative effectiveness values of 421.64% and 87% in Fashola, IITA and Onne soil respectively, with an average relative effectiveness of 64%. Thus cultivar TVsu 1231 had the least effective symbiosis with native populations of rhizobia in IITA and Fashola soils and would require inoculation with the appropriate rhizobia to fix nitrogen adequately in these soils.

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Très récemment, les essais d'inoculation du voandzou (*Vigna subterranea* (L.) Thouars) ont débuté en station expérimentale. Le voandzou est une légumineuse à graines de haute qualité nutritive (les graines contiennent 17% de protéines), résistante aux maladies et aux insectes, et parfaitement adaptée aux conditions semi arides des régions tropicales (NAS, 1979). Le voandzou est cultivé au sud-est du Sénégal dans les petites exploitations agricoles, seul ou en association avec une céréale.

Ce présent rapport décrit les premiers résultats obtenus sur l'effet de l'inoculation avec des souches de *Rhizobium* préalablement sélectionnées (Guèye et Bordeleau, 1988) sur le rendement en grains de deux variétés de voandzou.

Matériels et méthodes

Un essai d'inoculation du voandzou au champ a été conduit en station expérimentale au sud-est du Sénégal à Sinthiou Malème en 1986 sur le site A (pluviométrie : 794 km) et en 1988 sur le site B (pluviométrie : 873 mm). Les caractéristiques physicochimiques du sol des deux sites sont indiquées au Tableau 1.

Tableau 1 : Caractéristiques physico chimiques du sol de Sinthiou Malème

	ppm		Composition (%)					
			Passimilable					
Sites	C total	N total	P total	(Olsen*)	Argile	Limon	Sable	pH (H2O
A	4380	450	557	156,5	4,8	2,5	92,7	6,40
B	4540	470	402	109,5	4,3	2,3	93,4	6,64

*Olsen et al. (1954)

Effet de l'inoculation avec des souches de *Rhizobium* et de la fertilisation azotée sur le rendement en grains du Voandzou (*Vigna subterranea* (L.) 'Thouars) au Sénégal

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Résumé

Des essais au champ ont été conduits au Sénégal en 1986 et 1988 en station expérimentale pour étudier la réponse du *voandzou* (*Vigna subterranea* (L.) Thouars) à l'inoculation avec deux souches de *Rhizobiwn :* MAO 113 et TAL 2.2. L'inoculation des deux variétés étudiées a augmenté leur rendement en grains de 27 à 58%. L'amélioration de. rendement la plus significative (58%) a été obtenue en 1986 avec la variété 83-131 inoculée avec la souche TAL 22. En 1988, l'ion avec la souche MAO 113 a amélioré le rendement de la variété 79-l de 52%.

Introduction

L'inoculation des légumineuses à *graines* avec des souches de *Rhizobium* n'est pas une pratique courante au Sénégal bien que ses effets bénéfiques y aient été mis en évidence sur le soja en particulier. L'inoculation du soja au champ avec la souche de *Rhizobium* USDA 138 conditionnée dans de la tourbe a augmenté significativement le rendement en grain du soja (Larcher et al., 1984) ; au laboratoire, Dommergues *et al.* (1979) avaient également obtenu une augmentation très significative du poids sec des nodules et de l'azote total des parties aériennes du soja inoculé avec la souche de *Rhizobium* USDA 138 incluse dans un polymère de polyacrylamide.

Deux variétés de voandzou (79-l et 83-131) ont été inoculées avec deux souches de *Rhizobium* : MAO 113 isolée du voandzou au Sénégal (Guèye et Bordeleau, 1988) et TAL 22 fournie par le projet Niftal à Hawail. De plus, l'essai comportait deux traitements supplémentaires *sans* inoculation avec *une* souche de *Rhiwbium :* un traitement consistant en une application d'engrais azoté à raison de 50 kg urée/ha et un traitement sans azote. L'essai a été conduit en blocs complètement randomisés avec quatre répétitions. Chaque parcelle élémentaire (2,4 m x 1,5 m) avait reçu une fertilisation de fond de 60 kg P_2O_5/ha sous forme de supertriple et de 120 kg KCl/ha. L'inoculum a été apport.& au sol sous forme de tourbe (5g/poquet) contenant approximativement 10^9 *Rhizobium/g. Le* voandzou a été semé à raison d'une graine par poquet selon un écartement de 30 cm entre les lignes et 15 cm sur la ligne.

Le rendement en grains du voandzou a été évalué à la récolte. Les analyses statistiques ont été effectuées en utilisant le test de Duncan (1955). .

Résultats et discussion

En comparaison avec les parcelles témoins (Tableau 2), le rendement en grains du voandzou n'a pas été significativement affecté par un apport de 50 kg urée/ha bien que sur le site B en 1988, il y ait eu 30% d'augmentation avec la variété 83-131. Par contre, l'inoculation du voandzou avec *les souches* de *Rhizobium MAO* 113 et TAL 22 a augmenté le rendement en grains des variétés 79-1 et 83-131. En 1986, l'inoculation avec la souche MAO 113 a augmenté le rendement de la variété 83-131 de 51% ; l'inoculation aveclasoucbeTAL22aaugmentélerendementdesdeuxvaritWs79-1 et83-131 respectivement de 50 et 58%. En 1988, l'inoculation avec la souche MAO 113 a amélioré le rendement des variétés 79-1 et 83-131 respectivement de 52% et 33% ; avec la. souche TAL 22, les améliorations ont été de 33% sur la variété 79-1 et 27% sur la variété 83-131.

Ces résultats montrent que la différence génétique entre les deux variétés utilisées influence fortement la réponse 'au champ du voandzou à l'inoculation avec les souches de *Rhizobium : la* réponse de la variété 83-131 est plus stable que celle de la variété 79-l. Cela était prévisible car une expérience précédente avait montré que l'indice de nodulation de ces deux variétés révélait une symbiose plus efficiente de la variété 83-131 en association avec les souches MAO 113 et TAL 22 (Guèye et Bordeleau, 1988). Ces résultats confirment donc les travaux de Dadson *et al.* (1988) qui avaient mis en évidence l'influence du génotype des variétés de voandzou à l'inoculation avec des souches de *Rhizobium.* L'effet bénéfique de l'inoculation avec les souches de *Bradyrhizobium sur* le rendement en grains du voandzou était également prévisible cardes études antérieures (résultats non publiés) avaient montré que le sol de Sinthiou Malème renferme très peu de *Rhiwbium (c* 200/g) et que le voandzou a souvent besoin d'être inoculé (Johnson, 1968). On pourrait donc recommander l'inoculation du voandzou dans les sols du Sénégal renfermant très peu de *Bradyrhizobium.* Cependant, on doit garda présent à l'esprit le fait que l'efficience d'une association *Rhizobium* x plante-hôte varie considérablement d'un site à un autre.

Tableau 2 : Effet de-l'azote et-de-l'inoculation avec les souches de **Bradyrhizobium MAO 113** et TAL 22 sur le rendement en grain **du voandzou (variétés 79-1** et **83-131)** cultivé au champ, à la station **expérimentale** de Sinthiou **Malème.**

Variétés	Traitements	Rendement en grain (kg/ha) Site A (1986)	Site B (1988)
79-1	Témoin	2989,40 b	3062,50 b
	Azote (50 kg urée/ha)	2991,90 b	3933,00 ab
	MAO 113	3512,10 ab	4645,50 a
	TAL 22	4492,00 a	4068,40 ab
83-131	Témoin	3080,30 b	3555,40 b
	Azote (50 kg urée/ha)	3832,90 ab	4624,10 a
	MAO [illegible]	[illegible]	[illegible]
	TAL 22	4863,90 a	4524,40 a

Dans chaque colonne **et pour chaque** variété, les valeur suivies d'une: **même lettre** ne **sont significativement différentes** d'après. le test de **Duncan (1955)** au **seuil de 5 %.**

Remerciements

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FIXATION BIOLOGIQUE DE L'AZOTE CHEZ LES LEGUMINEUSES FOURRAGERES

Nitrogen fixation of the forage legumes and their -residual effects on wheat growth and yield

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Abstract

The symbiotically fixed nitrogen of various forage legumes was evaluated for two seasons under upland soil conditions in Ethiopian highlands using ^{15}N technique and *Avena sativa (oats) as* a non nodulating reference crop. Forage oats gave higher dry matter yield than *Vicia dasycarpa* (vetch), however, the latter gave feeding stuff of higher N concentration and yield (169 kg N/ha/year for vetch vs. 73 kg N/ha/ year for oats). In 1985. 78, 78 and 73% of the total N in vicia, clover (*Trifolium steudneri*) and medics (*Medicago polymorpha*) originated from symbiosis. Vicia, medics and trifolium fixed 131, 77 'and 61 kg N/ha respectively. In 1986. the dry matter and grain yield of wheat following vicia, medics and trifolium was significantly higher than that following oars. Between 12 and 31 kg of additional N was taken up by wheat straw and grain following legumes and fallow ascompared with those following Oats.

Introduction

Of the many possible nutrient deficiencies, lack of N imposes the most widespread and strongest restrictions on plant and animal production in sub-Saharan Africa (Nnadi and Haque, 1988a). While the N status of soils can be improved by the addition of N

fertilizer, it is expensive input and is reflected in its low consumption in the region (IFDC, 1986).

In Ethiopia, the average yields of most of the cereals are below 1000 kg/ha. In order to improve the productivity of feed and food in various production systems and to sustain it at reasonable level, there is an urgent need for alternate sources to increase the influx of N in the system. Use of legumes in various production systems to benefit from biological nitrogen fixation (BNF) contribution to following cereal crops may attract quick adoption by resource poor farmers. Legumes also contribute to soil productivity by improving soil structure (Andrew, 1965 ; Stoveman, 1973) and can control pests and diseases of cereals in legume-cereal rotations (Moore *et* al. 1982).

Owing to the multiple facets of the contribution of legumes to cereals and the difficulty of isolating these factors, the contribution of legumes is often studied by measuring the yield of following crops (Musa and Burhan, 1974 ; Papastylianou *et al.* 1981 ; Singh *et* al. 1985 ; Ayoub, 1986 ; Osman and Nersoyan, 1986 ; Mohamed-Saleem *et* al. 1986 ; Papastylianou, 1987 ; Papastylianou and Samios, 1987 ; Keatinge *et al. 1988).*

This communication reports BNF by annual forage legumes and their residual effect on subsequent wheat crop on an upland soil in Ethiopian highlands.

Materials and methods

Climate

Rainfall, potential evapottmspirattion (PE) and water balance. The rainfall exhibits strong scasonality and of the total annual rainfall of 866 mm, 84% is recorded in the rainy season extending from June to September (Fig. 1). Short season is from March to May, when a total of about 140 mm rainfall is received. PE is 1320 mm (Fig. 1) and values are lower (2-3 mm/day) during the rainy season as compared with short rainy season (34 mm/day).

The water budget (Fig. 1) shows that in the months of July and August there is a surplus of about 300 mm of water. Daniel (1977) reported that potential runoff at Debre Zeit, in an average year, is likely to be 100 mm which could be collected and reuse for crop and livestock production in the post rainy season.

Temperature and radiation. Temperature and radiation are two factors that limit crop and livestock production in Ethiopian highlands. Mean maximum, minimum and average monthly and annual temperatures. are shown in Fig. 2 Relative sunshine hours are about 50% during the rainy months and are 70 to 80% during the rest of the year. Total radiation is around 500 cal/cm²/day and somewhat lower during the rainy months (300450 cal/cm²/day) and higher in the dry period from February to May (500-550 cal/cm²/day).

Soils

Debre Zeit is situated 8" 44"N, 39" 02"E at an altitude of about 1850 m above sea level, about 50 km south-east of Addis Ababa in Ethiopian highlands. The soils are mainly vertisols and some alfisols. The experiment was conducted on an alfisol.

Along the toposequence, alfisol, soil with vertic properties, and vertisol stored 113, 187 and 278 mm water per meter depth respectively. During the maturity stages of forages

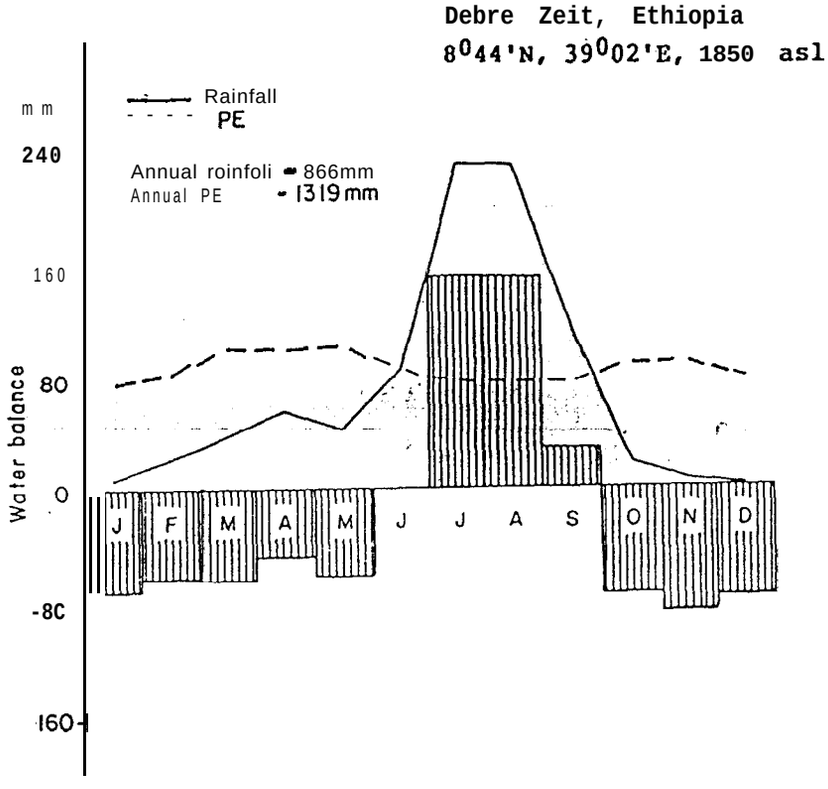


Figure 1: Rainfall, pan evapotranspiration (PE) and water budget at Debre Zeit for five years.

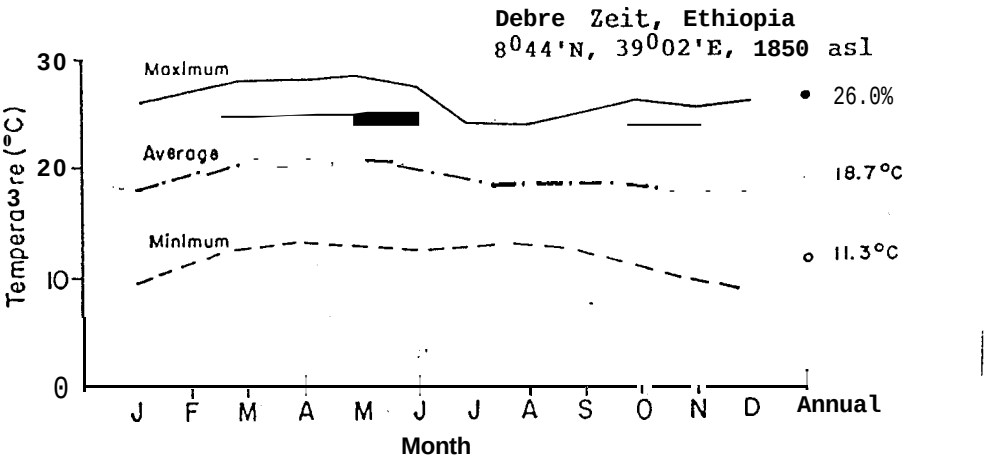


Figure 2: Maximum, minimum and average temperatures at Debre Zeit.

and crops, vertisol stored 57% more moisture than alfisol and remained moist throughout the year below 50 cm depth (Kamara and Haque, 1988). Some of the physico-chemical properties of experimental soil are given in Table 1.

Agronomy

A total of five treatments were tested along with a «fallow» and the non-nodulating reference (oats) treatments (Table 2). A randomized complete block design with four replications was adopted. The plot size was 6 x 4.50 m and there were 2 and 1 m paths between replications and plots respectively. All the legume treatments as well as non-nodulating standard crop received 20 kg N/ha as urea at planting except in microplots. Within each of these plots, a microplot of 2 x 1.5 m received 20 kg N/ha of 5 % ^{15}N a.e. in the form of urea solution.

All these treatments received a uniform application of 30 kg P/ha as triple superphosphate. Medics and clover were planted at 25 cm apart at a seed rate of 10 kg/ha. Vetch was planted at 50 cm apart at 25 kg/ha while oats, 25 cm apart at 100 kg/ha. The experiment was planted on July- 11, -1985 and 20 kg N/ha of 5 % ^{15}N ae. urea was applied on July 15, 1985.

Plants were harvested 98 days after planting (DAP) for dry matter estimation and 1 m^2 was sampled from each of ^{15}N labelled microplots for total N and ^{15}N analysis. The proportion of plant N derived from symbiotic N fixation was calculated according to the procedure given by Hardarson (1984). The legume residues were removed from the plots.

The following year, wheat cv. Buhe at 25 cm apart was planted on 18 June, 1986 at seeding rate of 100 Kg/ha. Two N rates (0 and 60 kg N/ha) in the form of urea were superimposed on each plot before planting. Wheat was barvested from 2 x 2.5 m at 113 DAP for dry matter and grain estimation. Composite samples of straw and grain were analysed for total N to estimate N uptake.

Table 1 : Physico-chemical properties of experimental soil

Properties	Values
Sand (%)	49
Silt (%)	19
Clay (%)	32
pH (H_2O)	6.7
Organic matter (%)	2 . 2
Nitrogen (%)	Ø.Ø5
Available P (Bray II - ppm)	7
Exch. cations (me/100 g)	
K	Ø.65
Ca	13.2Ø
Mg	9.76
Na	Ø.41

Results and discussion

Dry matter and nitrogen yield of legumes or oats

There were no significant differences among oats, vetch and medics with respect to dry matter yield 'and the legumes produced lower dry matter than oats (Table 2).

Significantly higher N yield was obtained from vetch as compared with other treatments and there were no differences in N yields among the others (Table 2). Papastylianou and Samios (1987) reported that wooly pod vetch gave higher N concentration and yield (86 kg N/ha/year for vetch vs 55 kg N/ha per year) 'than barley.

Biological nitrogen fixation

Table 2 shows the ^{15}N a&?&derived from fixation and N fixed by various legumes. ^{15}N enrichment of oats was significantly higher as compared with that of legumes. However, there was no significant differences among the legumes though lowest ^{15}N enrichment was noticed in vetch (Table 2).

Nitrogen derived from fixation varied from 73 to 78% in three legumes and no significant differences among various treatments were observed (Table 2). Total N fixed was 131, 77 and 61 kg N/ha in vetch, medics and clover respectively. Vetch significantly fixed higher N as 'Compared with clover (Table 2). Keatinge *et al* (1988) reported that vetch fixed 40 to 49 kg N/ha at Jindiress and Tel Hadya respectively in Northern Syria. They further observed that vetch fixed double the amount of N in improved management as Compared with traditional system. Higher N fixation by vetch in Ethiopian highland conditions might be due to more reliable rainfall, presence of indigenous rhizobia and higher fertility of upland soils as compared with northern Syrian'conditions.

Residual effect of legumes on wheat

Dry matter and grain yield. The residual effects of 1985 season cropping treatments on dry matter and grain yield of wheat at two N rates are given in Tables 3 and 4. The dry matter yield of wheat following vetch was significantly higher than that following oats or natural fallow. However, no significant difference in dry matter was observed -following vetch, medics and clover.

The grain yield of wheat following vetch was significantly higher than that following oats. No significant difference in wheat grain yield was observed in plots previously cropped to, the legumes. Yields in fallow and oats plots were the lowest (Table 4).

Table 2 : Dry matter yield, total N yield, percent ^{15}N atomic excess, percent N derived from fertilizer, N fertilizer yield, Ndf fixation and N fixed by forage legumes, Debre Zeit, 1985.

Treatments	Dry matter (kg/ha) non Isotopic	Dry matter (kg/ha) Isotopic	N yield	^{15}N a.e %	Ndf fert. %	N fert yield (kg/ha)	Ndf fix %	N fixed (kg/ha)
Vicia dasycarpa	4531b*	4900a*	169a*	0.099b*	1.852b*	3.015b*	77.93a*	131a*
Medicago polymorpha	4163b	3900ab	106b	0.123b	2.306b	2.425b	72.73a	77ab
Trifolium'steudneri	2150c	2625b	78b	0.102b	1.903b	1.475b	77.93a	61b
Avena sativa (oats)	5813a	5325a	73b	0.471a	8.827a	6.300a	-	-

*Values withincolumns followed by the same letter are not significantly different at 5% level according to Duncan's Multiple Range Test.

Table 3 : Effect of previous cropping and nitrogen application on dry matter yield of wheat, Debre Zeit, 1986

Previous cropping 1985	kg N/ha	
	6	6=
	kg/ha	
Vicia dasycarpa	4016a*	4185a*
Medicago polymorpha	3443ab	3351b
Trifolium steudneri	3543ab	4456a
Fallow	2746bc	3254b
Avena sativa	2321c	3070b

*Values within columns followed by the same letter are not significantly different at 5% level according to Duncan's Multiple Range Test.

Table 4 : Effect of previous cropping and nitrogen application on grain yield of wheat, Debre Zeit, 1986. .

Previous cropping 1985	kgN/ha	
	kg/ha	60
Vicia dasycarpa	2612a*	2810a*
Medicago polymorpha	2531a	2280bc
Trifolium steudneri	2600a	2661ab
Fallow	2090ab	2046c
Avena sativa	1753b	2119c

*Values within columns by the same letter are not significantly different at 5% level according to Duncan's Multiple Range Test.

Nitrogen yield of wheat straw and graln. In this investigation, we wish to quantify the residual benefit in N derived from legume crops. It can be seen from Fig. 3 that between 1 and 8 kg/ha of additional N are taken up by wheat straw following legumes aud fallow compared with those following oats. Preceding vetch crop resulted iu 53% higher N uptake by wheat as compared with control.

It is clear from Fig. 3 that between 11 and 23 kg/ha of additional N are taken up by wheat grain following legumesand fallow compared with following oats. Vetch produced 58% higher N uptake as compared with control. Nnadi and Haque (1988b) reported that vetch species could contribute .a considerable amount of N to following crops even when tops are not returned to the soil. Mineralization of root N occured in vetch species where root N content exceeded 2%.

Conclusion

The results of the present study could be of importance where farmers cannot afford to apply fertilizers which may often be unavailable, The choice as to which is the most beneficial forage to be grown should depend on feed production, nutritive value for animals, N economy and rotational value for following crops.

Recommendation for cereals in Ethiopian highlands is 18 kg N/ha (EPID, 1975a). However, trials with cereals on farmers' fields and research stations show up to 80% increase in yield over current recommendations with application of 46 kg N/ha (Christensen, 1973 ; EPID, 1975b). The amount of N fixed and Contributed by vetch used in this study could raise substantially the yield of cereal crops and livestockproduction on upland soils ill Ethiopian highlands.

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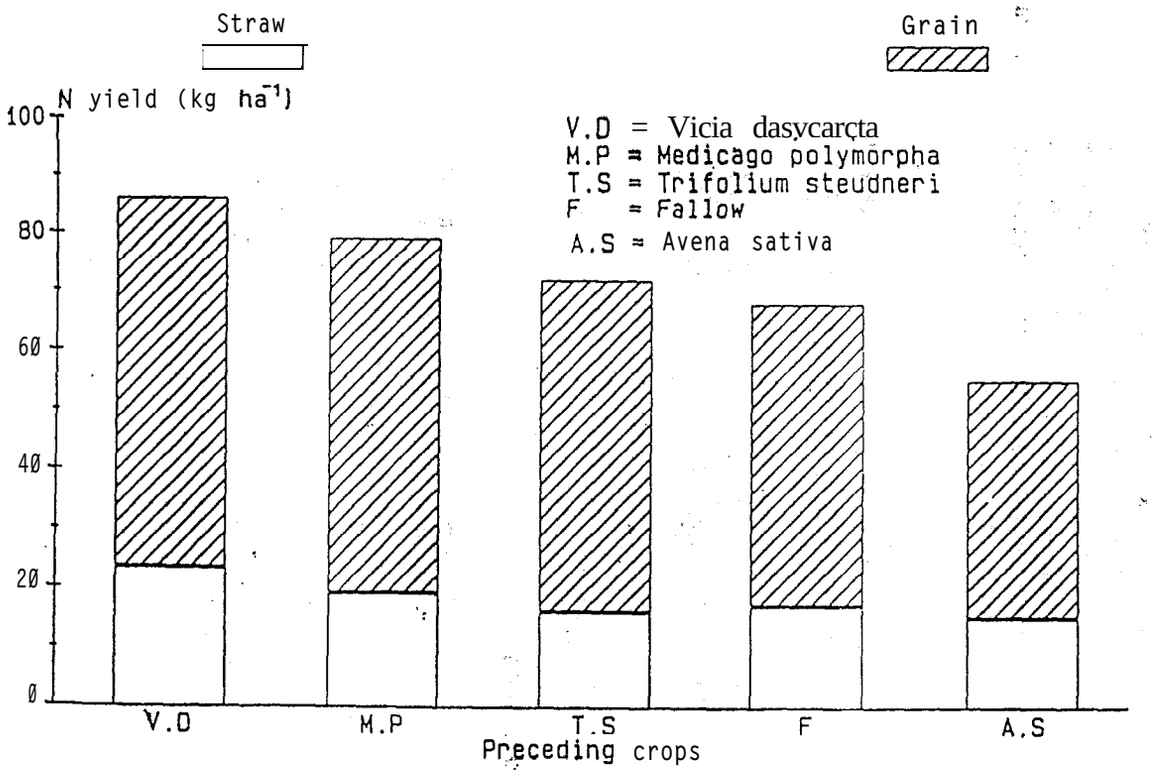


Figure 3: Effect of preceding crops on nitrogen yield of wheat straw end grain in unfertilized plots, Debre Zeit, 1986.

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FIXATION. BIOLOGIQUE DE L'AZOTE CHEZ LES ARBRES

Effect of the addition of phosphorus and *Rhizobium* inoculum on the nodulation and the growth of two species of gum arabic *trees Acacia senegal* L. Willd and *Acacia laeta* R. Br. ex Benth

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Abstract

Four treatments were applied to seedlings of *Acacia senegal* and *Acacia laeta* grown in containers filled with sandy soil poor in available phosphorus (11 ppm.). Compared to the control, *Rhizobium* inoculum treatment *induced* an increase, not always statistically significant, of the measured parameters of growth and nodulation. The foliar and nodule biomasses of *Acacia Senegal were* increased upto 132% and 283% respectively, and upto 21% and 66% for *Acacia laeta.*

Rhizobium inoculum and addition of 30 or 60 ppm P enhanced very significantly, all the parameters of growth and nodulation of the two gum arabic species. In the ease of *Acacia senegal,* the *addition* of phosphorus increased the growth parameters from 50% to 200%, the foliar biomass about 400%, the nodulation parameters from 1100% to 1400%. In comparison, for *Acacia laeta,* the addition of phosphorus increased the growth parameters from 40% to 131%. the foliar biomass about 150% and the nodulation parameters from 250% to 350%. However, there was no difference between these both treatments. Addition of 30 ppm P per plant seems to be sufficient to assure optimal response.

Further research is planned to determine the complete response curve of both trees to phosphorus in the presence or absence of *Rhizobium.*

Introduction

Among the roughly one thousand acacia species *(New,* 1984), *Acacia senegal* was used, like many other woody leguminous trees, to reforestation in the Sahelian zone. (Badji et *al.,* 1989).

The Sahalian ecosystem has been severely degraded due to the combined effects of an extended period of drought and the excessive exploitation of natural resources by man and *his* livestock. *The* natural population of *Acacia senegal has* decreased dramatically. This species is an ecologically beneficial tree because of its important role in the reconstitution of the 'vegetative cover of the zone.

Acacia senegal is able to fix atmospheric nitrogen through rhizobial nodulation (Basak and Goyal, 1980 ; Dreyfus and Dommergues, 1981), and hence may contribute to the improvement-of the soil. Found naturally throughout the semi-arid Sahel, this species seems to be well adapted to the edaphic and climatic conditions of the region (Giffard, 1975). The tree has many useful products for local populations, such as aerial fodder, firewood, wood for tools and construction. Moreover, the tree exudes gum arabic, a product of considerable economic value.

It may be possiile *to* introdue the *Acacia laeta in Senegal,* onother gum arabic producing species commonly found in the Sudan ; the major gum arabic exporting countries.

Because a positive effect of phosphorus on *Acacia holosericea* and *Acacia raddiana* has been shown earlier in Senegal (Comet and Diem, 1982 ; Cornet *et* al. 1982). the aim of this study is to determine the influence of the addition of phosphorus with *Rhizobium* inoculum on *the* nodulation and the growth of these two acacia species grown on sandy phosphorus poor soils found in the Sahel.

Materials and methods

The seeds of *Acacia senegal* belonged to the «light grey» phenotype (Dione, M. pers. comm). As with the seeds of *Acacia laeta, they were furnished* by the «Direction des Recherches sur les Productions Forestières» (DRPF) of the «Institut Senegalais de Recherches Agricoles» (ISRA). The seeds were putted in concentrated sulphuric acid for 14 mn, rinsed thoroughly in sterile water, pre-germinated in sterile vermiculite, and afterwards transferred into individual containers filled with'soil sampled from sand dunes located near Kebemer. This soil has a sandy texture (94,3% sand) and a low available phosphorus level (11 ppm,). Each container was filled with a 1.5 kg mixture of soil and polystyrene beads (2:1, v/v).

The factorial experiment, with randomization within the blocks, had three replicates and three controlled factors. The first factor *is* species *with two levels : the Acacia senegal and the Acacia laeta. The* second is soil sterilization, also divided into two levels : non-sterilized soil and autoclaved soil (120°C, 60 mn). The third control factor is fertilization with four levels : 1) no inoculation ; 2) rhizobial inoculation ; 3) rhizobial inoculation plus 30 ppm of phosphorus ; 4) rhizobial inoculation plus 60 ppm of phosphorus. Phosphorus is supplied as KH_2PO_4 in single application. Each elementary plot contained fours plants. The mean values for each plot was used in the statistical analysis.

At the *transfer to the greenhouse, the seedlings* were *inoculated with 2* ml of *Rhizobium* suspension (10^9 cells/ml). A fast growing strain (ORS 1007) selected previously (Badji *et al.,* 1988) was employed. It was isolated from nodules of *Acacia laeta and* it is effective on the two acacia species. The cross nodulation was demonstrated (Badji et *al.* 1988).

Twenty five weeks after planting (WAP) nodulation and growth were recorded using the following parameters : diameter at the collar (A); height of the main stem (B); total length of branches (C); number of shoot nodes (D); dry biomass of the leaves (E); number (F) and dry weight (G) of the nodules.

Results and discussion

Species effect

Table, 1 shows that during the first months of growth and development, *Acacia laeta* and *Acacia senegal* presented some significant differences with regards to the parameters A, B, C, E, and F. One non significant increase occured for number of shoot nodes (D) and dry weight of nodules (G).

Table 1 : Differences in growth and nodulation between 25 weeek old seedlings of Acacia Senegal and Acacia Laeta

	Measured Parameters*						
	Growth					Nodulation	
SPECIES	A	B	C	D	E.	F	G
Acacia Senegal (AS)	4.85 b	24.50 b	40.90 b	-35.38 -	1558 b	14.06 b	307 -
Acacia Senegal	5.61 a	35.85 a	63.25 a	38.17 -	2356 a	17.09 a	309
(AS-AL) 100 / AS	+16	+46	+55	+ 8	+51	+22	+1

* per plant, A = collar diameter in mm ; B = height of plant in cm ; C = total length of stem and branches in cm ; D = number of shoot nodes ; E = dry weight of leaves in mg ; F = number of nodules ; G = dry weight of nodules in mg. For each parameter data in each column without letter or followed by the same letter are not significantly different (P = 0.05 - Newman Keuls test).

Soil sterilization effect

For both species and for all growth parameters (A to E), the values were higher on non-sterilized soil than on sterilized soil (Table 2). However, these differences are not significant for the collar diameter (A) and the height (B). They are significant for total length of stem and branches (C) and, in the case of *Acacia laeta*, for the number of shoot nodes (D) and the dry foliar biomass (E). The differences were not significant for the number of nodules (F) and for the nodule dry weight (G). To conclude, the effect of soil sterilization was rather depressive but not significant (Newman and Keuls test, p = 0.05). Soil sterilization lolled the native rhizobium and the endomycorhizal propagules; this might explain the 'depressive effect.

Fertilization effect

The statistid analysis showed that generally, the effects of the fertilizing treatments were very significant (Table 3) on the growth parameters (A to E) and the nodulation parameters (F and G.). *Acacia senegal* responded better than *Acacia laeta. There was* no interaction between soil sterilization and fertilizing treatments.

Inoculation with rhizobium, in comparison with the control induced an increase of both growth and nodulation parameters with the exception of parameter B for *Acacia laeta.* However, these increases were not always significant. The significance threshold *was* reached *only* for *the* parameters *A,* F and *G* by *Acacia laeta* and for B, C, D, and E by *Acacia senegal.* Rhizobial inoculation plus 30 ppm or 60 ppm of phosphorus induced important increases for all the parameters. The values reached were significantly different from those of the other treatments. No significant differences occurred between the phosphorus treatments for both acacia species. One may suppose that in this case, the economically optimum level for the application of phosphorus is 30 ppm.

Table 4 shows the percentage of increase of all parameters for the treatments 2, 3, and 4 in comparison with the control treatment. They were low for treatment 2, they *ranged* from *20%* to 132% for the *growth* parameter for *Acacia senegal* and no more than 21% for *Acacia laeta.* For the number and dry weight of nodules, the increase ranged respectively from 190% to 283% and 66% to 142%.

For treatments 3 and 4, with phosphorus supplements to the rhizobial inoculation, these increases were higher ; for the growth parameters (A to D) of *Acacia senegal they* ranged from 50% to 200% and 35% to 131% for *Acacia Zueta. The* gain for the dry foliar biomass *was 506%* for *Acacia senegal* and 177% for *Acacia Zueta.* For the number (F) and dry weight (G) of nodules, the mean increase was approximately 1250% for *Acacia senegal* and 300% for the others species. Given what is known about the role of phosphorus in the structure of essential molecules, in the energy process, and in the metabolism of the plant and recent new information (Bielesky, 1973 ; Heldt *et al.,* 1977; Preiss, 1982 ; Mengel, 1984), this effect of phosphorus is not surprising. On the phosphorus deficient *soil* from Kebemer, P fertilization combined *with* by *Rhizobium* inoculation had a positive effect on the growth of both gum arabic trees and probably on N_2 fixation. Indeed the phosphorus treatments largely increased the nodulation. As nodule formation and nitrogen fixation require energy and thus ATP, one might assume that the increase of the available phosphorus level in the deficient soils induces a better functioning of the rhizobial symbiosis.

Table 2 : Effect of soil sterilization on growth and nodulation parameters of Acacia Senegal and Acacia laeta

Species (factor 1)	Soil sterilization (factor 2)	Measured Parameters *						
		Growth					Nodulation	
		A	B	C	D	E	F	G
Acacia Senegal	a. sterilized soil	4.69	24.10	37.86b	33.50	1462	14.32	281
	b. non sterilized soil	5.01	24.99.	43.93a	37.27	1654	13.80	328
	(a - b) . 100 / a	+11	to4	+16	+11	+13	- 0	4t17
Acacia laeta	a. sterilized soil	5.56	35.13	59.13b	34.66b	1958b	18.64	304
	b. non sterilized soil	5.66	36.56	67.36a	41.69a	2755a	15.53	307
	(a - b) . 100 / a	+02	to4	+14	+20	+41	-17	+01

* As in Table 1 ; each data for each species and in each column without letter or with the same letter are not significantly different (P = 0,05 - Newman Keuls test).

Table 3 : Effect of fertilizing treatments on the growth and nodulation of Acacia-Senegal and Acacia Laeta

Species	Fertilizing treatments	Measured Parameters*						
		A	B	C	D	E	F	G
A. senegal	No inoculation	3.63b	17.00c	19.50c	16.83c	423c	1.67b	39.6b
	Rhizobial inoculation	4.35b	22.97b	30.93b	25.87b	981b	4.85b	151.5b
	Rhizobial inoculation + 30 ppm P	5.61a	28.55a	56.93a	50.90a	2232a	25.85a	475.5a
	Rhizobial inoculation + 60 ppm P	5.73a	29.07a	55.28a	47.27a	2566a	23.43a	552.4a
A. laeta	No inoculation	4.52c	30.17b	40.17b	23.17c	1310c	5.50c	117.4c
	Rhizobial inoculation	5.14b	29.35b	47.13b	28.17c	1580c	13.32b	195.4b
	Rhizobial inoculation + 30 ppm P	6.34a	42.75a	79.40a	47.38b	2840b	26.82a	462.6a
	Rhizobial inoculation + 60 ppm P	6.35a	40.85a	85.42a	53.43a	3631a	22.37a	444.9a

* As in Table 1 ; for each species, in each column, data follow by the same letter are not significantly different (P = 0,05 - Newman Keuls test).

Table 4 : Variations in percentage for each parameter due to fertilizing treatments (2, 3, 4) versus control (1)

Species	Fertilizing treatments**	Measured Parameters*						
		A	B	C	D	E	F	G
A. senegal	2	20	35	59	54	132	190	283
	3	55	68	192	202	428	1447	1100
	4	58	71	183	181	506	1302	1294
A. laeta	2	14	-3	17	22	21	142	66
	3	40	42	98	104	117	388	294
	4	40	35	113	131	177	307	279

* Parameters A to G as in Table 1

**Fertilizing treatments as in Table 3.

Further investigations are planned to determine the influence of lower phosphorus levels on the growth of acacia in the presence or absence of rhizobii inoculation. Such experiments should help to solve the problem of costs and benefits of phosphorus application during afforestation programmes.

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Nodulation survey of leguminous and non leguminous trees growing at ICRAF's field station

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Abstract

A survey of nodulation was made on 49 leguminous and non-leguminous trees representing *Mimosoideae, Simaroubaceae, Casuarinaceae, Caesalpinoideae* and *Papilionoideae* families. This was done in order to identify those species which are self nodulating under ICRAF's field station conditions. Nodulation was observed on 24 species. Only 9 out of the 24 nodulated species were inoculated at the time of planting. Some of the observed species had been reported to nodulate in literature. The nodule shapes varied between species ranging from globose to coralloid. Successful nodulation in these plants indicates the presence of potential autochthonous rhizobial strains in the soil at this site.

Introduction

Substained productivity, of land is very much dependent on nitrogen input to the soil from both biological and non-biological sources. Legumes have been used in building and conserving soil fertility since the beginning of agriculture because of their ability to fix nitrogen from the atmosphere. It is estimated that this symbiotic process between bacteria and host plant accounts for approximately 20% of the total nitrogen fixed annually in the world (Dazzo and Hubbel 1974).

Most legumes grow as weeds (shrubs and trees), thus very little knowledge in the extent of nodulation is known. The presence of nodules is the preliminary and visual confirmation of nitrogen fixation in legumes. Nodulation among legumes is affected by

several factors among which include soil temperature (Santo and Dobereiner, 1970 ; Habiih, 1970 ; Gibson, 1977), soil moisture (Habish, 1970), absence or presence of excess minerals (Jutse and Haque, 1984). *All these* factors and *the* presence of the right *Rhizobium* affect the rate and position of nodulation.

About 49 trees (Table 1) grow at ICRAF's field station (some indigenous and introduced). Most of these species are likely to be used in agroforestry interventions which focus on fertility restoration. Up to date, no quantitative or qualitative information on the nodulating ability of these species has been given under the sub-humid, moderately acidic condition of Machakos field station, the center of ICRAF's field trials.

This paper reports the findings of the nodulating survey of some leguminous (47) and non-leguminous (2) trees being grown 'at the field station in a sub-humid and semi-arid environment.

Materials and methods

The study site lies between 1°33"S latitude and 37°15"E longitude at an altitude of 1560m in the sub-humid to semi-arid zone with bimodal annual rainfall of about 700mm. Mean annual air temperature of 22°C, with deep soil profile, well drained, moderately leached, weakly acid soils with pH rate 6.0-6.5 and medium nutrient levels. Soils generally classified as alfisols.

Forty nine plants were examined for the presence of nodules. These plants were leguminous and non-leguminous woody shrubs and trees Some of these 49 species have been reported to nodulate in various reports (Table 1) (Corby, 1974 ; Allen and Allen 1981). The species examined were both indigenous as well as introduced exotics (Table 2).

Periodic sampling for nodules was carried out during the wet active growing season (and'dry season) of the trees/shrubs. This was done on diverse dates during April and May in 1986, 1987 and 1988 after digging up a hole near the tree trunk to expose the root system. The excavated roots were washed under running tap water and the root systems were floated in trays of water to facilitate detection of nodules. The presence or absence and degree of nodulation on the root systems were noted. The morphology of mature nodules was described and their sizes (diameter) measured in mm. Special care was taken to distinguish root nodules from root malformations, such as those caused by nematodes, insects or other root inhabiting pathogenic microorganisms, by disecting the nodules to establish whether they are red, brown or green in internal colour.

Results

Observations on nodulation are presented in Table 2, which also lists whether the plants are indigenous or introduced species The recorded presence of root nodules on the examined species was checked against published lists of Allen and Allen (1961, 1981), Corby (1974), Lim (1979) and Mohammad and Mahmoud (1985). Table 2 also presents species which nodulated during the three seasons of sampling from May 1986 to May 1988. Nodulation ocurred *in 24* species, represented by *: Mimosoideae, Caesalpinoideae, Papilionoideae,* and non-legumes representing *Simaroubaceae* and *Casuarinaceae.* Majority of the species were moderately to heavily nodulated, some were very heavily nodulated, specially *Gliricidia sepium, Leucaena leucocephala, Samanea saman, Sesbania sesban,*

Table 1 :List of Nitrogen fixing trees growing at ICRAF's Field Station Machakos - Kenya

Species	Nodulation	
	Present Study	Previous Record (in literature1
Acacia albida		Y
Acacia deamii		Y
Acacia farnesiana		Y
Acacia holosericea		NI
Acacia mangium	-	Y
Acacia mellifera		Y
Acacia paniculata		NI
Acacia pennatula		Y
Acacia stuhlmanii	t	Y
Acacia saligna	t	Y
Acacia tortilis	+	Y
Acacia victoriae		Y
Acacia xanthoplea		Y
Albizzia longepedata		Y
**Alvarodoa amorphoides	+	Y
Ateleia herbert-smithii		NI
Caesalpinia velutina	+	NI
Caesalpinia coriaria	-	NI
Caesalpinia eriostachys	t	NI
Crescentia olata		NI
Cajanus cajan	+	Y
Calliandra calothyrsus	+	Y
Cassia alata		B
Cassia siamea		Y
**Casuarina equisetifolia	+	Y
Cordeauxia edulis	-	Y
Enterolobium cyclocarpmum	+	Y
Erythrina abyssinica	+	Y
Gliricidia sepium	+	Y
Haematoxylon brasilleto	-	B
Leucaena diversifolia	+	Y
Leucaena shannoni	+	Y
Leucaena leucocephala (Cunningham).	+	Y
Leucaena leucocephala (Peru)	+	Y
Leucaena leucocephala (Hawaiian giant - K8)	+	Y
Mimosa tenuiflora	+	Y
Myrospermum frutescens	+	Y
Parkinsonia aculeata	-	B
Pithecellobium dulce	+	Y
Prosopis juliflora	+	Y
Prosopis nigra	-	Y

Table 1 : (continued)

Species	Nodulation	
	Present Study	Previous Record (in literature)
Prosopis **pallida**		Y
Prosopis alba		Y
Prosopis chilensis		**Y**
Samanea **saman**	**+**	**Y**
Sesbaniamacrantha	+	Y
Sesbania grandiflora	+	Y
Sesbania sesban	+	**Y**
Senna atomeria		NI

** Non-leguminous
+ Presence of nodules
- absence of nodules
Y Nodulation previously observed in other studies
B Species previously investigated but nodulation never observed
NI No information available.

Table 2: Nodule morphology, degree of nodulation, average nodule diameter and standard errors of legume species

Species	Species	Family	Nodule morphology	Inoculation status	Degree of nodulation	Number of nodules sampled	Average nodule diameter (mm)	Standard error of nodule diameter (mm)	Remarks
	Acacia saligna	Mimosoideae	Fan shaped	-	+	-	-	-	Introduced
	Acacia tortilis	Mimosoideae	Fan shaped	-	++	-	-	-	Native
	Acacia Stuhlmanii	Mimosoideae	Fan shaped	-	++	-	-	-	Native
*	Alvarodoa amorphoides	Simaroubaceae	Coralloid	+	+++	30	2.7	0.9	Introduced
*	Casuarina equisetifolia	Casuarinaceae	Clusters (Coralloid)	-	+++	-	-	-	Introduced
	Caesalpinia velutina	Caesalpiniodeae	Globose	+	+	-	-	-	Introduced
	Caesalpinia eriostachys	Caesalpiniodeae	Globose	+	+	-	-	-	Introduced
	Calliandra calothrysus	Mimosoideae	Elongate lobed	-	+++	-	-	-	Introduced
	Erythrina abyssinica	Papilionoideae	Globose	-	+++	-	-	-	Introduced
	Enterolobium cyclocarpmum	Mimosoideae	Globose	+	+++	14	2.2	0.6	Introduced
	Gliricidia sepium	Palilionoideae	Fan shaped	-	++++	10	2.5	0.4	Introduced
	Leucaena diversifolia	Mimosoideae	Bifurcate	+	+	-	-	-	Introduced
	Leucaena shannoni	Mimosoideae	Bifurcate	+	+	-	-	-	Introduced
**	Leucaena leucocephala (Peru)	Mimosoideae	Bifurcate	-	+	14	1.7	0.7	Introduced
**	Leucaena leucocephala (Hawaiian giant K.8)	Mimosoideae	Bifurcate	-	+	10	2.1	0.8	Introduced
**	Leucaena leucocephala (Cunningham)	Mimosoideae	Bifurcate	-	+	10	1.9	0.4	Introduced
	Mimosa tenuiflora	Mimosoideae	Elongate and lobed	+	+	10	4	0.5	Introduced
	Myrospermum frutescens	Papilionoideae	Globose	+	++	-	-	-	Introduced
	Pithecellobium dulce	Mimosoideae	Bifurcate	+	+++	10	3.5	0.9	Introduced
	Prosopis juliflora	Mimosoideae	Globose	-	+	-	-	-	Introduced
	Samanea saman	Mimosoideae	Globose	-	++++	28	2.4	0.5	Introduced
	Sesbania sesban	Papilionoideae	Globose	-	++++	15	2.2	0.9	Native
	Sesbania macrantha	Papilionoideae	Globose	-	++++	14	3.7	0.6	Introduced
	Sesbania grandiflora	Papilionoideae	Globose	-	++++	10	4	0.7	Introduced

Descriptions of nodule morphology are taken from the drawings of Lim, G. and Ng, H.L. (1977).

Only mature nodules sampled ; all other species had nodules which were very young
Note sampling for nodulation done April/May 1986, 1988.
Diameter measurements done on samples which nodulated in May 1988.

* Indicates sparse nodulation
** Indicates moderate nodulation
*** Indicates heavy nodulation
**** Indicates very heavy nodulation

\- Not innoculated
\+ Innoculated

* Non legume
** Provenances

Sesbania macrantha and *Sesbania grandiflora*. Heavy nodulation ocurred in species *Alvarodoa amorphides*, *Casuarina equisetifolia*, *Calliandra Calothrysus*, *Erythrina abyssinica*, *Enterolobium cyclocarpomum* and *Pithecelobium dulce*. Sparse to moderate nodulation occurred in species *of Acacia saligna*, *Acacia tortilis*, *Acacia stuhlmanii*, *Prosopis juliflora*, *Caesalpinia velutina*, *Caesalpinia eriostachys*, *Leucaena diversifolia*, *Leucaena shannoni*, *provenanees of Leucaena leucocephala* (Hawaiian giant *K.8)* and *Cunningham* and *Mimosa tenuiflora* and *Myrospermum frutescens*.

Most of the nodules were located on the upper parts of central tap root systems in clusters as well as on lateral roots (Fig.1). Most of the young nodules were round, white and smooth-surf&xl while older nodules were of various shapes, mostly brown in internal colour and rough-surfaced. Nodule shapes were classified into different types as described by the drawings of Lim and Ng (1977). The nodule shapes in *Mimosoideae* were fan shaped, elongated, lobed, globose and bifurcate. Carolloid types were found in *Simaroubaceae* and *Casuarinaceae*. *In Caesalpinoideae, the* nodules were globose. and *in Papilionoideae the* nodule shapes were globose and fan-shaped (Table 2). Fig. 2 shows globose *shaped* nodules for *Sesbania sesban* occuring in clusters.

Table 2 also shows average diameters for mature nodules. The nodule diameters *are* very variable, ranging from 1.7 mm *in Leucaena leucocephala* (Peru) *to* large diameters of 4 mm in *Mimosa tenuiflora* and *Sesbania grandiflora*.



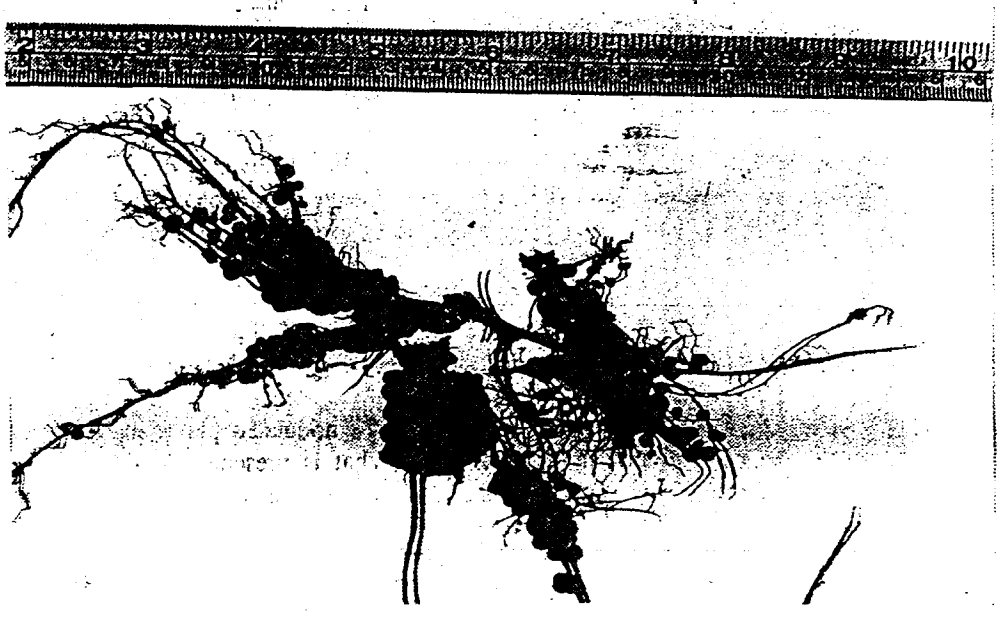
Sesbania macrantha and *Sesbania grandiflora*. Heavy nodulation ocurred in species *Alvarodoa amorphides, Casuarina equisetifolia, Calliandra Calothrysus, Erythrina abyssinica, Enterolobium cyclocarpomum* and *Pithecelobium dulce*. Sparse to moderate nodulation *occurred in* species of *Acacia saligna, Acacia tortilis, Acacia stuhlmanii, Prosopis juliflora, Caesalpinia velutina, Caesalpinia eriostachys, Leucaena diversifolia, Leucaena shannoni, provenances* of *Leucaena leucocephala (Hawaiian* giant K.8) and *Cunningham* and *Mimosa tenuiflora* and *Myrospermum frutescens.*

Most of the nodules were located on the upper parts of central tap root systems in clusters as well as on lateral roots (Fig.l). Most of the young nodules were round, white and smooth-surfaced while older nodules were of various shapes, mostly brown in internal colour and rough-surfaced. Nodule shapes were classified into different types as described by the drawings of Lim and Ng (1977). The nodule shapes in *Mimosoideae* were fan shaped, elongated, lobed, globose and bifurcate. Carolloid types were found *in Simaroubaceae* and *Casuarinaceae. In Caesalpinoideae, the* nodules were globose, and *in Papilionoideae* the nodule shapes were globose and fan-shaped (Table 2). Fig. 2 shows globose *shaped* nodules for *Sesbania sesban* occuring in clusters.

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Figure 1 : Exposed root system of *Sesbania bispinosa* showing nodules confined to the upper parts of the tap root and in laterial roots.

Figure 2 : *Sesbania sesban globose* shaped nodules in clusters



Discussion

In this survey, 24 out of the 49 species were recorded for presence of nodules. However, the absence of nodules on other species is not a conclusive evidence for lack of nodulating ability. The findings of this survey are in broad-agreement with the summary of the world-index of nodulation by Allen and Allen (1961). However, some of these species which were not observed to nodulate in this study have been reported elsewhere to be nodulating (Table 1). Successful nodulation in these plants indicates the presence of potential infective autochthonous rhizobial strains in the soil. The degree of nodulation was sparse in some species ; thii can be attributed to the fact that most of these species were not inoculated at the time of planting (Table 2). Those nodulating species somehow formed a symbiotic relationship with the indigenous rhizobia in the soil. Conclusive evidence cannot be drawn on whether there is correlation between those introduced and indigenous species in nodulation. There was a general absence of nodules on these trees/shrubs during the dry season. This confirms the inability of species to nodulate under extreme or adverse environmental conditions. Nodules were generally active during the wet seasons when there was a general active vegetative and reproductive growth in these species. However, the nodules during the dry season were generally inactive. Nodulated legumes and non-legumes can contribute significantly to the nitrogen status of the soil. In order to exploit the potential of fixed nitrogen, it is necessary to successfully identify nitrogen fixing nodulated legumes and non-legumes.

In this study, the non-legumes *Alvarodoa amorphoides* and *Casuarina equisetifolia* nodulated prolifically at this site, thus indicating a great potential of non-leguminous plants with nodules possessing nitrogen fixing capacity. In future, more and more nitrogen-fixing tree species with multiple benefits will be used in farming systems . So, more information is required on the agronomic and agricultural aspects of nodulated plants to enable the symbiotic system to be exploited to its maximum. What is presented in this paper is an account of the potential contribution of these nodulated plants to the nitrogen cycle in the biosphere.

The study shows prolific nodulation which varies among species. The next stage is to select the most prolific nodulating species for quantitative nitrogen fixation studies to be carried out at different periods of the year.

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Effect of inoculation- with *Rhizobium*, P application and liming on early growth of leucaena (*Leucaena leucocephala* Lam. de wit).

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Abstract

The effects of P application (0 and 50 mg kg' soil), inoculation with soil from a field with well nodulated plants of *Leucaena leucocephala*, and with a water suspension of a *Rhizobium strain* IRC 1045 specific for leueaena, on establishment of leueaena were studied in four soils, two Psammentic Ustorthents, one Oxic Paleustalf and one Typic Paleudult. Application of P resulted in improved leueaena growth and nodulation in all four soils. The sharpest response was obtained in the Psammentic Ustorthent from a farm where leucaena established Poorly. Soil inoculation with rhizobia resulted in improved leueaena growth increased dry matter yield and nodulation except on the Oxic Paleustalf from the International Institute of Tropical Agriculture (IITA) on which leueaena normally performs well. The soil inoculant performed better than the water suspension of strain IRC 1045. Dicalcium phosphate affected the early growth of leueaena on the Typic Paleudult because of its liming properties.

Introduction

Alley cropping is an agroforestry system in which food crops are grown in alleys formed by hedgerows of suitable trees or shrubs which can be pruned periodically to prevent excessive shading and which provide mulch and green manure for companion food crops. Leucaena (*Leucaena leucocephala var.* K *28)* appears to be a suitable hedgerow tree for alley cropping on Alfisols and Inceptisols of southwestern Nigeria (Kang and Wilson, 1987). The problems of establishment and of slow initial growth of leucaena

remain however a major limitation for adaptation alley cropping by farmers in some areas, particularly when the direct seeding method is used. The poor establishment experienced in farmers' fields has been attributed to competition with companion crops or weeds (Jones and Bray, 1983 ; Atta-Krah and Kolawole, 1987), to lack of effective strains of *Rhizobium* for nitrogen fixation (Ruaysoongnern *et al.,* 1984 ; Sanginga et *al.,* 1988) to P deficiency (Sanginga et *al.,* 1988) and to acidity in highly weathered soils (Hutton and Andrew, 1978 ; Olverra et *al.,* 1982).

The purpose of this study was to evaluate some of the soil factors that are responsible for the poor establishment of leucaena in southern Nigeria. The effect of lime and dicalcium phosphate on leucaena early growth in an acid soil was also compared

Materials and methods

A greenhouse pot experiment was conducted at the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria. The experimental design was a split-plot with four soils, two from on-farm sites near Alabata village, approximately 15 km Northwest of Ibadan (Psammentic Ustorthent), one from the IITA main station (Oxic Paleustalf) Ibadan, southwestern Nigeria, and one from the IITA high rainfall station (Typic Paleudult) at Onne, southeastern Nigeria, as main plot treatments. Leucaena establishment was relatively good at one on-farm site (farm-l) and poor at the other site (farm-2). The soil from IITA station usually supports good leucaena growth whereas leucaena performance in the acid soil is normally very poor. Chemical properties of these soils are shown in Table 1. The subplot treatments were two rates of dicalcium phosphate (0 and 50 mg P kg^{-1} soil) and three inoculation treatments (uninoculated control, inoculation with a water

Table 1 : Selected chemical properties of the soils used for the pot experiment

Soil	pH	Organic C	Total N	Bray-1 P	Ca	Mg	K	Total acidity
		%	% kg	mg^{-1}	------	meq/100g----------		
IITA (Oxic Paleustalf)	6.3	1.49	Ø.121	11.2	0.74	Ø.32	Ø.29	Ø.16
Farm-1 (Psammentic Ustorthent)	5.8	Ø.65	Ø.Ø52	7.2	Ø.53	Ø.17	Ø.3Ø	Ø.Ø5
Farm-2 (Psammentic Ustorthent)	5.6	Ø.51	Ø.Ø52	2.2	Ø.52	Ø.15	Ø.24	Ø.ØØ
Onne (Typic Paleudult)	4.9	1.14	Ø.Ø89	62.2.	Ø.73	Ø.25	Ø.3Ø	Ø.36

suspension of *Rhizobium strain* IRC 1045 and inoculation with a soil from a field with *well* nodulated leucaena) *in* a *factorial arrangement, Rhiwbium strain* IRC *1045 is an* elite strain isolated from nodules of leucaena grown on an Alfisol in southwestern Nigeria (Sanginga *et al.*, 1988). Ten milliliters of the water suspension of this strain (10^9 cells ml^{-1}) or 150 g of the' «soil inoculant» (10' leucaena rhizobia g^{-1} soil) were mixed with the surface soil in the pots. The final weight of soil in each pot was 3 kg.

A second pot experiment was carried out to study the effect of lime ($CaCO_3$) and dicalcium phosphate fertilizer on early growth of leucaena in the acid soil. The treatments were 0 and 2000 mg $CaCO_3$ kg^{-1} soil, and 0 and 50 mg P kg^{-1} soil in a factorial arrangement.

Six seeds of leucaena (Leucaena leucocephala var. K-28) previously scarified in sulfuric acid were sown in each pot Seedlings were thinned to three after one week. The pots were uniformly watered with 100 ml of deionised water daily for the first 6 weeks and twice daily afterwards. Bi-weekly height measurements were made until 14 weeks after planting (WAP). At harvest (14 WAP), above-ground dry matter was determined. Roots were washed in tap water over a sieve to minimize losses. Nodules were separated from the roots, counted and dried.

The Statistical Analysis Systems (SAS) package was used for statistical evaluation of variances. Least significant differences (LSD) are given only when the overall F-test is significant ($p = -0.05$).

Results and discusslon

Helght measurements

The beneficial effect of P application on leucaena growth was observed from 4 WAP and the effect remained significant for the rest of the experiment (Table 2). Application of 50 mg P kg^{-1} as dicalcium phosphate increased leucaena height by 18 - 42%. The largest effect was observed at 6 and 8 WAP.

Table 2 : Effect of P application on leucaena height at different times (Means of four soils, 3 inoculation treatments and four replications)

Phosphorus', rate	Weeks after planting						
	2	4	6	8	10	12	14
mg kg^{-1}	(cm)						
0	7	9	13	17	22	27	34
50	7	11	17	24	28	33	40
LSD(0.05)	NS	0.4	0.4	0.8	1.2	1.6	2.6

Response of leucaena to inoculation is shown in Table 3. Leucaena growth on the IITA station soil showed no reponse to inoculation except at 4 WAP. This indicates that effective rhiiia capable of infecting leucaena were not limiting at this site. In contrast, growth on other soils showed variable response to iuoculation. The two on-farm showed a delayed response to inoculation compared to 'the Onne soil but the initial growth of leucaena in the latter soil which is acidic was much slower than in the other soils. The soil inoculant *perform* better *than Rhizobizun* strain IRc 1045 on all soil types. Soil inoculauts are also known to supply other useful microorganisms like endomycorrhizal fungi and some soil nutrients.

The better response to soil inoculant can explain the improved establishment of transplanted leucaena compared with directly seeded plants in areas where leucaeua is introduced for the first time. Soils used in nurseries are likely to be from sites with favorable nutrient aud microbiological properties.

Dry matter yield and nodulation

Above ground and root dry matter were significantly enhanced by P application with significant interactions between soil type and P in root dry matter yield (Table 4). Without phosphorus application, leucaena growing on Farm-2 soil produced significantly less biomass compared with the other three soils. The sharp response to P by leucaena on Farm-2 soil indicates that available P was limiting leucaena growth on this soil. This was confirmed by the soil analytical results (Table 1).

Nodulation was not significantly enhanced by P application except on the acid soil. Significant interactions between soil type and inoculation were noted in dry matter yield and nodulation with the acid soil producing much sharper responses compared to the other soils (Table 5). The IITA soil did not show response to inoculation. There was a general tendency for increased nodulation with inoculation although the effects were significant only in the acid soil. Nodule counts were highly variable particularly in the sandy soils due to the difficulty of collecting the small nodules.

Effect of lime and dicalcium phosphate

In the aid soil, leucaena dry matter yields and nodulation showed significant interactions between lime and P (Table 6). Growth and nodulation were increased by the application of either lime or dicalcium phosphate. Lime produced greater increases. Extractable P is high in this soil (Table 1). With the application of lime, phosphorus produced no additional increase in native leucaena growth and nodulation. The explanation for this is that application of lime to this soil makes the native soil phosphorus more available.

Table 3 : Effect of inoculation with a soil inoculant and a water-suspension of Rhizobium strain IRc 1045 on height of leucaena on four soils at different times.

Inoculation treatment	Weeks after planting						
	2	4	6	8	10	12	14
	(cm)						
	IITA						
Control	7	12	16	22	27	33	39
Soil inoculant	7	9	15	21	27	34	39
IRc 1045	7	12	16	23	30	35	42
LSD(0.05)	NS	1.8	NS	NS	NS	NS	NS
	Farm 1						
Control	7	11	15	21	25	31	39
Soil inoculant	7	11	15	23	28	33	40
IRc 1045	7	10	14	20	24	27	34
LSD(0.05)	NS	NS	NS	NS	2.6	3.3	NS
	Farm 2						
Control	7	11	14	17	20	23	28
Soil inoculant	7	10	14	19	24	28	33
IRc 1045	7	11	14	18	22	26	30
LSD(0.05)	NS	NS	NS	1.6	2.3	3.6	NS
	Onne						
Control	5	8	13	18	22	27	35
Soil inoculant	6	9	15	23	29	34	42
IRc 1045	5	9	14	21	25	31	40
LSD(0.05)	0.5	0.8	1.4	2.1	3.1	4.5	NS

Table 4 : Effect of P application on dry matter production and nodulation of leucaena on four soils at 14 weeks after planting.

Phosphorus applied (mg kg-1)	Top weight	Root weight	Nodules number	Nodule weight
	g plant^{-1}	g plant^{-1}	No. plant^{-1}	mg plant^{-1}
		IITA		
Ø	5	5	34	1Ø7
5Ø	7	7	42	1ØØ
LSD(0.05)	Ø.5	Ø.6	NS	NS
		Farm 1		
Ø	5	5	36	14Ø
5Ø	6	6	39	11Ø
LSD(0.05)	Ø.6	Ø.7	NS	NS
		Farm 2		
Ø	3	3	28	6Ø
5Ø	5	5	39	a3
LSD(Ø.Ø5)	Ø.3	Ø.5	NS	NS
		Onne		
Ø	5	5	42	a3
5Ø	7	9	74	157
LSD(0.05)	Ø.8	1.Ø	25.Ø	6Ø.Ø

Table 5 : Effect of inoculation with a soil inoculant and a water-suspension: of Rhizobium strain IRc 1045 on leucaena dry matter yield and nodulation on four soils, at 14 weeks after planting.

Inoculation treatment	Top weight	Root weight	Nodule number	Nodule weight
	g plant^{-1}	g plant^{-1}	No: plant^{-1}	mg plant^{-1}
		IITA		
Control	6	6	32	120
Soil inoculant	6	6	42	90
IRc 1045	6	6	40	97
LSD(0.05)	NS	NS	NS	NS
		Farm 1		
Control	6	5	28	87
Soil inoculant	6	6	43	157
IRc 1045	5.	5 .	42	130
LSD(0.05)	0.7	NS	NS	5 3
		Farm 2		
Control	3	3	19	53
Soil inoculant	4	4	39	87
IRc 1045	4	4	42	73
LSD(0.05)	0.3	NS	NS	NS
		Onne		
Control	5	5	30	167
Soil inoculant	7	7	97	153
IRc 1045	5	5	50	97
LSD(0.05)	1.0	1.2	30.6	NS

Table 6 : Effects of lime and dicalcium phosphate on the dry matter yield and nodulation of Leucaena leucocephala in a pot experiment with an acid soil from Onne, at 14 weeks after planting.

P applied ($mg\ kg^{-1}$)	$CaCO_3$ applied, $mg\ kg^{-1}$ Ø	2 Ø Ø Ø	Means
		Tops, (g plant-')	
Ø	4	10	7.Ø
5Ø	6	10	8.Ø SE ± Ø.54
Means	5.Ø	10.Ø	
	SE ± Ø.54 CV 21%		SE (1 cell) = ± Ø.76
		Roots, ($g\ plant^{-1}$)	
Ø	4	7	5.5
5Ø	6	6	6.Ø SE ± 5.53
	SE ± Ø.55 CV 27 %		SE (1 cell) = Ø.78
		Nodule number ($No.plant^{-1}$)	
Ø	18	112	65.Ø SE ± 10.0
5Ø	62	97	79.5
Means	4Ø.Ø	104.5	
	SE ± 10.0 CV 39 %		SE (1 cell) = 14.2

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A preliminary study on the compatibility between indigenous rhizobia and some tree? legumes in Kenya

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Abstract

Cross inoculation study with indigenous rhizobia was carried out on seven host species of isolation : *Acacia albida, Acacia mearnsii, Calliandra calothyrsus, Leucaena leucocephala. Prosopis juliflora, Sesbania grandiflora* and *Sesbania sesban. The Rhizobium* strains *were highly infective* and effectiveness was variable across the host-strain combinations. There occured four cross-inoculation groups within the range of *Rhizobium strains and* host species studied. The two members of the genus *Sesbania* were more specific in their rhizobial partners in terms of beneficial association. Nodule shape and distribution was consistent on the root system of each host species in effective symbiosis.

Introduction

Acacia albida, Acacia mearnsii, Calliandra calothyrsus, Leucaena leucocephala, Prosopis juliflora, Sesbania grandiflora and Sesbania *sesban* are grown in Kenya to provide green manure and mulch in intercropping systems, browse or fodder for livestock and fuelwood. These nitrogen fixing trees (NFT) nodulate sporadically with indigenous rhizobia in the forest nurseries and field conditions (Odee, unpublished data).

Despite the increasing use of these NET in the country, little is known about their symbiosis with the naturally occurring rhizobia. Nodulation in both exotic and indigenous

legumes by natural rhizobii has been described as erratic (Keya and Van Eijnatten, 1975 ; Keya, 1977), but most of the work reported on natural nodulation have been of grain and pasture legumes (McDonald, 1935 ; Bumpus, 1957 : Morrison, 1966 ; Souza, 1969).

This paper reports on the results of cross-inoculation study of selected Rhiwbium strains on the given host spectrum and discusses the salient features.

Materials and methods

Eight strains of *Rhiwbiwn isolated* from geographically different areas were selected for *the* study. *Strain NUM 777 was* acquired from *the* Nairobi *Rhizobium* MIRCEN. Each strain was characterized by streaking on plates of yeast extract mannitol (YEM) agar. The composition of the medium was as described by Bergensen (1980) as follows (g l^{-1}) : mannitol, 10 ; yeast extract, 0.4 ; K_2HPO_4, 0.5 ; $MgSO_4$, $7H_2O$, 0.2 ; NaCl, 0.1 and distilled water. 0.5% alcoholic solution of bromothymol blue (BTB) was incorporated into the medium at the rate of 5 ml per liter for pH *reaction* of *the Rhiwbium strains.*

Streaked plates were incubated at 26°C for 10 days. Growth rates were described by cultural and colony size (Vincent, 1970). Fast growers achieved moderate to abundant growth with colony sizes of equal or greater than 2 mm within 5 days. Slow growers achieved slight to moderate growth with colony sizes of less than 2 mm after 5 days. The characteristics of the strains are shown in Table 1.

Leonard jar assemblies (Vincent, 1970) with vermiculite as the growth medium were used to grow seedlings. The composition of the nutrient solution for the assemblies was as described by Somasegaran and Hoben (1985) as follows (µM) : $CaCl_2,2H_2O$: 1000 ; K_2HPO_4 : 500 ; $C_6H_5O_7Fe$, H_2O : 10 ; $MgSO_4$, $7H_2O$: 250 ; K_2SO_4 : 250 ; $MnSO_4$, H_2O : 1 ; H_3BO_3 : 2 ; $ZnSO_4$, $7H_2O$: 0.5 ; $CuSO_4$, $5H_2O$: 0.2 ; $CoSO_4$, $7H_2O$: 0.1 ; Na_2MoO_2, $2H_2O$: 0.1 and 0.05% KNO_3 (w/v) was added to the nutrient solution in the plus nitrogen (+N) control assemblies. The pH was adjusted to 6.8 with 1N NaOH. The entire assemblies with nutrient solution and vermiculite medium were autoclaved at 121°C for 1 h.

Seed of the tree legumes obtained from the Kenya Forestry Seed Center were scari*fied* with concentrated sulphuric acid for *the* following times (mn) *: Acucica albida* and *Acacia mearnsii, 20 ; Leucaena leucocephala, Calliandra calothyrsus* and *Sesbania sesban,* 15 ; *Prosopis julijlora* and Sesbania *grandiflora,* 10. After treatment, the seeds were washed in several changes of sterile distilled water until all traces of the, acid were removed. The seeds were then aseptically placed onto 0.75% (w/v) wather agar plates and incubated at 28°C for 72 h.

Two pm-germinated seedlings of similar size and radicle length were grown per assembly. The seedlings were inoculated by dispensing 1 ml of a fully grown test culture around *theii roots* using a fresh pipette for each strain of *Rhizobium.* Sterilized *dry* gravel was spread over the surface of the vermiculite. Plants were thinned to one per assembly by excising the shoot of the unwanted plant with sterilized scissors.

Each treatment of host-strain combination and uninoculated plus nitrogen (+N) and minus nitrogen (-N) controls were replicated four times and grouped according to host species. The plants were then grown in the glasshouse for 10 weeks. Replenishment of water and nutrient solution was done once every fortnight.

Table 1 : Growth characteristics of eight Rhizobium strains isolated from seven tree legumes

Rhizobium strain	Locality	Host of Isolation	Growth	Reaction on Bromothymol Blue (BTB)	Colonial morphology
18b	Magarini (Coast Province)	Sesbania randiflora	Slow	Alkaline	Flat, dull, cream coloursd'with translucent margin differentiating into greater opacity at the center of the colonies
11a	Turbo (Rift Valley)	Sesbania sesban	Fast	Acidic	Flat, shiny, white coloured and evenly opaque colonies
2c	Mbita point (Nyanza Province)	Leucaena leuceuphela	Fast	Acidic	domed,; shiny, cream coloured with translucent margin differentiating into greater opacity at the center of the colonies
6b	Kisumu '(Nyanza Province)	Leucaena leuceu hala	Fast	Acidic	Domed, shiny, cream coured with translucent margin differentiating into greater opacity at the center of the colonies.
15b	Katangi (Eastern Province)	Prosopis juliflora	Fast	Acidic	Flat, shiny, white coloured and evenly opaque colonies.
16c	Wundanyi (Coast Province)	Calliandra calothyrsus	Fast	Acidic	Domed, shiny, white coloured with translucent margin differentiating into greater opacity at the center of the colonies
22	Mbita Point (Nyanza Province)	Acaci albida	Fast	Acidic	Domed, shiny, cream coloured with translucent margin differentiating into greater opacity at the center of the colonies
NM 777*	Eldoret wattle extractin f ctory (Rift Val ey	Acacia mernsii	Fast	Neutral	Flat, dull, white coloured and evhnly opaque colonies

* Source : The Nairobi Rhizobium MIRCEN.

Infectiveness and effectiveness of each host-strain combination was described by visual growth ratings. An effective association (E) produced nodulated dark green healthy plants showing excellent growth ; partially effective association (PE) produced nodulated plants with light green colour and restricted growth ; ineffective association (e) produced nodulated plants which were pale green and stunted ; and plants which were not nodulated were designated non-infective (ni). Nodule shape and distribution were described for each host-strain association according to Somasegaran and Hoben (1985). The dry weight of the plant shoots were determined after oven-drying at 70°C for 48 h.

Results and discussion

The results of the cross-inoculation tests are given in Table 2. Uninoculated control treatments remained free of nodules in all host species tested.

The Rhizobium strains *exhibited* a high degree of infectiveness with the test plants. All strains were infective on their own host of isolation. Strains isolated from *Calliandra calothyrsus, Leucaena leucocephala* and *Prosopis juliflora* nodulated all the hosts whereas *those* from *Sesbania grandiflora, Sesbania sesban* and *Acacia mearnsii* variably nodulated five host species each.

There were four cross-inoculation or infective groups within the scope of this study. These were as follows : (i) first group *: Acacia mearnsii* and *Prosopis juliflora ; (ii)* second *group : Leucaena leucocephala. Calliandra calothyrsus* and *Prosopis Juliflora ; (iii)* third group : *Acacia albida, Acacia mearnsii, Sesbania grandiflora* and *Sesbania sesban* ; (iv) fourth group : *Acacia Albida, Leucaena leucocephala, Sesbania grandiflora and Sesbania sesban.*

Rhizobii in each infective group were mutually interchangeable between or among the plant host members as defined by Graham (1976). Given the narrow range of *Rhizobium* strains and host species, and the fact that strains isolated from a given tree can also be different, the apparent infective groups may be regarded as atypical because of the evident overlap. Howevertheconcept of «infective group>> is not to be emphasized particularly in the tropics because even in the recognized groups, infection across the boundaries have been shown to be prevalent (Lange, 1961 ; Habish and Khairi, 1968 ; Trinick, 1968).

Symbiotic effectiveness as rated by visual appearance of test plants gave the following own host associations : effective symbioses in *Sesbania sesban, Sesbania grandiflora, Calliandra calothyrsus* and *Acacia albida* ; partially effective symbioses in *Leucaena leucocephala* and *Acacia mearnsii* ; *ineffective* association *in Prosopis juliflora.* Effective cross-inoculation symbioses were obtained by *Prosopis juliflora strain* 15b on *Leucaena leuwcephala* and *Calliandra calothyrsus* ; *Calliandra calothyrsus* strain 16c on *Leucaena leucocephala,* and *Acacia mearnsii* strain *NUM 777 on Acacia albida.*

On the basis of beneficial symbiosis, *Sesbania sesban* and *Sesbania grandiflora* were more specific in their rhizobial requirement. Others were less specific because in addition to forming effective or partially effective own host symbioses, they also exhibited varying degrees of *effectiveness in* cross-inoculation with *the* exception of *Prosopis juliflora.* The ineffective own host nodulation *in Prosopis juliflora* is not an isolated case. Miettinen *et al* (1968) working in Bura (Eastern Kenya) reported a similar occurrence and attributed it to cross-inoculation with indigenous soil rhizobia compatible with *the* indigenous *Acacia Senegal.* This would seem possible because *Prosopis juliflora* is not endemic or as naturalized as the other species in Kenya and thus may merely be acting as trap-host for indigenous

fable 2 : Ineffectiveness and effectiveness ratiqgs of host-straln combinations

Rhizobium strain	Host of Isolation	Sesbania grandiflora	Sesbania sesban	Leucaena leucocephala	Calllandra calothyrsus	Acacia albida	Acacia mearnsii	Prosopis juliflora
18b	Sesbanla grandiflora	E (1)	e	PE (2)	nt (4)	e (3)	PE	nt
11a	Sesbanla sesban	e	E	e	nt	e	e	nt
2c	Leucaena leucocephala	e	e	PE	e	PE	PE	e
6b	Leucaena leucocephala	e	e	PE	e	e	E	e
15b	Prosopis juliflora	e	e	E	E	PE	PE	e
16c	Calllandra calothyrsus	e	e	E	E	e	e	PE
22	Acacia albida	e	e	e	nt	E	PE	nt
NUM 777	Acacia mearnsii	e	e	nt	nt	E	PE	e

(1) E = Effective, (2) PE = Partially effective, (3) e = Ineffective, (4) nt = non infective.

rhizobii Although *the Rhizobium strains were* isolated from effective (pink) nodules, the partially effective or ineffective own host associations reported in this study emphasizes the need to re-authenticate strain effectiveness on host of isolation prior to use in cross-inoculation studies. The response to inoculation in terms of shoot dry weight on own host and cross-inoculation combinations gave similar trend as in visual ratings. Own host association of strains 11a, 18b, 16c and 22 (Fig. la, b, c, e) gave shoot dry weights that were significantly higher ($p = 0.05$) than the uninoculated -N control. Shoot dry weights of cross-inoculation association of strains 15b on *Leucaena leucocephala* and *Calliandra calothyrsus*, 16c on *Leucaena leucocephala* and *NUM 777* on *Acacia nlbida* (Fig. lc, f, e) were also significantly higher than the uninoculated -N control. Other beneficial symbioses of strain 16c on *Prosopis juliflora* (Fig. 1d) and strains 6b, 18b, NUM 777, 2c, 15b, 22 on *Acacia mearnsii* (Fig. lg) were not significant. The insignificant response of the Iatter two host species could be attributed to their growth rates which were observed to be relatively slower than the other host species. There also occurred a marked increase of shoot dry weight by the following symbioses and magnitudes relative to +N control : strains lla on *Sesbania sesban, x 6.0 ; strains* 16c and 15b on *Leucaena leucocephala, x 4.7* and 3.8 respectively ; strain NUM 777 on *Acacia albida, x* 1.6 ; and strain 15b on *Calliandra calothyrsus, x 3.0.* Despite the fact that the amount of inorganic N applied in +N control may not have been optimum, the magnitudes of the comparisons indicate some potential in biological nitrogen fixation of these NFT with the indigenous rhizobia.

The plant host determined the shape and distribution of nodules on the root system in each host *species irrespective* of host of isolation of *the Rhizobium strain* forming the association (Table 3). However, consistency in nodule shape and distribution was maintained only in effective symbioses.

The results presented in this paper have shown varying degrees of infectiveness and compatibility of indigenous rhizobii with the NFT. These have in effect posed a great challenge. The challenge to assess the need of inoculation, select effective symbioses under simulated environmental conditions in the laboratory/glasshouse and evaluate the selected symbioses in the field sites where they are to be grown. Kenya Forestry Research Institute is addressing itself to these studies.

Table 3 : Nodule shape and distribution of effective host-strain associations 10 weeks after planting

Test plant	Rhizoblum strain and host of isolation (in brackets)	Nodule shape	Nodule distribution
Sesbania grandiflora	18b (S. grandiflora)	Semi-globose	Prolific tap-root and occasional lateral root nodulation
Sesbania sesban	11a (S. sesban)	Globose	Occassional tap-root and large number of root nodulation
Leucaena leucecophala	16c (C. calothyrsus) 15b (P. juliflora)	Elongate and lobed	Occassional tap-root and lateral root nodulation
Prosopis juliflora	16c (C. calothyrsus)	Semi-globose and lobed	Occasslonal tap-root and lateral root nodulation
Calliandra calothyrsus	16c (C. calothyrsus) 15b (P. juliflora)	Semi-globose	Large number of tap-root and lateral root nodulation
Acacia albida	22 (A. albida) NUM 777 (A. mearnsii)	Semi-globose	Prolific tap-root and occas-. sional lateral root nodulation

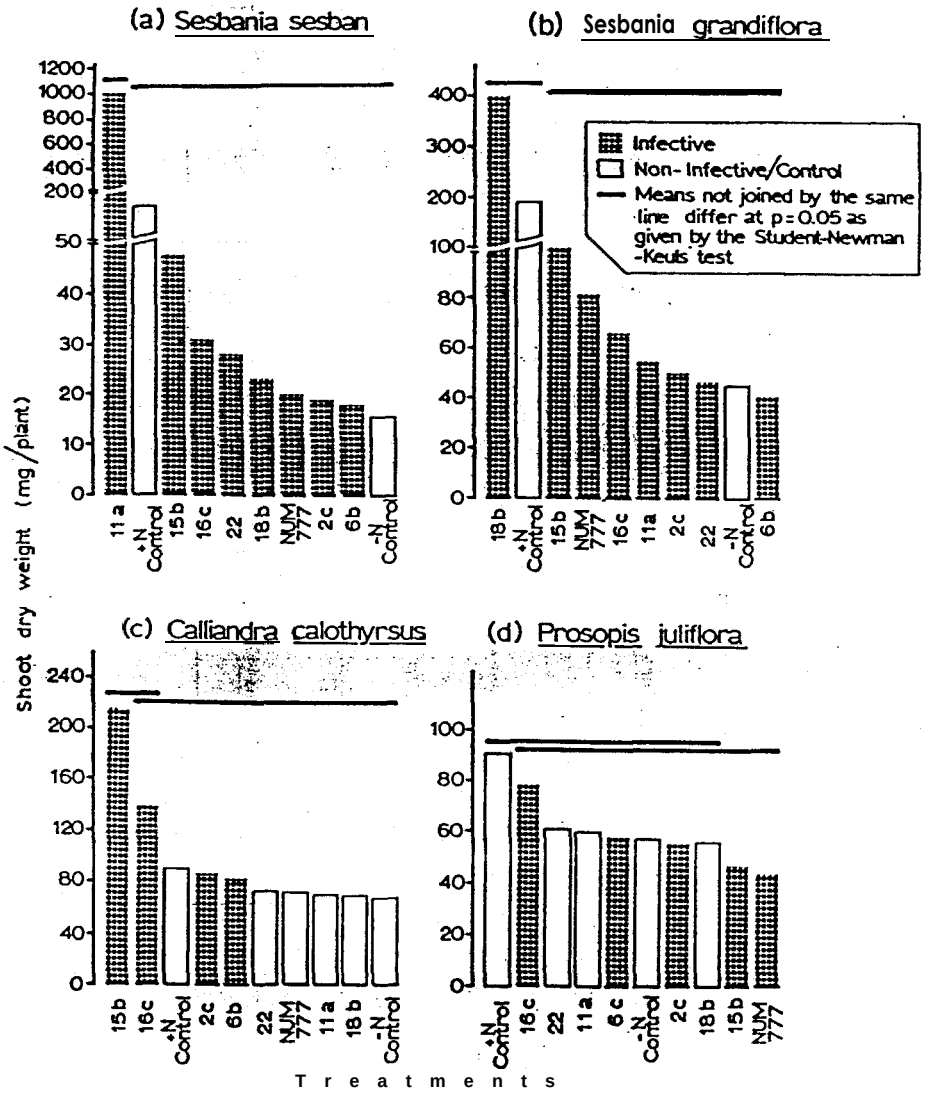


Fig.1. a b c & d. Effect of inoculation with strains of Rhizobium and application of inorganic N on shoot dry weights of various tree species in Leonards jars at 10 weeks after planting.

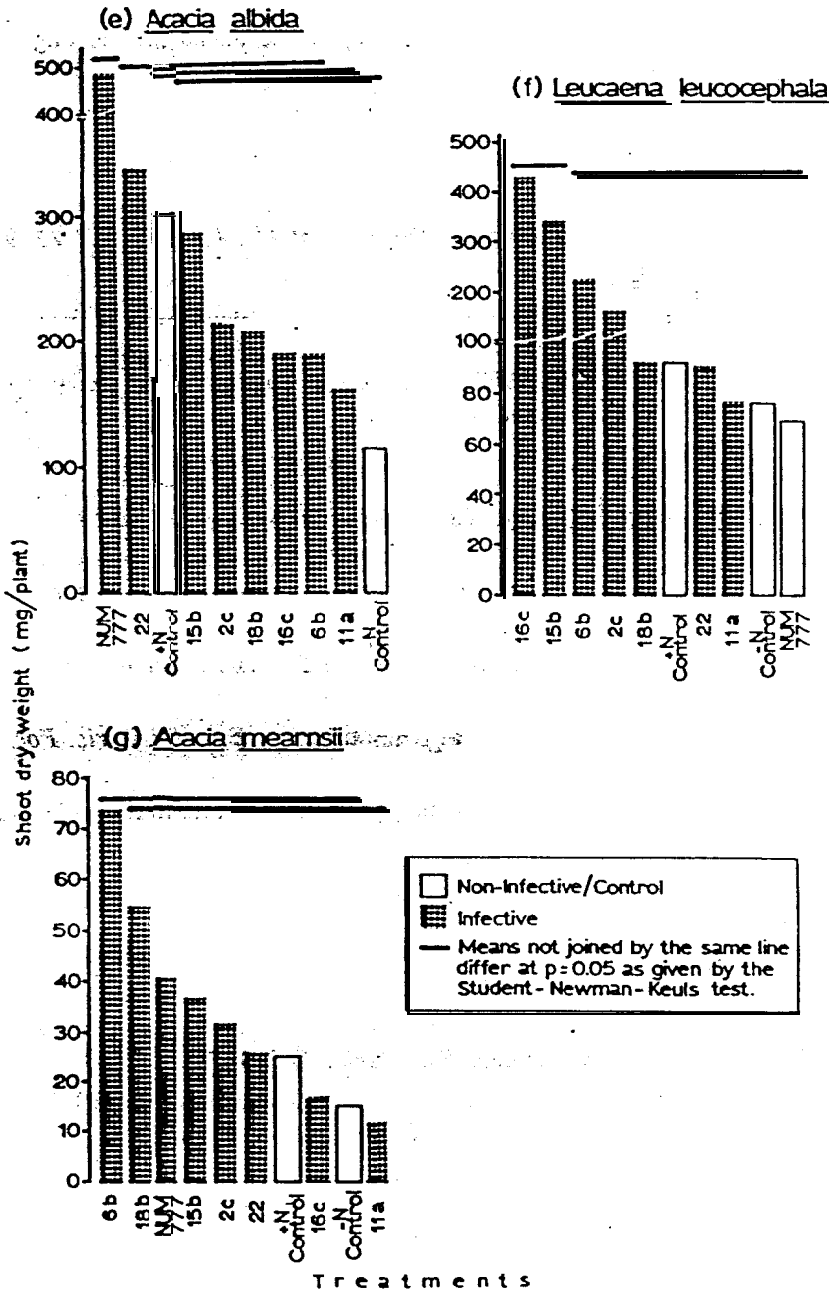


Fig.1. e,f,&g. Effect of inoculation with strains of Rhizobium and application of inorganic N on shoot dry weights of various tree species in Leonard jars at 10 weeks after planting

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FIXATION D'AZOTE ASSOCIATIVE

Effect of inoculation on the growth and yield of three maize cultivars

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Summary

Inoculation experiments were conducted using three maize cultivars (*Zea mays* L.) and three N_2-fixing plant-growth-promoting-rhizobacteria. The plants were grown to maturity and various vegetative and reproductive parameters were determined. The results obtained indicated significant increases in the shoot dry matter yield (34.2%) of one maize cultivar with only a small increase in seed dry weight. Significant increases in seed dry weight (38.4%-49.5%) were recorded for inoculated plants of two cultivars. No significant increase in the crude protein of the grain was observed in the. inoculated treatments.

Introduction

It has been suggested that increasing the proportion of rhizosphere diazotrophs in grass-bacteria associations, through inoculation, may result in increased N_2 fixation and plant yield (Patriquin, 1982 ; Okon, 1984). Such increases in the numbers of N_2-fixing rhizobacteria may be achieved by application of highly competitive bacterial strains, heavy inoculation or by the introduction of N_2-fixing genes to other root associated bacteria (Kleeberger and Klingmuller, 1980 ; Postgate and Kent, 1987). However results obtained in inoculation studies of cereal crops and forage grasses are very varied ranging from no response (Schank *et* al. 1980 ; Wright and Weaver, 1982) to highly significant responses (Kapulnik *et al.,* 1981 ; Mertens and Hess, 1984 ; Smith *et al.*, 1984). Differences in inoculation responses attributable to plant genotype effects have previously been demon-

trated in wheat and maize among other cereal crops (Kapulnik et al., 1981 ; Reynders and Vlassak, 1982 ; Millet *et* al., 1984).

In this study we report the response of three maize cultivars to inoculation with N_2-fixing plant-growth-promoting-rhizobacteria in a tropical soil.

Materials and Methods

Three facultatively anaerobic diazotrotrophs C, J and L previously isolated from maize plant roots were grown under static conditions at 24°C in a liquid nitrogen deficient glucose medium (Lindberg and Granhall, 1984 ; Mwaura and Granhall, 1986). The cultures were grown to stationary phase, centrifuged and washed twice in 0.05 M sterile phosphate buffer pH 7.0 (Berg *et al.*, 1980). The cells were finally resuspended in buffer and transferred into sterile petri dishes.

Seeds of three maize cultivars LG 11, Makueni and Katumani were washed under running water and in sterile distilled water to remove the fungicides. The seeds were germinated on moistened filter paper in the dark at room temperature. The seedling lot of each cultivar was divided into four batches. For each cultivar, one batch of seeds was used as a control while the other three batches were inoculated with the three bacterial strains C, J and L respectively. The seedlings were inoculated by placing them in petri dishes containing the bacterial suspensions for 30 mn. Control seeds were immersed in sterile buffer.

Four maize seedlings were sown in 30 1 plastic troughs containing well moistened soil from a weeded plot. Great care was taken to avoid cross contamination between the containers. Each treatment was replicated four times and all containers were randomly arranged in an open space but under a roof of clear plastic sheeting to prevent flooding of the containers by rain water. The soil in each container was covered with washed vermiculite lo minimise algal growth. All plants were watered every two or three days as necessary. The plants were thinned to three in each container 10 days after planting. After growing for 110 days, the plants were harvested and their shoot lengths measured. The shoot, ear and 1000-seed dry weights were determined in each treatment.

The plant materials were dried to constant weight in a forced-air oven at 80°C. The materials were then milled and the nitrogen content was determined by the Kjeldahl method (Hesse, 1971). The crude protein content of the maize grain was estimated by multiplying the nitrogen content with a factor of 6.25 (AOAC, 1960).

Results

The results are summarised in Tables 1 and 2. Improved vegetative growth was particularly evident in maize plants of cultivars LG 11 and Katumani inoculated with the three bacterial strains Substantial increases in plant height (11.6 - 43%) and shoot dry weights (3.1 - 342%) were recorded over the uninoculated plants (Table 1). However, little or no improvement in vegetative growth was observed in inoculated maize plants of the Makueni cultivar. Among the three inoculants tested, bacterial strain C was the most effective in increasing the shoot dry matter yield. Inoculation however did not increase the nitrogen content of maize plant shoots except for plants of cultivar LG 11 inoculated with bacterial strain C (Table 1).

Table 1. Effect of **inoculation** on the vegetative growth and N-yield of three **maize cultivars.**

Cultivar	Inoculant	Plant height (cm)	Shoot d.wt./plant (g)	% N	N-yield/plant (mg)
LG 11	control	130.2 ± 12.1	39.1 ± 4.9	0.35	136.9
	L	152.1 ± 5.4	51.1 ± **2.8***	0.34	**173.7**
	J	186.0 ± 5.6	47.7 ± 2.5	0.37	**176.5**
	C	180.3 ± 9.1	52.5 ± **4.4***	0.54	**283.5**
Katumani	control	184.7 ± 14.3	77.6 ± 7.0	0.65	504.4
	L	206.2 ± 6.6	± 8.1	0.64	**512.0**
	J	225.5 ± 15.4	**6.4**	0.61	**454.5**
	C	244.9 ± 9.2	**80.0 ± 9.2***	0.57	**554.0**
			74.5 ±		
Makueni	Lontrol	175.1 ± 8.9	75.4 ± 6.6	0.41	309.1
	J	196.8 187.7 ± 11.9 10	80.0 76.1 ± 7.3	**0.39**	296.8
			8.1	0.38	304.0
	C	162.7 ± 11.5	74.2 ± 6.0	q.39	289.4

* significant increases (P = 0.05').

Results are mean of six replicates plants t S.E.

With regard to the reproductive parameters, ear dry weight and the mean seed dry weight increased in inoculated plants compared to those of uninoculated treatments. The most significant responses were observed in Katumani and Makueni cultivars for which the 1000-seed dry weight increased by up to 49.5% and 38.4% respectively (Table 2). The results indicate that bacterial strains J and L were the most effective in promoting reproductive growth of the maize plants. Higher ear and seed dry weights were observed in maize plants inoculated with the two strains compared to those of plants inoculated with bacterial strain C. The crude protein content in the grain was however unaffected by inoculation except for the Makueni cuhivar in which higher crude protein levels were recorded in inoculated than in the uninoculated treatment (Table 2). It was also noted that the inoculated maize plants flowered and matured about 10 days earlier than the control plants.

Discussion

In addition to the inoculated plants maturing slightly earlier than the controls, inoculation increased plant height and dry matter yield Earlier heading and flowering has similarly been observed in wheat, *Setaria italica*, sorghum and *Panicum* sp. in the greenhouse and in the field after inoculation with N_2-fixing bacteria (Kapulnik *et al.*, 1981 ; Kapulnik *et* al., 1983 ; Lin et al., 1983 ; Yaholon *et al.*, 1984 ; *Sarig et al.*, 1984). Inoculation of the Makueni cultivar did not promote vegetative growth of the maize plants but the seed dry weight increased by up to 38.4%. Large increases in ear dry weight (31.9%) and seed dry weight (49.5%) were also recorded for the Katumani maize cuhivar inoculated with the test bacteria. The observed differences in maize cultivar response to inoculation with the N_2-fixing rhizobacteria were remarkable in that the third cultivar LG 11 responded best in terms of plant dry matter yield increases but recorded the lowest increase in seed dry weight among the three cultivars. Similar genotype effects have been established for maize (Kapulnik *et al.*, 1981 ; Hegazi and Monib, 1983) and other cereals (Millet *et al., 1984).*

In Israel, Kapulnik *et al.*,(1981) reported seed and cob dry weight increases of *28.2%* and 34.4% respectively in inoculated maize grown under field conditions. Few published reports on inoculation effects in maize and other cereals in Africa are presently available. In Egypt, Hegazi and Monib (1983) reported increases of 150-170%, 180-270%, 120-130% for straw yield, grain yield and total N yield respectively in maize inoculated with *Azospirillum sp.* under field conditions. More concerted efforts need to be made to study the potential for increasing grain and forage yield of cereal crops through inoculation with growth-promoting-rhizobacteria.

Table 2. Effect of inoculation on yield parameters of three maize cultivars.

Cultivar	Inoculant	ear d.wt. /plant (g)	1000-seed d.wt. (g)	mean seed d. wt. (mg)	% crude protein
LG 11	Control	57.3 ± 5.2	129.8	129.8	13.1
	J	56.7 54.0 ∓ 0.5 4.8	130.8 135.2	130.8	12.8
				135.2	9.5
	C	67.7 ∓ 13.∓	141.7	141.7	11.2
Katumani	Control.	32.9 ± 4.3	74.1	74.1	13.2
	J	43.4 38.4 ∓ 7.2 3.7,	1196.0	96.0	10.5
				110.8	11.6
	C	39.2 ∓ 4.1	76.5	76.5	14.1
Makueni	control	44.2 t 1.9	81.6	81.6	9.7
	L	46.4 ∓ 10.7	112.9	112.9*	10.5
	J	49.8 ∓ 6.6	100.8	100.8	10.5
	C	42.8 ∓ 2.5	79.5	79.5	11.4

* signlflcant'increases (P = 0.05).

Results are means of six replicate plants ± S.E. Ears of replicate plants were pooled, shelled and the grain dried to constant welght.

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Maximisez la FBA pour la Production Agricole et Forestière en Afrique

Population of nitrogen fixing bacteria in sweet potato fibrous roots

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Abstract

Fibrous roots of six sweet potato cultivars were evaluated for rhizosphere populations of N_2-fixing *Azospirillum*-like bacteria at three sampling dates. Populations of the bacteria were determined using culture tubes (pellicle formation and acetylene reduction activity), and colony plate counts. Plant growth parameters evaluated were : fresh and dry weights of storage roots, foliage, fibrous roots and total biomass. The bacterial population ranged from 10^4 to 10^6 g per dry root weight depending on the counting method used- The N_2-fixing bacterial populations were positively correlated (r = 0.67 to 0.88) with foliage and total biomass dry weight for cultivars TIS 70357 and TIS 8441 ; for the same parameters, negative correlations (r = -0.68 to -0.80) were found for cultivars TIS 9265 and TIb 4 and correlations were not significant for cultivars TIS 2498 and TIS 8504.

Introduction

Sweet potato cultivars from the breeding program at the International Institute of Tropical Agriculture (IITA) produce from 40 to 50 mt/ha of storage roots and up to 31 mt/ha foliage when grown in soils with low soil N indexes (Hahn et *al.*, 1984 ; Hill *et al.,* 1985). *Azospirillum*-like bacteria am widely distributed in tropical and temperate region soils and plants, and have been suggested as important associative N_2-fixing bacteria under the direct influence of plants. Several strains of A. *brasilense* and *A. lipoferum* associated with the roots of different sweet potato varieties have been isolated and characterized (Hill *et al.*, 1983 ; Hill and Bacon, 1984). Reviews of *Azospirillum* inoculation studies by Boddey and Dobereiner (1982) and Dart (1986) show inconsistent responses of cereals and grasses for several parameters including nitrogenase activity, nutrient uptake and top *dry matter.* When *sweet* potato *was* inoculated with *Azospirillum bra*dense, the root yield and N 'content were increased (Crossman and Hill, 1986).

The purpose of this study was to quantify naturally occuring nitrogen fixing bacteria in sweet potato rhizosphere and determine the effect of fertilizer nitrogen addition on growth and microbial parameters, and to evaluate relationships between sweet potato growth parameters and parameters used to measure associative N_2 fixation.

Material and Methods

Six sweet potato cultivars were selected from the IITA breeding program : TIb4, *TIS 2498, TIS 70357,* TIS 8441, TIS 8504 and TIS 9265 were planted in loamy sand at the IITA Ibadan, Nigeria. The experimental design was a split plot with two rates of fertilizer N (0 and 50 kg of calcium ammonium nitrate N ha^{-1}) as main plots and cultivars as subplot treatments. Each treatment consisted of four rows of a sweet potato cultivar, 13.2 m long and 1m wide each, with plants spaced 0.5 m within rows. At 6, 10 and 14 weeks after planting (WAP), four plants were collected at random from the left center row, and storage roots, foliage and fibrous root weights and associative N_2 fixation parameters were evaluated. At each sampling date, sub-samples of 2 g of fibrous roots plus adhering soil were analysed for number of *Azospirillum*-like bacteria, using the serial dilution method described by Wollum (1982) for colony, plate count and the most probable number method described by Knowles (1984). Malate N free media was used for both solid plates and semi-solid tubes. Acetylene reduction assay (ARA) was carried out on all tubes -showing pellicle formation, using a Carle Age 211 Gas Chromatograph equiped with a flame ionisation detector.

Results and Discussions

Storage root weight was higher for TIS 8504 and 8441 than for the other cultivars at 6, 10 and 14 WAP irrespective of fertilizer treatment (Table 1). Differences in total dry biomass and storage root weights were most apparent at 14 weeks after transplanting for both 0 and 50 kg N ha^{-1}. Fertilizer N addition tended to depress storage root yield and foliage weight of most cultivars. Acetylene reduction activity was detected in culture tubes at 10' dilution for all six cultivars at all three sample dates suggesting the presence of N_2-fixing bacteria in the rhizosphere of all the cultivars studied. The most probable numbers (MPN) of N_2-fixing bacteria were low, and significant differences were not apparent.

Table 1 : Effect of fertilizer N (50 kg N ha-') on sweet potato storage roots and foliage growth.

Cultivars	Tuber wt (kg ha^{-1})		Foliage wt (kg hi-11	
	N = 5Ø	N = O	N = 5Ø	N = O
TIS 8504	824 a	635 a	1822 b	2162 a
TIS 8441	492 b	548 b	2291 ab	2Ø75 b
TIS 2498	319 cd	33Ø c	1547 bc	1439 bc
TIb 4	39Ø c	374 c	1322 c	1172 c
TIS 9265	156 d	147 d	1967 b	17Ø5 bc
TIS 70357	188 d	151 d	25Ø2 a	2227 a

Means with the same letter in the same column are not significantly different at the 5 % level by the Duncan's multiple range test.

The lowest yielding cultivar TIS 70357 had a significantly higher MPN and percentage of N_2-fixing bacteria (Table 2). Significant correlations (r = 0.67 to 0.88) were found for foliage and total dry biomass weight, with MPN of *Azospirillum*-like bacteria in the rhizosphere (Table 3).

Correlations between N_2-fixing bacterial population and foliage weight or total biomass were positive for TIS 70357 and 8441 suggesting that *Azospirillum*-like *bacteria were* responsible for the increase in foliage production. Correlation for the same parameters were negative for TIb 4 and TIS 9265 (r= -0.68 to -0.80) and not significant for cultivar TIS 2498 and 8504.

These results suggest that associative N_2 fixation in sweet potato is cuhivar dependent, and that foliage and total biomass production of some sweet potato cultivars on soils with low N indexes may be related to the presence of N_2-fixing bacteria However the association between *Azospirillum*-like bacteria and sweet potato fibrous roots seems to be regulated by complex mechanisms that need to be more investigated.

Table 2 : Number of Azospirillum bacteria in sweet potato fibrous roots by the acetylene'reduction assay (ARA) and the most probable number (MPN)

Cultivars	ARA 105 /g roots	Log ARA	MPN 105 /g roots	Log MPN
TIS 8504	8.20 b	3.25 abc	0.090 a	3.75 a
TIS 8441	9.86 b	3.17 bc	0.067 a	3.62 a
TIS 2498	11.79 b	2.63 c	0.042 a	3.50 a
TIb 4	5.77 b	3.34 abc	0.055 a	3.67 a
TIS 9265	7.07 b	4.09 ab	0.089 a	3.72 a
TIS 70357	88.31 a	4.57 a	0.010 a	3.72 a

Means with the same letter in the same column are not significantly different at the 5 % level by Duncan's multiple range test.

Table 3. Correlation coefficients between selected growth parameters and rhitosphere Np-fixing bacterial count for six sweet potato cultivars.

CULTIVAR	MPN-A[1]	MPN-N[2]	CPC[3]	Log HPN-A	Log WPN-N	Log CPC
TIS 8504						
FDW[4]	NS	NS	NS	NS	NS	NS
TDB[5]	NS	NS	NS	NS	NS	NS
TIS 8441						
FDW	NS	0.75*	0.84***	NS	NS	NS
TDB	NS	0.77**	0.88***	NS	NS	NS
TIS 2498						
FDW	NS	NS	NS	NS	NS	NS
FDB	NS	NS	NS	NS	NS	NS
TIb 4						
FDW	-0.80**	NS	NS	-0.78**	-0.76*	-0.70*
TDB	-0.72*	NS	NS	-0.78**	NS	NS
TIS 9265						
FDW	NS	NS	NS	NS	NS	NS
TDB	NS	NS	NS	-0.75**	-0.68**	NS
TIS 70357						
FDW	0.71*	NS	0.75*	0.72*	0.67*	NS
TDB	NS	NS	NS	NS	NS	NS

1. Most probable numbers of Azospirillum
2. Most probable numbers of Micronitrophilic bacteria
3. Colony plate counts
4. Foliage dry weight
5. Total dry biomass

*Significant at the 5% level
**Significant at the 1% level
***Significant at the 0.1% level

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ASSOCIATION AZOLLA / ANABAENA

La recherche sur *Azolla* et ses applications : évolution et tendances actuelles

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Summaty

Azolla uses as biofertilizer and food, traditional in some parts of China and Vietnam, have been ignored or neglected in other countries (and even in wide areas of these countries) until the sixties, when research programmes were launched with a view to increasing areas under *Azolla* cultivation and improving cultural practices adapted to various ecozones. Since then research, pure and applied, has blossomed, and efforts to introduce *Azolla* in many Asian countries but also in other parts of the world have increased, with varying successes. The purpose of this paper is to outline the major steps of our knowledge on *Azolla*, to bring out the main present research trends, to descrîble the various possible *Azolla* uses and their constraints, with special emphasis on Africa. Suggestions are finally presented, aimed at promoting *Azolla* introduction in this continent, especially for small farmers, who are the main potential beneficiaries.

Introduction

L'objectif du présent document est de dresser un bref historique des principales connaissances acquises sur la fougère aquatique *Azolla*, de dégager les grands axes de recherche en cours, de décrire les diverses possibilités de valorisation d'*Azolla* en agriculture

et les problèmes poses par son utilisation, l'accent étant mis sur le cas particulier de l'Afrique. Des suggestions sont enfin émises en vue de promouvoir l'azolliculture dans ce continent, spécialement auprès des petits paysans qui en sont les principaux bénéficiaires potentiels.

L'évolution de la Recherche

Des origines à la crise pétrolière

La valeur fertilisante et alimentaire de la fouragère aquatique *Azolla* est connue et mise à profit depuis de nombreux siècles dans certaines régions très limitées de Chine et du Vietnam ; situées le long de la côte entre le 18ème et le 30ème degré de latitude Nord, ces régions se caractérisent par un climat subtropical humide, à variations saisonnières de température souvent considérables. Jusqu'en 1960, la maîtrise d'*Azolla* y était essentiellement empirique.

Dans le monde occidental par ailleurs, dès le début du dix neuvième siècle apparaissent quelques études fondamentales sur *Azolla* ; ce n'est cependant qu'au début du vingtième siècle, en grande partie grâce à l'intervention de chercheurs travaillant en Indochine, que l'intérêt pratique d'*Azolla* est signal& C'est ainsi qu'il est fait mention, sans d'ailleurs que des arguments probants soient. toujours présentés, de l'effet dépressif d'une couverture de plans d'eau par *Azolla*, sur le développement des moustiques (Smith, 1910), de la capacité de réduction de l'azote (Oes, 1913), du rôle fertilisant d'*Azolla* en riziculture et de sa valeur alimentaire pour des animaux d'élevage (Chevalier, 1926), de l'action inhibitrice d'*Azolla* sur le développement des adventices (Braemer, 1927) ou enfin de son influence négative sur les pertes d'eau par évapotranspiration (Nguyen, 1930). On avait donc, dès cette époque, relevé l'essentiel des proprités pratiques d'*Azolla* connues aujourd'hui, mais il faut bien reconnaître que ces informations ne suscitèrent pas grand intérêt.

En Crient par contre, dès la fin des années 50, les gouvernements vietnamien et chinois décidèrent de promouvoir la recherche scientifique ayant trait à *Azolla*. Il en résulta un foisonnement de travaux, en général à orientation appliquée. En Chine, Lumpkin et Plucknett (1982),par la mise au point de techniques diverses de conservation en saison froide, permit d'étendre considérablement l'aire de culture vers le Nord, jusqu'au delà de Beijing ; dans le Sud par ailleurs des techniques de protection contre les températures élevées de l'été permirent l'utilisation d'*Azolla* pour les cultures automnales.

L'étude des principaux insectes prédateurs d'*Azolla* (qui constituent un autre problème majeur en Asie) et des modes de lutte contre ceux-ci, ainsi que le développement de techniques d'enfouissement d'*Azolla* entre les Lignes de riz améliorèrent quant à eux l'efficacité de l'azolliculture.

Du côté vietnamien (Dao and Thuyet, 1979). les efforts portèrent sur la sélection d'écotypes (indigènes) d'*A. pinnata* var. *imbricata* et sur l'amélioration des pratiques culturales en association avec la culture du riz d'hiver, avec pour résultat un accroissement considérable des surfaces couvertes. Dès 1965, se développa la monoculture hivernale d'*Azolla*, dans divers systèmes de rotation. En même temps, de nombreuses recherches portant sur les prédateurs, les exigences en phosphore, la conservation d'*Azolla en* saison chaude, etc..., étaient entreprises.

L'explosion des années 1970

C'est durant les années 1970, en particulier grâce aux publications vietnamiennes plus aisément accessibles que celles provenant de Chine, que les milieux scientifiques extérieurs à ces deux pays prirent réellement conscience de l'intérêt potentiel d'*Azolla* en agriculture ; la monographie de Moore (1969) joua certainement un rôle important à cet égard. La mise au point en 1967 de la technique de mesure de l'activité nitrogénasique (C_2H_2) par chromatographie gazeuse suscita par ailleurs un regain général d'intérêt pour l'étude de la fixation biologique de l'azote. La crise pétrolière de 1973 enfin fut un stimulant puissant à la recherche sur les engrais biologiques A cette époque commencent dès lors à se multiplier les programmes de, recherche sur *Azolla* de par le monde.

Parmi les thèmes de recherche initiés et les acquis majeurs des années 1970, on retiendra particulièrement :

- la confirmation de la localisation de la nitrogénase au sein d'*Anabaena* (Peters and Mayne, 1974) ;
- les premières applications de la méthode isotopique au ^{15}N pour l'étude des relations entre les partenaires de la symbiose (Peters et *al.*, 1977) ;
- la description détaillée du cycle de développement d'*Azolla* (Konar and Kapoor, 1974) ;
- l'amélioration des connaissances sur les relations morphologiques et physiologiques entre *Azolla* et *Anabaena* (Peters, 1984) ;
- *les* tentatives *de culture in vitro* d'*Anabaena azollae (Bai et al.*, 1978 ; Newton and Herman, 1979) ;
- la confirmation de l'importance du phosphore (Subudhi and Watanahe, 1979) ;
- la confirmation de l'effet «herbicide» d'*Azolla* (Ngo, 1973 ; Talley *et al.*, 1977) ;
- le développement des recherches sur la composition chimique d'*Azolla* et sur son utilisation comme aliment (Buckingham et *al.*, 1977, 1978; Subudhi and Singh, 1978, 1979) ;
- l'étude de la décomposition d'*Azolla* dans le sol (Brotonegoro and Abdulkadir, 1978) ;
- les essais de compostage d'*Azolla* (Tran and Dao. 1973) ;
- l'introduction et la diffusion d'*Azolla filiculoïdes* en *Chine* en 1977, et des autres espèces en 1979 (Lumpkin and Plucknette, 1982)
- l'organisation des premiers essais coordonnés de l'International Network on Soil Fertility and Fertiliser Evaluation for Rice (INSFFER) sur *Azolla* (Watanabe, 1987) ;
- l'initiation des premières grandes collections de référence d'*Azolla* à l'International Riche Research Institute, aux Philippines et à l'Unviversité Catholique de Louvain, en Belgique.

Les années 1980 : l'Age d'Or ?

La stimulation de la recherche sur *Azolla* pendant les années 1970 et les espoirs qu'elle suscite sur le plan des applications donnent lieu à l'organisation de conférences

internationales à Porto Rico (Silver and Schröder, 1984) et en Chine (IRRI, 1987) et de cours de formation sur *Azolla*, au P&ou et au Sénégal en 1983, en Thaïlande en 1984. en Chine en 1987.

En 1982, la FAO et l'Agence Internationale de l'Energie Atomique développent un programme de recherche coordonné sur *Azolla, tandis* que paraît une excellente et très complète monographie (Lumpkin and Pluknett, 1982). qui fait encore autorité à ce jour. En Chine, le Centre National de Recherches sur *Azolla* (NARC) *est* inauguré, à Fuzhou, en 1985. Le rythme des publications continue à s'accélérer et des progès décisifs enregistrés :

- le problème de l'identification d'*Azolla* au niveau spécifique et subspécifique, crucial pour les utilisateurs, s'il n'est pas résolu, fait l'objet d'études approfondies basées sur les méthodes classiques (Perkins et *al.*, 1985 ; Dunham and fowler, 1987), et de chimiotaxonomie (Mai Kodomi et *al.*, *1984* ; *Zimmerman et al.*, 1984 ; Zimmerman *et al.*, 1988) ;
- la caractérisation des *Anabaena* présents *dans* les *diverses* espèces d'*Azolla* progresse grâce à la génétique moléculaire (Cohen - Bazire and Franche, 1985 ; Franche and Cohen - Bazire, 1985), et aux techniques des anticorps monoclonaux (Gates et *al.*, 1981 ; Ladha and Watanabe. 1982 ; Tang et *al*, 1987) ;
- l'intervention d'un troisième partenaire, probablement obligé, de la symbiose, à savoir une bactérie (du genre *Arthrobacter* ?) semble se confirmer (Gates et *al.*, 1980 ; Wallace and Gates, 1986 ; Formi and Grilli Caiola, 1988.) ;
- l'insensibilité relative de la nitrogénase du complexe *Azolla - Anabaena* aux composés azotés est mise en évidence, avec de fortes différences spécifiques et' subspécifiques (Okoronkwo *et al.*, 1989) ;
- des différences considérables quant à la valeur alimentaire des diverses espèces d'*Azolla* sont démontrrées (Antoine et *al.*, 1986 ; Sanginga and Van Hove, 1989) ;
- les premières hybridations entre espèces d'*Azolla* sont réalisées (Wei *et* af., 1986) ; dans le même ordre d'idée, de nouvelles combinaisons sont obtenues par transfert d'*Anabaena* provenant d'une espèce d'*Azolla* à *une* autre- espèce préalablement débarassée de son propre symbionte (Lin *et al.*, 1988) ;
- des travaux sur la production de biogaz à partir d'*Azolla* sont entrepris *(charlier et al.*, 1983) ;
- un système *de* culture intégrée Riz-*Azolla*-poisson *est testé,* apparemment avec des résultats très positifs (Liu, 1987) ;
- le pouvoir accumulateur de potassium d'*Azolla* est mis en évidence ainsi que divers avantages de la culture d'*Azolla sur* sol humide (Liu, 1987) ;
- enfin, les échanges de souches entre centres de recherche et les essais d'adaptation dans diverses écozones s'intensifient.

Les principaux modes actuels d'utilisation d'*Azolla*

Azolla comme engrais vert

C'est principalement en culture irriguée qu'*Azolla* est utilisé comme engrais vert. Il y est cultivé en association avec le riz et enfoui à une ou deux reprises au cours du développement de ce dernier ; lorsque le calendrier cultural et la disponibilité en eau le permettent, une monoculture d'*Azolla* et un enfouissement avant repiquage du riz peuvent précéder la culture associée.

Ces pratiques fournissent de l'azote au riz mais en outre inhibent les adventices, améliorent la structure du sol et diminuent les pertes d'eau par évapotranspiration.

D'autres cultures irriguées, telles que celles du Lotus et de la Colocase (taro), sont également fertilisées de cette manière.

Azolla peut -enfin servir d'engrais vert en cultures maraîchères ou horticoles de pleine terre (par exemple jardin potager jouxtant les pépinières de maintenance) ; il-peut y être incorporé au sol à l'état frais ou sec (en vue de facilitez le transport ou de différer son emploi), ou après compostage, seul ou en mélange avec d'autres composants tels que la paille de riz.

Azolla comme aliment

L'amélioration des connaissances sur la valeur alimentaire d'*Azolla*, la nécessité de valoriser la biomasse produite dans les pépinières en dehors des saisons d'utilisation en rizière, l'extention de l'azolliculture dans le nord de la Chine, région à traditiond'élevage, ont eu pour effet de stimuler l'emploi d'*Azolla* comme aliment pour divers animaux, principalement porcs et volaille mais aussi ruminants et plusieurs espèces de poissons (Subhudhi and Singh, 1978 ; FAO, 1985a, 1985b ; Querubin *et al.*, 1986). En règle générale, il semble qu'*Azolla* puisse se substituer à environ 20% des raisons classiques, mais il faut reconnaître que des études complémentaires sont indispensables pour affiner ces estimations. Quoi qu'il en soit cette situation d'*Azolla* est en forte expansion actuellement.

Azolla en systèmes d'exploitation complexes

Comme signal6 plus haut une technique prometteuse consistant à associer riz, poissons et *Azolla* a été récemment mise au point, chacun des partenaires bénéficiant de la présence des autres. Cette--technique se développe actuellement dans plusieurs provinces de Chine (Liu Chung Chu, Comm. Pers.).

Un système intégré impliquaut culture d'*Azolla*, élevage de volailles en volières sur pilotis surplombant des étangs à Tilapia et élevage de porcs est également à l'essai en Côte dIvoire.

La production de biogaz enfin, par fermentation anaérobie d'un mélange d'*Azolla* et de paille de riz, et la récupération de l'effluent du digesteur pour fertiliser les cultures voisines semblent donner des résultats positifs aux Philippines (Johnson, 1988).

La situation en Afrique

Les premiers résultats portant sur le potentiel d'*Azolla* en agriculture africaine (Roger et Reynaud, 1979) suggéraient qu'*Azolla africana* (*A. pinnata var. pinnata*) pourrait être valorisé dans la vallée du fleuve Sénégal pour autant que le problème du manque d'eau

en saison sèche soit résolu. Des essais d'adaptation de cette souche en rizière montrèrent cependant sa sensibilité aux basses températures de décembre à février (Ton That, 1980).

En 1980, l'ADRAO lance, en collaboration avec l'Université Catholique de Louvain (Belgique), un programme derecherche ayant pour objectif de tester le potentiel de valorisation d'*Azolla* dans diverses conditions écologiques d'Afrique de l'Ouest. Les premiers essais sont réalisés à Richard Toll (Sénégal) et à Rokupr (Sierra Leone) ; ils portent sur l'adaptation d'une-vingtaine d'écotypes représentant les sept espèces et deux variétés d'*Azolla*. 11 apparaît rapidement que certains écotypes s'adaptent bien dans chacune des stations, et que l'introduction d'espèces étrangères est essentielle, les souches d'origine locale étant en général peu performantes.

Suite à ces résultats encourageants, la collection de référence est progressivement enrichie en vue d'identifier des souches à haute productivité ; des méthodologies adaptées à l'étude comparée de ces souches en conditions contrôlées et en champs sont mises au point.

Les premiers essais de fertilisation -des rizières sont entrepris en 1981 ; les effets escomptes (apport d'N, inhibition des adventices) sont manifestes

Un cours et des stages de formation sont organises à l'intention des chercheurs des pays membres de l'ADRAO et une plaquette sur *Azolla* est diffusée *dans* les milieux intéressés (Van Hove *et al.*, 1983).

Ces actions, ainsi que diverses interventions de la FAO, sont suivies de la mise en place de programmes de recherche dans plusieurs pays d'Afrique de l'Ouest, ainsi qu'au Burundi, au Rwanda et en Tanzanie.

Plus récemment enfin des actions de vulgarisation et d'encadrement en milieu paysan 'ont débuté.

De l'expérience ainsi acquise (Diara and Vand Hove, 1983 ; Diara *et al.*, 1984a, 1984b, 1987 ; Van Hove, 1987 ; Van Hove and Diara, 1987a, 1987b ; Van Hove *et al.*, 1983, 1987) se dégagent. les conclusions suivantes :

- les conditions climatiques de nombreuses régions d'Afrique sont favorables à une bonne, ou même à une excellente productivité d'*Azolla*, et ce pendant toute l'année, pour autant qu'un choix approprié d'écotypes soit effectue ;
- les prédateurs d'*Azolla* qui empêchent ou contrarient souvent l'utilisation d'*Azolla* en Asie, ne provoquent pas de dégât sérieux en Afrique. Cette situation n'est cependant sans doute que momentanée et il faut s'attendre, surtout dans les zones tropicales humides, à ce que certains de ces prédateurs au moins, déjà présents en Afrique, posent problème au fur et à mesure que l'azolliculture se développera ;
- le manque de maîtrise de l'eau. dans une proportion élevée des zones agricoles africaines. constitue probablement le facteur physique limitant le plus habituel ; on doit espérer cependant une amélioration progressive de la situation dans ce domaine, amélioration d'ailleurs requise pour une meilleure production de riz, indépendamment d'*Azolla*.
- pour autant que la culture d'*Azolla* soit bien pratiquée, ses principaux effets en riziculture sont manifestes : augmentation sensible de la productivité (chaque enfouissement d'*Azolla* étant équivalent à l'application de 30 à 40 unités d'azote/ha), et diminution du développement des adventices (et

donc des travaux de désherbage) ; il faut y ajouter l'économie en eau et l'amélioration de la structure du sol ;

- les tentatives d'introduction de l'azolliculture en milieu paysan sont accueillies diversement selon la structure des exploitations agricoles. Dans les zones à fort encadrement technologique (distribution des intrants assurée, mécanisation poussée...) elles semblent avoir peu de chance d'aboutir, la motivation des agriculteurs étant insuffisante pour soutenir un effort prolongé de maîtrise des pratiques culturales requises. Ailleurs par contre, les réactions sont souvent positives, et dans quelques cas, très limités à l'heure actuelle il est vrai, les paysans pratiquent déjà effectivement et de leur propre initiative l'azolliculture.

Discussion et conclusions

Les recherches sur *Azolla* ont considérablement progressé ces 20 dernières années en même temps que s'amélioraient et se diversifiaient les pratiques culturales destinées à tirer profit de cette plante et que s'accroissaient les superficies cultivées.

Depuis quelques temps cependant, un courant d'opinion peu favorable à la poursuite des efforts dans ce domaine se dessine dans certains milieux, les arguments invoqués plus ou moins explicitement étant l'effondrement du prix des engrais de synthèse sur le marché mondial, la diminution de l'emploi d'*Azolla* comme engrais vert en Chine, ainsi que le faible pourcentage de réussite dans les tentatives d'introduction de l'azolliculture dans d'autres pays.

Si l'on considère le cas particulier de l'Afrique, il faut cependant reconnaître que ces arguments sont peu convaincants. Même au prix actuel (qu'il serait sans doute imprudent de considérer comme immuable) l'engrais commercial restera sans doute encore longtemps inaccessible au petit paysan éloigné des centres de distribution et dans ce cas *Azolla* peut représenter une alternative intéressante.

Quant à la régression de l'azolliculture en Chine, il faut signaler qu'elle effecte principalement l'utillisation d'*Azolla en* monoculture, et dans une moindre mesure en culture associée avec le riz, tandis que la production pour l'alimentation animale et en système intégré est au contraire en expansion. Les raisons justifiant cette régression partielle sont l'amélioration de la situation économique qui facilite l'accès aux engrais commerciaux, la diminution de la main d'oeuvre disponible dans les campagnes et la nécessité de consacrer la totalité du calendrier à la production alimentare, compte tenu de la forte densité de population ; c'est ainsi que la monoculture hivernale d'*Azolla* y est de plus en plus souvent remplacée par des cultures maraîchères ou de blé d'hiver. De telles raisons ne peuvent pas être invoquées dans les conditions générales actuelles de l'agriculture africaine.

Il est vrai enfin que les programmes de recherche tendant à introduire l'azolliculture dans plusieurs pays d'Asie n'ont pas souvent (mais parfois) été couronnés de succès. Les causes en sont sans doute diverses. Dans certains cas, des conditions similaires à celles prévalant actuellement en Chine peuvent être invoquées ; dans d'autres, les conditions du milieu sont effectivement défavorables au développement d'*Azolla* (abondance de prédateurs, température et humidité excessives, alcalinité du milieu favorable à la prolifération des algues...), dans d'autres, enfin c'est l'insuffisance des moyens mis en œuvre pour la recherche...), la formation et/ou l'encadrement qui est en cause. Pour de nombreuses régions d'Afrique les deux premières raisons ne peuvent certes être invoquées,

et les résultats de la recherche sont en outre dés à présent suffisamment positifs pour y justifier l'introduction de l'azolliculture.

La question se pose dès lors de savoir quels sont les moyens à mettre en œuvre pour que le paysan africain tire effectivement parti de ces acquis. L'expérience *montre* qu'un encadrement suivi est essentiel; au moins dans un premier temps. Un personnel compétent doit donc être formé et des conditions de travail lui permettant de se tenir en permanence à la disposition des paysans doivent lui être fournies. La prise en charge de ce personnel sera assurée selon les cas par des réseaux nationaux de vulgarisation, des coopératives, des organisations non gouvernementales. Si on se refère aux pays où *Azolla* est effectivement valorisé à l'heure actuelle; 'force est de constater que le développement de l'azolliculture y a toujours été promu de manière très directe par les pouvoirs publics qui y ont vu un investissement, non négligeable, sans doute, mais rentable à terme ; en cas de réussite de l'opération les dépenses consenties présentent en effet le triple avantage de ne pas requérir de devises étrangères, de n'être pas récurrentes (contrairement aux dépenses de subsidiation des engrais) et de toucher une tranche de paysannat particulièrement défavorisée. Il faut noter enfii qu'une telle stratégie ne comporte pas de risques élevés, compte tenu de ce qu'elle peut fort bien être testée, à titre probatoire, dans des régions limitées. Son succès dépendra du choix des zones d'introduction ; certaines sont en effet plus prometteuses que d'autres (maîtrise de l'eau, conditions climatiques, structures du paysannat) ; il dépendra peut-être surtout de la formation du personnel d'encadrement et de sa disponibilité sur le terrain.

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Phosphorous needs and accumulation potential of various *Azolla* species and strains

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Abstract

It is know-n that *Azolla* are able to accumulate phosphorus when grown on P-rich media, and it has been suggested to take advantage of this luxury consumption, loading up *Azolla* with P in the nurseries before inoculating them into the rice field, where they could continue to multiply using their excess P. The purpose of the present study was to identify *Azolla* ecotypes with high accumulation power and/or eventually low P needs, comparing 20 high temperature tolerant strains. Four *A. caroliniana* (ACA), 4 *A. microphylla* (AMI), 6 *A. pinnata var. imbricata (API)* and 6 *A. pinnata var. pinnata* (APP) have been grown first on N-free Hoagland solutions (dihlted 2/5) containing respectively 15, 30, 45 and 60 ppm P for 18 days. None, or only slight effects of the treatments are observed on productivity, but *Azolla* P content increases linearly with external P concentration, strongly in ACA and AMI, only slightly in API and APP, and varies widely from one strain to another, even *from the same species.* When such variously P-loaded *Azolla are* transferred (60 g/m^2) on a P-free medium, the biomass they reach at the plateau phase is strongly dependent on the pretreatment for ACA and AMI, not for API and APP ; at this stage *Azolla* P content varies from 0.023 to 0.106% (D.M.), but is not correlated with final biomass nor with species.

Introduction

Phosphorous is frequently the limiting factor of *Azolla* productivity (Tung and Watanabe, 1983 ; Lumpkin, 1987) ; on the other hand *Azolla* is able to make some luxurious P consumption (Lumpkin, 1987 ; Reddy and Debusk, 1984 ; Subudhi and Singh, 1979 ; Subudhi and Watanabe, 1981), and it has been suggested to make advantage from this property, enriching *Azolla* in the nurseries before field inoculation (Lumpkin, 1987).

Few data are nevertheless available on the growth potential of P-preloaded *Azolla* when cultivated on P deficient media.

The purpose of the present research is to identify *Azolla* ecotypes with high accumulation power and/or low P needs ; the investigation is restricted to a group of high temperature tolerant strains.

Material and Methods

Twenty *Azollu* straius from the ADUL collection (Van Hove *et* al. 1987). previously selected among 150 for their tolerance to high temperatures (unpublished results) are tested in this experiment : four *A. caroliniana* (ACA) : ADUL 8, 61, 109, 113 ; Four *A. microphylla* (AMI) *: ADUL 65,* 66, 104,175 ; six *A. pinnatu var. pinnata* (APP) : ADUL 98, 99, 111, 129, 136, 144 and *six A; pinnata var. imbricata* (API) *: ADUL 6, 26, 87,* 92, 100, 137.

Experiment 1 : four culture baths containing 40 plastic boxes with the bottom replaced by a mosquito net (Van Hove *et al.* 1987) are filled with 120 1 of diluted (4/10) Hoagland solution in which nitrates are replaced by chlorides and phosphorus is added as KH_2PO_4 at respective concentrations of 15, 30, 45 and 60 ppm P (initial pH : *6.00,* 5.29, 5.19, 4.89). Each box is inoculated with lg (F.W.) *Azolla,* every strain being duplicated, at random, in the four culture baths. Culture media are replaced after 9 days. The experiment is realized in a greenhouse in which conditions are : light intensity : natural + 65 $\mu E.m^{-2}S.^{-1}$; air t° : 35 ± 4°C ; water t° : 33 ± 4°C ; relative humidity : *47 ± 5%.*

On day 19, *Azolla* samples are harvested and their fresh weight measured after 1 mn centrifugation (16 g) of the *Azolla* containing boxes, followed by 20 s drainage on absorbent paper ; 1 g of each sample is set apart as inoculum for the second experiment ; the remaining biomass is used for dry weight measnrement and phosphorous analysis.

Experiment 2 : The device is similar to the preceding one except that the culture medium (initial pH : 6.05) is P-free, and is renewed every 7 days ; air t° : 38 ± 5°C ; water t° 37 ± 4°C ; relative humidity : 51 ± 5%. Each box is inoculated with 1 g of variously P-enriched *Azolla* from the first experiment (60 gm^{-2}).

Fresh weight is measnred as above, every 2, 3 or 7 days according to the growth stage until the plateau phase is reached (two successive not increasing biomass values). *Azolla* P content is measured at the end of the experiment.

Chemical analysis : Total phosphorous is determined colorimetrically as its reduced phosphomolybdate complex, after mineralization in diluted nitric acid.

Results

Experiment 1 : Effect of P concentration in the medium on *Azolla* growth and P content.

Productivity - Under the prevailing experimental conditions *A. caroliniana and A. microphylla are the* most productive species, followed by *the A. pinnata varieties* (Table 1). Increasing P concentration from 15 to 60 ppm has no or only slight effects on productivity.

Table 1 : Mean productivity and standard deviation (gFM/m2.day) of 4 Azolla species or varieties as a function of the external P concentration (ppm P).

Species	15 ppmP	30 ppmP	45 ppmP	60 ppmP
ACA	122.2 ± 4.8a	120.8 ± 3.7a	118.8 ± 5.0a	116.8 ± 5.3a
AMI	116.9 ± 6.6a	118.0 ± 7.6a	11955 ± 4.4a	111.0 ± 9.0a
API	75.9 ± 4.1b	'70.1 ± 5.5b	72.7 ± 8.8b	70.4 ± 7.6b
APP	62.7 ±14.9c	63.9 ± 3.0b	68.7 ±10.3b	66.3 ± 9.5b

In a column, values with the same letter are not significantly different at P = 0.05 (Duncan test).

Phosphorus content - Internal P concentration at the end of the experiment (Fig. 1) varies considerably from one strain to another in a given species or variety. At low (15 ppm) external P concentration, there is nevertheless no specific or varietal difference (Table 2) but P content increases linearly with increasing external concentration, slightly in *the* two *A. pinnata* varieties. for which angular coefficients of the regression line (internal P content (%)/medium P content (ppm)) vary from 0.003 to 0.009, strongly *in A. caroliniana* and *A. microphylla (angular* coefficients : 0.01 to 0.02).

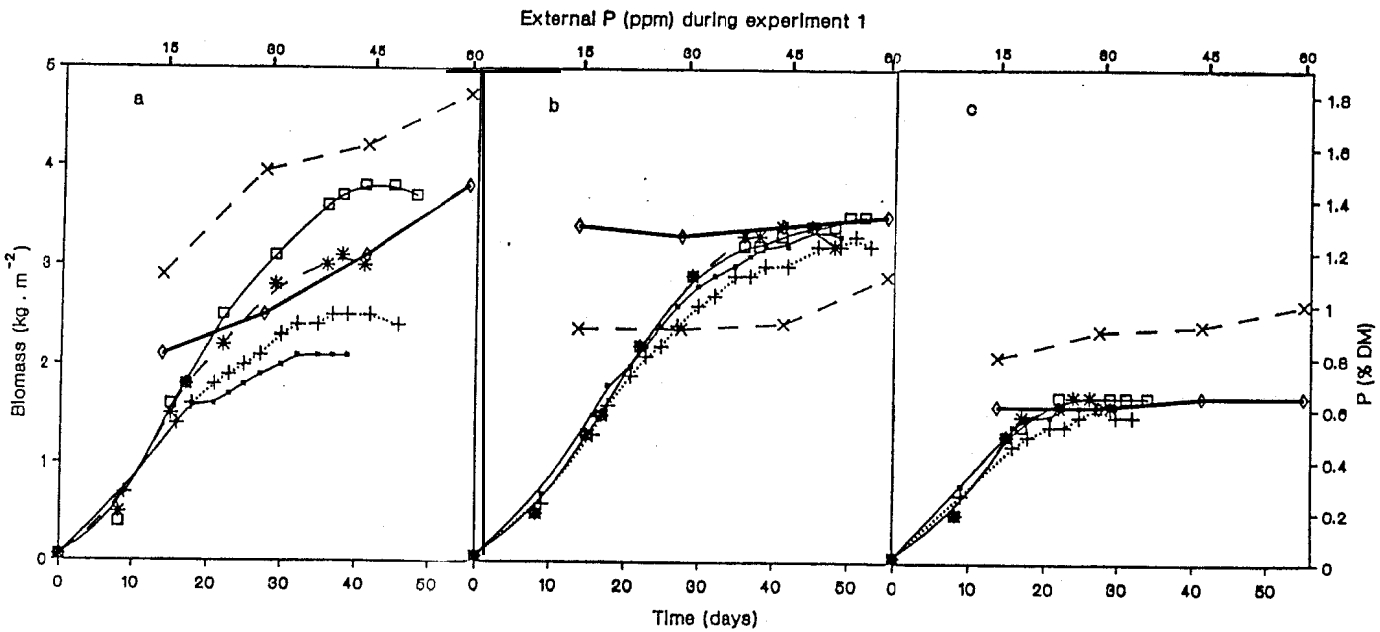

External P (ppm) during experiment 1
15
30
45
60
a
b
c
Biomass (kg . m -2)
P (% DM)
Time (days)
0
10
20
30
40
50
1
2
3
4
5
0.2
0.4
0.6
0.8
1.2
1.4
1.6
1.8

Figure 1 : Three typical behavior of 20 strains of *Azolla* species or varieties growing on a P deficient medium after a pretreatment on 4 P concentrations : ACA & AMI (a), best APP (b) and API or other APP (c).

Growth curves as biomass in $kgFM/m^2$ related to time in day after pretreatment at 15 (i)), 30 (+), 45 (✱) and 60 ppm P (◘).

Maximum biomass in $kgFM/m^2$ (◊) and final P content in %DM (✕) related to P concentration in ppm P of the pretreatment medium.

Table 2 : Final P content and standard deviation (%DM) of 4 Azolla species or varieties as a function of the external P concentration (ppm P).

Species	15 ppm P	30 ppm P	45 ppm P	60 ppm P
ACA	0.94 ± 0.14a	1.05 ± 0.07b	1.32 ± 0.12a	1.53 ± 0.16a
AM1	1.00 ± 0.19a	1.25 ± 0.17a	1.39 ± 0.18a	1.59 ± 0.15a
API	0.93 ± 0.16a	1.01 ± 0.18b	1.11 ± 0.20b	1.20 ± 16b
APP	0.88 ± 0.16a	0.96 ± 0.176	0.99 ± 0.13b	1.11 ± 0.11b

In a column, values with the same letter are not significantly different at p = 0.05 (Duncan test)

Experiment 2 : *Azolla* growth in P-free media as influenced by initial P content.

Maximum biomass - Maximum biomass for a given pretreatment (Table 3) is highly strain dependent *in A. pinnata* var. *pinnata* (high standard deviation), not in *A. pinnata var. imbricata* which always shows low plateau values, or in the two other species. As expected from results obtained in the first experiment, *A. caroliniana* and *A. microphylla* final biomasses are highly influenced by the pretreatment, whereas *A. pinnata* are practically not. Fig. la and lb illustrate these two typical growth patterns. Also for *A. caroliniana* and *A. microphylla* there is a good correlation, at the species level, between initial P content and maximum biomass. For *A. pinnatu* on the other hand such a correlation only exists at the strain level as illustrated in Fig. lb and c.

Final **Azolla P content** - Azolla P content at the plateau phase (Table 4) is generally higher *in A. microphylla* and *A. caroliniana than* in *A. pinnata*, which presents very high intraspecific variations. It must be mentioned that the observed values are higher than the calculated values (initial P content. initial biomass. final biomass') ; this has to be attributed to traces of P brought in the system by demineralized water compensating evapotranspiration.

Table 4 : Final internal P content and standard deviation (% DM) of 4 Azolla species or varieties cultured in a P deficient medium after a pretreatment at different P concentration (ppm P)

Species	Pf(%DM)
ACA	0.076 ± Ø.ØØ9 b
AMI	0.087 ± 0.013 a
API	0.056 ± 0.022 c
APP	Ø.Ø41 ± Ø.Ø18 d

In a column, values with the same letter are not significantly different at p = Ø.Ø5 (Duncan test).

Table 3 : Maximum biomass yield and standard deviation (kgFM/m2) of 4 Azolla species or varieties cultured in a P deficient medium after a pretreatment at different P concentration (ppm P).

Species	15 ppm P	30 ppm P	45 ppm P	60 ppm P
ACA	2.4 ± 0.4ab	2.7 ± 0.3a	3.4 ± 0.3a	4.1 ± 0.4a
AMI	2.0 ± 0.3bc	2.4 ± 0.3a	2.9 ± 0.4b	3.6 ± 0.5b
API	1.6 ± 0.3c	1.8 ± 0.2b	2.1 ± 0.3c	2.Ø ± 0.3d
APP	2.5 ± 0.8a	2.6 ± 0.6a	2.7 ± 0.6b	2.9 ± 0.6c

In each column, values with the same letter are not significantly different at p = Ø.Ø5 (Duncan test)

Discussion

Subudhi and Watanabe (1981) have *shown* that *A. pinnuta* growth does not improve when external P concentration increases from 5 to 10 ppm, whereas Singh *et al (1984)* obtained *growth* increases up to 15 ppm P (the highest concentration *tested)* for *A. filiculoides* and *A. mexicana,* to 10 ppm for *me A. pinnata* strain, and to 5 ppm for another. Cary and Weerts (1982, 1984) observed better *A. filiculoides* growth at 20 ppm than at 5 ppm ; for A. *pinnata* optimal growth was reached in the 5 to 20 ppm range, with a significant decrease at 40 ppm.

In the present study, the P concentration range was extended to 60 ppm, which is very high according to agricultural practice standards, with the view of testing phosphorus accumulation potential of various *Azolla* species and strains. Results are generally in agreement with previous information, except for the fact that no depressive effects of high P concentration were generally noticed

Subudhi and Watanabe (1981) also found that P content *in A. pinnata increases* linearly with increasing external P concentration between 0 and 10 ppm. We observed the same behaviour for the 20 strains tested between 15 and 60 ppm P ; the fact that productivity is not affected in this range of concentration confirms the luxury consumption capacity of *Azolla* (Lumpkin and Pluchnett, 1982). Significant interspecific differences nevertheless exist, *A. caroliniana* and *A. microphylla* having a higher accumulation power *than A. pinnata ;* considering that they are also characterized by a good correlation between P content and maximal biomass, over fertilization of these two species at the nursery level seems justified. If P fertilizer availability is restricted on the other hand, some *A. pinnata var. pinnata* should be preferred.

Looking at the *Azolla* P content at the plateau phase in P deficient culture media, it appears once more *that A. caroliniana* and *A. microphylla* differ from *A. pinnata, with* a higher P content and a lower intraspecific variability. The extreme values are 0.023% and 0.106% ; assuming a 1.2% P content in the inoculum, which is easily obtained with a good P fertilization in the nursery, this means biomass multiplication potentials, in a P-free medium, of respectively 52 and 11. *Azolla* strains with low minimal P content have thus certainly to be considered in selection program. More information should nevertheless be necessary concerning N content of *Azolla* in these extreme conditions since it is known (Watanabe *et* al., 1980) that P and N contents are correlated.

Furthermore, the capacity of a given strain to reach a high biomass in P deficient culture media is not attributable to lower P needs, final biomass and final P content being poorly correlated (r = *0.35);* this means that strains having similar initial and final P contents can differ by their final biomass, due probably to differential capacities of their root system to extract traces of phosphorus present in the culture medium, as suggested by Subudhi and Watanabe (1981).

Conclusion

The aim of the present research was to estimate the range of phosphorus needs and phosphorus accumulation power of various *Azolla* species and strains, with the view of introducing, eventually, such criteria in *Azolla* selection program.

Based on a limited number of strains, the conclusion is that the two criteria have to be considered, somme *strains* from the *A. pinnata* group being characterized by their low treshold P level, some *others,* from *the A. caroliniana* and *A. microphylla* group having a high phosphorus accumulation power.

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Sensitivity to Aluminium of various *Azolla* species and strains

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Abstract

High aluminium concentration is a major problem in many acidic tropical soils, where it interferes among others with phosphorus availability, an element especially important for *Azolla*. Information on *Azolla* sensitivity to Al is nevertheless missing, and it was the purpose of this preliminary research to estimate the range of Al concentrations allowing proper *Azolla growth* and to select, eventually, Al tolerant strains. Twenty high temperature tolerant strains, four A. *caroliniana* (ACA), four *A. microphylla (Ah@, six A. pinnata var. imbricata (API)* and six *A. pinnata* var. *pinnuta* (APP), were grown on N-free 2/5 Hoagland solutions with respectively 0, 2, 5 and 8 ppm Al, and their biomass (fresh matter) was measured after 11 days. Results show that, according to the strain productivities decrease with increasing Al concentration either ± linearly or exponentially. At the species level API is the most sensitive, followed by ACA and AMI and then by APP. Intraspecific differences are nevertheless high, except for API strains. According to the strain, productivities at 2 ppm vary from 100 to 50% and at 8 ppm from 73 to 16% as compared to the control.

Introduction

Aluminium toxicity is an important growth limiting factor for plants in many acidic soils, in which Al is easily solubilized. Al toxicity can induce mineral nutrition disturbances, such as P (Randall and Vosc, 1963) and Ca (Awad *et al.*, 1976), uptake decrease ; it also inhibits cell division (Horst *et al.*, 1983).

As far as the aquatic fern *Azolla* and its endosymbiont *Anabaena are* concerned, only scarce information is available (Lumpkin and Plucknett, 1982). The purpose of the present research is to estimate the range of Al concentrations allowing satisfactory *Azolla* growth, and to select eventually Al tolerant ecotypes (among a group of high temperature tolerant strains).

Material and Methods

Azolla strains as well as the experimental device are identical to those described by Desmadryl *et* al., (page 118 in this volume). Culture media contain 0, 2, 5 and 8 ppm Al as Al, $(SO_4)_3.18H_2O$), their respective pH being 4.80 ± 0.41, 4.14 ± 0.11, 3.72 ± 0.04 and 3.59 ± 0.06 during all the experiment. Each box is inoculated with 5 g (FW) *Azolla* (± 300 g/m^2) and each strain is duplicated. The conditions in the greenhouse are : light intensity : natural + 65 $\mu E.m^{-2}.s^{-1}$; photoperiod 12/12 h; air t° : 31 ± 2°C; water t° : 30 ± 2°C; relative humidity : 55 ± 5%.

After 11 days *Azolla* samples are harvested and their fresh weight measured after 1 mn centrifugation (16 g) and 20 s drainage on absorbent paper.

Results

In the conditions of the experiment, the productivity of the controls (0 ppm Al) varies strongly between strains, but *also* between species and varieties (Pig. 1) *A. caroliniana* and *A. microphylla* are very similar and the most productive, followed by *A. pinnata var. pinnatu* and finally by *A. pinnata var. imbricata.* Productivity of all the strains decreases with increasing Aluminium concentration, either exponentially, for the most sensitive strains, or more or less linearly for the others. Concentrations of 2 and 5 ppm Al only slightly affect growth of tolerant strains, while for the sensitive ones, growth is already strongly depressed. At 8 ppm Al only one strain (ADUL 136 PP) grows very poorly. *A. pinnata var. pinnata* is the most tolerant variety (Table 1), *A. pinnuta var. imbricata the* most sensitive, the two other species being intermediate. Intraspecific and intravarietal differences (except for *A. pinnatu var imbricata)* are nevertheless high.

Discussion and Conclusion

Sensitivity to Al varies widely among plants. *Gossypium hirsutum* root growth for example stops above 0.5 ppm Al (Rios and Pearson, 1964), other plants are affected at 1.5 ppm Al (Foy and Brown, 1964) whereas *Vigna unguiculata* sustains 2 ▪ 4 ppm (Horst *et al.*, 1983), *Sorghum bicolor* 8 ppm (Furlani and Clark, 1981). *Pennisetum typhoides*, 16 ppm (Long *et* al., 1973) and *Camelia Sinensis* up to 27 ppm at pH 6 (Matsumoto

et al., 1976). *Oryza sativa* which is of special concern here, is not depressed at 10 ppm at the adult stage (Howeler and Cadavid, 1976), whereas seedlings are quite sensitive (Thawomwong and Van Diest, 1974).

Compared to these plants, *Azolla* in general thus seems moderately tolerant to Al. It must be mentioned that growth inhibition observed in this experiment at high Al concentrations could be partly attributed to pH effects ; nevertheless it is well known that *Azolla* is able to grow properly in a very wide range of pH (Nickell, 1961 ; Lumpkin and Plucknett, 1982 ; Van Hove *et al.,* 1983).

In view of the wide *Azolla* species and strains Aluminium sensitivity differences, selection based on this characteristic has to be considered for *Azolla use* on Al rich soils.

Table 1 : Productivity as compared to the control and standard deviation (%) of Azolla species or varieties as influenced by the external Al concentration (ppm Al).

Species	2 ppm Al	5 ppm Al	8 ppm Al
APP	90.51 ± 11.81a	63.46 ± 16.60a	46.52 ± 15.27a
ACA	80.42 ± 13.52ab	45.07 ± 16.16b	33.76 ± 14.19b
AMI	76.20 ± 13.50b	30.33 ± 13.84b	23.01 ± 7.67bc
API	61.74 ± 8.70c	33.06 ± 7.77b	21.70 ± 5.09c

In a column, values whith the same lettre are not significantly different at P = 0.05 (Duncan test)

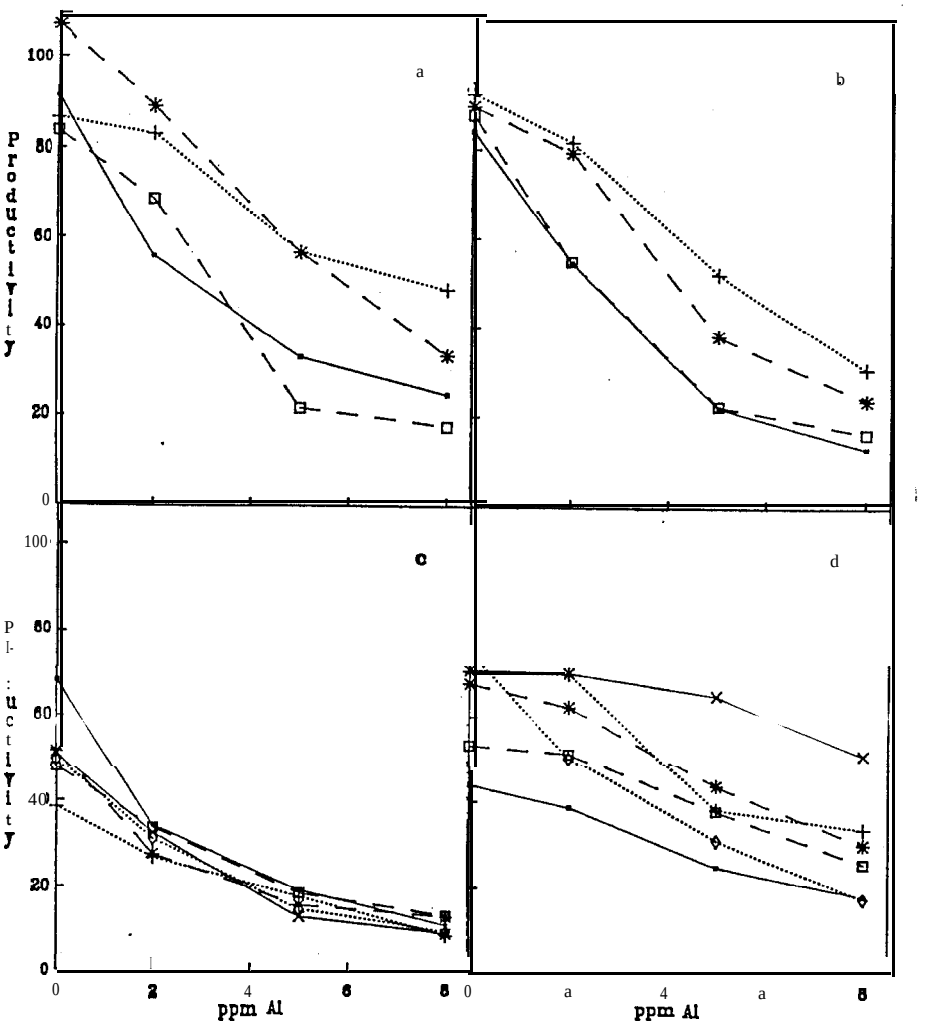

a
b
c
d
Productivity
Productivity
100
80
60
40
20
0
0
2
4
6
8
ppm Al
ppm Al

Figure 1 : Productivity (g/m². day) of 20 *Azolla strains as influenced by the* external Al concentration (ppm Al). Strains are characterised by their entry number in the ADUL collection

(a) ACA	:	8, 65,	61,	109	113.		
(b) AMI	:		66,	104:	175.		
(c) API	:	6,	26,	87.	92,	100,	137.
(d) APP	:	98,	99.	111.	129,	136.	144.

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INOCULATION :
PRODUCTION D'INOCULUM
ET ESSAIS D'INOCULATION

à couper –

Maximisez la FBA pour la Production Agricole et Forestière en Afrique

Production and uses of rhizobium inoculants

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Abstract

The inoculation of legumes with *Rhizobiwn* inoculants *is one* of the few cases of man's successful exploitation of microorganisms for avp production. This biotechnology is outstanding in its wide applicability in different agricultural environments. We outline here background information on the various aspects of legume inoculant technology, namely : strain selection and characteristics, preparation and properties of carriers, growth and survival of rhizobia, quality control program from strain selection through preparation and distribution of final products. Criteria used in the selection of *Rhizobium strains* for inoculation include prompt effective nodulation over a wide range of field conditions and root temperatures, ability to grow well in culture, to grow and survive in carrier and to survive on the seed. Other characters are competi-tiveness in nodule formation and survival and multiplication in the soil, pH tolerance, pesticide tolerance and nodulation in the presence of combined nitrogen The success of legume inoculation depends on both the quality of inoculant preparation and the methods used to introduce rhizobia into soil at sowing. Inoculant carriers must be selected accord+ to availability of material and technologies, and must permit the introduction of the highest possible number of infective cells in the soil where the host plant will first accept an infection Finely ground peat is the rhizobial carrier most extensively used. New technologies such as polymer-entrapped microorganisms, and concentrated freezedried cells powder inoculants are arriving on the market A quality control program is an essential part of inoculation technology.

Introduction

Food and fibers production is often limited by low soil fertility and nitrogen appears to be one of the most limiting factors. Industrially manufactured nitrogenous fertilizers are not afordable for most of developing countries that want to intensify their agricultural production. Therefore, the alternative of using some microorganisms to convert atmospheric nitrogen to a form available for the nutrition of higher plants was extensively exploited

The nitrogen-fixing symbiosis between leguminous hosts and their rhizobia is an outstanding biotechnology because of its wide applicability in diverse environments and agricultural systems. The *inoculation* of legumes with *Rhizobium inoculants is one* of the few cases of man's successful exploitation of microorganisms for agricultural purposes. This biological system has attracted considerable attention of scientists having diverse backgrounds because of the complexity of the phenomenon which demands the application of many skills for its understanding and more successful utilization. However, there is a real risk when the enthusiastic non-microbiologist moves in to invest&ate a system in which a bacterium, which may be more difficult to control, plays its own major role in an intimate association with the more tractable host. Whether he is plant physiologist, biochemist, geneticist, soil scientist, or even a microbiologist unfamiliar with rhizobium, a worker coming into this field cannot afford to take this microorganism too lightly. Difficulties can be encountered by the relatively slow development of even the faster-growing rhiiobia, leaving them open to overgrowth by a contaminant, by the gummy nature of its growth, increasing the likelihood of an associated organism remaining undetected even with single colony isolation. Furthermore, the difficulty one has to definitively distinguish rhizobia from some other genera, apart from demonstration of nodulating capacity, adds up to the confusion due to contamination or wrong identification and may lead to a whole body of developmental work which is bias to the practice of legume inoculation. In such a case, either the newcomer takes the trouble to familiarize himself sufficiently with the nature of the microorganism he is dealing with, and the technique needed for its purification and maintenance, or he works cooperatively with someone who has this knowledge and technical capacity.

In this presentation, we will outline background information on the various aspects of legume inoculant technology, including factors affecting quality and technique for quality control

Selection of rhizobia for inoculants

Many of the failures to establish rhizobia in soil successfully may be attributed either to insufficient or unsuitable rhizobia in the inoculant. As agriculture extends further and further into regions which are marginal because of low and/or erratic rainfall and poor fertility, rhizobia will be called upon to perform with a wider range of host plants and environmental and edaphic conditions. The selection of suitable strains of rhiiobia is the first step in the produciton of legumes inoculants. At one time, nitrogen fixation efficiency was the only criterion seriously considered for a good inoculant strain, but now there are many that should be considered as well : (i) competitive ability with other strains, including indigenous population, for prompt effective nodulation of the host legumes ; (ii) nitrogen fixing ability over a wide range of environmental conditions ; (iii) nodule-

forming and nitrogen-fixing abilities in the presence of soil nitrogen ; (iv) ability to multiply in broth and to survive in carriers ; (v) ability to survive on the seed ; (vi) ability to survive in adverse physical and physiological conditions such as desiccation, heat, freezing, low soil fertily ; (vii) tolerance to pH changes, pesticides ; (viii) genetic stability during storage and growth.

In the context of inoculant production, strain competitiveness and specificity refer to effectiveness in forming rapidly an effective association with a particular host (Antoun et al., 1979). The host legume exerts a strong influence on the effectiveness of the symbiotic association in fixing N_2, and strain specificity exists for particular hosts (Gueye and Bordeleau, 1988), while interactions between the symbiotic association and the environment have been also described (Brockwell, 1980 ; McLoughlin et *al.,* 1984). These create a dilemma in deciding wether to produce numerous inoculants with highly effective strains for individual host or to use a «wide-spectrum» strains that vary from good to excellent with a range of legumes. The use of multiple strain inoculants should be avoided due to possible antagonistic and competitive effects within the culture and the likelihood of competition in nodule formation from less effective strains in the inoculant (Bordeleau and Antoun, 1977). This problem poses a delicate situation for microbiologists who must decide between practical aspects of inoculant production and marketing, and scientific *excellence* in *providing the best* available material for inoculant production.

As a rule, for each legume that we want to improve yields through nitrogen fixation, it is preferable to select strains of rhizobia directly from the cultivar we intend to use and then check the efficiency. The main criterion used in selection is the amount of N_2 fixed, either assayed directly, or indirectly by determining plant dry weight (Bordeleau et *al.*, 1977). The latter method integrates all the factors that influence the symbiotic system. With grain legumes, pediodical measurements of biomass and nitrogen content of plant parts during the development and at maturity will permit to calculate the harvest index of a strain, since it is known that plant inoculation with *Rhizobium can* modify the nitrogen distribution in the plant. Tests in controlled environment provide reliable comparative information on the ability of a number of strains to fix N_2, but they do not evaluate ability to colonize the rhizosphere, to compete for nodule sites, or to persist in the soil. In the other hand, field tests cm evaluate only a limited number of strains because of demands on time, labor and equipment. Both systems, therefore, are commonly used in sequence, the former to select a set of strains with above average N_2-fixing capacity and the latter for an overall evaluation of the strains' ability to fix N_2 under a variety of field situations. Specific separate tests can be conducted if a particular character is bemg evaluated. However, field trials provide the ultimate test of a strain's performance, since N_2 fixation and the proportion of nodules formed by the inoculum are the end result to the interactions of all the factors involved.

Large-scale production of rhizobia

The first step in the production of legume inoculant is massive production of a selected *Rhizobium* species in liquid medium. Rhizobia are heterotrophic and generally not highly demanding in their nutrient requirements.

Different media have been proposed, they are composed of a carbon source, a source of nitrogen and growth factors, and different minerals such as potassium phosphate, magnesium sulfate, and sodium chloride. Mannitol and sucrose are the conventional

carbon sources used in routine media, although growth of slow growers on mannitol is variable. The most satisfactory carbohydrate is glycerol, on which slow growers have shorter generation tunes than on glucose, mannitol, galactose, or sucrose (Stowens, 1985). Yeast extract can be used as the sole carbon and nitrogen source ; however, high concentrations cause cell distortion and even growth inhibition of certain *Rhizobium*, which can be counteracted by calcium. The economy of large-scale production is largely governed by the price and availability of a suitable carbon source. Corn-steep liquor, proteolysed pea husks, malt sprouts, whey and industrial-grade yeast extract have been proposed as media for *Rhiwbium* production at the industrial level (Meade et *al*., 1985 ; Bissonnette et *al*., 1986). All of these materials ate industrial by-products which contain growth factors, nitrogen and carbon. Efficient utilization of a carbon source often depends on aeration, method of sterilization and equipment available. For the production of inoculants, a high-count broth is required to ensure a highquality inoculant ; increased numbers of viable cells may be achieved by varying the source and concentration of materials according to the individual strain requirements. A- short fermentation time for the broth culture is desirable in order to reduce contamination risks. A large inoculum, providing 10^6-10^7 rhizobia/ml culture medium at the beginning of fermentation, reduces the time required to reach maximum viable numbers, and, therefore, reduces the chance and effects of contamination. Fast-growing strains reach maximum viable number in 30-40 hours, compared with 80-120 hours for slow-growing strains. Temperature of incubation for rhizobia is generally kept at 26-28°C, although few strains can be produced at 32-35°C without affecting their nodulation ability.

Facilities for multiplication of rhizobia in liquid culture vary from elaborate large-capacity (10002000 liters) industrial fermentors. to simple flasks or drums (10-100 liters) (Date and Roughley, 1977). Good fermentors can be fabricated from steel drums fitted with brass inlet, outlet, inoculation and sampling ports The air inlet extends to the bottom to provide stirring of the broth. Proper filtration systems sterilize the air from an external supply. The vessel with filters and outlet tubes attached and two-thirds filled with medium is sterilized by autoclaving. Maximum infective rhizobia after incubation should be in the order of 10^9-10^{10} cells/ml.

Carriers of rhizobia and their preparation

High-quality legume inoculants is dependent on selection of suitable carrier materials and their pre-treatment (Kremer and Peterson. 1983). An assessment of the literature on carriers of rhizobia leaves the net impression that peat is still unchallenged as a carrier and that it is relatively easy to devise a substrate from a variety of materials that would support satisfactory growth and survival of rhizobia (Burton, 1976 ; Brockwell, 1980). As a rule, carriers must have a high water-holding capacity, provide a nutritive medium for growth of rhizobia and enhance survival during storage, distribution and when inoculated onto seed. Various carriers of organic origin have been investigated These include : soil and peat mixtures, soil plus charcoal, Nile silt plus nutrients, soil plus coir dust, coal with and without nutrients, composted maize cobs, bagasse, filter mud from sugar cane, cellulose powder, rice husk, bentonite, ground common talc.

The most important factors to consider before selecting a particular carrier are the availability and characteristic of the material. Whereas the choice may be aided by a chemical analysis, actual multiplication and *survival* studies of *Rhizobium* strains in

it are essential. Finely ground peat is the rhizobial carrier used most extensively in conventional legume inoculant production. After proper neutralisation with $CaCO_3$, and milling to as fine a particle size as possible, broth culture of rhizobial strain containing at least 10^9 rhizobia/ml are mixed with dry unsterilized peat in bulk and then matured in shallow trays for 72-96 hours at temperatures near 26°C. Inoculants prepared with unsterilized peat usually contain up to 100-fold fewer rhizobia than sterilized peat Packaging is done in polythene bags, moisture content corresponds to 35-40 % on a wet weight basis. Rhizobia grow and survive better in sterile than in unsterile peat. The extent of this difference is dependent on the suitability of the peat and the temperature and duration of storage. The choice of a method for sterilizing peat depends on the type of container in which the culture is to be packed, the number of cultures to be prepared, and the availability of sterilizing facilities, Autoclaving, ethylene oxide treatment, and gamma irradiation are used. Sterilized peat in sealed polythene bags is inoculated by injecting enough broth culture to raise the moisture content to 50-60 %. The puncture is sealed with adhesive tape, the peat and broth are blended by manipulation and then incubated at 26°C for 2 weeks followed by storage at 4°C. High quality inoculants containing up to 10^{10} rhizobia/g can be prepared by this method (Roughley and Pulsford, 1982).

Alternative carriers to *prepare Rhizobium* inoculant are polymer gels ma& of polyacrylamide, alginate, or xanthan. They were successfully tested 10 years ago by Dommergues *et al.* (1979). Alginate-based polymer-entrapped *Rhizobizun* inoculant contain 10^9 to 10^{11} cells/g dry material of high survival ability *Rhizobium* (Diem et al., 1988). However, this technology requires specialized equipment and a certain level of expertise that are not yet adapted for use in developing countries.

Improved technologies for dried microbial cells preparation through freeze-drying processus, in which various protectants are added, were recently applied for *Rhizobium* inoculants. Thus, high number of rhizobia can be added to seed because of the very high count of the inoculant so prepared. *Rhizobium can* survive for a very long time in these concentrated inoculants (E. Brochu, Rosell Institute, Montreal, Canada, pers. comm.).

Survival of rhizobia

Survival ability of rhizobia throughout the manufacturation and uses of inoculants are very important, since the inoculum must contain very high number of rhizobia if *success* of nodulation *is to* occur in field situation. Survival of *Rhizobium has* been studied in culture media (Bordeleau and McLoughling, 1982), in carriers (Kremer and Peterson, 1983), in the seed environment (Burton, 1986) and in soils (Bushby and Marchall, 1977). Cultural factors and conditions may affect survival ability ; it is generally recognized that younger *cells* of *Rhiwbiwn* survive better when subjected to stress condition because they have more endogenous energy available to them to overcome the adverse conditions (Bordeleau and McLoughlin, 1982). In liquid medium, survival ability of fast growing *Rhizobium* is not significantly affected by limited amounts of carbon, phosphorus and potassium in growing medium. However, under desiccation, survival is poor unless cells are protected by agents such as lactose. It appears that cell leakages, and therefore cell damages, are limited by lactose and the exact nature of this protection is unknown. Commercial production of fast-growing rhizobia on lactose rich by-product such as whey is now becoming a practice (Bissonnette et *al.*, 1986), not only because this medium permits the production of high concentrated broth (10^{10} rhi-

zobia/ml), but also acts as a good protectant and contributes to high survival. Strains of rhizobia varied in their susceptibility to desiccation, and they have to be selected accordingly.

Survival of organisms plays an important part in microbial ecology, especially when the environmental stress may adversely affect the cell. These environmental stresses are generally the lack of nutrients (energy) in the environment. lack of moisture, presence of other organisms, changes in pH, Presence of chemicals, etc. When applied to the rhizobii, the ability of legumes to be properly infected with desirable strain depends on the rhizobia to survive in the carrier, on the seed, and in the soil under adverse conditions until it is ready to infect the host. With the current practice of inoculating the seeds, ability of the rhizobia to survive throughout the process is of the first importance. As mentioned earlier, carriers can be manipulated to sustain high number of cells. However, exposure of inoculants to hot and dry conditions during shipping, storage, and planting often results in decreased numbers of N_2-fixing effectiveness of the rhizobia. Variable inoculant quality is a common problem in the tropics and subtropics where inoculants are often subjected to unpredictable handling and storage during distribution and use. Therefore, inoculant formulation and packaging cannot be overlooked from a practical point of view.

The survival of rhizobia on seed is of the greatest importance since seed pelleting as a method of legume inoculation has received widespread attention and it is now a standard agricultural practice. Rhizobia die quickly on freshly inoculated seed particularly with broth inocula (Burton, 1976 ; Brockwell, 1980). Therefore, protective agent assuring survival of rhizobia and a durable coating the seeds without affecting their germination must be used Lost of humidity is an important factor for the death of rhizobia on the seeds (Salema et al., 1982) and, on the other hand, the coating has to be dried enough to stay stable and to equilibrate with the moisture level of the seed. Other factors must be taken into consideration such as toxicity of the coating, pH and nutrient value. Pelleting of leguminous seeds with organic based inocula and pulverized limestone and nutritive sticking agents such as lactose, gum arabic and cheesewhey provides both a good survival of rhizobia and uniformity of coating. Pelleted seeds are often treated with fungicides to protect them against fungal diseases. Problems arise when legumes inoculants are used in conjunction with pesticide treatments (Tu, 1981). Such treatments may affect the survival and/or the infectivity of *Rhizobium* inoculants, unless resistant strains and compatible doses of pesticides are used (Odeyemi and Alexander, 1977).

Quality control program

The quality of legume inocula depends on both the number of rhizobia they contain and their effectiveness in fixing nitrogen with the intended host. Quality control tests measure the number (quantitative) and identify the type (qualitative) of rhizobia in the inoculant during its recommended useful life (Roughley and Pulsford, 1982). Quality, therefore, is determined by the various strain attributes mentioned earlier. The standards of quality adopted need to reflect both the number of rhizobia required for successful nodulation and the ability of manufacturers to produce such inoculants. The principal reason for quality control is consumer protection, a function normally performed by government agencies. However, such control applies only to the fmal products. Quality control during preparation and before sale is usually the responsibility of manufacturers.

It can be safely stated that the higher the number of infective rhizobia contained in the inoculant, provided the strain is efficient, the better the inoculant. All investigators recognize the importance of inoculant having at least a 10-fold more rhizobia when produced than required at the time of usage to compensate for die-off. Ideally the standard for an inoculant is based on the number of infective rhizobia/seed necessary to initiate prompt and effective nodulation. This number will vary considerably from the favorable conditions of aseptic culture in greenhouse to the highly competitive situation in the field. In practice, standards are strongly influenced by the level of rhizobia that manufacturers can consistently attain and maintain in their inoculants with available technology -Many investigators recommend an inoculum which would provide approximately 10^3 to 10^5 rhizobia per seed for a good nodulation. Even at this level of inoculation and where indigenous soil rhizobial population might be as high as 10^8 cells/g soil, the introduced *Rhizobium* *is so heavily* outnumbered that it needs special advantages in order to become established. In Canada, quality standards of inoculants were established at levels that provide at least 10^3 infective rhizobia/seed for small seeded legumes, and up to 10^5 infective rhizobia/seed for larger seeds such as soybean and peanuts (Bordeleau and Prevost, 1981).

Basically any program should involve both qualitative aud quantitative tests of the broth culture before impregnation of the carrier, fmal carrier culture immediately after manufacturation and before release, and inoculants obtained from the retail outlets. Tests on broths and carriers before release are essential for all batches of culture. Broth cultures should be examined for authenticity by appropriate immunological tests to ensure the identity of the inoculant strain, and for freedom from contamination by absence of growth on glucose-peptone agard and gram-stained smear free of positive cells. Results of these tests are available within 24 hours. The strain in the broth should also be checked for infectiveness and effectiveness by inoculating plants growing on N-free medium in an aseptic environment.

After maturation of the inoculant in the carrier and before distribution, plate counts on sterile carriers should be made to determine the number of rhiibii present and to ensure that contamination is low. Where unsterile peats are used, plate counts are not feasible because no specific media exist to differentiate rhizobial cells, plant-infection tests should be made (Bordeleau and Prevost, 1981). Although, labor tune, space and material consuming, the plant-infection test provides a useful tool to assess identity and number of infective rhizobia in non-sterile carriers. Only after completion of these tests should the inoculant be released for use and an expiry date assigned. The length of the useful life of the inoculant will depend on the characteristics of the packaging and carrier materials, storage conditions, methods of distribution and sale. Therefore, it is impossible to set general expiry date.

The quality control program should include testing of inoculants sampled from retails outlets. Such a program was established in Canada and it contributed greatly to improve the quality of legume inoculant products offered to the farmers. This program provided information on the effect of storage conditions, on the performance of particular inoculant and a check on the labelling and claims by manufacturers. It also provided maximum consumer protection aud more direct assistance to manufacturers by early detection of problems such as poor survival in carrier culture and on pm-inoculated seeds, or failure to produce nodulation on the recommended host-plant.

Field applications of legume inoculants

The act of inoculation concentrates the inoculum close to the position where tbe host plant will first accept an infection whereas competing soil organisms, sometimes much more numerous, are distributed randomly. A major advantage enjoyed by inoculant rhizobii is proximity to a developing legume rhizosphere where it can multiply. Moreover, the time between sowing and germination is a critical period. If the inoculant mortality rate is high due to a soil environment that is physically, chemically, or microbiologically unfavourable, delayed germination can lead to a situation where the rhizobial population is too low to colonize the rhizosphere. The likelihood of nodulation can be increased by reducing the mortality rate of the inoculant, by raising the rate of inoculation, or by decreasing the time to seedling germination. Increased or massive rates of inoculation, may lead to the establishment of the inoculant in the soil micro-habitat by weight of numbers. Alternatively, seed pelleting procedures protect the rhizobia against deleterious environmental factors. We will present here a few methods in usage for inoculant application in the field, with a relative appraisal of each method.

Seed Inoculation.

Dusting. At one time some manufacturers recommended that inoculant be merely mixed with the seed immediately before sowing or sprinkled on to the seed in the seed box. Some culture adheres to the seed by lodging in the micropyle and in scratches and irregularities on the seed coat, and by electrostatic attraction. However, much of it is shed particularly during passage of the seed through machinery. Dusting is certainly the simplest method of inoculation but it is also the least efficient with conventional products, and unless break-through in this method proves a better efficiency. by the use of new products, it is not a recommended method.

Slurry inoculation. *In* order to increase the amount of inoculant adhering to the seed, the culture is applied as a water suspension ; alternatively the inoculant is mixed with moistened seed. The seed must be dried before sowing (not in direct sunlight), but as it dries a proportion of the inoculant is lost. More inoculant can be attached to seed by using an adhesive in the slurry ; e.g. a 10 % solution of household sugar (but sugar lacks tenacity). Adhesives such as gum arabic and substituted cellulose compounds are more tenacious. Caution must be exercised to avoid samples of gum arabic which contain preservatives lethal to rhizobia. The celluloses tend to be rather cheaper and more readily available than gum arabic.

Seed ***pelleting.*** Coating legume seeds with lime to promote nodulation apparently had its origin in Maine, U.S.A., more than 40 years ago. The development of the process as a technique for establishing pasture species on acid soils was begun in Australia in the 1950"s (Roughley and Pulsford, 1982). Its use is now widespread extending beyond more protection against soil acidity. Advantages include protection of the inoculant rhizobii against rhizobiotoxic substances contained in some legume seed coats, unfavourable physical and chemical conditions in soil, competition from the soil microflora, the effects of acid fertilii, and against seed-harvesting ants. Pelleting makes aerial sowing of inoculated seed a practical proposition and ensures better survival of the rhizobia when delays between inoculation and sowing are unavoidable.

Materials used for pellet coatings include calcium carbonate in many forms, dolomite, various grades of gypsum, bentonite and other clay minerals, rock phosphate and other phosphorus compounds, inert materials such as titanium dioxide and talc, soil and humus, and activated charcoal. The main requirements for a good coating material are that it should be relatively close to neutrality in pH and finely ground (90 % at least *passing* 300 mesh). Adhesives used for attaching the coating material to seed include synthetic glues, glues of vegetable or animal origin, gelatin, various sugars, and honey. The adhesive should have sufficient tenacity so that the coating material does not slough off, but should not be so tenacious that the cotyledons are damaged during germination. Adhesives must be free of preservatives.

Seed bed inoculation

There are several situations in which seed application of rhizobia may be an inefficient means of inoculation : (i) preemergence diseases or insect attack may make it necessary to use seed dressings of fungicides or insecticides all of which have some effect on the viability of rhizobia and some of which are very toxic indeed (Odeyemi and Alexander, 1977 ; Tu, 1981) ; (ii) inoculation for broad-acre sowings of crop legumes with high seeding rates is a major task, limiting the speed of the sowing operation ; (iii) some *seeds are too* fragile *to be inoculated, e.g. shelled peanuts* (*Arachis hypogaea* L.) ; (iv) some legumes such as soybean (*Glycine max* (L.) Merr.) and subterranean clover (*Trifolium subterraneum*) *lift the seed coat* out of the soil during emergence so that the rhizobia on the seed coat are not deposited in the soil ; (v) the dimensions of the seed surface place a restriction upon the number of rhizobia which may be applied ; sometimes, especially when small-seeded legumes are used or where naturally occurring competitive rhizobia are present, it is necessary that rates of inoculant application should exceed this Limit ; (vi) the seed coats of some species contain substances toxic to rhizobia. In these circumstances, alternative means of inoculation are needed.

Solide inoculant - Solid inoculant is made by coating solid, granulated material with peat inoculant in an adhesive. Suitable adhesives include a 25% aqueous solution of gum arabic (no preservatives) or a 5% solution of methyl cellulose. Tenacity of the adhesive solution can be improved by chilling overnight Peat inoculant is thoroughly stirred into the adhesive and this suspension poured on to the beads and mixed together until all beads appear evenly coated. The beads should be dried by spreading in a thin layer. When dry and any lumps have been broken up, the material is ready for use. other inert particulate, freely flowing material such as marble chips, coarsegraded sand, polyethylene beads, clay granula, can be used for preparing solid inoculant. Availability and economic factors are the main points to consider in choosing material. For large-scale application, solid inoculant can be applied through the fertilizer box or insecticide attachment of a seed drill.

Liquid *(spray)* inoculation - Excellent nodulation can be obtained by spraying inoculum into the row beside or beneath the seed. A peat culture of rhizobia or frozen, concentrated broth cultures, is mixed into a paste with water, diluted to a slurry, then added to a water-filled tank prior to spray application. Any spray equipment is satisfactory provided it has not *been* used previously for toxic chemicals. This method provides very good results because high number of rhizobia can be applied. However, marketing

problems exist especially with frozen concentrated cultures which deteriorate during handling if proper conditions are not given to avoid thawing of the preparation.

Pre-inoculation before sale

Pre-inoculation is the inoculation of seed prior to sale or distribution for farmers; it implies a period of storage of the inoculated seed before it is sown. The main objective is to allow seed merchants to build up a stockpile of inoculated seed in order to meet predictable heavy demand by farmers at time when sowing deadlines are imposed by climate. Bacteriologically, the objectives should be to use cultures containing very high populations of rhizobia and to preserve their viability so that, at sowing, the level of inoculum on pre-inoculated seed is equal to that on freshly inoculated seed. New technology exists to produce pelleted, pre-inoculated seed with excellent mechanical properties and large population of viable rhizobia. Pre-inoculated seeds should meet the same standard in the quautitative terms of infective rhizobii by seed as that provided by inoculation at sowing time. Seed pelleting represents the major aud probably the only real advance in the technology of inoculant application, and appears to offer the best prospects for the development of a reliable inoculation.

Concluding remarks

The introduction of a legume seed inoculant strain into soil aud its establishment as a permanent member of the soil community is an exercice in applied microbial ecology. Quantitatively, the number of rhizobii associated with inoculated seed represent only a very small fraction of the total soil microflora. The choice of a strain, the quality of the inoculant and its method of application are important to insure that the introduced *Rhiwbium* strain *survives* and establishes an effective symbiosis with its designated host. Unless proper methods of inoculaut manufacturation and application are used, inoculant will fail to meet the objectives of its practice, regardless of the strain. Quality control programm is an essential part of inoculation technology.

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Caractérisation et choix de supports pour inoculum

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Résumé

L'étude réalisée avait pour but de tenter de relier quelques unes des caractéristiques physico-chimiques de supports possibles d'inoculum avec leur capacité réelle à permettre la survie des rhizobiums.

Les modalités de conditionnement des inoculums ont également fait l'objet de mises au point visant à l'obtention de produits de qualité élevée.

Les supports utilisés proviennent de pays où des unités de production d'inoculum pour légumineuses ont été installées dans le cadre de programme de coopération technique de la FAO : Madagascar, Tunisie, Zaïre, Bhutan, Nicaragua, Turquie., France.

Les résultats obtenus ont permis de montrer : (i) L'importance du pH initial du support, l'optimum se situant entre 6 et 6,5. Lorsque œ n'est pas le cas, une neutralisation peut être effectuée avant l'utilisation ; (ii) les conditions de stérilisation des apports ont des conséquences primordiales sur la survie des rhizobiums ; (iii) l'existence dans la majorité des cas d'une légère multiplication des rhizobiums au cours des premières semaines suivant l'ensachage puis une décroissance plus ou moins rapide selon les conditions de conservation (température de stockage).

Ces résultats ont conduit à des recommandations sur les méthodes de production des inoculums dans des unités de petite taille. Leur mise en œuvre fait également l'objet d'une présentation.

Introduction

Le nombre de rhizobiums contenus dans un inoculum conditionne en grande partie son efficacité. Lors de la production à l'échelle pilote, pour les besoins de l'expérimentation en milieu paysan, la qualité du produit utilisé joue un rôle primordial et doit être aussi élevée que possible. La multiplication des rhizobiums dans des petits fer-menteurs simplifiés mis au point par l'IRAT et baptises UPIL (Unité de Production d'Inoculums pour Légumineuses) est maintenant bien maitrisée et adoptée dans plusieurs pays (Saint Macary et *al*, 1986). L'étape suivante de la production, dans laquelle la culture liquide est mélangée à un support nécessite la recherche, au niveau local, de composés permettant une bonne survie des rhizobiums. Dans le cadre de projets de coopération technique de la FAO, de tels supports ont été recherches et leur aptitude à être utilises dans les unités a été évaluée.

Matériel et méthodes

Les principales caractéristiques physiques et chimiques des supports utilises sont présentées dans le Tableau 1.

L'ensemble des supports a été séché à l'air puis à l'étude à 50°C pour obtenir une humidité finale de 12 à 15%. Ils ont ensuite été broyés. passes au tamis de 0,5 mm puis conditionnés dans des sachets en plastique autoclavables (Polyéthylène moyenne densite ; épaisseur 150 µ) à raison de 80 g par sachet. L'ensemble des sachets contenant les supports a été stérilise par trois passages à l'autoclave (120°C, 20 mn), les autoclaves étant espacés de 24 h.

Les cultures de rhizobium utilisées pour injection dans les sachets ont été réalisées en fioles d'erlenmeyers, agitées à 30°C, contenant du milieu yeast extract mannitol (YEM), (Vincent, 1970). Les inoculums ont été préparés en injectant en conditions stériles dans les sachets une quantité de culture liquide correspondant à une valeur située entre 40% et 60% de la capacité maximale de rétention en eau du support (Date et Roughley, 1977).

Après différents temps, et, selon les traitements appliqués (stérilisation ou non, neutralisation ou non, température de stokage), le nombre de rhizobiums contenus dans les sachets a été déterminé selon les méthodes de suspension dilution puis étalement en boite de pétri (FAO, 1984).

Résultats et Discussions

Neutralité des supports

Les résultats obtenus sur la tourbe de Betafo (Madagascar), représentés dans la Fig. 1 montrent clairement que la neutralisation par du carbonate de calcium d'un support très acide peut considérablement améliorer sa capacité à maintenir une population microbienne *en* vie. La tourbe neutralisée, actuellement utilisée pour la fabrication d'inoculums *permet la survie* de *Bradyrhizobium japonicum* pendant près de deux mois, ce qui permet d'envisager un délai entre la fabrication des produits et leur utilisation.

Tableau 1 : Caractéristiques chimiques des supports utilises

Pays d'origine	Nature	Lieu de prélèvement	Identification sur Fig. 4	pH (eau)	Carbone (%)	C/N	Conductivité à 25°C (micromhos)
Madagascar	Tourbe	Betafo		3,78	15,86	16	1180
Turquie	Tourbe	Bolu	B	5,60	41,06	23	1650
		Trabzon	T	4,60	24,65	25	600
		Kayseri	K	7,60	9,90	17	1700
Bhutan	Tourbe	Parø		4,60	21,80	13	-
Nicaragua	Tourbe	Las Playitas	A	6,35	14,70	64	176
			N	6,68	6,38	36	106
			C	6,90	7,56	34	116
		San Carlos	D	5,90	19,02	20	104
	Balle de riz		R	5,14	27,70	69	397
	Ecume de canne		S	5,92	37,62	51	2750
	Bagasse de canne		Z	5,54	14,74	79	350
	Bagacillo de canne		L	6,18	15,60	80	335
	Pulpe de café		P	7,37	46,94	42	2330

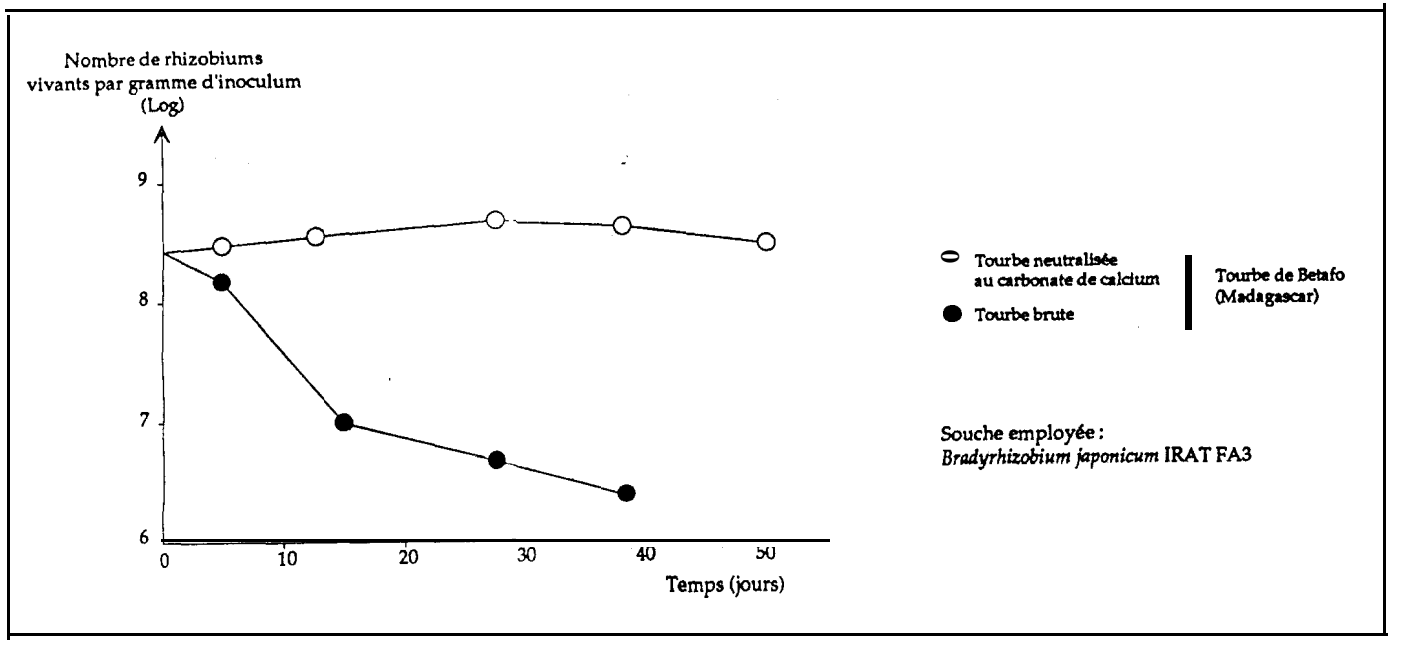


Figure 1 : Effet de la neutralisation du support sur la survie des rhizobiums

Stérilité des supports

Le traitement par la chaleur humide de tourbes de Turquie (Fig. 2) améliore la conservation des rhizobiums. Il est naturel, en effet, qu'en l'absence d'autres microorganismes, les rhizobiums ne rencontrent aucune compétition pour les sources d'éléments nutritifs contenus dans le support. L'hypothèse souvent exprimée d'un risque de dégradation du support pendant la stérilisation et d'une libération de composés toxiques pour les rhizobiums ne se trouve cependant vérifiée pour aucun des deux supports comparés dans cette étude. Entre ces deux supports cependant, des différences notables existent et la tourbe extraite de Bolu fait aujourd'hui l'objet d'une utilisation par l'industriel de fabrication d'inoculum turque.

Température de conservation des supports

L'étude réalisée sur une tourbe de Paro (Bhutan) comparant l'effet du stockage d'inocnlnms à 4°C et 25°C a permis de montrer (Fig. 3) qu'il n'existait pas, pour la période considérée de différence importante entre les traitements. D'autres résultats (non présentés ici) ont montré que la conservation des inoculums à température ambiante ou à 30°C pouvait résulter, dans un premier temps en une multiplication des rhizobiums et l'obtention de meilleurs inoculums que ceux conservés dès leur conditionnement à basse température.

Relations pH, matière organique, survie

La *survie* de *la* souche de *Bradyrhizobium japonicum* IRAT FA3, après 50 jours de conservation dans différents supports est représentée dans la Fig. 4, où sont également indiquées deux caractéristiques chimiques des supports : le pH et la teneur en carbone. On constate qu'il n'existe pas de règle générale, au moins pour les deux facteurs pH et C, permettant de prédire la valeur d'un support, Les meilleurs résultats ont Cd obtenus avec des supports proches de la neutralité (pH = 5 à 7) et pour des teneurs en C moyennement élevées. Ces conditions peuvent sembler nécessaires mais ne sont pas suffisantes puisque certains supports, comme la bagasse de canne (Z) ou la tourbe de Las Playitas (C) ne donnent que des résultats médiocres. On peut remarquer cependant que les meilleurs résultats sont souvent obtenus avec des composés organiques comme la tourbe (échantillons D, B, K, N), les matières organiques fraîches (Z, R, S) étant souvent de mauvais supports d'inoculums.

Conclusion

L'ensemble des résultats obtenus dans ces études et dans d'autres essais non présentés ici, montrent la difficulté qu'il y a à prévoir, par une simple analyse chimique, le comportement d'un support vis-à-vis des rhizobiums. Parmi les règles pouvant être énoncées pour la fabrication d'inoculums dans lesquels les rhizobiums possèdent une probabilité de survie élevée, celles de la neutralité et de la stérilité du support semblent primordiales. Par la suite, une évaluation bas& sur des tests biologiques de survie des microorganismes semble obligatoire pour déterminer les meilleures conditions de préparation et de conservation des produits. En la matière, une bonne connaissance de l'évolution du nombre de rhizobiums dans le support permet de déterminer la durée maximale d'utilisation des inoculums, de prévoir leur durée de production en conséquence, et de préciser les conditions de leur distribution, la réfrigération ne constituant pas toujours une obligation.

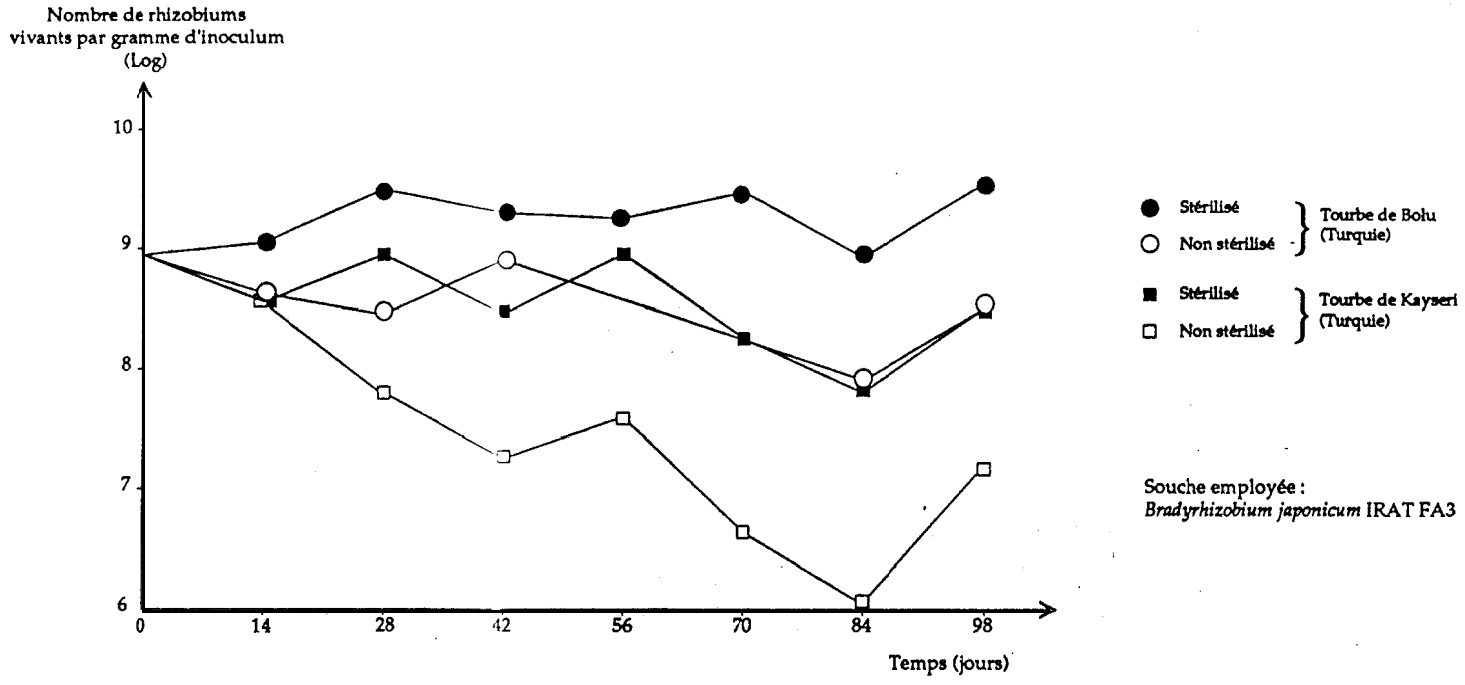


Figure 2 : Effet de la stérilisation de supports sur la des rhizobiums

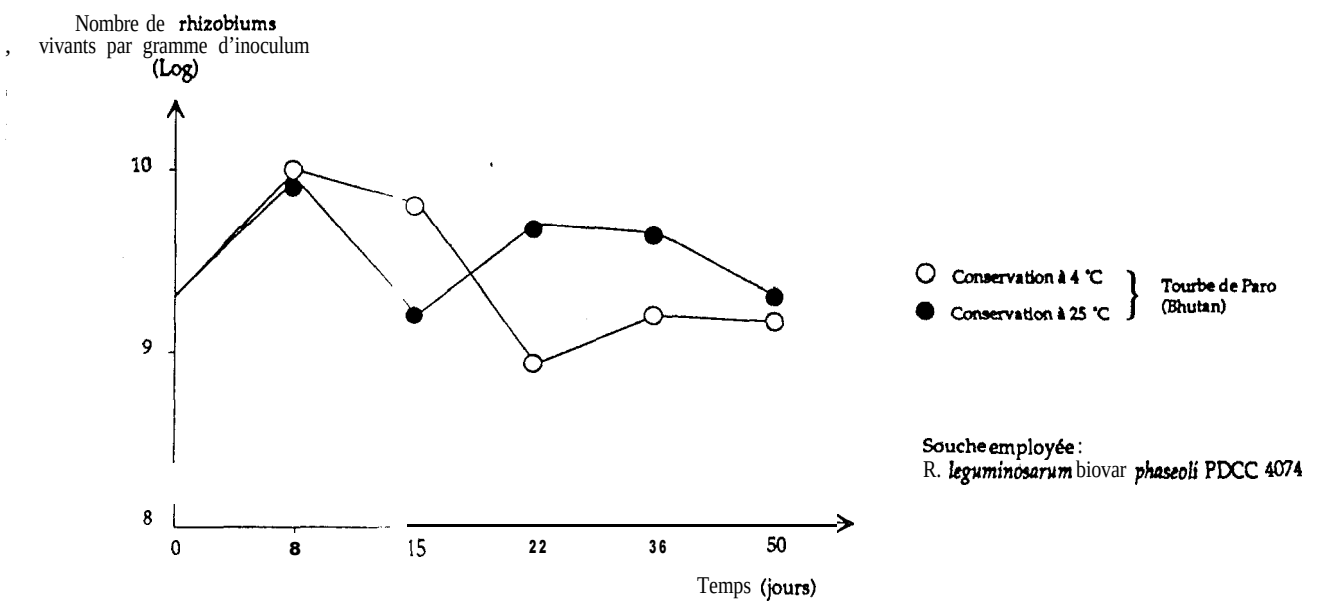


Figure 3 : Effet de la température de conservation des inoculums sur la survie des rhizobiums

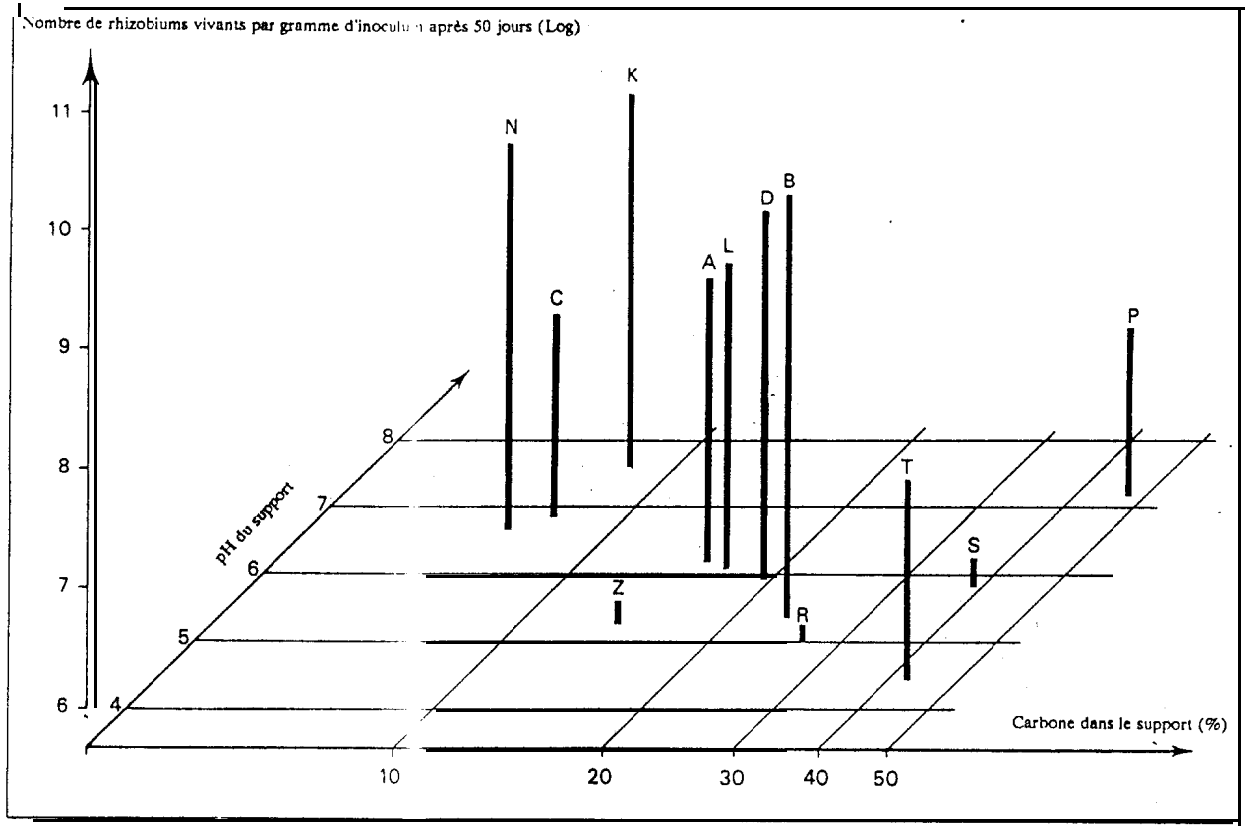


Fig. 4 : Nombre de rhizobiums contenus dans les différents supports après 50 jours. La signification des lettres représentant les supports est donnée dans le tableau 1

Cette étude n'a pris en compte, comme critère de qualité des inoculums, que le nombre de ***Rhizobium*** vivants. Il n'est pas certain que dans tous les cas des modifications de l'efficacité des ***Rhizobium*** ne se soient pas produites. L'obtention et l'application d'un nombre aussi élevé que possible de ***Rhizobium*** viables constitue cependant un préalable indispensable à une bonne qualité des inoculums. C'est pourquoi le critère de nombre a été seul considéré dans l'étude.

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Progrès récents dans la technologie des inocülums utilisés en agriculture et en foresterie

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Summaty

The preparation of inoculants requires (i) mass production of the target microorganism to be introduced in the agrosystem, and (ii) an appropriate formulation of the microbial culture ensuring long survival during storage and ease of application

The principles of mass culture methods for growing rhizobia, ectomycorrhizal fungi, *Frankiae*, and VAM fungi are briefly presented. Emphasis is specially placed on the difficulties encountered when growing *Frankiae* and on the solutions adopted to increase its biomass production, which includes biphasic culture, two stage culture, and growth in a polymeric matrix.

The modem technique of processing the inoculants is based on the inclusion of bacteria in a polymeric matrix (generally alginate). Two types of polymeric inoculants can be prepared : PEM inoculants made of microbial cells entrapped in the polymeric matrix ; PEC inoculants made of microbial colonies entrapped in the matrix (the colonies resulting from the growth of entrapped isolated cells). PEM and PEC inoculants are usually dried, then applied as a powder onto seeds or soil, or rehydrated before beiig used. Polymeric inoculants were shown to be quite effective and reliable in field experiments with rhizobia, VAM mycorrhizae and frankiae.

Introduction

On peut classer en deux catégories les microorganismes rhizosphériques et phyllosphériques qui améliorent la production végétale.

Le premier groupe est constitué par les microorganismes qui agissent directement sur le végétal en contribuant à sa nutrition azotée ; par *ex. Rhiwbium ou Frankia,* en améliorant l'absorption des minéraux et de l'eau, par ex. *Azospirillum* (Okon, 1984 ; Nayak et *al.,* 1986 ; Boddey et Dobereiner, 1988), champignons mycorhiziens (Menge, 1983; Gianinazzi-Pearson e t G i i , 1986) ; en solubilisant certains éléments, par ex. bactéries solubilisant les phosphates (Barea et *al.,* 1967 ; Azcon et *al.,* 1976) ; en synthétisant des substances de *croissance,* par ex. Awtobacter* (*Brown,* 1982), *Azospirillum* (Okon, 1987 ; Boddey et Dobereiner, 1988).

Le deuxième groupe affecte indirectement la croissance du végétal en luttant contre les microorganismes pathogènes, par ex. *Trichoderma* sp., *Agrobacterium radiobacter* ; en luttant contre les arthopodes, par ex. *Beauveria bassiana* ; en luttant contre les mauvaises herbes (microorganismes pathogènes de ces plantes notamment mycoherbicides), par ex. *Phytophthora palmivora* ; en préservant des effets du gel, par ex. *Pseudomonas syringae* (Okon et Hadar, 1987).

En plus des microorganismes rhizophériques et phyllosphériques, des microorganismes endophytiques (c'est-à-dire vivant dans le xylème des plantes) pourraient être utilisés à l'avenir, après manipulation génétique, comme agent de lutte biologique contre différents ennemis des plantes, spécialement des insectes (Carlson, 1988).

On pense que l'inoculation des plantes effectuée dans le but d'améliorer les récoltes est un type de biotechnoiogie élémentaire ; en fait il s'agit d'une opération relativement compliquée (Bashan, 1986). Les techniques conventionnelles donnent des résultats irréguliers et les méthodes d'inoculation sont souvent primitives. C'est ainsi que dans certains pays, on inocule encore les plantes actinorhiziennes avec des broyats de nodules. On doit interdire une telle pratique en raison du risque d'introduction de souches ineffectives (non fixatrices d'azote) et surtout du danger de contamination des semis ou des boutures avec des agents pathogènes, tels que *Rhizoctonia solani ou Pseudomonas solanacearum dans* le *cas* de *Casuarina equisetifolia* (Liang Zichao, 1986) ou des nématodes lorsque l'on a affaire à des plantes sensibles à ces derniers parasites, telles que les acacias australiens introduits en Afrique de l'Ouest.

Cette communication fait le point des progrès les plus marquants réalisés récemment (i) dans la culture des quatres types les plus importants de microorganismes symbiotiques, *Rhizobium, Frankia,* champignons ectomycorhiziens (EMC) et vésiculo-arbosculaires (VAM), et (ii) dans le conditionnement du microorganisme utilisé pour l'inoculation, opération fond& sur l'inclusion du micmorganisme dans une matrice de polymére, par exemple alginate. On donnera enfin les résultats d'essais d'inoculation au champ portant sur ce nouveau type d'inoculum.

Culture en masse du microorganisme

La production d'inoculum repose sur la culture en masse du microorganisme que l'on souhaite introduire dans l'agrosystème. Alors que l'on peut cultiver facilement certains microorganismes *in vitro* (rhizobiums, champignons ectomycorhiziens), il est beaucoup plus difficile de produire des quantités importantes d'autres microorganismes (par ex. *Fran-*

kia). En outre on n'est pas encore parvenu à cultiver *in vitro* un certain nombre de microorganismes, en particulier les champignons VAM.

Rhizobiums

On peut cultiver facilement les rhizobiums dans de nombreux types de fermenteurs, du type «batch» ou semi-continu (Williams, 1984).

On peut aussi cultiver ces bactéries à l'état inclus dans une matrice polyémrique suivant le procédé pour les frankias (cf: infra). Contrairement aux frankias dont les colonies sont régulièrement distribuées à l'intérieur des billes, les rhizobiums, comme les autres microorganismes aérobies tels que *Bacillus subtilis* (Baudet *et al.*, 1983), poussent seulement dans la couche externe des billes, dont le diamètre ne devrait donc pas dépasser 1 mm.

On sait maintenant que, dans la symbiose rhizobienne, nombre de composés apparentés aux flavones stimulent l'expression des gènes *nod, y* compris les gènes *nod ABCEF* (seul le gène *nod* D est constitutif chez les rhizobiums à l'état libre), et accélèrent la nodulation, ce qui rend plus compétitifs les rhizobiums incubés en présence de ces substances. Ces composés se trouvent dans les exudats des plantes-hôtes, mais la quantité exsudée peut ne pas être suffisante pour éliciter la nodulation (Halverson et Stacey, 1986 ; Redmond *et al.*, 1986 ; Phillips *et al.*, 1988). L'addition d'exsudats ou de substances inductrices réduit le temps nécessaire à la nodulation (Rolfe *et al.*, 1987 ; Wijffelman *et al.*, 1987). Ce traitement sera probablement commercialisé dans un proche avenir.

Champignons ectomycorhiziens

On sait cultiver les champignons ectomycorhiziens (par ex. *Suillus granulatus* , *Rhizopogon luteus*, *Thelephora terrestris*. *Pisolithus tinctorius*) soit en milieu liquide, dans différents types de fermenteurs y compris de grands fermenteurs industriels (Marx et Kenney, 1982), soit sur substratum solide imprégné de milieu nutritif. Dans le second cas, on met à incuber le substratum inoculé avec le champignon dans des sacs de plastique de 5 à 10 litres dotés sur une face d'une membrane permettant les échanges gazeux tout en empêchant des contaminations éventuelles (Le Tacon et Garbaye, 1986 ; Gagnon *et al.*, 1987).

Frankias

La culture *in vitro* de *Frankia* soulève plusieurs problèmes (Diem *et al.*, 1988) dûs aux caractéristiques de cet actinomycète : (i) une croissance très lente, le temps de doublement étant de 50 à 60 h, (ii) l'inhibition de la croissance en batch lorsque la biomasse de *Frankia* dans le milieu atteint le seuil anormalement bas de 10 µg de protéines cytoplasmiques par ml c'est-Mire 70-100 µg de protéines totales par ml de milieu, (iii) une diminution très rapide de la biomasse physiologiquement active à la fin de la phase active de croissante (Fig.1), cette phase de déclin étant caractérisée par une lyse marquée des différentes structures de *Frankia*, (iv) une sporulation souvent irrégulière et instable. Ce dernier point mérite d'être étudié car on peut penser qu'en obtenant une sporulation plus abondante, on améliorera la viabilité de *Frankia* dans l'inoculum pendant le stockage.

Des expériences récentes effectuées dans notre laboratoire suggèrent que l'on peut stimuler la sporulation de *Frankia* en utilisant un milieu de culture limité en N ou en apportant certains acides aminés (leucine, alanine, phénylalanine, arginine et valine).

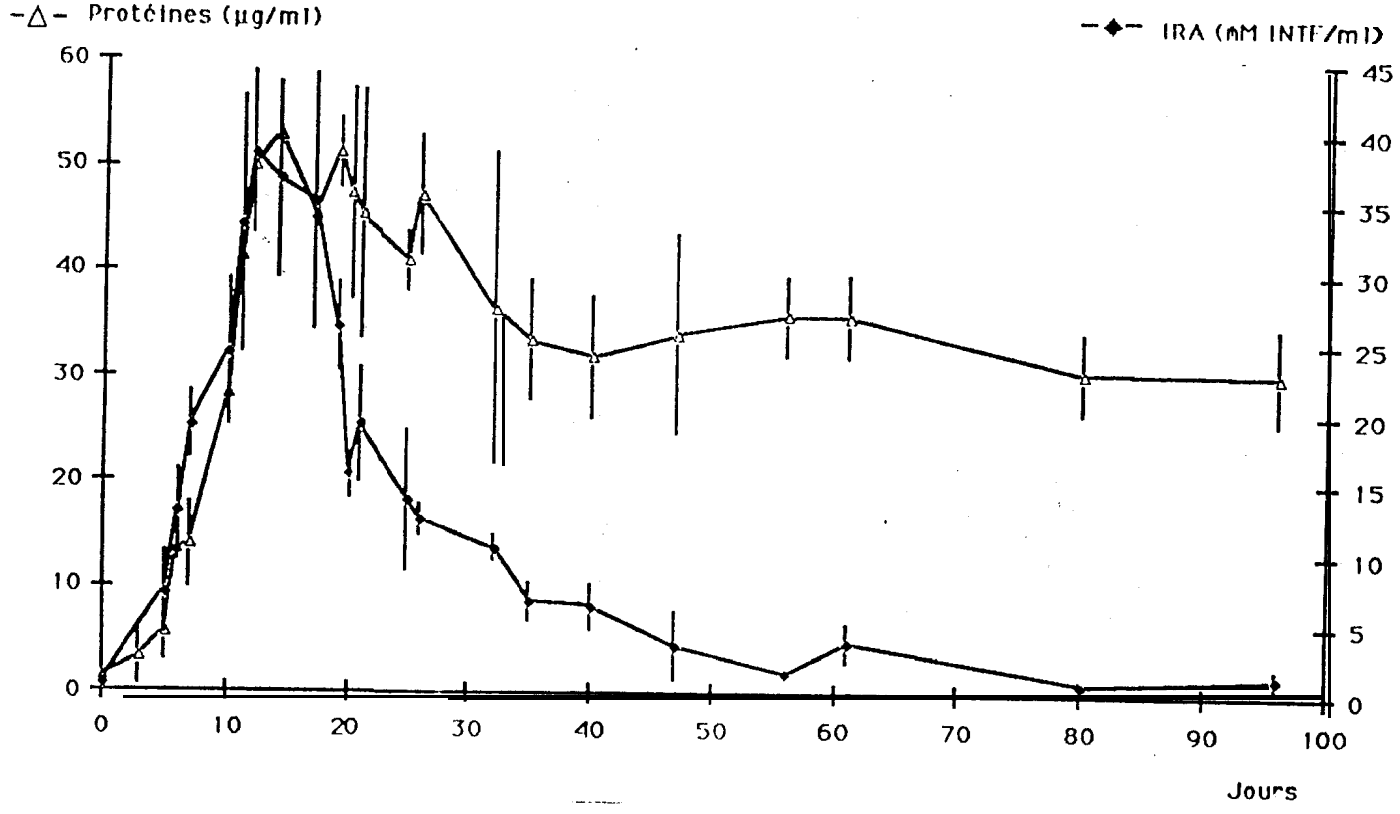

-△- Protéines (µg/ml)
-◆- IRA (mM INTF/ml)
60
50
40
30
20
10
0
45
40
35
30
25
20
15
10
5
0
0
10
20
30
40
50
60
70
80
90
100
Jours

Figure 1 : Courbe de croissance de la souche de frankia ORS 020607. La courbe en fonction du temps de la teneur de la culture en protéines comporte trois phases : (i) phase de latence (jusqu'au 5ème jour), (ii) phase de croissance active, que l'on suppose de type linéaire, du 5ème au 15ème jour et (iii) une phase plus ou moins stationnaire jusqu'au 96ème jour.

Jusqu'à présent, on a utilise surtout les cultures en batch, avec ou sans agitation, avec ou sans aération. Comme on ne peut utiliser ces méthodes pour produire les quantités importantes de *Frankia* nécessaires à la préparation des inoculums, nous cherchons actuellement à mettre au point trois nouvelles techniques de production en masse.

Culture biphasique

Etant donné que *Frankia* pousse bien lorsqu'il est attaché à une surface solide, Diem et Dommergues (1985) ont mis au point une méthode de culture biphasique dans laquelle on inocule le milieu liquide avec des fragments de gélose provenant de l'écrasement d'une culture de *Frankia* ayant pousse sur un milieu nutritif gélosé, les fragments de gélose constituant des matrices où sont incluses de nombreuses structures de *Frankia*. Cette méthode permet d'obtenir une croissance satisfaisante de *Frankia* à l'interface entre la courbe de gélose écrasée (phase solide) qui tombe au fond du flacon de culture contenant le milieu liquide (phase liquide). On obtient ainsi la formation, à l'interface solide-liquide, d'un enchevêtrement homogène d'hyphes de *Frankia* ayant 2 mm d'épaisseur. Une telle amélioration de la croissance au niveau d'une interface liquidesolide a aussi été observée dans le cas d'autres bactéries (Bauder et *al.*, 1983). Ce système de culture biphasique permet en outre (i) de réactiver la croissance *de* vieilles cultures *de Frankia* qui ne pouvaient redémarrer à la suite du simple transfert sur les milieux solides ou liquides habituels, (iii d'assurer le développement des microcolonies obtenues au cours dé l'isolement de *Frankia* à *partir* des nodules, la biomasse initiale de ces microcolonies étant trop faible pour en assurer leur croissance ultérieure dans les milieux solides ou liquides habituels.

Culture à deux étapes

Neyra a mis au point dans notre laboratoire une méthode de culture à deux étapes (résultats non publiés). Dans la première étape, on cultive *Frunkia dans un* chémostat conçu par Belaich, J.P. (comm. pers.) pour la culture des microorganismes à croissance lente. Dans la deuxième étape, la culture de *Frankia* qui sort du chémostat est utilisée pour inoculer des batchs ancillaires contenant du milieu frais.

Culture en billes d'alginate

Le principe de la mtthode est le suivant : les cellules de *Frankia* sont incluses aseptiquement dans des billes d'alginate (qui constituent la matrice polymérique) obtenues conformément au protocole décrit au paragraphe consacre au conditionnement des inoculums et légèrement modifie. Cette modification consiste à remplacer l'eau distillée utilisée pour faire la suspension d'alginate à 5 % par un milieu de culture renfermant moins de phosphate que le milieu usuel. Ces billes sont ensuite lavées dans l'eau stérile et incubées pendant 10-15 jours dans le milieu de culture. Les colonies de *Frankia* qui se développennt à l'intérieur des billes (Fig. 5) renferment de nombreux sporanges au milieu de chacune de ces colonies. Lorsque les colonies formées à l'intérieur des billes sont suffisamment développées, les billes sont séchées et constituent ainsi l'inoculation PEC en poudre (cf : infra).

Dans le cas des plantes actinorhiziennes, l'expression des gènes *nod* est probablement induite par des substances du même type que celles qui interviennent dans la symbiose *Rhizobium*-légumineuse. En effet, Prin et Rougier (1987) ont montré que les exsudats de l'*Alnus* sont impliqués dans le processus d'infection.

Champignons mycorhiziens vésico-arbosculaires (VAM)

Ainsi qu'on l'a signalé antérieurement, les champignons VAM ne peuvent être cultivés *in vitro,* c'est-a-dire en l'absence de la plante-hôte. Contrairement aux rhizobiums et frankias, ces champignons sont si peu spécifiques que presque toutes les espèces végétales dotées d'un système racinaire abondant peuvent être utilisées comme plantes-hôtes.

La méthode habituelle, encore largement utilisée, consiste à cultiver la plante-hôte dans un sol ou un substratum stérile place dans un récipient ou directement sur le plancher de la serre. On inocule le sol ou le substratum avec des spores ou des racines déjà infectées avec le champignon VAM à multiplier, provenant de cultures en pot effectuées antérieurement. Linoculum ainsi prépare est volumineux et.. difficile à manipuler-

Récemment, Mosse et Thompson (1984) ont mis au point une méthode appelée NFT (Nutrient Film Technique). Cette méthode consiste à faire pousser la plante-hôte dont les racines ont été préalablement infectées avec le champignon VAM à multiplier dans un canal étroit où coule la solution nutritive pour le végétal. Le réseau dense de racines formé dans ces conditions constitue un milieu idéal pour la multiplication du champignon VAM. L'avantage de l'inoculum ainsi obtenu est qu'il suffit de 60 kg de racines infectées pour traiter 1 ha, poids bien inférieur à celui de l'inoculum classique décrit ci-dessus. En admettant que le rendement en racines soit de 300 g (poids frais) par m de canal, on peut considérer qu'avec 200 m de canal on peut inoculer 1 ha (Elmes et *al.,* 1983). Il est évident que le matériel ainsi obtenu n'est pas exempt de contaminants, les plus fréquents étant des protozoaires ou des nématodes saprophytes (Mosse et Thompson, 1984). La méthode NFT a été expérimentée avec succès sur haricot (*Phaseolus vulgaris*), maïs (*Zea mays*) et d'autres hôtes infestés avec *Glomus mosseae, G. fasciculatus, G. caledonium et Acaulospora laevis* (Elmes *et* Mosse, 1984).

Une autre. méthode est fondée sur l'emploi de plantes-hôtes cultivées aéroponiquement. Hung et Sylvia (1988) ont confirmé des études antérieures montrant que cette méthode peut être utilisée avec succès pour multiplier les champignons VAM. car une bonne aération de la rhizosphère stimule la sporulation.

Enfin, Mugnier et Mosse (1987) ont proposé une approche originale pour améliorer la culture des champignons VAM : au lieu d'utiliser la plante entière, on utilise des racines transformées par le T-DNA d'*Agrobacterium rhizogenes. Les* racines transformées de *Convolvulus sepium,* que l'on peut cultiver dans des fermenteurs usuels, sont infectées par des spores de *Glomus mosseae* et assurent une bonne croissance du champignon. Ce procédé, qui donne satisfaction au niveau du laboratoire, n'a pas encore été commercialise.

Inoculants constitués par des microorganismes inclus dans une matrice polymérique

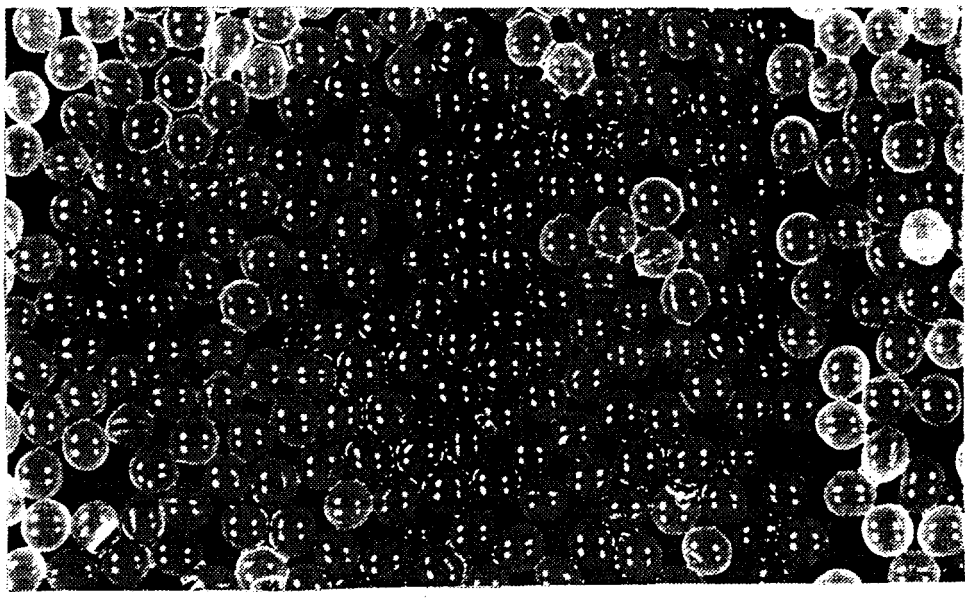
Le conditionnement des inoculums microbiens utilisés en agriculture est très important. Le succès de ces inoculums dépend en effet non seulement de la survie du plus grand nombre possible de cellules microbiennes pendant le laps de temps s'écoulant entre la fabrication et l'application, mais aussi de la formulation qui doit être simple mais permettant un transport facile et une application aisée. Le conditionnement classique est fondé sur l'adsorption de la culture microbienne sur un support protecteur, en général la tourbe (Date 1977 ; Burton, 1979 ; Williams, 1984). Des supports de remplacement ont été pro-

posés et utilisés tels que lignite, charbon, paille, cellulose, bagasse, résidus de récolte broyés en poudre et sol (Chao et Alexander, 1984). Dans certains cas, la culture et le conditionnement du microorganisme sont effectues en même temps. C'est ainsi que les inoculums d'ectomycorhizes sont faits d'un mélange d'hyphes fongiques et du substrat de vermiculite imprégné du milieu spécifique sur lequel le champignon a pousse (Max et Kenney, 1982).

Dès 1977 et 1979, Dommergues et *al.*, ont suggéré que l'on pouvait utiliser des gels de polymères pour inclure les microorganismes bénéfiques pour préparer des inoculums à usage agricole. Les premiers inocuhnns faits de microorganismes inclus dans des matrices polymériques ont été testés sur des rhizobiums il y a 10 ans. Ce concept est maintenant appliqué avec succès, sur une échelle expérimentale, à d'autres groupes de microorganismes, par ex. champignons VAM (Ganry *et al.*, 1982), champignons ectomycorhiziens (Jung et *al.*, 1981 ; le Tacon *et al.*, 1985), *Azospirillum* (Bashan, 1986) et aussi divers chamignons utilises en lutte biologique (Lewis et Papavizas, 1985). Nombre de substances utilisées pour I'inunobilisation des cellules, enzymes, anticorps et autres protéines (Scott, 1987) ont été testées pour préparer des inoculums polymériques : polyacrylamide, alginate, xanthane (Jung et *al.*, 1982) et de nombreux autres gels naturels tels que le carrageenan.

Cellules microbiennes incluses dans une matrice polymérique (PEM)

On peut utiliser divers polymères pour inclure les cellules microbiennes dans une matrice polyémérique, le produit obtenu étant désigné sous le terme d'inoculum polymérique PEM ou plus brièvement inoculum PEM (Polymer-entrapped microbial cells). Dans le cas de l'alginate de sodium (par ex. Alginate S 170 fabrique par SATIA, 15 avenue d'Eylau, 75116 Paris, France), qui est un polymère très largement employé, on prépare une solution à 5% (poids/volume) de ce compose dans l'eau distillée et on mélange cette solution avec le même volume de la culture microbienne à la fm de sa phase active de croissance. On laisse tomber goutte à goutte le mélange constitue par la solution d'alginate et la culture microbienne dans une solution 0,1-0,2 M de $CaCl_2$ sous agitation magnétique. En entrant en contact avec la solution de $CaCl_2$, chaque goutte forme une bille sphérique indépendante (diamètre 14 mm) résultant du pontage de l'alginate par les ions Ca^{2+} (Fig. 2). On maintient les billes dans la solution de $CaCl_2$ pendant 45 mn, puis on les récolte et on les lave plusieurs fois dans l'eau stérile ; finalement on les sèche pendant 24 h sous hotte à flux laminaire (Fig. 3). On peut broyer les billes sèches pour en faire une poudre de 50-200 µm. L'inoculum ainsi fabriqué est stocke dans des récipients fermés. Dans le cas du rhizobium, chaque g d'inoculum PEM séché renferme 10^9 à 10^{11} unités formant des colonies (CFU).



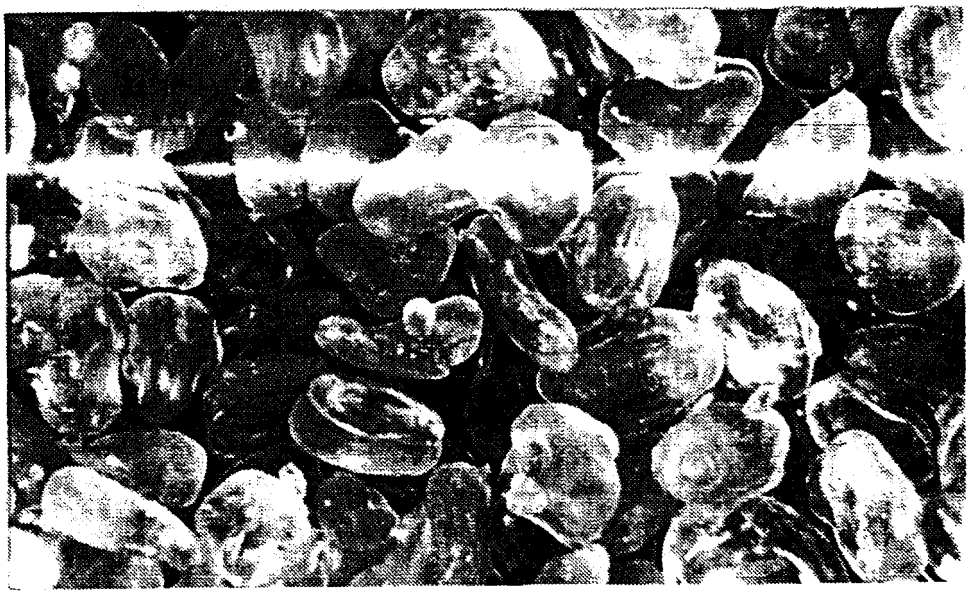


Figure 2 : Inoculum PEM : billes d'alginate fraîches (non séchées) où sont inclus des rhizobiums (diamètre : environ 3 mm)

Figure 3 : Inoculum PEM : billes d'alginate séchées où sont inclus des frankias (longueur : environ 1,5 mm)

Dans le cas des champignons VAM, le matériel microbien inclus dans la matrice polymérique n'est pas seulement constitué de structures fongiques (spores, hyphes, vésicules) mais aussi de fragments de racines infectés par le champignon (Fig. 4). Toutes ces structures doivent être soigneusement lavées avant d'être mélangées avec la solution d'alginate. Les dernières phases de la préparation de ces inoculums sont les mêmes que celles concernant la préparation des inoculums bactériens (Jung *et al.*, 1981 ; Ganry *et al., 1982).*

Figure 4 : Inoculum PEM : billes d'alginate fraîches (non séchées) où sont inclues des structures de champignon VAM et des fragments de racine (diamètre : environ 3 mm)

L'iuoculum PEM est appliqué aux plants (généralement sur les graines ou dans le sol de la raie de semis) soit sous forme séchée, soit sous forme d'une pseudo-solution obtenue en mettant l'inoculnm séché dans une solution tampon (0,03 M KH_2PO_4 ; 0,17 M K_2HPO_4 ; pH 7,4) pendant 4-8 h.

L'inoculum sec peut être fixé sur les graines avec une substance adhésive (Elegba et Rennie, 1984), telle que la gomme arabique (40% poids/volume). On peut ainsi fixer entre 1 et 2 mg d'inoculum PEM sec sur une graine de soja, soit plus de 10^6 à *2* x 10^8 CFU par graine.

Les principaux avantages des inoculums PEM à base d'alginate (avantages probablement partagés avec les autres inoculums PEM) sont les suivants : (i) leur faible poids qui les rend faciles à manipuler et à transporter de l'unité de fermentation au site d'application ; (ii) ils peuvent facilement être mélangés les uns aux autres ou avec différents produits chimiques tels que des pesticides (Dreyfus *et al., 1983) ou* des substances protectrices ; (iii) après un séchage convenable de l'inoculum, de nombreuses espèces de microorganismes peuvent survivre longtemps *:* On peut conserver *Frankia* et les champignons VAM pendant 2-3 ans à 20-25 C sans qu'ils perdent leur pouvoir infectif.

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De même il est clair que certains champignons ectomycorhiziens bénéficient grandement de leur inclusion dans l'alginate (Jung *et al.*, 1981 ; le Tacon *et al.*, 1985).

La survie des rhizobiums (Jung *et al.*, 1982). et plus généralement des bactéries ne formant pas de spores, dépend de la tolérance des souches au processus d'inclusion (gélification du polymère), de la nature des carbohydrates présents dans la matrice et de la teneur finale en eau (en fait l'activité de l'eau : a_w) de l'inoculum séché. Mugnier et Jung (1985) ont montre que les carbohydrates à faible poids moléculaire (C_3 à C_5) avaient un effet défavorable sur la survie, alors que les composes de poids moléculaire plus élevé (C_6 à C_{12}) protégaient les microorganismes. Avec les souches testées par ces auteurs, le nombre de cellules vivantes (10^{10} par g d'inoculum sec) reste constant pendant un stockage d'une dur& supérieure à 3 ans à 28°C quand a, de l'inoculum est maintenue au dessous de 0,069. Dans l'intervalle de a_w 0,069-0,830, le taux de survie diminue d'autant plus vite que a, est élevée. La tolérance des rhizobiums à la dessiccation varie en fonction de la souche considérée et d'autres facteurs tels que la phase de croissance (Mary *et al.*, 1985). Lorsque a_w de l'inoculum tombe au-dessous d'un certain seuil, la perte d'eau des cellules peut devenir irréversible de sorte que les bactéries incluses meurent

Il est ensentiel que le nombre de bactéries viables dans l'inoculum se maintienne à un niveau élevé entre le moment de la production et celui de l'application. Une bonne conservation de la viabilité pendant le stockage permet au fabricant de préparer les inoculums bien avant qu'ils ne soient réclamés par l'utilisateur, ce qui est tout particulièrement important lorsqu'on doit disposer d'un large éventail de souches.

Des recherches sont encore nécessaires pour améliorer la survie des microorganismes dans les inoculums PEM et PEC. Différentes approches peuvent être employées pour assurer la survie des microorgauïsmes pendant l'inclusion, la dessiccation et le stockage :

(i) utilisation d'un agent gélifiant approprie, par ex., dans le 'cas de l'alginate, remplacement de $CaCl_2$ par le gluconate de Ca ($C_{12}H_{22}CaO_{14}$) (Fravel *et ai.*, 1985) ;

(ii) incorporation dans la matrice de substances protectrices telles que les argiles (Jung *et al.*, 1982 ; Fravel *et ul.*, 1985) ou des carbohydrates de poids moléculaire élevé comme on l'a signalé plus haut ;

(iii) stimulation de la production in vitro de structures de résistance telles que les sporanges (*Frankia*), ascospores et conidies (champignons) ou sclérotes *(par ex. dans* le *cas* de *Phlebopus soudanensis*, (Thoen, D. Comm. pers.) ;

(iv) stockage à basse température.

Colonies incluses dans une matrice polymérique (PEC)

Ce type nouveau d'inoculum est obtenu à partir de billes d'alginate dans lesquelles on a laissé croître les microorganismes, qui forment donc des colonies à l'intérieur des billes (Diem et Dommergues, 1985). On désigne cet inoculum sous le terme d'inoculum polymétrique PEC ou plus brièvement inoculum PEC (Polymer-entrapped colonies) (Fig. 5). La préparation de ces inoculums a été décrite ci-dessus. D'après les recherches préliminaires en cours, les inoculums PEC renferment un plus grand nombre de CFU que les inoculums PEM, chez lesquels il n'y a pas eu de croissance des microorganismes

après l'inclusion dans la matrice polymérique. Des expériences au champ sont actuellement en cours pour comparer la réponse des légumineuses et des plantes actinorhiziennes à l'application des inoculums PEC et PEM.

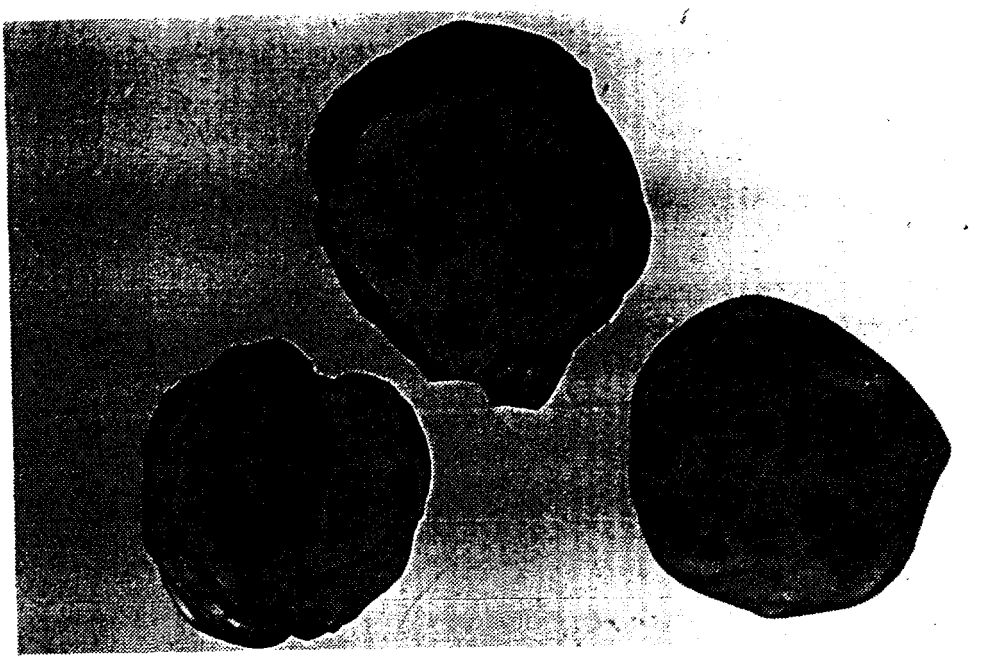


Figure 5 : Inoculum PEC : colonies de frankia poussant dans des billes d'alginate (diamètre : environ 3 mm)

Expériences au champ

Inoculum polymérique PEM de rhizobium

En 1979, on a commencé des expériences portant sur l'utilisation d'inoculums polymériques PEM renfermant des rhizobiums, aussi bien pour l'application au sol dans la raie de semis que pour l'enrobage des graines. Une de ces expériences a été mise en place à Guérina au Sud du Sénégal, dans un sol sableux déficient en N (0,026% N) pendant la saison des pluies, c'est-à-dire de Juillet à Octobre, 1980. L'objectif de cette expérience était de comparer la réponse du soja à l'inoculation avec trois types d'inoculums, deux inoculums polymériques (PEM-alginate et PEM-xanthane) et un inoculum liquide (culture liquide de rhizobium). Le sol utilisé n'avait pas été cultivé en soja auparavant de sorte qu'il ne renfermait pas le *Bradyrhizobium* spécifique du soja. La fertilisation avait consiste dans l'apport de 30 kg P (superphosphate) et de 100 kg de K (KCl) par ha.

On a comparé cinq traitements : pas d'inoculation (témoin) ; inoculation des graines enrobées avec la poudre d'inoculum PEM-alginate ; inoculation des graines enrobées avec la poudre d'inoculum PEM-xanthane ; inoculation du sol avec une culture liquide de *Rhizobium* appliquée à une dose très élevée ; application d'engrais azoté à la dose de 300 kg N par ha. Le dispositif expérimental comprenait quatre blocs randomisés constitués de parcelles élémentaires de 8 m x 8 m. Le Tableau 1 montre que les inoculums PEM

après l'inclusion dans la matrice polymerique. Des expériences au champ sont actuellement en cours pour comparer la réponse des légumineuses et des plantes actinorhiziennes à l'application des inoculums PEC et PEM.

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En 1979, on a commencé des expériences portant sur l'utilisation d'inoculums polymériques PEM renfermant des rhizobiums, aussi bien pour l'application au sol dans la raie de semis que pour l'enrobage des graines. Une de ces expériences a été mise en place à Guérina au Sud du Sénégal, dans un sol sableux déficient en N (0,026% N) pendant la saison des pluies, c'est-a-dire de Juillet à Octobre, 1980. L'objectif de cette expérience était de comparer la réponse du soja à l'inoculation avec trois types d'inoculums, deux inoculums polymériques (PEM-alginate et PEM-xanthane) et un inoculum liquide (culture liquide de rhizobium). Le sol utilise n'avait pas été cultivé en soja auparavant de sorte qu'il ne renfermait pas *le Bradyrhizobium* spécifique du soja. La fertilisation avait consiste dans l'apport de 30 kg P (superphosphate) et de 100 kg de K (KCl) par ha.

On a compare cinq traitements : pas d'inoculation (témoin) ; inoculation des graines enrobées avec la poudre d'inoculum PEM-alginate ; inoculation des graines enrobées avec la poudre d'inoculum PEM-xanthane ; inoculation du sol avec une culture liquide de *Rhizobium* appliquée à une dose très élevée ; application d'engrais azoté à la dose de 300 kg N par ha Le dispositif expérimental comprenait quatre blocs randomisés constitues de parcelles élémentaires de 8 m x 8 m. Le Tableau 1 montre que les inoculums PEM

sont aussi efficaces que l'inoculum liquide appliqué directement à très forte dose. En ce qui concerne la nodulation, l'inoculum PEM-xanthane est apparu supérieur à l'inoculum PEM-alginate ; mais pour les rendements en graines, il n'y a pas eu de différence significative entre ces deux inoculums (Jung et *al.*, 1982).

On a utilisé ultérieurement avec succès les inoculums PEM dans le cas des légumineuses à nodules de tige, plus particulièrement celui de *Sesbania rostrata* (*Dreyfus et al.,* 1983).

Tableau 1 : Réponse du soja à l'inoculation des graines avec l'inoculum PEM-alginate et l'inoculum PEM-xanthane et à l'inoculation du sol avec une culture liquide. Expérience effectuée à Guërina, Sénégal (Jung et al., 1982).

Traitements	Rendement en grain (kg/ha)		Poids sec des nodules (g/plante)
	Poids sec	N total	
Pas d'inoculation	1919 c	107 b	0,08 c
Inoculation des graines			
PEM-alginate	2752 ab	190-a	1,6 b
PEM-xanthane	2773 a	176 a	5,0 a
Inoculation du sol culture liquide	2445 ab	160 a	2,2 b
Engrais azotë (300 kg N/ha)	2366 bc	130 ab	0,02 d

Les chiffres suivisdes mémes lettres ne diffërent pas significativement pour P = 0,01.

Inoculum polymérique PEM de *Frankia*

On a mis en place au Sénégal une expérimentation sur une échelle relativement grande *pour évaluer la* réponse de *Casuarina equisetifolia* à l'inoculation avec un inoculum PEM de *Frankia.*

Le 15 mai 1984, on a plante des semis âgés d'un mois dans des gaines de polyéthylène remplies d'un sol très pauvre (0,4% C ; 0,026% N) stérilisé au préalable au bromure de méthyle. La moitié des semis a été inoculée ; l'autre moitié (témoins) ne l'a pas été. L'inoculum PEM de *Frankia* a été fabriqué avec la souche de *Frankia* ORS 021001 (Diem *et al.,* 1983) incluse dans des billes d'alginate préparées comme il est indiqué ci-dessus, ces billes ayant été séchées à l'air et conservées pendant 2 ans à la température du laboratoire (20-25°C) avant d'être utilisées. On a procédé à l'inoculation en introduisant dans chaque gaine une suspension de *Frankia* dans un tampon phosphate pendant 4-8 h (15 g d'inoculum PEM-alginate sec dans 1,5 1 de tampon) et en homogénéisant la gelée liquide ainsi obtenue. Pendant tout leur séjour en serre, chacun des semis de *Casuarina equisetifolia* a reçu une fois par semaine 100 ml d'une solution Hoagland sans azote diluée au 1/4. Le 23 juillet 1984, lorsque les plantes avaient environ 20 cm de haut, elles ont été mises en place à Notto, à 80 km au Nord de Dakar et à 5 km de la Côte. Le sol qui était un sol dunaire sableux très pauvre (0,047-0,121% N), n'avait jamais été plante auparavant en *Casuarina* et ne contenait pas de *Frankia. La* plantation a été faite après que les pluies eussent mouille le sol jusqu'à une profondeur de 30-40 cm. Le dispositif expérimental comportait huit parcelles élémentaires de 416 m^2 (quatre témoins, quatre inoculées) avec 84 plants par parcelle, ce qui correspond à environ 2 000 plants par ha. Une fertilisation phosphatée a été appliquée aux doses suivantes ; première année, 5 g K_2HPO_4 ; deuxième année 10 g ; troisième année 15 g. Les pluies ont été respectivement de 215, 375 et 473 mm en 1985, 1986 et 1987. Au cours de la première année, on a apporte 200 1 d'eau à chaque plant, mais cette irrigation n'a pas été répétée au cours des deux années suivantes.

Pendant trois années consécutives, soit en Juillet 1985, Juillet 1986 et Juillet 1987, dans chacune des huit parcelles, on a arraché cinq arbres repérés au hasard. Apres les avoir mesures et pesés, on a divise chacun des arbres en quatre fractions : rameaux assimilateurs, branches,' racines et nodules. Chaque fraction a été séchée et broyée. Après échantillonnage, on a déterminé N total. Le Tableau 2 montre que la réponse de *Casuarina equisetifoliu* à l'application d'inoculum PEM de Frankia est significative ($P < 0,05$) ou très significative ($P < 0,01$) sauf en ce qui concerne le paramètre hauteur. L'effet favorable de l'inoculum a porte sur la biomasse des différentes fractions des arbres exprimée en poids sec ou N total. Le rapport parues aériennes/racines calculé sur la base du poids sec ou de N total a été également favorablement modifié par l'inoculation (Sougoufara *et al.,* 1989).

Les résultats indiquent clairement que l'inoculum PEM de *Frankia* est *un* inoculum efficace et qu'il peut être stocke pendant 2 ans sans perdre ni son inefectivité ni son effectivité.

Inoculum polymérique (PEM) de champignon VAM

En 1982, Ganry et *al.* ont mis en place au Sénégal une expérience destinée à évaluer la réponse du soja à l'application d'un inoculum PEM de *Glomus mosseae* fait de spores, mycelium et racines mycorhiennes homogénéisées, ces différentes structures

Tableau 2 : Réponse de Casuarina equisetifolia à l'inoculation avec l'inoculum PEH-alginate de frankia 1, 2 et 3 ans après la mise en place au champ. Expérience effectuée à Notto. Sénégal (Sougoufara et al., 1989).

I. (1)	Haut. (2)	Poids sec (g/arbre) (3)				N total (g/arbre)				Rapport(1) P.A./Rac. basé sur	
		br.	ras.	rac.	nod.	br.	ras.	roc.	nod.	PS	N
Juillet 1985 (1 an après la transplantation au champ)											
Ø	151	163	89	266	Ø	Ø.63	1.46	1.24	Ø	Ø.95	1,69
+	182 *	324 **	119 **	309 *	2.55	1.99 **	2.17 **	1.60 *	Ø.Ø4	1.42 *	2,54 **
Juillet 1986 (2 ans après la transplantation au champ)											
Ø	291	890	628	507	Ø	5,64	9,03	2,52	Ø	2,99	5,82
+	313 NS	1349 **	871 **	626 **	12.18	9.39 **	16.24 **	3.21 **	Ø.2Ø	3,48 *	7,52 *
Juillet 1987 (3 ans après la transplantation au champ) (5)											
Ø	500	1451	1000	840	Ø	7,32	15,57	4.74	Ø	2,91	4,85
+	575 NS	2220 **	1356 **	1031 **	22.75	12,20 **	23.35 **	5,12 NS	Ø.4Ø	3,39 **	6,47 *

Seuil de signification : ** P = Ø, Ø1 ; * P = Ø, Ø5 ; NS = non significatif

(1) I. = inoculation. Ø : pas d'inoculation (témoins) ; + : inoculation avec l'inoculum PEM-alginate de la souche de frankia ORSØ21ØØ1.

(2) Hauteur moyenne des arbres en cm.

(3) br. = branches y compris les troncs ; ras = rameaux assimilateurs ; rac. = racines; nod. = nodules.

(4) Rapport "Parties aériennes/Racines" exprimé en poids sec (PS) ou N total (N).

(5) Au cours de la 3ème année des attaques de borers ont très sensiblement ralenti la croissance des arbres.

ayant été incluses dans des billes d'alginate. *Glomus mosseae* avait été au préalable multiplie sur niébé (*Vigna unguiculata*). Le champ d'expérience, situe à la station ISRA (Institut Sénégalais de Recherches Agricoles) de Séfa, au Sud du Sénégal, avait été choisi en raison du faible potentiel infectieux de son sol en ce qui concerne les champignons VAM. Ce sol deficient en N (environ 0.04% N) n'avait jamais éd cultivé auparavant en soja. Le dispositif expérimental utilise était du type split-plot avec huit répétitions. Les trois traitements principaux (chaque parcelle principale avait 40 m^2) ont été les suivants : T (témoin), inoculation avec une souche ineffective de *Bradyrhiwbium sur support tourbe ;* B, inoculation avec une souche effective de *Bradyrihizobium sur* support tourbe ; BG, inoculation avec *la* souche effective de *Bradyrhizobiwn sur support tourbe* (comme dans le traitement B) et inoculation avec inoculum PEM-alginate de *Glomus mosseae* (conserve à la chambre froide pendant plus d'un mois) appliqué à la dose de 15-20 billes fraîches (non séchées) à 3-4 cm de profondeur autour de chaque semis de soja, lorsque ceux-ci avaient 15 jours. Les deux sous-traitements ont été les suivants : OP, pas d'application de fertilisation phosphatée ; P, application de superphosphate à la dose de 20 kg par ha. L'azote marqué (^{15}N) a été appliqué sur des microparcelles de 1,65m^2 pour mesurer la fixation de N_2 par la méthode de la valeur A. On a analyse séparément feuilles, tiges, gousses et graines. Par souci de simplification, les données concernant les différentes parties aériennes des plantes, à l'exception des graines ont été regroupées sous le terme de «parties aériennes». Les chutes de pluie ont été de 170 mm avant le semis (ler Mai - 17 Juillet) et de 692 mm pendant le cycle cultural (17 Juillet - 10 Octobre). La distribution des pluies a été assez régulière, sans période sèche marquée.

Le Tableau 3 montre clairement que l'inoculum PEM-alginate de *Glomus mosseae* peut être utilise avec succès au champ, en tant que traitement complémentaire de l'inoculum de *Bradyrhizobium*, ce qui confirme les expériences publiées antérieurement par Ganry et *al.*, (1982).

Quand on a appliqué une fertilisation phosphatée légère (20 kg P/ha) l'inoculum PEM-alginate de *Glomus* mosseae a augmenté la fixation de N_2 de 109 (inoculation avec *Bradyrhizobium* seul) à 139 kg N_2/ha, ce qui constitue une amélioration substantielle. Dans les parcelles ayant reçu la fertilisation phosphatée, l'inoculum PEM-alginate de *Glomus mosseae* a augmente significativement le rendement en grain exprime en poids sec, et N total, et en outre améliore l'indice de récolte. Ces résultats positifs revêtent une grande importance pour le fermier.

Conclusion

II est maintenant clair que l'on peut améliorer considérablement la réponse des plantes à l'inoculation en faisant appel à la technologie fondée sur l'utilisation des microorganismes inclus dans une matrice polymérique.

Etant donné que la production de ces inoculums perfectionnes exige un certain niveau d'expertise, les usines de fabrication d'inoculums polymériques devraient étre installées clans un nombre limite de centres d'excellence. En outre, les expériences au champ, qui sont capitales pour toutes les souches, devraient être mises en place dans les différentes régions écologiques où l'inoculation est reconnue indispensable. Ceci est particulièrement vrai pour les arbres fixateurs de N_2

On peut penser que le développement de l'utilisation d'inoculum de haute qualité n'accroîtra pas seulement la fixation de N_2 et la croissance des plantes, mais aussi ren-

Tableau 3 : Réponse du soja à l'inoculation avec une souche non effective (non fixatrice de N2) de Bradyrhizobium (traitement T), d'une souche effective de Bradyrhizobium seule (traitement B) ou associée à Glomus mosseae, ce dernier inoculum étant un inoculum PEM-alginate (traitement BG). Dans la moitié des parcelles on n'a pas apporté de P (traitement PO), dans l'autre moitié on n'a apporté P sous forme de superphosphate à la dose de 22 kg P/ha (traitement Super). Expérience effectuée à Séfa, Sénégal (Ganry et al., 1985)

Traitement	Rendement en grain (kg/ha)		Grains et parties aériennes (kg/ha)		Indice de récolte sur la base de N total	N2 fixé (kg/ha)
	PS	N	N	P		
T OP	1023	101,6	127,2	5,6 11,1	3,98	0 0
T Super	1023					
B OP	1423	90,3	112,0	6,0	4,18	73,1
B Super	2017	133,8	161,0	10,0	4,71	109,0
BG OP	1431	98,2	120,0	6,9	4,42	80,2
BG Super	2290	154,7	183,7	11,8	5,33	139.3
PPDS entre les traitements'						
T, B et BG	192	12,8	16,2	0,8		14,5
OP et Super	197	13,5	17,0	0,8		13,4

forcera leur résistance aux parasites et pathogènes, ce qui permettra une diminution des besoins en engrais et pesticides, d'où une réduction des sources de pollution.

Actuellement, on met au point en France la fabrication à l'échelle industrielle d'inoculums PEM et PEC pour les rhizobiums, *Frankia* et différents microorganismes impliques dans la lutte biologique. Cette technologie devra être adaptée aux pays en voie de développement dans un proche avenir.

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ECONOMIE DE L'AZOTE DANS LES SYSTEMES DE CULTURE EN ASSOCIATION ET EN AGROFORESTERIE

Biological nitrogen fixation and nitrogen transfer in multiple cropping in tropical Africa

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Abstract

Traditional farming systems throughout the humid and subhumid tropics are characterized by multiple cropping, the association of crops in time and space on a piece of land during one calendar year. These systems usually involve leguminous crops and trees. Legumes can harness atmospheric nitrogen (N_2) into the systems and benefit non-leguminous crops through the transfer of nitrogen (N) in the root systems and decomposition of their residues. Biological mechanisms such as competition for light, water and nutrients that determine the yield levels for the individual crops can affect the processes involved in N_2 fiiation. Legumes can improve soil N status with resulting N and grain yield benefits to the succeeding nonleguminous crops. Even in the absence of net N benefit, intercropped nonlegumes have been shown to derive important fractions of their N from N_2 fixed by leguminous component(s). Only few studies have been published on economic profits of using legumes in cropping systems in the tropical Africa. Although they show interesting returns relative to the use of chemical fertilizers, they usually do not consider risk factors. A quadratic programming approach is thus proposed as a more appropriate method to appraise the economic potentials of legumes in multiple cropping in sub-saharan Africa.

Introduction

Traditional agriculture in tropical Africa is based on shifting cultivation and related bush fallow systems. The restorative success of these systems depends largely on the litter cover from the fallow vegetation and on the nutrient recycling function of the deep-rooted trees and shrubs (Sanchez,, 1976). In recent years, however, due to increasing populations and/or the scarcity of labor in areas with low population densities, fallow periods have been systematically reduced or suppressed, leading to more intensive cultivation of marginal lands. Continuous cultivation of the same fields has resulted in soil erosion, increased weed populations, and decreasing soil fertility and crop yields (Kang et *al.*, 1985 ; Lal, 1987).

Earlier research approaches towards alleviating the food deficit problem in tropical Africa were based on the Green Revolution which was associated with high-yielding varieties, sole cropping, monoculture and enormous inputs of non-renewable resources to extend the cropping season, increase soil productivity and control pests. The failure of the Green Revolution, some fifteen years ago, led «developers» to revise their approach for the promotion of food production in the tropics. They began to emphasize the needs for crop production stability, resource conservation, reduction of external inputs and more complete use of natural resources. This tendency has stimulated a lot of research effort in the area of multiple cropping aiming at defining plant populations of the component crops, planting patterns, plant structure, relative duration of the life cycles of the component crops, and fertilizer managements that would result in combined yield advantages (Okigbo and Greenland, 1976).

Legumes are usually included in multiple cropping systems for three basic reasons. Low-growing legumes can be integrated into the architecture of tall cereals without excessive reduction in yields of component crops, but with significant increases in protein and crop yields per hectare (Norman, 1982). Legume-cereal mixtures are advantageous because legumes can fix atmospheric N_2 symbiotically and transfer this additional N to other crops in the system. Finally, farmers are risk averters who base their farming decisions not on profit maximization but on the judicious choice and arrangement in time and space of various crops to suit their microecologies and conditions and ensure crop production with minimum risk.

There is however a dearth of information about how much N is fixed ; how this process is affected by the various plant interactions such as shading in the cropping system ; how leguminous plants and, in general, N_2-fixing systems contribute to the yield advantages and the maintenance of soil fertility in multiple cropping. Economic evaluation of N_2 fixation and N transfer is also lacking partly because of the poor knowledge of complex N transfer processes in multiple cropping systems. Although, a great deal of research work on legumes in multiple cropping has been conducted in tropical Asia, comparatively little work has been conducted on this topic in tropical Africa.

In this paper, we briefly review the major ecological factors determining yield levels in multiple cropping systems and their influence on biological N_2 fixation. We then discuss N contribution of legumes in selected types of multiple cropping. Our discussion does not include associative N_2 fixation and pasture systems to simplify the presentation of the paper. However, principles described for other systems are also valid for these systems. Finally, we briefly review the economics of using legumes in multiple cropping and consider the quadratic programming approach that includes the risk mini-

mixing attitude of farmers as a possible tool for economic assessment of the potential of legumes in sub-saharan Africa.

Some definitions

Before addressing these issues, it is useful to define certain concepts. Multiple cropping is the general term which is used to describe systems where two or more crops are grown on the same field in a year. The system is said sequential when the crops are grown in sequence. In this case, the intensification of crop production is only in the time dimension. Sequential cropping is rare in traditional african farming systems. It characterizes areas where on-farm agricultural technology is geared towards commercial production (Andrews and Kassam, 1976).

Intercropping, the growing of two or more crops simultaneously on the same field, is widespread in tropical Africa with the highest complexity in compound gardens of the rainforest region where are intercropped annual staples, vegetables and perennial fruit trees (Okigbo and Greenland, 1976). Mixed intercropping, the simultaneous growing of two or more crops without distinct row arrangement, and relay intercropping, the growing of two or more crops simultaneously during part of the life cycle of each component, are the predominant practices in the tropics. In row intercropping, two or more crops are grown simultaneously with one or more crops planted in rows. Strip intercropping is the growing of two or more crops simultaneously in strips. Both row and strips intercropping are rare in Africa except where animals or tractors are used in cultivation (Okigbo and Greenland, 1976 ; Steiner, 1982). In the live-mulch system, food crops are grown in an establish field of a low-growing perennial leguminous cover crop (Akobundu, 1980). The live mulch prevents soil degradation and erosion, suppresses weeds and provides biologically fixed N_2

Trees such as oilpalm, coconut, cocoa are also often intercropped to a range of annual crops in the early years of plantation (Okigbo and Greenland 1976). This multistorey cropping is considered as the ideal farming system in the humid tropics as it resembles the natural vegetation. Related to the multistorey cropping system is alley cropping, the growing of food crops between hedgerows of woody species that are periodically pruned to prevent shading of the associated crops and to make available mulch materials and the nutrients locked up in the prunings (Kang et *al.*, 1981).

Multiple cropping is generally more complex in the forest regions than in the savanna areas. In the forest region, crops are more diverse, farmers' holdings arc relatively small and the growing season is longer (Okigbo and Greenland, 1976). Within the same ecological zone, the diversity in component crops and their spatial arrangement increases with soil fertility which in turn improves with the proximity to the homestead (e.g. compound gardens).

Factors determining yield levels in multiple cropping systems

The major factors determining yield levels in multiple cropping systems are the interactions of component crops during the use of growth factors, the N_2-fixing ability of intercrops and stresses interfering with biological N_2-fixation. We discuss each of these processes subsequently.

Interactions of component crops

When plants are intercropped or grown in sequence, they interact through various forms of modifications imposed on the environment. These interactions and their effects on crop yields were reviewed by Trenbath (1976). Willey (1979a) and Palaniappan (1985). The interactions are said non-competitive when the associated crops share growth factors that are present in adequate quantities, such that some positive level of output from one crop is possible without any reduction in the output of another crop. Competition occurs when component crops share a limited pool of growth resources. In this case, one output must be forgone in order to produce more of the other output.

In an intercropping situation, 'when the component species are able to exploit the supply of growth factors in different ways in time or space, or if one species is able to help the other in the supply of a factor, the interaction is said to be complementary. The process by which growth factors are used is referred to as annidation. Other synonyms found in the literature include facilitation, cooperation aud compensation. Another form of interaction observed among plants grown in intercropping is the influence of one component crop on pests that may affect the performance of other crops in the system.

Plant growth factors that are likely to play important role in multiple cropping are light, water and mineral nutrients. Light is absorbed by the leaves, and water and nutrients mainly by the roots. In healthy young plants, growth is responsive to the rate of absorption of any one of the growth factors when it is in relatively short supply (Trenbath, 1976). Competition for light occurs when the photosynthetic canopy of one component is set higher than that of another. Thus taller components compete more effectively for light. But leaf area and leaf inclination are likely to determine the magnitude of competition. If shading is partial, the shaded plants can adapt to low light conditions by reducing their rate of dark respiration, lowering their root/shoot ratio, or increasing the leaf area/leaf weight ratio. These adaptative changes can be part of selection traits in breeding genotypes suitable for multiple cropping. Intense shading will reduce growth considerably and induce early senescence of leaves. Light is different from other growth factors as it cannot be much influenced by man, Therefore, by maximizing light interception, multiple cropping can suppress weeds and reduce evapotranspiration losses from the soil surface effectively.

The uptake of water, dissolved nutrients and oxygen (O_2) by a root surface establishes a concentration gradient in the vicinity of the root The zone of depletion of water and mobile ions such as nitrates will expand rapidly as compared to that of P and cations like ammonium, calcium and potassium that are strongly adsorbed onto the surfaces of soil particles. Thus in multiple cropping particularly in intercropping, a component crop can induce water and nutrient stress for the other crops. Experiments on competition for O_2 have not clearly demonstrated the magnitude and consequences of this interaction. Competition for carbon dioxide (CO_2) has been demonstrated in sealed containers, but it is unlikely to occur in the fields owing to the turbulence of air within canopies (Trenbath, 1976).

Yield advanfages of nitrogen-fixing intercrops

Nitrogen-fixing intercrops affect crop yields through annidation in space, in time and with respect to nitrogen. Spatial differences between crops are related to shoot height, plant architecture, and root depth and distribution. Willey and Rao (1981) demonstrated

the importance of spatial interactions in intercropping by comparing the performance of 17 genotypes of pigeon pea in association with sorghum. None of the genotypes affected sorghum yields adversely but their own yields ranged between 36 and 73% of sole crop yields. Genotypes that were compact in the early growth stage and that spread later to fully utilize the resources after sorghum harvest minimized competition with sorghum significantly. They were found to be most suitable for high combined yields.

Another example of annidation in space is the multistorey cropping involving planting leguminous shade trees or low-growing cover crops such *as* ***Pueraria*** or ***Centro-***sema in cocoa and tea plantations. Trenbath (1976) noted that where the taller intercropped component has steeply inclined leaves and the shorter component has prostrate leaves, there is a possibility of a land equivalent ratio (LER) > 1. In alley cropping, ***Flemingia congesta*** *is* one of the few nitrogen-fixing shrubs that perform well under the shade of plantain. Its N-rich pruning can improve plantain (International Institute of Tropical Agriculture, 1985) and maize gram (Table 1) yields . The principle of exploiting different vertical space layers is also found in live-mulch system. However, cover crop species currently *used in the* system such as ***Psophocarpus palustris*** and *Centrosema* ***pubescens*** tend to climb and strangle the associated crop if they are not sprayed with growth retardants.

Table 1. Effect of prunings from Cassia siamea, Flemingia congesta, Gliricidia sepium and Leucaena leucocephala on maize' N content and grain yield (Mulongoy, unpublished data).

Pruning	Pruning N applied[2]	Maize N	Maize grain yield
	kg ha^{-1}		
Cassia	168	16.7	2430
Flemingia	90	13.9	1540
Gliricidia	180	21.8	2870
Control	0	8.0	1100

1 On the basis of 40000 plants ha-'. Nitrogen content was determined at 8 weeks after planting.

2 Prunings were applied only at maize planting

Some degree of root stratification must be common in intercrops of dissimilar components on deep soils (Trenbath, 1976). But only few reports have been published on this topic. In alley cropping, Kang et al. (1985) showed that both maize and leucaena had distinct root feeding zones with leucaena extracting moisture and nutrients from deeper layers. However, hedgerow trees established from cuttings may have shallow root systems (Atta-Krah et al. 1985) and thus compete with the associated crops.

Two intercrops of widely different maturities will likely reach their peak demands for growth factors at different times. This dissimilarity will reduce interference between them. A minimum maturity difference of 30-40 days for yield advantage has been suggested (Palaniappan, 1985). After the early crop is harvested, the other crop will fully exploit the growth factors. Yield advantages in relay cropping are partly based on this principle. Another temporal effect occurs in situations where senescent plant materials, litter or prunings could make nutrients available to another crop. Norman (1982) notes for instance that up to 70% of the aboveground materials of guar (*Cyamopsis tetragonoloba*) returns naturally to soil as leaf fall. Charreau and Vidal (1965) also noted the leaf fall of *Acacia albida* and its contribution to soil N and N uptake of cereals planted around it.

In an intercropping system involving N_2-fixing and non-N_2-fixing systems, the N_2-fixer will obtain part of its N from biological fixation of atmospheric N_2, while the other component(s) will utilize exclusively soil mineral N. If the non-N_2-fixing component is very competitive for soil N, a depleted soil in N will favour nodulation and nitrogen fixation of the N_2-fixer intercrop. Pigeon pea seems to nodulate better where the roots intermingle with those of intercropped sorghum (Willey, 1979a). Ennik cited by Trenbath (1976) reports that white clover intercropped with grass on N-poor soil gives a LER up to 6.7 due to the interactions between the component crops. Some investigators explain the positive effect of added legumes on LER by some transfer of the N_2 fixed to the non-leguminous component (Trenbath, 1976). But more data are needed to corroborate this explanation.

interference stresses on biological nitrogen fixation

Various stresses can impair biological N_2 fixation in multiple cropping systems. Biological N_2 fixation is markedly dependent on current photosynthesis regulated by light This is shown by diurnal variations in nitrogenase activity (Sloger *et al.,* 1975) and by shading effect on N_2 fixation. Gibson (1976) reported that *nitrogenase* of *Trifolium subterraneum* lost 40% of its activity within five hours when the plants were transferred from an illuminance of 620 $\mu Em^{-2}s^{-1}$ to one of 165 $\mu Em^{-2}s^{-1}$. Nutman (1976) reported also that 50% shading of soybean plants at the end of flowering decreased fixation from 125 to 91 kg N ha^{-1}. He concluded in his review that there is a direct relationship between light intensity and N_2 fixation through the amount of photosynthate produced. Similarly, growth and CO, uptake of intercropped azolla declines in response to a developing rice canopy (Lumpkin and Plucknett, 1982). Photosynthesis by leaves of legumes seems saturated at 30-50% of maximum sunlight (Ludlow cited by Henzell, 1981). As stated earlier, plants exhibit various phenotypical **adaptations to low light to ensure maximum light interception.**

The detrimental effect of water stress on the host-plant, the rhizobia and symbiotic **effectiveness is well** documented (Sprent, 1971, 1972 ; Eaglesham and Ayanaba, 1984).

Rhizobial populations are reduced in size when soils become desiccated. Slow-growing rhizobia tend to survive desiccation better than fast-growing rhizobia *Soil* type influences the desiccation tolerance of rhizobia. Nodulation tends to fail at low soil moisture levels not only as a result of death of the rhizobia, but essentially because root hairs assume a configuration incompatible with the infection process (Eagle&m and Ayanaba, 1984). The water potential levels allowing nodulation of legumes should be defined. Sprent (1971) noted that soil moisture loss below 80% of the maximum resulted in irreversible damage of soybean nodules. Some legumes such as clovers can recover more rapidly from drought than others like soybeans that shed nodules under dry conditions. Water stress can also reduce photosynthesis by reducing leaf area and inducing leaf fall ; this in turn will reduce symbiotic N_2 fixation.

It is accepted that N_2-fixing plants require more P than plants supplied with mineral N. Phosphorus deficiency will limit nodule development, nitrogenase acrivity and host-plant growth. A number of experiments have demonstrated the important role of vesicular-arbuscular mycorrhiza in increasing P uptake of infected plants. Also, dual inoculation with effective strains of rhizobia and mycorrhizal fungi shows synergistic effects on nodulation and reduction of atmospheric N_2 in Pdefrcient soils. Recent studies reveal genetic diversities within plant species in tolerance to low soil P (Sanginga *et* al.. 1990).

The effect of K on the *Rhizobium* - lugume symbiosis seems to be indirect and to act through the physiology of the plant (Jardim Freire, 1984). There are however reports showing nodulation response to K under field conditions (Ayanaba and Dart, 1977).

Danso *et al.* (1987) observed a reduction in dry matter yield, total N and N_2 fixed by fababeans intercropped with barley. Mulongoy (1986a) found a similar reduction in dry matter of intercropped cowpea and maize that resulted in reduced total plant N. With increasing plant population densities of either fababeans in the sole crop or barley in the intercrop, Danso *et al.* (1987) obeserved increases in the proportion of N derived by fababeans from fixation. Greater N_2 fixation may either increase the total N yield in the legume or just the proportion of N from fixation as compared to N derived from mineral sources by the legume. Danso *et al.* (1987) found in their study that the presence of barley decreased soil and fertilizer N uptake by fababeans and enhanced symbiotic N_2 fixation of the legume component. The mechanisms governing these processes were not described. However, since the reduced soil N uptake by the legume was not reflected in increased soil or total N in barley, there was therefore no evidence of N-sparing effect by the legume for the associated non legume. Data presented by Danso et al. (1987) do not support the hypothesis of N transfer by the legume to maize.

Mulongoy and Nkwiine (1987) observed that populations of rhizobia decreased when maize was included in a sequence of soybean monoculture (Table 2). Whether the reduction in rhizobia populations was due to allelopathic effects was not investigated. Reduction in the size of rhizobial population can result in poor nodulation and nitrogen fixation. It is important to note that poorly or not nodulated legumes can deplete soil N just like cereals. Mulongoy and Nkwiine showed in the same experiments that crop ping cowpea or soybean after maize increased the population of *Bradyrhizobium japonicum.* It is important to note that poorly or not nodulated legumes can deplete soil N just like cereals.

From this discussion, it is evident that various types of interferences occurring in multiple cropping can reduce the number of rhizobia and affect nodulation and nitrogen fixation adversely. Few investigations have been carried out to monitor changes in

these factors and how they affect biological nitrogen fixation of leguminous components in multiple cropping.

Table 2. Effect of cropping sequences of soybean(s) and maize on the log10 of the numbers of soybean rhizobia per gram of soil recorded after the last cropping season, on a loamy sand. (Mulongoy and Nkwiine, 1986a)

Cropping sequence	Bradyrhizobium japonicum (log10 number)
Soybean - Soybean - Soybean	3.710 a
Soybean - Soybean - Maize	3.492 b
Soybean - Maize - Soybean	3.710 a
Soybean - Maize - Maize	3.118 c

Nitrogen contribution of legumes in multiple cropping

Nitrogen contribution of legumes and, in general, of N_2-fixing systems in multiple cropping can occur through (1) exudation or excretion of nitrogenous substances by active nodules and roots, (2) leaching of soluble N from living leaves and plant parts during rainfalls, (3) litter fall and decomposition, (4) root and nodule decay, (5) decomposition of crop residues and prunings, and (6) animal manure. All these modes of N transfer are possible in an intercropping situation, but only residual effects will be involved in sequential cropping. In this section, we examine successively N contribution in legume-non-legume intercropping, livemulch systems, alley cropping and sequential farming.

Nitrogen contribution in legume-nonlegume intercropping

Various reports give evidence that the legumes benefit the associated crops in intercropping systems (Evans, 1960 ; Ckiru and Willey, 1972). But Trenbath (1976) compiled data (607 values) from various sources and showed that the effect of legumes on LER was not necessarily better than adding nonleguminous intercrops. He concluded that the main advantage from the legume components was in the saving of N-fertilizer. He also noted that while competition for N would be miuimized in mixtures involving legumes, competition for other growth factors could occur.

Early scientific reports on active N tranfer from the root nodules to associated crops were published in the 1930s, in pot experiments (Willey, 1979a). It was noted that shading favours N excretion from the legumes. This may however be significant only when the legumes have already fixed N_2, as shading will reduce N_2 fixation. Defoliation can also promote N release in the root system. Whitney and Kanehiro (1967) found N

contribution of 0.1 to 0.6 kg ha-' from the root system of defoliated *Desmodium intortum* to pangola grass.

Although direct evidence of active N excretion from the root nodules of-legumes is lacking, other data suggesting N transfer from the legumes include the lack of response of cereals to fertilizer N when they are intercropped with legumes (Agboola and Fayemi, 1972 ; Remison, 1978), higher N uptake of the cereals in intercropping than when they are grown as sole crops (De, 1980 ; Eaglesham et *al.*, 1981) and ^{15}N studies (Eaglesham, 1982 ; Patra et *al.*, 1986). Short cycle legumes seem to contribute more nitrogen to the associated crops than late maturing species. Maize derived more benefits from *Vigna radiata* than it did from cowpea (Agboola and Fayemi, 1972), and from *Lathyrus* sp. and early maturing cowpea or groundnut than it did from *Phaseolus vulgaris* (Waghmare and Singh, 1984). This suggests that N contributions might occur mostly through senescence and decomposition of the short cycle legumes.

Nitrogen transfer from legumes to nonlegumes in intercropping is not always translated in N (Fig. 1) or grain'yield (Danso *et al.*, 1987) advantages. Nitrogen-15 studies provide the most reliable data on the occurence and magnitude of N transfer in intercropping involving legumes. Eaglesham (1982) found that intercropped maize had access to a source of N not available to sole maize crop. He presented ^{15}N data consistent with N transfer from cowpea to the intercropped maize. In experiments conducted by Patra *et* al. (1986), 28% of maize N representing 21.2 kg N ha^{-1} was obtained from the atmosphere through transfer of fixed N by cowpea grown in association with maize. They confirmed their data in pots with either maize or wheat intercropped with cowpea.

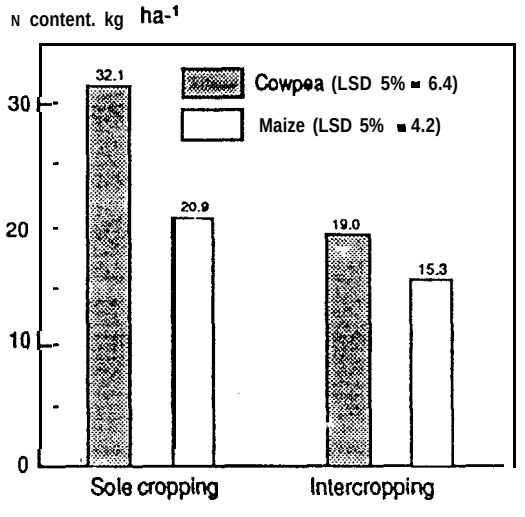


Figure 1: Response of wwpea and maize N content to intercropping. Cowpea values are average for 6 wwpea cultivars ; for maize, average of maize intercropped with 6 wwpea cultivars (Adapted from Mulongoy, 1986a).

Nitrogen contribution in live-mulch systems

Live-mulch is a crop production system in which food crops are planted into a low-growing cover crop with minimum soil disturbance (Akobundu, 1980). The cover crop smothers weeds and protects the soil. Akobundu (1980) observed that after five seasons of continuous cropping, maize did not respond to fertilizer N in live-mulch plots of *Psophocarpus palustris* and Centrosema *pubescens* whereas maize yields in bare plots under minimum or conventional tillage increased when N fertilizer (60 kg ha^{-1}) was given. Mulongoy (1986b) compared N contributions to maize of newly established and 3-years old live-mulches of *Psophocarpus, Centrosema* and *Arachis repens. In the* newly established field, cover crops and maize competed for N to the disadvantage of maize. In the longterm plot, *P. palustris* was the best live-mulch with N contributions averaging 30.7 kg ha-'.

In another experiment, nitrogen uptake by maize was monitored for five cropping seasons in a live-mulch of *P. palustris.* During the first four cropping seasons, *P. palustris* and maize competed for soil N in the live-mulch, and maize grown in the live-mulch contained less N than maize grown by itself. In the fifth season, the live-mulch contributed 15 kg N ha^{-1} to maize in the plots that were cropped continuously for five seasons. Maise grown in a psopho livemulch that had already been established for four cropping seasons contained 36 kg ha^{-1} more N than maize grown by itself. This positive N contribution came, at least in part, from increases in microbial biomass and in organic matter derived from the cover crop in the form of litter and earthworm casts (Mulongoy, 1986b).

The live-mulch system, however, needs refinement because, at present, a net contribution of N and an increase in food crop yield occur only after the cover crop has been established for some cropping seasons. This lag in benefits has to be shortened if the live-mulch system is to be accepted by farmers. One way to shorten the lag is to minimize competition for soil N between the cover crop and the food crop during live-mulch establishement. Also, if the pathways of N transfer from the live-mulch to the food crop were understood, it might be possible to manage the system so as to increase the amount of N contributed by the legume.

Simpson (1976) and Ladd *et al.* (1981) also found little N transfer from low-growing legumes to associated crops, in pastures. The main N value from the legume seems to be the building-up of soil organic N for adequate N supply to subsequent crops. Müller and Sundman (1988) who monitored the fate of ^{15}N released during decomposition of leguminous plant materials buried in the field found that 2746% of input N was retained in the soil. A succeeding crop of barley took up 6-25% of input.

Nitrogen contribution in alley cropping

In the alley cropping, leguminous trees are preferred to monleguminous trees for the hedgerows. They can bring atmospheric N_2 into the soil system. Well-nodulated trees or shrubs can yield more than 500 kg N ha^{-1} in a year (Guevarra, 1976) owing to their high N_2-fixing ability. Estimation of N_2 fixation of trees is difficult. But current data indicate that nodulated trees can fix symbiotically more than 50% of their N (Roskoski, 1981 ; Hogberg and Kvarnstrom, 1982 ; Rinaudo *et* al., 1982 ; Sanginga et al., 1986; Mulongoy et *al.,* 1988 ; Ndoye and Dreyfus, 1988). The mojor proportion of hedgerow tree N is found in the prunings consisting of leaves, twigs and succulent plant parts including immature pods. Application of legume prunings increases N and grain yields in the

associated crops (Table 1 ; Kang, 1987). However, N contribution of the prunings to the associated crop may not always be very efficient. Kang (1987) and Mulongoy and Van der Meersch (1988) reported it in the range of 10 and 40% of the input N, and Kang et *al.* (1985) found that up to 80 kg fertilizer N was needed for optimum maize grain yields. Some results suggest that part of the N from prunings is retained in the soil organic-N pool (Mulongoy and Van der Meersch, 1988). In a hydromorphic soil crop ped to rice, N contribution of *Sesbania rostrata was estimated* equivalent to 40 kg N ha^{-1} representing about 30% of the N applied in the prunings (Mulongoy, 1986c). Management practices such as incorporation of prunings in the soil can increase the efficiency with which N is transferred from prunings to the associated crops (Read et al., 1985 ; Wilson *et al.*, 1986).

Nitrogen contribution from belowground parts can be considerable. Sangiga *et al.* (1986, 1988) estimated it at about 30% of total N contribution of leucaena to maize in a rotation experiment. Nitrogen contribution from roots of hedgerow trees needs to be investigated.

Nitrogen contribution in sequential cropping

The residual effect of leguminous crops in a sequence will greatly depend on several factors including the legume N at harvest time, N harvest index, management of the residues (Whether retained on the soil surface, incorporated in the soil or partly removed for domestic use or for feeding animals) and on the time interval between crops in the sequence. Kang (1987) discussed some examples of N returned to soil in crop residues of pigeon pea, cowpea and soybean. The following generalizations can be drawn : (1) legume N yield i.e. the sum of atmospheric nitrogen fixed and N absorbed from soil, is high, varying in these examples between 73 and 205 kg N ha^{-1} ; (2) N in fallen leaves and root system is usually low ; but it can be as high as 40 kg N ha^{-1} in well nodulated shrubs at harvest ; (3) N removed in crop residues, pods and seeds can be relatively low ; it ranged in these examples between 26 and 85 kg N ha^{-1} ; (4) N balance can be very low even negative indicating that the legume can deplete soil N particularly when N_2 fixation is low and/or N harvest index is high.

The N returned to the soil in the residues will then be mineralized before it is used by the succeeding crops. Plowing the residues under may reduce the probability of N loss by volatilization and erosion, and may increase N contribution to the succeeding crops.

Kang (1987) reports a number of experimental data showing that the legumes improved soil N status, with resulting N and grain yield benefits to the succeeding non leguminous crops. There were differences among plant species in the level of N contribution. Preceeding non-leguminous crops with a legume increased their yields more than intercropping. Some investigators however reported no or negative N contribution from legumes in sequential cropping. A good knowledge of the N cycle in the system will allow to devise practices that can maximize the resources for low-input agriculture suitable for the smallholder farmers.

Methods for evaluating the economic potential of legumes In multiple cropping systems

The purpose of this section is to examine methods by which to appraise the economic potential of legumes in multiple cropping systems. The basic question is what are the best systems (with or without legumes) capable of raising or maintaining the level of soil fertility while satisfying the farmer's goals and resource limitations. The need to answer this question is pressing as results from past agronomic studies show that multiple cropping systems (especially when legumes are associated with non leguminous food crops) are much more efficient than sole cropping (Willey, 1979a and 1979b ; Steiner, 1982 ; Horwith, 1985 ; Spencer, 1985). Leguminous crops have been shown to improve soil fertility (through nitrogen fixation and transfer) thus contributing to yield stability (risk reduction) over different seasons and higher yields in a given season.

Few economic studies are available which evaluate the role of leguminous crops in multiple cropping systems. Mittal *et al.* (1985) conducted a study to determine yield, total production and net returns by intercropping legumes (black gram, green gram, cowpea and groundnut) with maize. Their results show that maize intercropped with groundnuts is the most profitable and suitable system for rainfed conditions in Shiwalik region, India. Singh and Chand (1980) also studied the income effect of intercropping grain legumes with maize. Their results show that maize intercropped with soybean is the most remunerative system with 120 kg N ha^{-1}.

Although these studies show interesting results, they are limited for a number of reasons. First, they were conducted essentially in tropical Asia and therefore their results may not be applicable to sub-saharan Africa where the rate of demographic change is higher and the physical conditions which determine technical potential are more difficult (Matlon and Spencer, 1984). Most of these studies use the partial budgeting approach in which the market value of land and labor are unknown or included in an ad-hoc manner. Yet the real economic prices (i.e. shadow prices) of these resources are essential for evaluating the profitability of a cropping system and how for example labor and different land types are allocated in the cropping seasons and among crops. Partial budgeting approach ignores the substitutability of inputs and how they are allocated based on fixed endowments and the implicit prices of the resources. Finally, these studies do not consider risk factors. Farmers in sub-sahamn Africa are risk-averse. They hedge against risk by diversifying into as many crops as possible through multiple cropping. Crop yields and prices vary from one growing season to another thus affecting the expected income of the farmer. If this is true, then an economic evaluation of multiple cropping systems using riskless models is inappropriate.

For appraising the economic potential of legumes in multiple cropping, a moderne theory based on utility maximization and risk programming is proposed. This is a much more appropriate model to describe farmers behaviour in sub-sahamn Africa than the standard profit maximization models. Farmers practice diversified farming not only to take advantage of the complementary nature of the various crops but also to minimize risk in aggregate farm income given a level of expected income. Quadratic programming which is based on the expected-income variance criterion is a practical way of selecting income-risk efficient plans. It assumes that the farmer's preference among alternative farm plans is based solely on expected net farm income, E and variance, V (Freund, 1956). Quadratic programming model was first applied to agriculture by Freund (1956) and has since been

recommended for analysis of uncertainties in gross margins, gross returns and net variable costs in farm planning (Heraedy and Candler, 1958).

The problem of resource allocation under risk can be mathematically formulated as :

$$\text{Max } EV(X) = RX - (\alpha/2)X'QX$$

Subject to $AX<B$, where X is an (n x 1) vector of activities (e.g. hectares planted to maize/cowpea ; sole maize, maize/cassava/leucaena etc.)

- R = (n x 1) vector of expected net returns associated with the vector of activities
- t = transpose of R and X
- A = (n x n) matrix of technical coefficients (e.g. labor, rotational or land requirement etc.)
- B = (n x 1) vector of resource constraint level (e.g. available labor or capital, or land etc.)
- α = is the risk averse coefficient
- Q = (n x n) matrix of tbe variance-covariance matrix of gross returns.

The special feature of the quadratic programming approach is the variance covariance component of the objective function, EV. When the risk aversion coefficient cc is zero, the problem collapses to a standard linear programming problem indicating that farmers are risk-neutral. A positive (negative) value of a indicates the farmer is risk averse (lover). Although there is no clear method by which to estimate a the latter is usually obtained by eliciting farmers' preferences. Application of the quadratic programming approach also requires that expected returns and variance and covariance of returns for all enterprises be known. Covariances are fundamental for efficient diversification among farm enterprises as a means of hedging against risk (Heady, 1952). For example combination of activities that have negative covariate gross margins will usually have a more stable aggregate return that the return from more specialized strategies. A case in print in sub-saharan Africa is the intercropping versus the monocropping system. Returns of crops in an intercropping may covariate negatively thus providing in the aggregate a stable return, compared to monocropping systems.

The quadratic programming approach assumes that the farmer is rational and thus restricts his choice to those farm plans for which the associated income variances are minimum for given expected income levels. This assumption is based on the premises that individuals like increases in expected returns and dislike increases in variance of returns. The variance and covariance of return can be obtained using standard statistical packages or a culculation using the variance formula

$$V = \Sigma_j(R_j - r)^2/n-1$$

where R_j denotes the gross margin of activity j, and r is the average return on activity j over n cropping seasons. Other data needed depend on the problem specification and are usually obtained by means of survey techniques, direct measurements and secondary sources of information.

Conclusions

Multiple cropping is typical of agricultural practices in the tropics. Depending on the level of agricultural technology, multiple cropping involves the growing of sole crops in sequence or various mixtures of rain-fed crops. Intercropping is the predominant practice by small-holder farmers producing relatively low crop yields. It does however make better use of the resources than sole cropping, and help minimize variability in returns.

Legumes are usually included in multiple cropping as sources of protein. This is because they fit the temporal and spatial requirements of the system. It is indeed believed that legumes competeonly little with other crops for N, and that they can reduce the need for fertilizer N in the system since they can fix atmospheric N_2

There is a dearth of information in multiple cropping on the dynamics of rhizobia and other nitrogen-fixing microorganisms when they are living in the rhizosphere of non-host plants, on how nodulation is affected by the various interactions observed between component crops, and on how much N_2 legumes and other N_2-fixing systems fix in mixtures or in sequence with non-leguminous crops. The response of legumes and other crops such as maize to crop mixture and high population densities have been widely studied utilizing the «replacement» series (De Wit, 1965) or, most commonly, the « additive » technique (Chang, 1981). The same techniques can be used to monitor the changes in biological N_2 fixation parameters. Also, only little quantitative data is available on the process of N cycling in multiple cropping. Nitrogen-15 studies will provide the most accurate information about N_2 fixation and N transfer. They allow a distinction on one hand between N derived from atmosphere, soil and fertilizer and, on the other hand, between N transfer and competition (Patra et *al.*, 1986).

Although, research results have demonstrated the N and grain yield advantages of including legumes in multiple cropping, analyses are laking which determine the economic potential of nitrogen fixation and transfer of legumes in multiple cropping systems in Africa. In this paper, a method of economic analysis is highlighted which takes into account the risk minimizing behaviour of farmers in sub-saharan Africa.

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AMELIORATION DE LA FIXATION SYMBIOTIQUE DE L'AZOTE PAR LA SELECTION DES PLANTES

Importance de la plante-hôte et amélioration génétique de la fixation biologique de N_2 chez les arbres tropicaux

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Summaty.

The host plant plays a most important role in the process of symbiotic N_2 fixation Some of host gene products involved in the development and maintenance of the symbiosis have already been identified. But further studies are necessary before attempting the transfer of the symbiotic genes to novel plants.

Improvement of the host plant performances to increase its N_2-fixing potential and tolerance to environmental stresses could be achieved by : exploiting natural genetic variability, expanding genetic variabiity through somaclonal variation and introducing new genes in the plant genome by genetic transformation.

The results discussed in the paper support the recommandation that selection of host plant should take into account various traits, specially N_2-fixing potential, nitrate assimilation, and tolerance to combined nitrogen inhibition.

Introduction

Les stratégies d'amélioration des arbres forestiers reposent, comme chez les plantes cultivées annuelles, sur une amélioration génétique des individus et également sur l'utilisation de méthodes culturales adaptées. Ce qui diffère chez les ligneux, c'est tout d'abord, la dur& de la vie de la plante qui peut être parfois considérable et ensuite, la grande variabilité génétique naturelle qui existe dans les peuplements, par suite de l'absence de

travaux de génétique sur ces végétaux à cycle de génération très long. De surcroît, dans les associations symbiotiques. l'étude du partenaire végétal a été longtemps délaissée au profit de celle du microorganisme. Il n'est donc pas étonnant que l'arbre, en tant que plante-hôte des associations symbiotiques forestières, ait été peu utilise en tant que système fixateur de N_2

Les récents progrès obtenus ces dernières années dans les technologies avancées de transfert de gènes aussi bien chez les microorganismes que chez les plantes herbacées ont permis d'obtenir des résultats tout à fait remarquables (Shah et *al.*, 1986 ; De Blok et *al.*, 1987). Qu'en est-il des systbmes forestiers fixateurs de N_2 ?

Avant de faire l'état actuel de nos connaissances, nous rappellerons l'importance du partenaire végétal en dégageant quelques-uns des rôles connus de la plante-hôte dans le fonctionnement de la symbiose fixatrice de N_2. Nous envisagerons ensuite les approches possibles que l'on peut mettre en œuvre pour améliorer la fixation de N_2 en faisant appel à la plante-hôte, et les premiers résultats concernant cette stratégie seront présentés. Enfin, l'amélioration génétique de la plante-hôte sera discutée en fonction des autres approches **Utilisées.**

Importance du rôle de la plante-hôte dans la fixation de N_2

Qn sait que la plante-hôte fournit l'énergie suffisante au métabolisme de la fixation de N_2 et à la survie du microorganisme symbiotique. Elle contrôle l'activité de l'hydrogénase et participe aussi à la protection de la nitrogénase. Depuis un demi-siècle, de nombreux travaux ont démontré abondamment que chacune des étapes du processus de l'infection et de la nodulation est contrôlée par la plante, en relation étroite avec le microorganisme symbiotique. Le contrôle porte en particulier sur le nombre, la taille, la morphogénèse du nodule et la potentialité de fixer N_2. On peut mettre en évidence ce rôle, non seulement dans l'expression phénotypique, mais aussi, de plus en plus, au niveau génomique.

L'infection

L'existence des groupes d'inoculation croisée entre l'hôte et la bactérie symbiotique est une manifestation évidente du rôle de la plante-hôte dans la symbiose. Une espèce donnée de *Rhizobium ou de Frankia* ne nodule pas toutes les espèces de légumineuses ou de plantes actinorhiziennes mais seulement un ensemble d'espèces végétales appartenant à un groupe dit «d'inoculation crois&». Ce groupe peut comprendre un grand nombre d'espèces (qui sont dites non spécifiques) ou un petit nombre d'espèces ou même de variétés (groupe spécifique).

Sion s'en tient au système Légumineuse-*Rhizobium* qui constitue le modèle symbiotique le mieux connu actuellement, on peut souligner les rôles du génome de la plante qui interviennent lors de l'infection de la plante par *le Rhizobium :*

émission de substances chimiques excrétées par la racine.

Ces substances jouent le rôle de signaux pour l'activation ou non des gènes *nod* de la bactérie. Certaines de ces substances, hydroxyflavones et isoflavones (Firmin et *al.*, 1986 ; Peters et *al.*, 1986) seraient stimulatrices, tandis que d'autres, coumarines et isoflavones, réprimeraient l'expression de ces mêmes gènes (Djordjevic et *al.*, 1987a). Les substances stimulatrices seraient secrétées dans la zone d'émergence des poils absorbants, les substances inhibitrices proviendraient des autres zones de la racine (Djorvdjevic et *al.*, 1987b).

activation des systèmes de reconnaissance. Sous l'influence de polymères polysaccharidiques varies, exopolysaccharides, lipopolysaccharides, polysaccharides capsulaires (Leigh et *al.*, 1985) synthétisés dans la paroi bactérienne, le génôme de la plante reconnait ou non le *Rhizobium* spécifique. En cas de reconnaissance il participe à la courbure du poil absorbant et à la formation du cordon d'infection. Dans le cas contraire il induit une réaction d'incompatibilité.

mise en place de l'autorégulation de la nodulation. Ce phénomène déclenché par le génome de la plante permet de limiter le nombre des nodules et définit leur répartition sur la racine (Nutman, 1952 ; Pierce et Bauer, 1983). La zone de la racine susceptible d'être infect& est limitée à la région d'émergence des poils absorbants. Par ailleurs, une première infection inhibe un deuxième point d'inoculation situe à proximité (Rolfe et Gresshoff, 1988).

Etablissement de la symbiose

La variabilité dans les réponses obtenues concernant la nodulation est le résultat de l'interaction du génome des deux partenaires. Par l'intermédiaire des mutants obtenus à la fois chez la bactérie et la plante-hôte on a mis en évidence une série d'anomalies dans la nodulation qui ont comme support le génôme des deux partenaires. Nous nous attacherons ici à présenter celui de la plante-hôte.

On résumera dans le Tableau 1 quelques exemples de caractéristiques contrôlées par la plante en indiquant, lorsqu'ils sont connus, les gènes responsables.

Fixation de N_2

Chez de nombreuses associations Légumineuses-*Rhizobium*, *Pisum* (Cousin et *al.*, 1985), *Trifolium* (Nutman, 1971). *Medicago* (Viands et *al.*, 1981 ; Barnes et *al.*, 1986) on a pu mettre en évidence une large variabilid génétique dans l'activité fixatrice. Une part importante de cette variabilité revient à la plan-hôte.

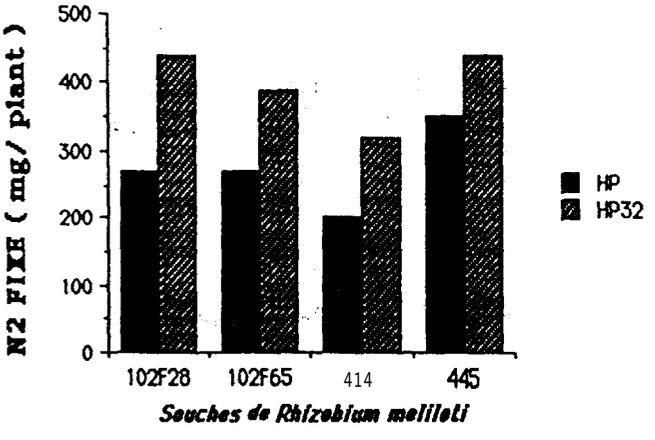
Rôle de l'hôte dans la quantité de N_2 fixé. On choisira comme exemple, une méthode d'amélioration de la fixation biologique de N_2 développée chez la luzerne (Phillips *et al.*, 1985) basée sur la sélection des génotypes de la plante. Deux *génotypes HP* et HP32, sont inocules avec quatre *souches* de *Rhizobium.* Il apparait (Fig. 1) que la quantité de N_2 fixé est de 22 à 53% plus élevée dans le génotype HP32. Les performances symbiotiques sont donc nettement améliorées si on choisit le génotype de la plante-hôte.

Influencede l'hôte dans la variabilité de potentialité de fixation de N_2. En utilisant le modèle expérimental décrit dans la Fig.1, Phillips et *al.*, (1985) ont montre que plus de 70 % de la variabilité relative au poids sec des tiges, au poids sec total, et à l'azote total peut être attribuée aux génotypes de la plante. Les souches de *Rhiwbium* n'interviennent que pour 10 à 15 % dans la variabilité.

Au cours de la fixation de N_2, on sait que le génôme de la plante intervient également. Des gènes végétaux sont exprimes uniquement dans le nodule conduisant à la formation de substances spécifiques appelées nodulines. Dans les symbioses L&umineuses-Rhizobium on en connait au moins une vingtaine (Govers et *al.* 1985) qui interviennent dans le fonctionnement de la symbiose.

Tableau 1 : Rôles de l'hôte dans la nodulation

Caractéristique de la symbiose	hôte	gène de l'hôte responsable	Phénotype observe	Références
pas de nodule	Glycine max	rj1	pas de cordon d'infection	CALDWELL, 1966
pas de nodule	Pisum sativum	syml	pas de cordon	LIE, 1971
taille et nombre des nodules	Trifolium sp.	-	complexe	in NUTMAN, 1981
nombre des nodules	Glycine max		nombre x 10 NO_3^- tolérant = super-nodulants	CARROLL et al., 1985
structure et forme des nodules	Lupinus sp.(L.) Ornithopus sp.(O)	-	t R. Lupini nodules en anneau (L) 1 bactéroïde/mb nodules cylind. (O) pl. bactéroïdes/mb	KIDBY et GOODCHILD, 1966
mode d'infection	Eleagnus Myrica		Frankia infection interc. (E) infect. poil abs. (M)	MILLER et BAKER, 1986



Fiiure 1: N_2 total fixé par deux génotypes HP 32 et HP de luzerne inoculés avec quatre souches de *Rhizobium meliloti*. (Phillips et coll.. 1985).

Les approches pour l'amélioration génétique de la plante-hôte chez les arbres

Exploitation de la variabilité génétique naturelle

Approches conventionnelles. Une technique simple et efficace consiste à utiliser les variations génétiques spontanées obtenues dans un lot de graines d'une même provenance (c'est à dire, d'une même aire géographique) ou de provenances différentes. L'identification des individus ayant un potentiel fixateur de N_2 élevé doit être basée sur des tests non destructeurs, comme de la numération des nodules ou des tests de réduction d'acétylène. Ensuite, les individus sélectionnés sont propagés par des techniques de multiplication végétative classique (Sougoufara et *al.*, 1987) et testes au champ pour leur aptitude à fixer l'azote. Les auteurs ont pu ainsi identifier un clone ß qui fixait 1.8 fois plus d'azote que le clone a inoculé avec la même souche de *Frankia*.

Approches modernes. Dans ce cas, l'ensemble des opérations est effectuée au laboratoire. L'inoculation et la nodulation des plantes est obtenue in vitro dans des systèmes expérimentaux adaptes aux arbres (Dhawan et Bhojwani, 1987 ; Alabarce, 1987). L'inoculation expérimentale en conditions aseptiques permet d'assurer le contrôle de nombreux paramètres et d'optimiser la nodulation.

La sélection des génotypes repose sur les comparaisons des tests d'effectivité et les mesures de biomasse des nodules et des plantes. Les conditions d'asepsie permettent de réaliser une micropropagation efficace des plantes sélectionnées. La multiplication végétative est assurée par les techniques de culture in vitro, en procédant au développement des bourgeons axillaires végétatifs chez *Alnus* (Tremblay et Lalonde, 1984 ; Simon *et al.*, 1987), *Eleagnus* (Bertrand et Lalonde. 1986), *Casuarina* (Sougoufara *et al.*, 1986), *Acacia albida* (Gassama et Duhoux, 1986), *Acuciu mangium* (Galiana, com. pers.), ou au developpement de bourgeons axillaires d'inflorescences immatures de *Casuarina* (Duhoux *et al.*, 1986).

Induction par culture de tissus d'une nouvelle variabilité génétique par modification globale du génôme

La variation somaclonale. La culture in vitro des cellules végétales s'accompagne très souvent d'anomalies génétiques. Plus la phase de désorganisation tissulaire (cal) est longue, plus fréquents sont observes les variants lors de la régénération. Ces anomalies relèvent de plusieurs catégories : chromosomique (aneuploïdes, polyploïdes), épigénique (d'origine nucléaire et/ou cytoplasmique) (Mestre et Benbadis, 1985).

La variation somaclonale représente ainsi une méthode pour produire une variation génétique en Cvitant la voie de la reproduction sexuée. Ces dernières années, on a pu appliquer ce procédé pour l'obtention de divers variants chez les plantes herbacées (sélection de plantes tolérant aux toxines de pathogènes, sélection de plantes résistantes aux herbicides, Larkin. 1987).

Chez les arbres fixateurs de N, cette technique ne peut-être mise en œuvre que chez les plantes capables de régénérer à partir de cals : *Alnus* (Hutinen *et al.*, 1982 ; Tremblay *et al.*, 1985) ; *Acacia koa* (Skolmen et *Mapes,* 1976) ; *Dalbergia latifolia* (Rao, 1986) ; *Allocasuarina*, (Duhoux, résultats non publiés).

Hybridation somatique. Avec la fusion de protoplastes, deux partenaires (espèces ou genres différents) peuvent procéder à un échange d'information génétique (nucléaire et/ou cytoplasmique) en contournant aussi une partie des obstacles de la reproduction sexuée.

Cette technique pourrait conduire à la création d'hybrides somatiques possèdant de nouvelles caractéristiques quant à leur nodulation, ou présentant une tolérance accrue à certaines contraintes de l'environnement. Actuellement, l'obtention de protoplastes d'arbres fixateurs de N_2 demeure rare ; *Alnus glutinosa, A. incana* (Hutinen *et al.*, 1982) et *A. incana* (Tremblay *et al.*, 1985) et l'absence fréquente de leur pouvoir de division et de régénération demeure encore une limitation de l'emploi de ces techniques.

introduction dans le génome de la plante d'un ou de quelques caractères nouveaux.

Les récents succès en biotechnologie ont montre des réussites remarquables pour des plantes modèles comme le tabac avec laquelle Shah *et al.*, (1986), De Block *et al.,* (1987) ont obtenu des plantes transgéniques résistantes à un herbicide, le glyphosate. L'obtention d'arbres transgéniques résistants à certains herbicides est envisagée. Des telles plantes possèderaient ainsi un avantage sélectif certain, vis à vis de plantes herbacées qui entrent en compétition au moment de la mise en place des plantations.

Les techniques de transfert de gènes utilisent soit, des vecteurs biologiques (*Agrobacterium fumefaciens, A.* rhizogenes, le virus de la mosaïque du chou fleur), soit le transfert direct (électroporation, liposomes, microinjection, microprojectiles enrobes de DNA...). Le nombre de plantes transgéniques obtenues chez les arbres est encore faible ; un gène de tolérance à un herbicide a été transféré et exprimé chez un peuplier (Fillati *et al., 1987).* Chez les arbres fixateurs de N_2, des travaux ont débuté chez l'Aulne (Mackay et *al.*, 1987a) et les Casuarinas (Phelep, 1988).

Il est à noter que si les travaux de génétique moléculaire concernant la fixation de N_2 sont maintenant bien avancés pour le microorganisme (*Rhizobium*), *il* n'en est pas de même pour la plante-hôte (légumineuses et plantes actinorhiziennes). Il en résulte que nos connaissances actuelles de la biologie moléculaire de la plante-hôte ne sont pas encore suffisantes pour envisager l'utilisation du génie génétique dans l'amélioration de la potentialité de fixer N_2.

D'autres stratégies, comme celle de transférer les gènes de la nodulation à des arbres (le bouleau) non fixateurs de N_2, mais proches d'un point de vue systématique des plantes actinorhiziennes (aulnes), sont envisagées (Tremblay *et al.*, 1985).

Application de l'exploitation de la variabilité naturelle à l'amélioration de la fixation de N_2 chez les arbres

Les résultats expérimentaux

Les travaux concernant les arbres sont peu nombreux. Ils seront illustres ici par un exemple d'amélioration basée sur l'exploitation de la variabilité naturelle chez une plante actinorhizienne (Sougoufara et *al.*, 1987, 1989).

Un criblage a été effectué sur de jeunes plants issus de semis de C. *equisetifolia* et inocules avec la souche de *Frankia* ORS 021001 selon la méthode déjà décrite (Sougoufara et *al.*, 1987). Trois individus a. ß et S qui présentaient des différences signifi-

catives en ce qui concerne la biomasse de leurs parties aériennes, leur nodulation, et leur aptitude à fixer N_2 ont été retenus. Des boutures âgées de 3 mois de ces individus ont été plantées dans un sol stérile déficient en N, marqué avec de l'urée ^{15}N afin de comparer les quantités d'azote de la plante provenant de la fixation de N_2 (Ndfa) et du sol (Ndfs). Une moitié des plantes a été inoculée avec la même souche de *Frankia*, l'autre n'a pas été inoculée. Les clones inocules de 12 mois différaient entre eux, non seulement par le poids de leurs nodules mais aussi par leur aptitude à fixer N_2 (Ndfa) et à absorber N du sol (Ndfs), (Sougoufara *et al.*, 1989). Les clones non inocules différaient Cgalement entre eux par leur absorption spécifique de N du sol (Fig. 2).

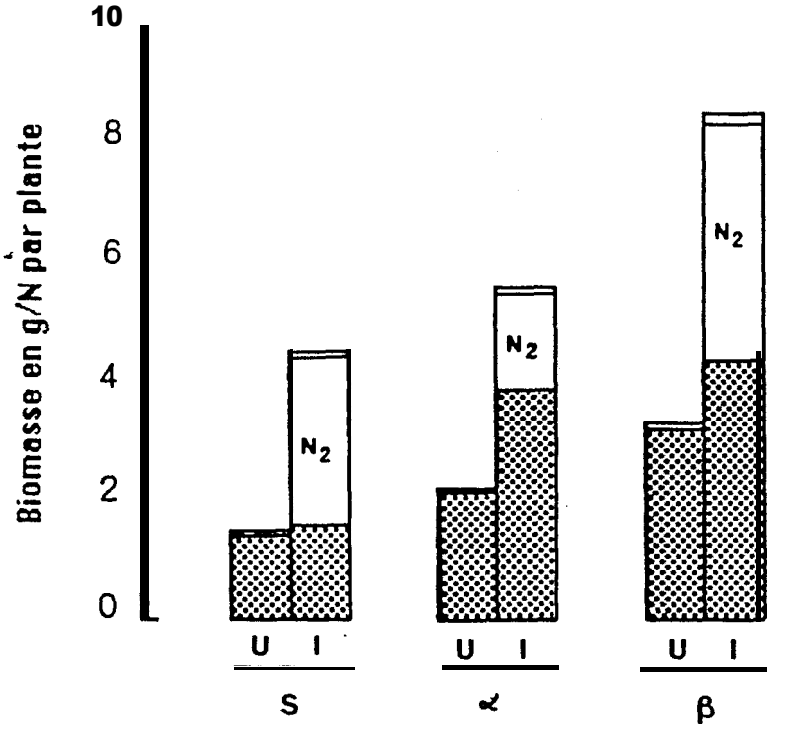


Figure 3 : Biomasse exprim6.e en g/N/plante de trois clones de *Casuarina equisetifolia* (S. α, β) 12 mois après leur transplantation (SOUGOUFARA et *al.*, 1989).
1: plants inoculés avec la souche de *Frankia* ORS 021001. La contribution du sol est représentée par les rectangles vides et celle de la fixation de N_2 par les rectangles avec l'indication "N_2".

On a pu ainsi mesurer, dans le cadre d'une variabilité intraspécifique de plantes-hôtes de C. *equisetifolia*, différents critères, Ndfa, Ndfs, biomasse des plantes (exprimée en N total), qui sont indépendants des expérimentations. Les différences observées sont uniquement le reflet de caractères phénotypiques de la plante. Autrement dit, chaque clone possède des caractéristiques propres en ce qui concerne la potentialité de fixer N_2, d'absorber N du sol ou de produire sa biomasse. Dans le cadre de cette expérience, les augmentations de rendement consécutives à l'inoculation sont spectaculaires : +200% pour le clone S, +158% pour le clone a, et +157% pour le clone ß. La micropropagation des clones sélec-tionnés et leur transfert au champ peut donc amener des gains de productivité considérable.

Choix des critères de sélection

L'amélioration de la plante-hôte doit tenir compte de la potentialité de fixer N_2 et Cgalement des critères d'adaptation à l'environnement (conditions climatiques, édaphiques, biotiques).

Propriétés symbiotiques. Les mesures de biomasse de nodules constituent un premier élément d'appréciation. Les techniques de mesure directe de la fixation de N_2 étant difficiles à mettre en oeuvre chez les arbres âgés, on se contentera d'individus de quelques années, voire même de jeunes plants. Les mesures sont réalisées avec le test de réduction de l'acétylène (ARA) ou mieux, par les méthodes isotopiques (Dommergues *et al.*, 1988).

Adaptation à l'environnement. Un des problèmes importants pour la mise en place d'un programme de sélection est de déterminer, afin de les éviter, les facteurs Limitant la croissance de la plante tek que la présence d'azote combine du sol, les contraintes hydriques, salines, et pathogènes. La tolérance aux herbicides peut constituer pour ces végétaux une caractéristique génétique à introduire quand on sait, qu'en zone aride, la compétition de la couverture herbacée est très forte pendant les premières années de la vie de l'arbre. Parmi les facteurs biotiques, il faut inclure l'influence de *la* compétition avec *les* souches sauvages de *Rhizobium* et de *Frankia* du sol. Considérant ce dernier point, il est recommandé (Dommergues *et al.*, 1988) de sélectionner des plantes-hôtes capables de noduler seulement avec des souches très effectives de microorganismes spécifiques, pour éviter l'établissement de la symbiose par des souches sauvages présentes dans le sol.

Critères de sélection propres aux arbres

(i) Utilisation de la multiplication végétative comme technique de propagation des plantes sélectionnées : il apparait peu réaliste d'envisager un programme d'amélioration bas& sur des expériences d'hybridation sexuée chez les arbres tropicaux fixateurs de N_2 qui présentent une très forte hétérozygotie. La multiplication végétative par les techniques conventionnelles et les techniques de culture in vitro s'avèrent être pour le moment, les seules méthodes pratiques de propagation des individus sélectionnés. On connait la perte progressive de l'aptitude au développement orthotrope et de la capacité rhizogène des arbres âges, il s'avère donc préférable d'utiliser les techniques de culture in vitro dont la puissance de multiplication est par ailleurs remarquable.

(ii) Sélection précoce des individus : la micropropagation est beaucoup plus aisée à partir de jeunes individus (meilleure réactivité, infections réduites...) que sur des sujets matures. Si on peut détecter. par ailleurs, les caractéristiques physiologiques des jeunes plants comme dans l'exemple du *Casuarina* (Sougoufara *et al.*, 1989) la sélection précoce des individus réduira la durée du processus d'amélioration de la plante.

(iii) Difficulté du choix des critères de sélection : compte tenu des résultats préliminaires que nous possédons, il est nécessaire de déterminer des parambtres simples comme la biomasse des plantes (exprimée en N total et tenant compte à la fois du potentiel fixateur de N_2 et de l'aptitude de l'arbre à utiliser N du sol) et de la biomasse des nodules (exprimée en N total) qui semble assez bien corrélés avec la fixation de N_2 (Sougoufara et *al.*, 1989).

Pour identifier les individus caractérisés par un bon potentiel fixateur de N_2, il est indispensable de faire appel à une méthode kotopique qui permet en outre, de mesurer l'aptitude des plantes à utiliser l'azote du sol. La sélection des clones selon leur aptitude à absorver N du sol, mais aussi selon leur susceptibilité à ce facteur extérieur semble être un paramère important. Par ailleurs, Mackay *et al.*, (1987b) ont montre que la performance des génotypes d'*Alnus glutinosa* inoculés avec des souches de *Frankia* étaient dépendants de l'apport en N combiné du substrat, certains clones étant plus dépendants que d'autres.

(iv) L' électrophorèse d' isozymes pourrait-elle permettre d' identifier des *génotypes bons fixateurs* de N_2 ? L'étude des profils électrophorétiques (protéines, DNA) constitue un outil puissant pour identifier et reconnaître à un stade précoce des clones (Feldmann *et al.*, 1983), pour contrôler l'efficacité d'un brassage génétique et en déduire le type de fécondation, auto ou allogamie, pour estimer la variabilid génétique (Vigneron, 1984). Cette technique peut-elle être utilisée pour l'identification de génotypes bons fixateurs de N_2 ? Nous sommes tentés d'en douter, car on ne connait pas encore le support génétique de la potentialité plus ou moins grande de fixer N_2 qui est complexe et vraissemblablement polygénique. Par ailleurs, la variation quantitative d'une fonction génique traduit la variabilité des gènes de régulation affectant les quantités de protéines et la variation qualitative correspond en majorité à la variabilité des gènes de structure (Damerval *et al.*, 1986). Une étude électrophorétique pourrait donc, sans doute, identifier et repérer des génotypes non fixateurs par comparaison avec des individus fixant N_2. Par contre, il semble plus improbable de mettre en évidence une liaison entre une variation dans le degré de fixation de N_2 chez un génotype et une variation de la nature des marqueurs enzymatiques ; les techniques électrophorétiques n'appréciant que des variations qualitatives des molécules (protéines, ADN) analysées. C'est d'ailleurs la raison pour laquelle les caractères enzymatiques ne sont que très rarement liés à la valeur agronomique des individus.

Conclusions

L'amélioration de la fixation biologique de N_2 à travers la sélection et la génétique est un problème complexe car ce phénomène relève des deux partenaires. Les travaux de recherche développés ces dernières années ont largement contribué à l'amélioration de nos connaissances sur la complexité des mécanismes mis en jeu. Les premières recherhes ont surtout été orientées vers le microorganisme et une première moisson de résultats très importants concernant le *Rhizobium* a déjà été récoltée.

Nous avons présenté ici l'état de nos connaissances concernant la plante-hôte dont le rôle joué dans la symbiose commence à être de mieux en mieux connu, même s'il reste encore beaucoup à faire en génétique et en biologie moléculaire. Le sélectionneur peut améliorer efficacement la biomasse produite en utilisant la grande variabilité génétique naturelle des arbres. Selon les résultats dont nous disposons actuellement, il apparait que l'objectif des travaux de sélection à venir serait d'identifier des génotypes présentant les trois caractéristiques suivantes : grand pouvoir de fixation de N_2, faible susceptibilité de la symbiose à l'égard de N combiné du sol, et accroissement de l'assimilation de N du sol. Ces trois caractéristiques sont des caractères indépendants sur le plan génétique ; elles peuvent vraissemblablement agir en synergie, et sont pour quelques cas connus, le *Casuarina* (Sougoufara et *al.*, 1989) et le soja (Imsande, 1985) corrélées avec une biomasse importante de l'appareil végétatif de la plante. Il serait Cgalement utile de sélectionner des arbres capables de continuer à fixer N_2 en présence de N combine du sol en particulier dans les peuplements denses (forêts) où N du sol s'accumule au cours du temps.

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Analysis of the plant genes involved in the nitrogen fixing symbiosis. *Sesbania rostrata* as a model

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Summary

During nitrogen fixing nodule formation on leguminous plants, several plant genes, so-called nodulin genes, are specifically expressed. In this paper the current knowledge on the expression, induction and regulation of nodulin genes in the different symbiosises studied so far is summarized, together with main results obtained *in the case* of *Sesbania rostrata* **on nodule-specific plant gene expression.**

Introduction

Each stage in the *Rhizobium*-legume symbiosis is characterized by a series of developmental events concerning both bacteria and plant, resulting in a complex, well organized and well coordinated plant organ. This has been welldescriifromamorphological point of view (Bauer, 1981).

By classical genetic experiments, several plant genes involved in nodulation and symbiotic nitrogen fixation have been identified in pea, soybean, clover and alfalfa (Nutman, 1981 ; la Rue et al., 1985). Mutations in the plant genome can result in disturbed nodule development, varying from the absence of nodules to the developement of wild-type-like but ineffective nodules (Vincent, 1980).

Theknowledge of nodule formation has made rapid progress since the rise of molecular biological techniques. It has been found that a number of genes in both plant and bacterium are only expressed in nodules. An effective symbiosis is accomplished by different&ion

of bacteria into bacteroïds on the one hand, and differenciation of plant cells into a root nodule on the other hand. The major part of research activity has concentrated on the nitrogen fixing bacteria, which are more easily accessible to genetic manipulation in comparison with legume plants. Most of the bacterial symbiotic genes (genes for nodulation and nitrogen fixation) have been identified and are well documented. The plant is equally important, providing the right environment, the energy and using the fixed nitrogen for its growth and development. Over the last few years, interest in the role of the plant in the symbiosis has considerably intensified, with the evidence of plant genome-encoded, nodulespecific proteins, called nodulins (Legocki and Verma, 1980) differentially expressed during nodule development.

Compared with other plant differentiation processes, root nodule development is unique in the involvement of a prokaryote in the induction and control of plant development Developmental program leading to root nodules, as an organ, is as complex as other developmental programs in plants and involves numerous genes. Nodulin gene expression is one of the most specific aspects of nodule differentiation and its study may be a useful approach to understand root nodule development.

Nodulin definition. By definition, nodulins are plant gene-encoded proteins, which are found only in root nodules and not in uninfected roots nor in other part of the host plant (Van Kammen, 1984). Nodulin genes are, by consequence, plant genes exclusively expressed during the development of the symbiosis. Around 10 to 40 different nodulins have been found in the various systems studied so far, like soybean (Legocki and Verma 1980). *pea* (Bisseling *et al.,* 1983), *Medicago sativa* (Lullien *et al.,* 1987), *Phaseolus vulgaris* (Campos *et al.,* 1987), *Sesbania rostrata* (*De* Lajudie and Huguet, 1988). Nodulin genes are differentially expressed during nodule development (Bisseling *et al.,* 1983 ; Gloudemans *et al.,* 1987). The majority of nodulin genes is expressed around the onset of nitrogen fixation, like leghemoglobin, and are called nodulin genes. They most p&ably function in establishing and maintaining a proper environment within the nodule that allows nitrogen fixation and ammonium assimilation to occur. Few nodulins are detectable at earlier stages of development when the nodule structure is being formed, which are called early nodulin genes.

Nodulin functions. In all systems studied so far, the use of mutated or engineered bacterial strains that arrest nodule development at different stages has enabled to establish parallels events at the levels of nodule structure and nodulin gene expression by coupling histological and molecular biological data. Very few nodulins have been identified Moreover, nodulins must play roles at every stage of nodule developement and function : (i) for plant-microbe recognition : lectins, enzymes of host origin capable of degrading the capsular polysaccharides of the bacteria (Batter, 1981) ; (ii) during infection process : root hair curling, infection thread formation, development of a meristem from cortex cells, release of bacteria in the meristematic cells and differentiation of host cells and bacteria into a nitrogen fixing nodule ; (iii) for the transport of metabolites including organic acids, amino acids and sugars, as well as N_2 and O_2 ; (iv) for the assimilation of fixed nitrogen produced by bacteroïds ; (v) roles in all the morphological, cytological and physiological changes that take place in the infected cell, like modification of endomembrane systems, increased abundance of free ribosomes. polyribosomes. proplastids and mitochondria ; carbon, nitrogen and oxygen metabolism changes dramatically during the differentiation of the meristem into a root nodule, as indicated by the increase in

activities of several enzymes such as PEP carboxylase, malate deshydrogenase, glutamine synthetase and uricase (for a review see Verma and Long, 1983).

A few early nodulin genes have been identified. Among them only one has been well characterized : ENOD2, a nodulin of soybean (*Glycine* mar.), which has been also found in other legumes like pea, vetch, alfalfa and clover (Govers et *al.*, 1987). The structural features of this nodulin, like its hydroxyproline-rich sequence, suggest that it is a cell wall protein closely related to the extensin (Nap, 1988).

Late nodulins are more documented The most famous is leghemoglobin, which constitutes 30% of the total soluble proteins. Leghemoglobin is a hemoprotein with high oxygen affinity, which provides high yield of oxygen to bacteroïds at a low partial tension, compatible with nitrogen fixation. It is generally detectable just before nitrogenase activity can be measured. Leghemoglobin is a true symbiotic protein since the heme is a product of the bacteroïd and protein is plant-encoded. In all legumes studied so far, there are several leghemoglobins, encoded by several genes (Marcker et al., 1984). Other late nodulius have been identifield. One is a soybean nodule-specific uricase, a key enzyme in the ureide biosynthetic pathway for ammonia assimilation (Bergman *et al.*, 1983). Two enzymes of glutamine synthetase. which catalyses the first reaction in the assimilation of ammonia into organic nitrogen, are present in nodules of *Phaseolus vulgaris*, one of which is nodule-*specific (Cullimore et al., 1983).* A sucrose synthase, an enzyme which catalyzes the degradation of sucrose into fructose and glucose, has been found specific to nodules of soybean (Thummler and Verma, 1987). Other nodule-specific forms of enzymes that differ in physical, kinetic and immunochemical properties from the corresponding enzymes in roots have been found : phosphoenolpyruvate carboxylase, choline kinase, xanthine deshydrogenase, purine nucleosidase, malate deshydrogenase (Nap, 1988). But there is still no evidence that they derive from nodule-specific gene expression or from nodule-specific modifications of root enzymes.

Several other nodulins, whose functions are yet unknown, have been found associated with peribacteroïd membrane, the site of closest interaction and exchanges between the bacteroïds and the host cell.

Molecular studies on nodulin genes have progressed a lot since the last few years : gene cloning, organization in the genome, regulation, sequence analysis (for a review, see Bisseling et *al.*, 1986).

Sesbania rostrata

Sesbania rostrata is a tropical legume which forms nitrogen fixing nodules on stem and roots. This makes *Sesbania* a very efficient system for nitrogen fixation and a real potential as green manure in tropical agriculture. Two bacterial genera are capable *to* induce *nodules* on *S. rostrata : Azorhizobium caulinodans* and *Rhizobium sp.* (Dreyfus et al., 1988). Due to its particular properties (in vitro nitrogen fixation), *A.caulinodans* has been extensively studied. Opposite to this, few data are available on the plant itself (Bogusz *et al.*, 1987 ; De Lajudie and Huguet, 1988, 1989). We focussed on *S. rostrata* to investigate plant genes activated during nodule development and function. *S. rostrata* constitutes a good model in such a study since, opposite to that of root nodules, age of stem nodules can precisely be known : stem nodules are homogenous in age, opposite to root nodules that form continuously.

Our approach consisted in measuring the level of translatable mRNA present in each tissue by comparing one-and-two dimensional polyacrylamide gel electrophoretic patterns of in vitro translation products of poly(A)+ RNA from uninfected stems and stem nodules, uninfected roots and root nodules.

Plant gene expression in stem nodules appears very similar to that in root nodules, except some quantitative differences in the intensity of certain spots which could be explained by the heterogenous age of root nodules. We found 16 nodule-specific polypeptides, in addition to around 20 other present at different relative intensities in nodules and in uninfected tissues. During stem nodule development, majority of nodule-specific genes are expressed 12 days after inoculation, concommitantly with leghemoglobin and initiation of nitrogen fixation. These could be correlated with transport of substrates towards bacteroïds and assimilation of fixed nitrogen. Some other genes are expressed either during the early stages of infection, or transiently, suggesting a role in plant-microbe recognition, infection process, and nodule morphogenesis. Some others, expressed later (day 18), could have a role in maintaining nodule structure and function, or in nodule senescence. All these observations indicate that there is a sequence in the activation of nodule-specific genes during nodule life, like in other described systems.

Among the nodule-specific polypeptides, we identified six in vitro translation products that cross reacted with a serum anti-leghemoglobin of *Sesbania rostrata*. During nodule development, these leghemoglobin components are differently expressed and their relative proportions vary : some of them are first major to become minor during the late stages of nodule development.

Root nodules induced either by *Rhizobium* sp. (ORS 51) or *A. caulinodans* (ORS 571) are very similar except for the expression of one more specific gene in the case of ORS 51. One of the great differences pointed out between stem and root nodules is the presence of chloroplasts and photosynthesis in stem nodules. We found interesting to compare plant gene expression in stem nodules developed either in the dark or under light : they show only minor differences ; moreover, stem nodules in the dark and root nodules show some striking similarities. This, together with other arguments like the same seven leghemoglobin isomeres (Bogusz *et al.*, 1987), the same infection process in stem and root nodules (Ndoye and Truchet, in preparation), or the fact that root nodules developed under light are photosynthetic, suggests that stem and root nodules are not of completely different nature.

We used different nitrogen fixation deficient mutant strains of *A. caulinodans* ORS 571 and observed that induction of nodule-specific gene expression is not dependant on nitrogen fixation. However the level of expression of leghemoglobin and several other nodulin genes is lowered when there is no nitrogen fixing activity in nodule. This is in agreement with all systems described so far.

Conclusion

Besides the agronomic aspect, nitrogen fixing symbiosises are a tools towards elucidating several fundamental biological problems. They constitute a model for the study of plant microbe interactions, which are diverse and numerous in nature, especially of parasitism and pathogenicity.

Opposite to *Agrobacterium tumefaciens*, the other genus of the *Rhizobiaceae* family, which induces tumor formation, a set of undifferentiated cells, *Rhizobium* is able

to bypass the plant defense mechanisms and to induce a de-differentiation of plant cells and the development of a nodule, a true organ specialized for nitrogen fixation. Therefore, *Rhizobium*-Legume symbiosis with *Frankia* actinorhii plants are an interesting and rare example for plant molecular biology of the induction by a prokaryote of an organogenesis in a eukaryote.

In less than a decade, our knowledge on plant gene expression during symbiosis has progressed a lot, and we begin to know more about the complex mechanisms of symbiosis, bacterial genes implied in the induction of nodulin genes, which is a first step in identifying bacterial signals to the plant that induces expression of the successive classes of nodulin genes. A better understanding of all the mechanisms implied in symbiosis will help in a better domestication for a practical use in the field

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Sesbania rostrata : un modèle pour la biologie moléculaire de la nodulation

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Résumé

Sesbania rostrata forme des nodules aussi bien sur les tiges que sur les racines. Ses micro-symbiotes appartiennent à deux genres différents : *Azorhizobium* et *Rhizobium*. Les souches telle que ORS 571 qui induiint des nodules de tige et de racine et qui fixent l'azote atmosphérique en culture pure appartiennent à la nouvelle espèce *Azorhizobium caulinodans*. Les souches isolées des racines de S. *rostrata* et qui forment des nodules effectifs sur d'autres espèces de *Sesbania* appartiennent au genre *Rhizobium*.

La mutagenèse *de* la souche d'*Azorhizobium caulinodans* ORS 571 a permis d'identifier deux loci *Nod1* et Nod2 qui sont impliqués dans la double nodulation racinaire et caulinaire. L'hybridation moléculaire et l'analyse des séquences du locus *Nod1* ont révélé l'existence de trois gènes ayant une forte homologie avec les gènes *nod ABC* de *Rhizobiwn meliloti*. L'analyse génétique a montré que l'expression de ces gènes est induite en présence des racines et des primordia racinaires situés sur la *tige de S. rostrata ;* elle a par ailleurs permis de découvrir, dans le génome de ORS 571, un troisième locus dont la présence est essentielle pour l'induction des gènes du locus *Nod1*. Ce troisième locus semble réguler l'expression des gènes de nodulation racinaire et caulinaire.

Chez S. *rostrata*, un mutant incapable de former des nodules de tige a été isolé. Ce mutant a une croissance aérienne normale mais sa racine principale est plus vigoureuse et ses racines secondaires moins abondantes que celles de la plante de type sauvage. Des différences dans la composition des protéines ont été mises en évidence entre le mutant et la plante normale. Une caractérisation moléculaire approfondie devrait permettre de rechercher chez S. *rostrata* les gènes impliqués dans la nodulation caulinaire.

Introduction

Sesbania rostrata est une légumineuse que l'on rencontre sur les sols hydromorphes de l'Ouest-africain. Son aptitude à former des nodules caulinaires lui confère un potentiel de fixation d'azote exceptionnel et lui permet de noduler et de fixer l'azote sur des sols inondés ou renfermant des quantités élevées d'azote combine (Dreyfus *et al.*, 1984).

Les nodules caulinaires se développent à partir de sites prédéterminés (Duhoux et Dreyfus, 1982 ; Tsien et *al.*, 1983). Ces sites sur les tiges sont une caractéristique particulière de *Sesbuniu rostrata*, de certaines espèces d'*Aeschynomene* et de *Neptunia* (Dreyfus et *ul.,* 1984) ; ils sont constitues d'ébauches racinaires adventices qui se développent en nodules lorsqu'elles sont infectées par des symbiotes spécifiques (Duhoux et Dreyfus, 1982).

Les bactéries qui sont associées à *Sesbunia rostrata* appartiennent à deux genres différents : *Azorhizobium* et *Rhizobiwn.*

Les souches d'*Azorhizobium cuulinodans* du type ORS 571 (Dreyfus *et al.*, 1988) induisent des nodules fixateurs d'azote aussi bien sur les tiges que sur les racines. Ces souches sont capables de fixer l'azote en culture pure. Bien que les souches d'*Azorhizobium* soient capables de former des nodules sur un grand nombre d'espèces du genre *Sesbania*, elles ne fixent l'azote qu'avec *Sesbuniu rostruta.*

Les souches de *Rhizobium* nodulent effectivement les racines *de Sesbania rostrata* et des autres *espèces* du genre *Sesbuniu* ; la plupart d'entre elles sont capables de former des nodules fixateurs d'azote *sur* les tiges de *Sesbania rostrata*, certaines y induisent des pseudonodules ou des nodules ineffectifs et quelques unes ne forment pas du tout de nodules sur les tiges.

Chez *Azorhizobium cuulinoduns, la* mutagenèse par transposon Tn5 de la souche ORS 571 a permis d'identifier et de cloner deux loci *Nodl* et *Nod2* essentiels pour la nodulation racinaire et caulinaire (Van Den Eede et *al.,* 1987). La technique d'hybridation moléculaire a permis de mettre en évidence une homologie de séquence entre le locus *Nodl* et les gènes de nodulation *nodABC* de *Rhizobium meliloti* (Kondorosi *et al.,* 1984). Les gènes homologues *nodABC* de la souche ORS 571 ont ensuite été isoles et caractérisés par le séquençage du locus *Nodl*.

Le présent article est principalement consacré à l'étude de l'expression des gènes *nod* de la souche ORS 571 ; nous nous intéresserons par ailleurs aux résultats préliminaires concernant la caractérisation d'un mutant de *Sesbunia rostrata* dépourvu *de* sites de nodulation de tige.

Induction et régulation des gènes de nodulation de la souche ORS 571

Chez *certains Rhizobium* à croissance rapide tel que *R. meliloti* et *R. leguminosarum*, les gènes *nod* ne s'expriment qu'en présence d'exsudats racinaires issus de la plante hôte (John et *al.*, 1985). Différents flavonoïdes isoles à partir de ces exsudats notamment la lutéoline chez la luzerne, sont capables d'induire l'expression de ces gènes. Par ailleurs, *il* a été mis en évidence *dans le* génome de *ces Rhizobium*, des gènes régulateurs indispensables à l'induction des gènes *nod* par des flavonoïdes de la plante hôte (John *et al.*, 1985).

Pour étudier l'induction de l'expression des gènes *nod* de la souche ORS 571, nous avons construit plusieurs fusions entre le locus *Nodl* et le gène *LacZ* de la B-galactosidase de *E. coli* à l'aide d'un transposon (Stachel *et al.*, 1985). Le principe de ces fusions consiste à insérer dans un fragment d'ADN le gène *LacZ* sans son promoteur afin que son expression soit sous la seule dépendance de l'expression du gène dans lequel il s'est inséré ; il faut pour cela que la fusion soit orientée dans la direction transcriptionnelle et qu'elle soit en phase de lecture. Les fusions construites dans le locus *Nodl* étaient ensuite transférées dans une population de ORS 571 et testées pour l'activité de la ß-galactosidase en présence et en absence de *Sesbania rostrata.*

Quelques unes des nombreuses fusions testées étaient induites par les fragments de tige et de *racine* de *Sesbania rostrata.* Ces fusions inductibles sont toutes localisées dans les gènes *nod* et transcrites dans la même direction ; de plus, ces gènes semblent dépendre de la même unité transcriptionnelle, ce qui indique une organisation sous forme d'opéron comme pour les gènes *nodABC* de *R. meliloti.*

Pour isoler la substance qui induit l'expression des gènes de la souche ORS 571, des exudats *racinaires de Sesbania rostrata* ont été analysés par chromatographie liquide. Le test d'activité de la ß-galactosidase à partir des différentes fractions issues de ces exudats a révélé l'existence d'un inducteur de type flavonoïde. Nous caractérisons actuellement cette substance. Parallèlement, nous préparons des extraits de tige afin d'isoler et de comparer la substance inductrice de tige à celle des racines. Nous avons par ailleurs teste les fusions inductibles en présence d'une série de flavonoïdes de provenance commerciale ; des niveaux élevés d'induction ont été observés avec la naringénine (Nar). Quelques mesures de l'activité de la ß-galactosidase sont présentées dans le Tableau 1.

Etude de la régulation de l'induction des génes Nod de la souche ORS 571

Comme l'induction des gènes de *R. meliloti* par la lutéoline est sous la dépendance d'un gène régulateur *nodD*, nous avons pense à une situation similaire chez ORS 571. Deux approches ont été choisies pour rechercher le gène régulateur homologue du gène *nodD* dans le génome de ORS 571.

(i) Après avoir introduit une fusion inductible *dam Agrobacterium tumefaciens*, nous avons recherche dans la banque d'ADN de ORS 571 les clones qui permettent son induction par la naringénine. Un clone ayant un fragment de 16 kilobases (clone pRG910-16) a ainsi été sélectionné.

(ii) Dans une deuxième approche, nous avons isolé un mutant spontané (ORS 571-IN-) qui ne permet plus l'induction des fusions *lacZ* par *les* fragments de *Sesbania rostrata.* Inocule aux *racines* et aux tiges *de Sesbania rostrata,* ce mutant induit des nodules anormaux et irréguliers. Lorsqu'on introduit dans ce mutant le clone pRG910-16 qui per-

Table 1 : Activités de la -galactosidase avec et sans induction des souches ORS 571 portant des fusions lacZ dans le locus Nodl

ORS 571 avec différentes fusions lac Z	Activités de la β galactosidase (unité β-gal)	
	-Nar	+Nar
ORS571-Mu63	22	650
ORS571-Mu3	11	205
ORS571-Mu22	17	240
ORS571-Mu21	18	470
ORS571-T20	23	1680

Les unités de β-galactosidase ont été mesurées (Miller 1972) 12 heures après l'addition de 10 µM de naringénine (Nar) à des cultures de ORS571 en phase logarithmique.

met l'induction des fusions *lacZ* dans *A. tumefaciens*, il est complémenté pour tous ses phénotypes mutants.

Ces deux approches indiquent la présence sur le fragment de 16 kilobases d'une fonction régulatrice nécessaire à l'expression des gènes de nodulation du locus *Nodl*. Pour isoler cette fonction, une matagenèse par transposon a été effectuée dans le clone pRG910-16 ; une population de clones mutés a été introduite dans le mutant ORS 571-IN- et les colonies qui n'étaient plus induites ont été sélectionnées. Les fragments mutés ont été extraits de ces colonies et sont actuellement cartographiés afii d'isoler les gènes impliques dans la régulation de la nodulation.

Caractérisation du mutant de *Sesbania rostrata* sans site de nodulation de tige

Chez *Sesbania rostrata*, les nodules de tige apparaissent sur des sites prédéterminés qui correspondent à des primordia racinaires. Nous avons récemment obtenu par mutagenèse chimique un mutant de *Sesbania rostrata* ne possédant plus de sites de nodulation sur sa tige.

Ce mutant ne présente aucune anomalie dans sa croissance aérienne ni dans la nodulation racinaire. Il a par contre une racine principale plus vigoureuse et des racines secondaires moins abondantes que colles de la plante normale. Dans les sols inondés,

la quantité d'azote fixé par le mutant décroît alors que celle de la plante normale est augmentée suite à la présence de nodules de tige.

Nous avons compare par électrophorèse en une dimension les protéines totales du mutant et celles de la plante normale. D'après les profils électrophorétiques, il existerait une ou deux protéines supplémentaires chez le mutant Nous procédons actuellement à une analyse approfondie qui devrait nous permettre de rechercher et d'identifier les fonctions qui gouvernent l'initiation et le développement des sites de nodulation de tige chez Sesbania rostrata.

Discussion et conclusion

L'analyse génétique et moléculaire de la souche d'*Azorhizobium* ORS 571 a permis d'identifier et de caractériser des gènes impliques dans la nodulation des racines et des tiges de ***Sesbania rostrata.*** Il a été montré que ces gènes ne sont induits qu'en présence de fragments racinaires ou caulinaires de ***Sesbania rostrata*** et que cette induction est sous la dépendance d'un gbne régulateur. Comme chez ***Rhizobium meliloti,*** l'interaction entre ce régulateur et la substance inductrice pourrait déterminer l'amorce de la nodulation. Nous pensons que la capacité de ORS 571 à exprimer pleinement ses gènes *nod* dépend de l'affinité entre la molécule inductrice et ce gène régulateur. Il se pourrait que les gènes ***nod*** des ***Rhizobium*** de racine soient induits par une substance présente dans les racines et pas dans les sites de nodulation de tige.

La découverte de quelques souches de ***Rhizobium*** qui n'induisent que des nodules non fixateurs ***d'azote sur*** les ***tiges de S. rostrata*** suggère que ***Azorhizobium*** ORS 571, contrairement à ces ***Rhizobium,*** posséderait des gènes ou des mécanismes spécifiques de la fixation ou de la transformation de l'azote atmosphérique dans les nodules de tige (gènes *nif*, *ntr* et *fix*). La mise en évidence et l'étude de l'expression de nombreux gènes *fix* et *nif* d'une part (Danèfle ***et al.,*** 1987 ; Pawlowski ***et al.,*** 1987 ; Kaminski ***et al.,*** 1988) et l'étude des mutants de la régulation de ces gènes d'autre part (de Bruijn ***et al., 1988)*** devraient permettre de rechercher et de comprendre les mécanismes spécifiques de la fixation d'azote dans les nodules de tige.

Parrallèlement, de nombreuses recherches ont été entreprises sur *S. rostrata afii* de mieux comprendre l'initiation des évènements précoces de la nodulation et le développement des nodules chez la plante hôte. Une étude récente (de Lajudie et Huguet, 1988) a révélé une différence entre les protéines végétales induites dans les nodules racinaires par la souche ORS 571 et celles induites par une souche de racine. Bogusz et *al.* (1987) ont par ailleurs montre qu'il n'y avait pas de différence significative entre les composants de la leghémoglobine extraits des nodules racinaires et ceux issus des nodules caulinaires. Les travaux de Trinh (com. pers.) ont permis de mettre au point un système de nodulation in vitro pour suivre avec précision les différentes étapes de l'infection à la morphogenèse des nodules

En ce qui concerne le mutant de *S. rostrata* sans site de nodulation caulinaire, nous espérons en continuant sa caractérisation moléculaire, parvenir à élucider les mécanismes de l'induction et du développement des nodules de tige et à isoler les gènes correspondants. Ces mécanismes seront nécessaires pour créer des nodules aériens sur d'autres espèces et particulièrement celles du genre ***Sesbania*** utilisées en agriculture et en agroforesterie.

L'association *S. rostrata-Azorhizobium* et ***Rhizobium*** apparait comme l'une des interactions symbiotiques présentant un intérêt majeur pour les études moléculaires. Le

développement des recherches fondamentales initiées dans plusieurs laboratoires sur cette association aura certainement des retombées sur la connaissance de l'ensemble des interactions symbiotiques. Par ses caractéristiques particulieres, ce système apparait de plus en plus comme un modèle pour la biologie moléculaire de la fixation d'azote.

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AMELIORATION
DE LA FIXATION SYMBIOTIQUE
DE L'AZOTE
PAR LES PRATIQUES CULTURALES

Amélioration de la Fixation Biologique de l'Azote (N_2) par les pratiques culturales

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L'augmentation de la production d'une légumineuse annuelle, telle que l'arachide, fait appel à des pratiques culturales appropriées qui doivent aboutir à une augmentation du rendement à l'hectare et à un maintien du niveau de fixation symbiotique de l'azote (N_2) le plus élevé possible. C'est en vue d'améliorer la quantité d'azote fixé par la légumineuse, qui se différencie du premier objectif (l'augmentation du rendement) en cc sens qu'il place la fixation de l'azote en priorité, que nous raisonnerons les différentes techniques culturales.

La diminution de la quantité d'azote fixé par une culture de légumineuse résulte de l'effet de contraintes qui affectent M des paramètres suivants ou les deux à la fois : (i) la croissance végétative avec une action indirecte sur la fixation de l'azote et (ii) la fixation de l'azote avec ou sans action dépressive sur le rendement. Pour lever ou éviter ces différentes contraintes, les agronomes doivent être en mesure de proposer des techniques culturales applicables dans l'agriculture du pays, et capables de maintenir ou de redresser le niveau de fixation de l'azote dans les conditions d'environnement de la légumineuse cultivée. Ceci pose le problème de l'applicatioon des méthodes de quantification de la fixation de l'azote in situ. Ces contraintes ont été relativement bien analysées dans les principales zones écologiques du Sénégal pour l'arachide et le soja (Dommergues et Ganry, 1986 ; Ganry, 1987).

Les méthodes de quantification in situ se résument à deux types : les méthodes fondées sur l'analyse chimique de l'azote seulement, et les méthodes fondées sur l'analyse chimique et le marquage isotopique. Les premières prennent en compte sur une période donnée de t années la variation du stock d'azote total du sol ($\Delta N = N_0 - N_1$) ainsi que les inputs et outputs connus d'azote dans l'agrosystème : elles permettent seulement de savoir si le système est le siège de pertes ou de gains d'azote, les gains d'azote (inputs non connus) étant dus essentiellement à la fixation de l'azote. Les secondes méthodes permettent la

quantification précise de la fixation de l'azote dans les parties aériennes et partant, une quantification de cette fixation dans les racines.

Analyse des contraintes pouvant affecter la quantité d'azote fixé

Ces contraintes peuvent affecter directement la fixation de l'azote, ou indirectement celle-ci par l'intermédiaire de la plante hôte, essentiellement par réduction de l'enracinement et de l'activité photosynthétique. Des techniques culturales appropriées devront être appliquées pour lever ces contraintes.

La contrainte climatique

Cette contrainte est essentiellement hydrique. Trois voies d'étude sont poursuivies pour résoudre le problème du déficit hydrique dont l'importance au Sénégal est croissante du sud ou nord :

(i) Choix des variétés à cycle végétatif adapté à la longueur utile de la saison des pluies (Dancette 1979) : Il s'agit là d'une action indirecte sur la fixation de l'azote.

(ii) Amélioration de la résistance à la sécheresse de la légumineuse, d'autant plus importante que la durée du cycle est raccourcie (Bockelee-Morvan *et al.*, 1974 ; Gautreau, 1977) : Il s'agit la également d'une action indirecte sur la fixation de l'azote.

(iii) Amélioration des techniques permettant une meilleure alimentation hydrique (Kuo and Boersma, 1971 ; Sprent, 1972 ; Pankhurst and Sprent, 1975 ; Pate 1975) : Il s'agit là d'actions directe et indirecte sur la fixation de l'azote. A titre d'exemple, nous analyserons l'effet d'un stress hydrique en cours de cycle sur une arachide. On sait d'une part qu'un stress hydrique en saison de culture provient davantage de l'épuisement du stock d'eau disponible dans le sol que de l'accroissement de la demande Cvaporative, de sorte que ce stress va affecter d'abord la zone racinaire et les nodosités. D'autre part, on sait que lorsque les conditions environnementales ne permettent plus à la plante de satisfaire à la demande évaporative, elle peut s'adapter en fermant ses stomates mais ce faisant, elle interrompt sa photosynthèse et prive particulièrement de chaines carbonnées les régions les plus éloignées de la source, notamment les nodosités. Ainsi, lorsque des symptômes de flétrissement temporaire apparaissent au champ à la période la plus chaude de la journée, la fixation de l'azote a déjà été fortement affectée par carence hydrique et par carence énergétique. Les interventions peuvent poursuivre deux objectifs complémentaires : l'accroissement du stock d'eau dans le sol en supprimant les pertes par ruissellement, par évaporation et par transpiration des adventices et l'amélioration de la capacité de la plante à utiliser cette eau par amélioration du système racinaire

La contrainte fertilité

En zone soudano-sahélienne, on observe depuis une quinzaine d'années une baisse inquiétante des rendements de l'arachide, imputable en grande partie à la baisse de la fertilité des sols. Nous savons que le phénomène de dépérissement de l'arachide est cause par une insuffisance de la fixation de l'azote liée vraisemblablement à une dégradation de l'environnement édaphique qui n'est pas sans rapport avec cette baisse de fertilité. Sans prétendre que toute la baisse de fertilité soit due aux pertes minérales, on peut les considérer comme essentielles (Blondel, 1970 ; Piéri, 1976 ; Wey et *al., 1977).*

La contrainte parasitisme

Elle est la charnière entre la contrainte climatique et la contrainte «techniques culturales». En effet, la virulence d'une maladie donnée dépend à la fois du climat et des techniques culturales adoptées sur l'exploitation (rotation, association d'espèces ou de variétés) favorisant le cycle des parasites. Par exemple, le climat humide de la Casamance dans le sud du Sénégal favorise la rosette de l'arachide ; de plus, les semis échelonnés qui y sont possibles, accentuent la maladie.

La contrainte «date de semis.

La nécessité de semer à la date voulue se heurte souvent à un problème de calendrier cultural sur l'exploitation ou le manque de matériel et de bétail de traction, conduisant à reporter une partie des semis au delà de cette date la plus favorable. Il en résulte des pertes de rendement dues essentiellement à un défaut d'enracinement Les pertes de rendement sur l'arachide ont pu être estimées dans le Centre Sud Sénégal (Sine-Saloum) à *50* kg/ha/jour de gousses dans la première quinzaine de juillet (Tourte, 1974)

La contrainte microbiologique

Parmi les microorganismes impliqués dans la fixation de l'azote des légumineuses, il y a bien évidemment les rhizobiums, partenaires obligatoires dans la symbiose, mais aussi, nous faisons l'hypothèse que dans les conditions édaphiques des sols pauvres étudiés, les champignons endomycorhiziens à vésicules et à arbuscules (MVA) pourraient jouer un rôle important (Dommergues, 1977).

L'intervention sur la biomasse rhizobiale du sol peut être réalisée pour trois raisons :

a) Les sols n'ont pas de rhizobiums : l'inoculation est alors nécessaire dans ces sols ; c'est le cas du soja au Sénégal étudié par Wey (1983).

b) Les sols sont défavorables à la nodulation et à la fixation de l'azote bien que possédant encore des rhizobiums : on doit y remédier afin d'éviter le dépérissement des cultures de légumineuses ; c'est le cas du dépérissement de l'arachide au Sénégal sur laquelle l'inoculation a été étudiée par Wey (1974) et par Drevon (1981).

c) Les sots sont bien pourvus en rhizobiums : dans ces sols où la fixation de l'azote est normale, on peut théoriquement améliorer l'efficience du système fixateur en substituant à ces souches autochtones une autre souche

à condition de résoudre le problème de la compétition : c'est le cas de l'arachide au Sénégal étudiée par Ganry et Wey (1975) et par Ganry et NDiaye (1977).

L'intervention sur la biomasse mycorhizienne peut être envisagée dans les sols où le potentiel d'infection mycorhizienne est réduit (Ganry et *al.* 1982. 1985).

L'Application de techniques culturales appropriées permet de réduire les effets néfastes des différentes contraintes

Ces techniques sont essentiellement la culture de variétés à cycle végétatif adapte, le semis à une date optimale, la fertilisation minérale, le travail du sol, l'amendement organique et calcique, l'inoculation avec des rhizobiums et éventuellement des MVA, la rotation.

Comment mesurer quantitativement l'effet des techniques culturales sur la fixation de l'azote et plus généralement sur l'économie de l'azote ?

Comme nous l'avons évoqué dans l'introduction, seules les méthodes isotopiques (traçage isotopique et abondance naturelle) permettent d'estimer avec une relative précision in situ, la fixation de l'azote. Pour atteindre l'objectif visé d'économie des engrais azotes, il faut considérer le système de culture dans lequel participe une légumineuse et mettre en oeuvre la démarche suivante : utiliser au maximum la légumineuse pour enrichir le pool d'azote du sol et d'autre part déplacer le flux de consommation de l'azote du sol, de la légumineuse (en lui assurant cependant le minimum vital pour sa productivité) vers la céréale qui doit exploiter au mieux ce pool mobilisable d'azote du sol en vue d'économiser les engrais azotés. Par ailleurs, il faut chercher à réduire le plus possible les pertes d'azote provenant de l'engrais. Cette stratégie s'applique au système de culture composé de la céréale et de la légumineuse en rotation.

Pour définir ce système de culture économe en engrais azoté, en l'occurrence la rotation légumineuse céréale, nous devons alors poursuivre trois objectifs :

a) Pour la légumineuse, il faut réduire le plus possible la fourniture d'azote par le sol durant la phase où elle est capable de fixer l'azote (NJ.

b) Pour la céréale, il faut : (i) accroître la fourniture d'azote par le sol incluant l'azote fixé (en vue de réduire l'apport d'engrais azoté) et (ii) réduire les pertes d'azote engrais.

Cette démarche méthodologique requiert la quantification des flux d'azote dans l'agrosystème, en particulier les pertes d'azote engrais, la fixation de l'azote et la quantité d'azote sol prélevé par la plante. Les méthodes isotopiques ^{15}N permettent ces trois quantifications. Au Sénégal, cette démarche a été appliquée aux systèmes mil-arachide et maïs-soja (Ganry, 1987).

Conclusion

En guise de conclusion, nous prendrons l'exemple de la culture arachidikre.

L'analyse des contraintes agronomiques et climatiques pouvant affecter la production arachidière met en relief : (i) l'importance de la contrainte hydrique, à caractère

non prévisible. avec comme première conséquence un déficit prononce de la fixation de l'azote ; (iii la contrainte fertilité, à caractère prévisible et graduellement menaçante en culture traditionnelle : la manifestation la plus importante de cette baisse de fertilité est la carence en azote, attribuée aux faibles réserves azotées des sols sableux tropicaux (faible teneur en matière organique) mais aussi et surtout à l'acidité du sol dont l'effet inhibiteur sur la fixation de l' azote en deçà de pH = 5,5 se manifeste aussi par une carence en azote.

Il apparait donc que dans des conditions de stress hydrique et d'acidité du sol (pH = 5,5), la quantité d'azote fixé par une culture d'arachide est doublement réduite en raison de l'action conjuguée de ces facteurs dépressifs sur la croissance végétative et sur la fixation de l'azote.

Les interventions culturales appropriees devront d'abord rechercher des types de plantes aptes à résister ou à Cviter la sécheresse et éventuellement résister à la toxicité aluminique. Une fois le choix variétal fait, un certain nombre de techniques devront être appliquées au sol : travail du sol et amendement et éventuellement inoculation avec des microorganismes. Il importe de savoir maintenant quel est le degré d'application et d'applicabilid de ces techniques en milieu paysan .

En ce qui concerne le choix des variétés adaptées et de la date de semis la plus précoce, il ne soulève pas de problèmes majeurs car chaque agriculteur mesure bien les risques qu'il encourt en ne respectant pas ces choix.

En ce qui concerne les techniques de labour, d'amendement organique et d'amendement calcique, leur application est capitale puisqu'elle conditionne la productivité du sol et le maintien du patrimoine foncier. Malheureusement, cette application se heurte à de nombreuses difficultés. En effet, très peu de paysans labourent leurs champs en fm de cycle cultural et y apportent de la matière organique par enfouissement des pailles ou apport de fumier. Ce problème doit être examine dans le cadre du système agraire ayant pour objectif l'intégration du troupeau dans l'exploitation.

Quant aux difficultés d'amendement calcique des sols, elles sont dues à un problème de coût et d'approvisionnement en chaux, qui, au Sénégal, malgré la présence d'une usine de chaux demeurent un obstacle à son utilisation par l'agriculteur.

En ce qui concerne l'inoculation par le rhizobium, elle n'a pas dépassé le stade expérimental pour l'instant au Sénégal. Des recherches sont menées en vue de son application par l'agriculteur. Néanmoins, nous considérons que l'inoculation ne devrait être envisagée, dans le cas de l'arachide que lorsque les techniques culturales préconisées auront été rationnellement appliquées.

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Response of *Phaseolus vulgaris* L. to inoculation with *Rhizobium* and fertilization with nitrogen and phosphorus in Northern Tanzania.

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Abstract

In this study we aim to identify soil factors limiting nodulation and bean (*Phaseolus vulgaris* L) production in Nothern Tanzania. Investigations carried out in 1987 *indicated that* large numbers (10^4 *cells* g^{-1} *soil)* of *Rhizobium leguminosarum* bv. *phaseoli* were *present in* most soils, and response to inoculation alone was poor. Chemical analyses of soil samples collected from these areas showed that pH, organic matter content and cation exchange capacity were reasonable, but amount of soil available P was very small (l-2 mg P kg^{-1}). In 1988, results based on two on-farm experiments showed that inoculation with *Rhizobium* (*strain* CIAT 899) and application of triple superphosphate significantly increased nodulation and yield. Application on Minjingu rock phosphate also increased nodulation and yield but to a much lesser extent.

Introduction

Bean (*Phaseolus vulgaris* L.) is an important grain legume widely grown in the highland areas (1000 meters above sea level) of East and Central Africa. The main bean producers are small farmers for whom bean consumption contributes up to 50% of dietary protein (Allen, 1986). The majority of these farmers grow the crop on low fertility soils with no fertilizer input. Generally under these conditions, effective nodulation with *Rhizobiwn* is rarely observed and amount of atmospheric nitrogen fixed is probably small. Many factors (e.g. genetic variability, eclaphic and agronomic factors) are known to affect nodulation in beans (Graham, 1981). Our objective has been to identify soil factors which limit nodulation and nitrogen fixation in the field. The investigations reported here were carried out during two growing seasons (March to August 1987 and 1988) in Northern Tanzania, one of the principal areas for bean production.

Materials and methods

Experiment I (1987)

On station, experiments on two contrasting soil types (volcanic soil at Salien, and brown *earth* at *Lambo)* were done *to* test *response to nitrogen* application and *Rhiwbium* inoculation. Two bean varieties of large seeded dwarf (Type 1) were chosen : Canadian Wonder, a popular variety grown throughout Northern Tanzania, and Lyamungu 85, a variety recently released by the Tanzania Agricultural Research Organisation (TARO) Bean Programme. Nitrogen was applied at 0 or 30 kg N ha^{-1} as $(NH_4)_2SO_4$ at time of planting, and seeds were uninoculated or inoculated (10^6 cells per seed) with peat-based mixed ***Rhizobium*** inoculant of strains CIAT 632 and 899. A basal dose of 60 kg P_2O_5 ha^{-1} as triple superphosphate (TSP) was applied to all treatments. Each treatment had five replicates, and a split plot design was used to reduce the risk of contamination between uninoculated and inoculated treatments. Plot size was 4 m x 6 m containing eight rows 0.5 m apart with one seed sown at 0.1 m interval to give 200,000 plants ha^{-1}. Nodulation (nodule number, nodule diameter and score for effectiveness) was recorded up to flowering, and seed yield was measured at harvest. Bean fields owned by research stations and farmers were visited to assess nodulation and plant vigour. Soil samples were collected for chemical analyses (MAFF/ADAS, 1986) to determine variation in soil fertility and to identify factors limiting the establishment of effective nodulation.

Experiment II (1988)

Two sites (Erente, Lushoto and Tropical Pesticide Research Institute (TPRI), Amsha) which had no record of previous fertilizer application were selected for an experiment to test response to *Rhizobium* inoculation, nitrogen application and phosphate fertilization. Lyamungu 85 variety was either uninoculated or inoculated with peat based inoculant (CIAT 899) to give 10^6 cells per seed and N applied at 0 or 30 kg ha^{-1} as nitrochalk (21% N). Two sources of P were used ; Minjingu rock phosphate (MRP) containing approximately 30% P_2O_5 or triple super phosphate (TSP) containing 45% P_2O_5 at equivalent application rates of 0, 60, 120 or 180 kg P_2O_5 ha^{-1}. The rock phosphate is a sedimentary guano deposit located at South West of Arusha. N. Tanzania (Scaife, 1968). and has

been recommended as an alternative source of P costing a third of the price of TSP. A split plot with two replicates was used to avoid contamination between uninoculated and inoculated treatments. Plot size was 4 m x 4 m containing eight rows 0.5 m apart with two seeds sown at 0.2 m interval to give 200,000 plants ha^{-1}. Measurements included nodule number before flowering, vesicular arbuscular mycorrhizal infection (Phillips and Hayman, 1970) at pod development, and final seed yield at harvest. Soil pH and bicarbonate soluble P (available P) were also determined at final harvest Rainfall was poor at TPRI, and moisture stress experienced after flowering reduced final yield and may offset potential effects of the treatments.

Results and discussion

Experiment I (1987)

At Salien, nodulation was excellent (up to 100 nodules per root system, 2-3 mm in diameter and effective, i.e. pink in colour) in all treatments including uninoculated plants which nodulated well *with* indigenous *Rhizobium. Earlier* on, nodulation was suppressed in treatment receiving N. However this effect was not found at later harvests. Mean seed yield was 1350 kg ha^{-1} with no increase due to N application or inoculation, suggesting that some other factors were limiting yield. A nutrient disorder resemblig potash deficiency was observed, and work is in progress to identify the cause. At Lambo, nodulation was poor in all treatment (up to 20 nodules, 1 mm in diameter and white in colour). Mean seed yield was 840 kg ha^{-1} and did not differ with treatments. This site experience prolonged moisture stress which would have offset any potential effect of N application or inoculation. In 1988, Lambo received good rainfall, and nodulation was exceellent.

Enumeration of *Phaseolus* rhizobia (Vincent, 1970) showed that up to 10^4 cells g^{-1} soil were present at both sites. Singleton and Tavares (1986) have shown that unless *the native* population of *Rhiwbium* is less than 10^2 cells g^{-1}, response to inoculation is not common. Hence it is not surprising to record no response to inoculation in our studies at Salien or Lambo where indigenous population exceeded this number using the strains CIAT 632 and 899 which had not been shown to have exceptional competitive ability.

Generally, bean plants had good nodulation and plant vigour when raised on fertile volcanic soils (e.g. Salien, Table 1). In contrast, nodulation and plant vigour were poor in the more common reddish brown soils. Chemical analysis of these soils (Table 1) showed that concentration of available P (bicarbonate-soluble) was small (l-2 mg kg ^{1}) and that this might be a limiting factor for plant growth and nodulation. No other single factor could be identified as the cause for poor nodulation, Soil acidity has been shown to depress nodulation, however our data indicated that most soils from this region were not very acidic (pH 5.5, Table 1). Organic matter content was average (3%) and cation exchange capacity also reasonnable (30 meq/100 g), and there was no indication that nodulation was related to either of these (Table 1).

In addition to low concentration of soil P, the data clearly showed that soils from research stations had unrealistically high levels of fertility and did not resemble those from farmers' fields (Table 1). Hence the results of Experiment I, done on sites belonging to research stations, are not comparable wth the situation in the farmers' field. It was also evident that although large numbers of *Phaseolus* rhizobia were often present in the farmers' fields, nodulation and plant vigour were poor and this might be due to the small amount of available soil phosphate.

Table 1 : Observations on plant vigueur and nodulation, and chemical characteristics of some representative soils from the survey.

Location	Plant Vigour	Nodulation	Soil Colour	Soil pH	Organic matter %	Bicarb Sol P_{-1} mg kg^{-1}	C.E.C. me/100g	Exchangeable cations me/100g				Base saturation %
								K^+	Na^+	Ca^{2+}	Mg^{2+}	
Salien (1300m) N. Tanzania	Good	Good	Black	6.6	3.7	17.1	40.6	7.17	0.37	27.08	5.15	98
Lambo (1020m) N. Tanzania	Moderate	Poor	Brown	6.5	4.2	36.9	28.9	2.56	0.09	16.35	2.75	75
Lyamungu (1270m) N. Tanzania	Moderate	Poor	Brown	5.4	4.7	58.7	30.6	1.66	0.12	9.58	1.68	43
Lushoto (1350m) N. Tanzania	Poor	Poor	Red	5.8	4.5	1.0	18.1	0.18	0.29	8.54	2.16	62
Morogoro (700m) C. Tanzania	Moderate	Moderate	Brown	6.5	3.5	3.3	20.6	1.24	0.29	12.92	3.19	86
Mbeya (1500m) S. Tanzania	Good	Moderate	Black	6.1	2.7	1.7	19.4	3.20	0.35	6.25	2.13	61

Experiment II (1988)

At both Erente and TPRI, inoculation with *Rhizobium* and P application as TSP markedly ($P < 0.01$) increased nodulation (Tables 2 and 3), and yield (Tables 4 and 5). Inoculation alone also increased nodulation and yield, but it was not statistically significant Nitrogen application suppressed nodulation (Tables 2 and 3) and sometimes increased yield (Tables 4 and 5). Phosphorus when applied in the form' of TSP increased nodulation and yield (Tables 2, 3, 4 and 5). Similar findings have been reported in Kenya (Ssali and Keya, 1983). MRP also increased nodulation and yield but to a lesser extent (Tables 2, 3, 4 and 5). The highest level of P applied (180 kg P_2O_5 ha^{-1}) gave the maximum yield (Tables 4 and 5) thus potentially yield might be increased with further application of P, although such amounts would not be economical and rarely used by the farmer. There was no change in pH, but amount of available P had increased following increasing rates of P application (Table 6), more so at TPRI than at Erente. On average, only 20% of the root system was colonized by vesicular arbuscular mycorrhizas, a fungal root symbiosis which plays an important role in uptake of P from the soil. There was no real difference in the amount of colonization between sites or treatments. The small extent of colonization is surprising. For bean, roots are highly susceptible to mycorrhizal infection (Holley and Peterson, 1979). When the soil was examined, a few spores of *Glomus monosporum* were present. It would be justifiable to test response to mycorrhizal inoculation, especially in association with rock phosphate, if such small spore populations are found to be common.

The preliminary results presented here are now part of a long-term investigation to study effects of phosphorus, in particular the residual effect of MRP, an important soil factor limiting bean production.

Conclusions

Large populations of indigenous *Rhizobium phaseoli* are present and yield responses due to *Rhizobium* inoculation are not common in these soils, even though nodulation may be increased.

Soil phosphate is a major factor limiting nodulation and *Phaseolus* production.

By raising the soil available P, it may be possible to form effective nodulation from the existing indigenous population of *Rhizobium,* which appear to be equally efficient as the inoculation strain used here.

In this first year of study, TSP was a more effective source of P fertilizer in comparison to MRP. Future studies will show whether the residual effect from MRP can give similar yields obtained with TSP.

Acknowledgement

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Table 2 : Nodule number (m-2) at Erente (28 OAP) comparing inoculation (± Inoc), application of nitrogen (+N = 30 kg N ha^{-1}) and Minjingu rock phosphate (MRP) or triple super phosphate (TSP).

Level of P Kg P_2O_5 ha^{-1}	Inoc	MRP		TSP	
		-N	+N	-N	+N
0	-	721	189	721	189
60		558	319	1031	412
120		1119	281	1812	357
180		779	476	1544	1235
0	+	759	300	759	300
60	+	584	256	1970	906
120	t	830	111	1248	596
180	+	952	241	1259	819

Standard Error 82.8

Table 3 : Nodule number (m^{-2}) at TPRI (37 DAP) comparing inoculation ($\pm$ Inoc), application of nitrogen (+N = 30 kg N ha^{-1}) and Minjingu rock phosphate (MRP) or triple super phosphate (TSP).

Level of P Kg P_2O_5 ha^{-1}	Inoc	MRP		TSP	
		- N	+ N	- N	+ N
0		689	300	689	300
60		1090	408	1631	633
120		1022	437	1858	812
180		1159	593	1873	740
0	+	604	226	604	226
60	+	828	259	1626	588
120	+	1013	367	1847	536
180	+	1234	533	2627	951
Standard Error			52.8		

Table 4 : Seed yield (kg ha^{-1}) at Erente (109 DAP) comparing inoculation ($\pm$ Inoc), application of nitrogen (+N = 30 kg N ha^{-1}) and Minjingu rock phosphate (MRP) or triple super phosphate (TSP).

Level of P Kg P_2O_5 ha^{-1}	Inoc	MRP		TSP	
		-N	+N	-N	+N
0		421	528	421	528
60		552	596	530	552
120		546	500	668	698
180		398	540	797	866
0	+	367	563	367	563
60	t	500	476	622	546
120	+	599	494	586	632
180	t	449	608	790	962
Standard Error			24.6		

Table 5 : Seed yield (kg ha^{-1}) at TPRI (93 DAP) comparing inoculation (± Inoc), application of nitrogen (+N = 30 kg N ha-1) and Minjingu' rock phosphate (MRP) or triple super phosphate (TSP).

Level of P kg P_2O_5 ha^{-1}	Inoc	MRP		TSP	
		-N	+N	-N	+N
0		158	274	158	274
60		249	221	314	294
120		221	279	323	389
180		276	269	391	438
0	+	181	246	-181	246
60	+	237	264	286	342
120	+	215	279	319	332
180	t	262	408	338	388

Standard Error 10.1

Table 6 : Change with application (kg P_2O_5 ha-1) of Minjingu rock phosphate (MRP) or triple super phosphate (TSP) in soil pH and bicarbonate soluble P (mg P kg-1) measured at Erente (109 DAP) and TPRI (93 DAP).

		Source and level of applied P							
		MRP				TSP			
		0	60	120	180	0	60	120	180
		-	-	-	-	-	-	-	-
Erente	pH	5.3	5.3	5.2	5.4	5.3	5.3	5.3	5.3
	P	2.2	2.8	4.7	6.2	2.2	4.2	4.7	6.5
TPRI	pH	6.6	6.8	6.6	6.7	6.6	6.6	6.7	6.7
	P	1.4	3.9	10.2	11.8	1.4	5.8	14.3	19.0

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Variation in acid-Al tolerance *of Bradyrhizobium japonicum*

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Abstract

Seventy-six strains of *Bradyrhizobium japonicwn (54* African *and 22 exotic)* were examined for tolerance of acidity (pH 4.5). low P (5 μM) and high Al (50 and 100 μM) by an agar plate method. Fortyfour strains were tolerant to acidity regardless of P levels (1.000 or 5 μM) of the medium and 22 were sensitive. The remaining 10 strains differed in tolerance of acidity depending on level P of the medium ; nine were tolelant of low P (5 μM) and one of high P (1.000 μM). All of 21 strains which grew on the most severe stress used consisting of low P and high Al (100 μM) at pH 4.5 showed tolerance of acidity at both levels of P. Isolates from a highly acidic soil (pH 4.6, H_2O) from Onne, southeastern Nigeria, showed different levels of acid-Al tolerance but tended to be more tolerant than those from a slightly acidic soil (pH 6.5, H_2O) from Ibadan, southwestern Nigeria However. 20 % of the Onne isolates and 30 % of the Ibadan isolates were sensitive to acidity. Thus, tolerance of strains was not necessarily determined by the acidity of the soils in which they reside. Gum production of certain strains occured at low P. but this had no relation to acid-Al tolerance.

The great variation in acid-Al tolerance observed among strains of *B. japonicwn* supports the strategy to select strains for use as inoculants in acid soils.

Introduction

Performance of inoculated strains of *Bradyrhizobium japonicum* in a highly acidic soil (Ultisol, pH 4.6, H_2O) from Onne, southeastern Nigeria, was good in terms of nodulation of soybeans (*Glycine max* (L.) Merr.) in the first year but was poor in the second (Bromfield and Ayanaba, 1980 ; IITA, 1981). It was suggested that some acid soil factors are detrimental to the ecological behavior of introduced rhizobial strains and that use of acid tolerant strains could lead to better utilization of the benefits of biological nitrogen fixation to grow soybeans under such acid conditions.

A great variation in tolerance of several acid soil factors has been shown among strains of *Rhizobium leguminosarum* biovar *trifolii* (Thornton and Davey, 1983 ; Thurman *et al.,* 1985). *R. leguminosarum* biovar *phaseoli* (Munns et *al.,* 1979 ; Graham et *al.,* 1982 ; Lowendorf and Alexander, 1983), *R. meliloti* (Howieson *et al.,* 1988) ; *R. loti* and *Bradyrhizobium sp. (lotus)* (Cooper, 1982), *Bradyrhizobium* sp. (cowpea group) (Date and Halliday, 1979 ; Keyser and Munns, 1979a and b ; Keyser *et al.*, 1979 ; De Carvalho v., 1981 ; Munns and Keyser, 1981 ; Ayanaba *et al.,* 1983) and *B. japonicum* (Keyser and Munns, 1979a and b ; Ayanaba *et al.,* 1983). Little information, however, is available on native African strains of *B. japonicum.* Ayanaba *et* al., (1983) reported that some African rhizobia isolates nodulating both cow-pea (*Vigna unguiculata* (L.) Walp.) and soybean cultivar TGm 344 showed wide variation in tolerance.

Tolerance of strains of rhizobia to acid soil factors is generally tested by growth in defined liquid media (Date and Halliday, 1979 ; Keyser and Munns, 1979a and b ; Howieson, 1985). Keyser and MUMS (1979a) reported that in the test of tolerance of acid soil factors with *Bradyrhizobium* sp. (cowpea group) and *B. japonicum,* Al (25-50 μM) was probably a more severe stress than low Ca (50 μM) and/or high Mn (200 μM). Thornton and Davey (1983) showed that in liquid culture media, growth of *R. trifoliti* was more limited by high Al (15 to 40 μM) than by low P (1 to 6 μM). Ayanaba *et al.,* (1983) proved that screening of *Rhizobium* for tolerance to acid-Al stress could be achieved by an agar plate method and that this method had an advantage in handling large numbers of rhizobia.

In the present paper we report variation in tolerance of acid-Al stress among strains of *B. japonicum* isolated mostly from African soils. Acidity, low P and high Al were factors examined by the agar plate method.

Materials and methods

Rhizobial strains

Seventy-six strains of *B. japonicum* from the culture collection of the International Institute of Tropical Agriculture (IITA), Ibadan Nigeria were used. Fifty-four of them were isolated from African soils, 20 of which were from either soybean promiscuous (Malayan, Orba or 4H/149/1) or non-promiscuous cultivars (TGm 294 or Bossier) grown in a highly acidic soil of Onne. Twenty were from the same cultivars grown in a slightly acidic soil (pH 6.5, H_2O) of Ibadan, southwestern Nigeria. Some of the African isolates nodulate cowpeas (IITA, 1979). Three wild-type strains and their antibiotic resistant mutants were included. One of them is resistant to spectinomycin and the other two to streptomycin. All strains were cultured on slopes of yeast extract-mannitol agar medium (YEM agar) (Vincent 1970).

Test agar media

Six media prepared according to Keyser and Munns (1976b) were used : full - defined ; acid ; low P ; low P, acid and two levels of high Al (50 and 100 µM). Each medium was uspplemented with 15 g/l ionagar (Oxoid Co. Ltd., England). Each medium contained the basal solution of Keyser and Munns (1979b). Five hundred µM each of KH_2PO_4 and K_2HPO_4 and 1.1 g/l Na-glutamate were added to the two full-defined media, whereas 5 µM KH_2PO_4, 1.5 mM KC1 and 1.1 g/l Na-glutamate were added to the other four media. Full-defined and low P media were adjusted to pH 6.8 with 1N-NaOH and the others were adjusted to pH 4.5 with 1N-HCl before addition of $AlK(SO_4)_2$ and growth factors (Keyser and Munns, 1979b). Furthermore, methyl red in ethanol was added as a pH indicator to a final concentration of 0.002%. The media were autoclaved (121°C, 15 mn.) before dispensing into plates (30 ml/plate). Autoclaving and the addition of these substances slightly changed the pH values of the media but it did not affect the testing.

Testing of acid-Al tolerance

Loopfub of rhizobia grown on YEM agar slopes were suspended in sterilized 0.85% saline to give similar turbidity among strains. The six different agar media, predried for 3 h at 45°C were inoculated by transferring the bacterial suspensions with sterilized cotton swabs. Twenty strains were tested in one plate and the experiment was duplicated. Obervations were made daily during a lo-day-incubation at 28°C. Tolerance of rhizobia to acidity or acid-Al stress was evaluated by comparing their growth on the stress media with that on the full-defined medium. The tolerance of Onne and lbadan isolates was as designated in Table 1, bases on the levels of severity of the stress.

Table 1 : Designation (I to VII) of the degree of response to acid-Al stress by Bradyrhizobium isolates from Onne and Ibadan soils ; media were at pH 4.5.[a]

		Designation						
P(uM)	Al (uM)	I	II	III	IV	v	VI	VII
1,000	0	S	S	S	T	T	T	T
5	0	S	T	T	S	T	T	T
5	50	s	s	T	S	S	T	T
5	100	s	s	S	s	s	s	T

a: Growth on each medium was compared with that on control medium (pH 6.8, 1,000 uM, P and 0uM Al) and designated as T (tolerant) or S (sensitive) accordingly.

Results and discussion

Most rhizobia grown on laboratory media are known to produce either acidic or alkaline substances (Norris, 1965 ; Jones and Burrows, 1969 ; Cooper, 1982, Hernandez and Focht, 1984), depending on the preferential use of sugars or organic nitrogen compounds, respectively, as the source of energy (Parker et *al.*, 1977). In evaluating the growth of ***Rhizobium*** on such media, therefore, changes in pH have to be monitored Methyl red was employed in the present experiment for that purpose. About half of the strains tested produced alkaline substances when grown on the agar media (Table 2), but. it was

Table 2 : Bradyrhizobium japonicum strains tolerant to acid-Al (100µM, pH 4.5) stress

IITA accession no.	Origin or source	Alkali production[a]	Colony type	
			High P (1,000µM)	Low P (5µM)
IRj 2001	cv. Malayan, Onne		nd[b]	nd
2002	cv. Halayan, Onne	+	white	watery
2004	cv. Halayan, Onne	+	white	watery
2007	cv. Orba, Onne	+	white	watery
2013	cv. Halayan, Ibadan	+	white	watery
2019	cv. Orba, Ibadan	+	white	watery
2020	cv. Orba, Ibadan	-	nd	nd
2025	cv. TGm294, Ibadan	+	white	watery
2033	cv. Orba, Yandev		white	watery
2035	cv. Orba, Yandev		nd	nd
2038	cv. Malayan, Samaru		nd	nd
2039	cv. Halayan, Samaru	+	nd	nd
2048	cv. TGm294, Samaru		white	watery
2049	cv. TGm294, Samaru		nd	nd
2052	cv. Bossier, Samaru	nd	nd	nd
2109	Univ. of Wisconsin, USA (SM 61A76)		nd	nd
2119	ENSA, Ivory Coast (G3)	+	nd	nd
2120	ENSA, Ivory Coast (G8)	nd	nd	nd
2123	USDA (311b138)	+	nd	nd
2128	Thailand (THA2)	+	white	not watery
2129	Thailand (THA5)	+	white	not watery

a Alkali production : +, producing ; -, not producing ; nd, not determined.

b nd, not determined.

observed that their growth preceded the shift in pH of the media. Ayanaba et al. (1983) observed that strains of rhizobia tolerant of acid-Al stress on agar medium grew significantly in liquid stress medium before a change in pH. Thus, it seemed that tolerance of rhizobia to acid-Al stress might not be directly related to their ability to produce alkali.

Rhizobial strains showed great variation in tolerance of acid-Al stress (Fig.1). Most strains tolerated acidity stress (pH 4.5) similarly regardless of P levels (1.000 or 5 μM)

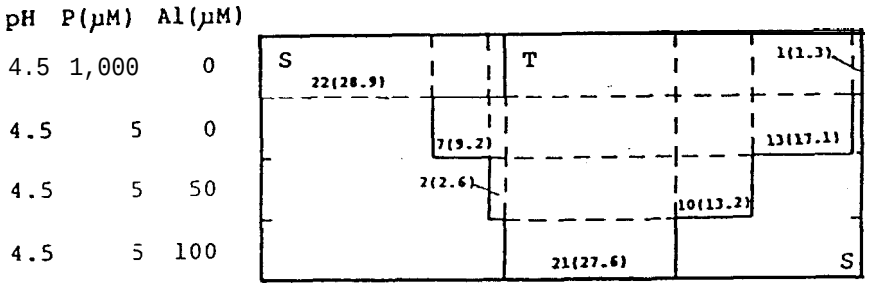


Figure I : Effects of acidity and various levels of P and Al on growth of *B. japonicum* inoculated on agar media. Growth on each medium was examined by comparison with that on control medium (pH 6.8. P 1,000 μM and Al 0 μM) and designated T (tolerant) or S (sentitive) accordingly. Results are shown with numbers of strains and their proportions in percent in parentheses.

of the medium ; 44 strains were tolerant and 22 were sensitive. The remaining 10 strains, however, differed in tolerance of acidity depending on the level of P. Nine required low P 5 μM) to be torerant and one required high P (1,000 μM) to be tolerant. Thus, tolerance of rhizobia to acidity seemed to differ depending on the level of phosphorus content of the medium. Table 3 shows the effect of P levels on the growth of rhizobia under two pH conditions (6.8 and 4.5). Over 90 % of the strains grew similarly at pH 6.8 regardless of P levels. By contrast at pH 4.5, 25 % or 19 strains grew less at the high P than at the low P. Nine strains which required low P to grow at pH 4.5 (Fig.1) were also in this group. Uptake and utilization efficiencies of phosphorus were reported to be greatly different among strains of rhizobia, which caused differences in low P tolerance of rhizobia (Cassman *et* al., 1981b ; Beck and Munns, 1984). The result shown in Table 3 suggests that the efficiencies in phosphorus uptake and utilization might change depending on the pH surrounding strains of rbizobia.

Growth of rhizobia at pH 6.8 and 4.5 was also compared at different P conditions (1.000 and 5 μM) (Table 4). Most strains responded similarly to pH regardless of P levels, suggesting that phosphorus is less influential on the growth of rbizobia than pH. This supports observations with *R. trifolii* by Thornton and Davey (1983).

As shown in Fig. 1, tolerance of acidity did not always mean tolerance of acid-Al, indicating that tolerance of acidity stress among rhizobia might be enhanced by the presence of aluminum. Thirty three of the 53 strains that tolerated acidity at the low P level could grow under acid-Al (50 μM, pH 4.5) stress ; 21 of these or 27.6 % were further tolerant of the most severe Al stress tested (100 μM). Similar results were obtained

Table 3 : Effet of P levels on growth of B. japonicum at pH 6.8 and 4.5[a]

pH	Growth in 1,000 µMP compared to that in 5 µMP		
	Larger	Similar	Smaller
6.8	2 (2.6)	70 (92.1)	4 (5.3)
4.5	2 (2.6)	55 (72.4)	19 (25.0)

a Growth of strains was compared after lo-day-incubation. Visual readings on a scale of 0-5 were made of colonies and the differences of the minimum 2-point-readings were considered significant. Results are shown with numbers of strains and their proportions in percent in parentheses.

by Keyser and Munns (1979b) Who found that three of the eight *B. japonicum strains* that were tolerant of pH 4.5 also tolerated acid-Al (50 µM) stress. The most tolerant strains (Table 2) were not restricted to one geographic region of the world

Does acidity of bulk soil determine the acid-Al tolerance of rhizobia isolated from the soil ? To investigate it, acid-Al tolerance of isolates from a highly acidic Onne or

Table 4 : Effect of pH on growth of B. japonicum at two different levels of P[a]

P (uM)	Growth at pH 6.8 compared to that at pH 4.5		
	Larger	Similar	Smaller
1,000	42 (55.3)	31 (40.8)	3 (3.9)
5	32 (42.1)	44 (57.9)	0 (0.0)

a See the footnote in Table 3.

slightly acidic Ibadan soil (pH 4.6 and 6.5 respectively) were compared (Fig. 2). The Onne isolates showed less varied levels of tolerance than those from Ibadan soil and tended to be more tolerant of acid-AI than the Ibadan isolates, indicating that the acidity of a bulk soil could affect the acid-Al tolerance of rhizobia residing in that soil. However, 20 % of the Onne isolates were sensitive to acidity (pH 4.5), and so were 30 % of the Ibadan isolates. Thus, the tolerance of strains seemed not to be necessarily determined by the acidity of bulk soil from which they were isolated. Jones and Burrows (1969) also reported no correlation between the final pH of the culture media in which isolates of *R. trifolii* grew and the pH of the soils from which they originated In soil, acidity or acid-Al conditions of a microenvironment could affect the acid-Al tolerance of the inhabitants, therefore, further research needs to be carried out in this connection.

Rhizobia nodulating non-promiscuous soybean cultivars are known to nodulate promiscuous ones, too, but the reverse is not always true (IITA, 1979 ; Bromfield and Roughley, 1980). It means there is a difference among strains of rhizobia in terms of compatibility with host cultivars. Promiscuity of soybean cultivars (Nangju, 1980 ; Pulver et *al.,* 1985) from which the Onne and Ibadan isolates were obtained and the acid-Al tolerance of these isolates are shown in Fig. 3. Acid-Al tolerance of the isolates was not dependent on the promiscuity of host plants from which they were isolated.

Certain strains formed watery and translucent colonies; regardless of P levels (1,000 or 5 μM) in the full-defined medium. However, some strains formed white colonies on the same medium when P was high, and watery and translucent colonies when P was low (Table 2). Watery colonies were formed presumably because production of copious amounts of gummy substances occurred when the P level of the medium was low. Cassman *et al.*, (1981a) also observed abundant production of external gum by strains of *B. juponicum* in defined liquid medium with low and moderate P, but not with the high P concentrations routinely supplied in laboratory media. These results suggest that gum production of certain rhizobia may be enhanced when the P level of the medium becomes low. In the study of cowpea rhizobia isolated from three locations in West Africa, two types of colonies, dry and pinpoint or large and gummy, were frequently observed when grown on YEM agar (IITA, 1981 ; Ahmad *et* al., 1981) and Ayanaba *et* al., (1983) found that rhizobia strains forming the former type of colonies were more sensitive to acid-Al stress than those forming the latter ones. Cunningham and Munns (1984) showed that high production of extracellular polysaccharide is positively related to the acid tolerance of rhizobia. In this study, the relation between gum production and acid-Al tolerance of rhizobia was not clear. In this connection, Howieson et *al.*, (1988) also reported that with *R. trifolii*, there was no distinct link between polysaccaride production and acid tolerance.

Three wild type strains and their antibiotic resistant mutants were included in this study. Mutation induced by two antibiotics seemed not to affect the acid-Al tolerance of rhizobia : all strains were as sensitive or tolerant as the wild types (data not shown). This result supports the observation by Ayanaba and Wong (1982) that antibiotic resistant mutants tolerated acidity to the same extent as their parents.

A consistent and stable property of tolerance of rhizobia to acid and aluminum stresses (Munns and Keyser, 1981; Munns, 1986) and a great variation in acid-Al tolerance among strains of *B. juponicum* observed in this study prove that strains can be selected for possible use as soybean inoculants in acid soils. It is obvious, however, that there are some other adverse, abiotic stresses like high temperature and/or desication (Osa-Afiana and Alexander, 1982) in acid soils to which rhizobial inocula are probably expo-

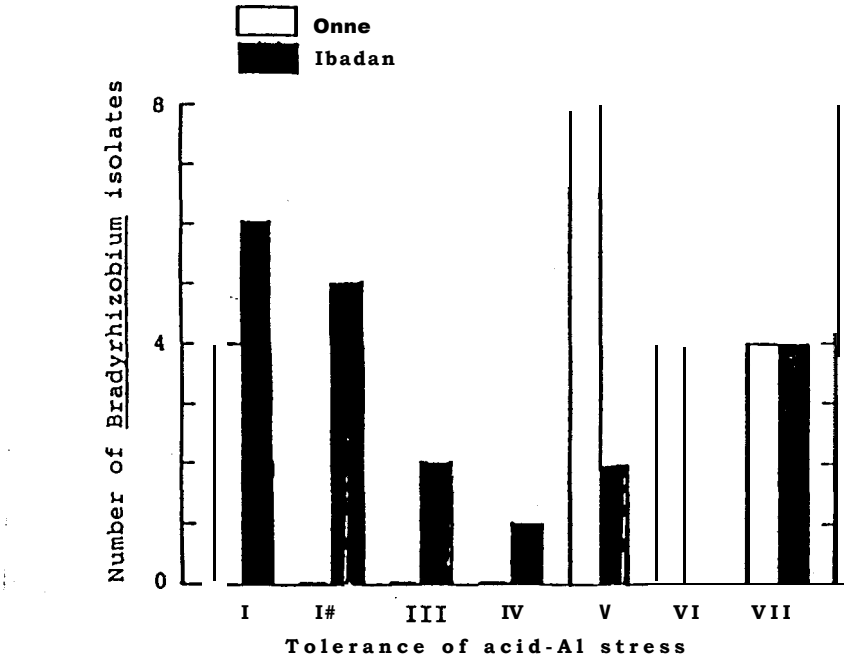


Figure 2 : Variation in *the* tolerance of acid-Al *stress* shown by *Bradyrhizobium* isolates from Onne and Ibadan soils. Twenty isolates each were tested. I - VII : See Table 1.

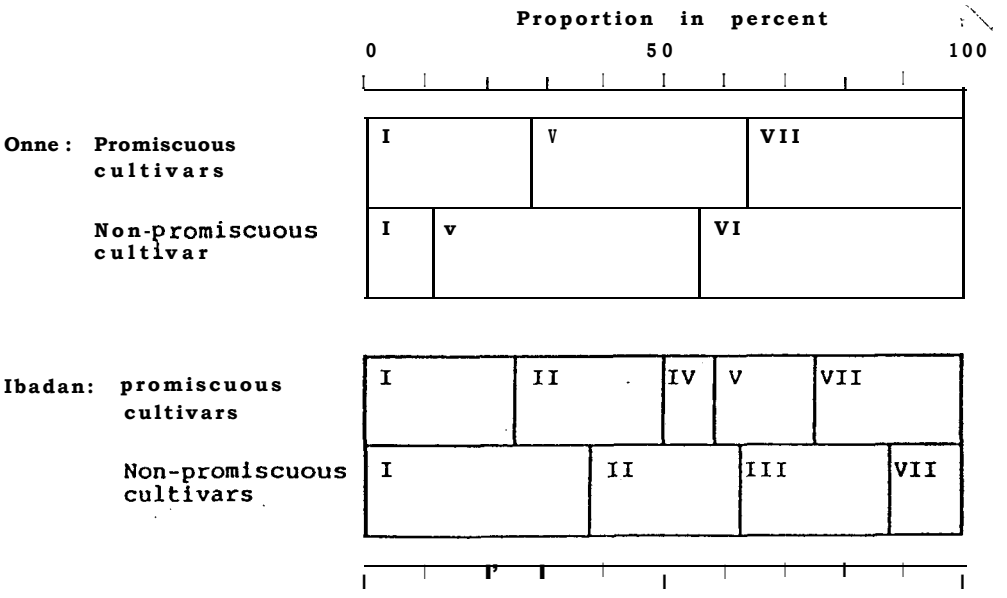


Figure 3 : Comparison of acid-Al *stress* tolerance between *Bradyrhizobium* isolates from promiscuous and non-promiscuous cultivars of soybean grown in Onne and Ibadan soils. Eleven and 9 isolates were tested for the promiscuous and non-promiscuous cultivars, respectively. in Onne soil and each 12 and 8 isolates, respectively. in Ibadan soil.

sed, particularly during fallow periods under tropical African conditions. Therefore, tests are required on the effects of those stresses on the saprophytic competence of rhizobia (Thornton and Davey, 1984) and their symbiotic effectiveness under field conditions. At the same time, screening or breeding of soybean cultivars adapted to acid soil conditions should not be ignored (Munns *et al., 1981).*

Acknowledgments

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The influence of some parameters of soil fertility on early growth of *Leucaena leucocephala* and *Cassia siamea*

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Abstract

Leucaena leucocephala and *Cassia siamea* were grown in concrete cylinders containing 0.35 t of a sandy, high base status Entisol. The nutrient content of the soils varied from one cylinder to another. Fertilizer was added to the rate 11 kg N, 88 kg P and 5 kg Zn per hectare. At 8 months after planting, *Leucaena* was well no-&late-d and produced on average seven times as much biomass as the non-nodulated *Cassia*. There were significant ($P = 0.05$) correlations with topsoil N. With *Cassia*, positive correlations were observed for plant height ($r = 0.73$) and total stem length ($r = 0.75$). With *Leucaena* there were significant negative correlations for plant height ($r = -0.85$) and estimated total biomass ($r = -0.90$). *Leucaena* plant height was also positively correlated with topsoil K ($r = 0.81$). Ninety eight per cent of the variation in height could be accounted for by the equation $Y = 4.88 + 117.37X - 14.94X^2$ where Y = plant height and X = K/N ratio.

Introduction

Trials of alley cropping in farmers' fields have run into problems on account of the very uneven establishment of the trees due to poorly understood factors. The work reported here provides indications of how certain parameters of soil fertility may influence early growth of *Cassia siamea* and *Leucaena leucocephala.*

Materials and methods

Two month-old plants of *Leucaena leucocephala* (K8) and *Cassia siamea* (local variety) were transplanted into concrete cylinders (60 cm i.d. and 90 cm deep) sunk in the ground and containing a sandy, high base status Entisol which had been previously used for various experiments for over a decade at Intemational Institute of Tropical Agriculture (BTA), Ibadan. Before planting, composite topsoil (15 cm) samples were taken by bulking five cores per cylinder, and their nutrient content determined at the university of Ibadan. At planting time, the cylinders were fertilized with 88 kg P ha^{-1} as single superphosphate and 11 kg N ha^{-1} as $(NH_4)_2SO_4$. Two months later 5 kg ha^{-1} Zn was applied as $ZnSO_4$.Plant height, total stem lenght (plant height plus the sum of branch lenghts) and leaf number were recorded 6 months after transplanting and a rough estimate of total aboveground biomass was made, using the formula of Anderson and Ingram (1987) to estimate stem and branch biomass. Leaf biomass was estimated by multiplying leaf number by the average weight per leaf of a sample of leaves.

Results

Soil analysis (Table 1) indicated marked differences in nutrient content between the cylinders with coefficients of variation ranging from 11 to 64%. Soil physical properties (not reported here) varied little. The soils could be described as having medium to high organic matter, low available P and medium to high exchangeable K. The plant growth data show that *Leucaena* produced on average about seven times more biomass than *Cassia.* There was considerable variation from one plant to another. For *Leucaena,* coefficients of variation for leaf number, total stem length, plant height and estimated total biomass were 51, 58, 17 and 48% respectively. For *Cassia* the variation was lower : 17, 40, 18 and 36% respectively. The form of different trees of the same species also varied appreciably. *Leucaena* had 3 to 24 branches, *Cassia* 1 to 7 branches. *Leucaena* was well nodulated in all the cylinders, but *Cassia* did not nodulate.

Regression analyses indicated that *Cassia* and *Leucaena* were differently affected by topsoil properties.

Cassia stem and leaf production were positively correlated with soil organic C, total N, available P, exchangeable Ca and Mg but negatively correlated with exchangeable K. Except for total N, these correlations were not significant. Total N was significantly correlated at the 5% level with total stem length (r = 0.74) and there was a high though not significant positive correlation with leaf number (r = 0.66). The correlation coefficient for leaf number and available P was not significant (r = 0.41). Stem and leaf production were positively correlated with exchangeable Ca (r = 0.58 and 0.19 respectively), and stem production was positively correlated with exchageable Mg (r = 0.34). On the other hand, plant height was negatively correlated with all these soil properties except

Table 1 : The range and variation of soil properties in cylinders planted with Leucaena leucocephala and Cassia siamea and the range and variation of plant growth parameters of the two species.

Characteristic	Leucaena leucocephala			Cassia siamea		
	Mean	Range	cv (%)	Mean	Range	CV (%)
Soil properties						
Organic C (%	1.8	1.8- 2.0	16	1.9	1.5-2.1	11
Total N (%)	0.13	0.07-0.24	43	0.12	0.10-0.14	13
Extractable P (ug g^{-1})			64	4.8	1.8-8.4	48
K (meq/100 g)	0.3	1.3- 8.4	38	0.3	0.2-0.4	55
Ca(meq/100 g)	7.8	05.5-13.0 4.6	35	6.9	4.3-12.0	44
Mg(meq/100 g)	3.8		28	3.7		28
K/N(meq/100 g)	2.8	2.61.5- 4.9 5.8	65	2.5	2.3-3.3	58
Plant growth parameters						
Leaf No. plant-'	113	23-190	51	54	39-62	17
Height (cm)	174	123-201	17	54	43-69	18
Total stem length (cm)	517	123-935	58	137	69-251	40
Estimated total biomass (g)	737	79-1056	48	96	74-157	36

Table 2 : Correlation coefficients between soil properties and plant growth parameters

Tree	Plant parameters	Soil property C	N	P	K	Ca	Mg	K/N
Leucaena leucocephala	Leaf No.	Ø.Ø5	-0.76*	-Ø.22	Ø.Ø5	-Ø.Ø4	-Ø.53	Ø.3Ø
	Total stem length	Ø.41	-Ø.69	-Ø.Ø2	Ø.19	Ø.22	-Ø.41	Ø.6Ø
	Height	-Ø.58	-Ø.85"	-Ø.31	0.81*	Ø.Ø1	-Ø.55	Ø.7Ø
	Estimated biomass	-Ø.13	-0.90*	-Ø.34	Ø.36	Ø.12	-Ø.49	Ø.53
Cassia siamea	Leaf No	Ø.Ø2	Ø.66	Ø.41	-Ø.41	Ø.19	Ø.ØØ1	-Ø.57
	Total stem length	Ø.3Ø	0.73*	Ø.32	-Ø.2	Ø.58	Ø.34	-Ø.18
	Height	-Ø.45	0.75*	-Ø.56	-Ø.Ø1	Ø.25	-Ø.43	-Ø.Ø3
	Cstimated biomass	Ø.Ø9	Ø.47	Ø.Ø3	-Ø.4Ø	Ø.2Ø	-0.09	-Ø.25

* Significant at P = Ø.Ø5

total N. There was no correlation between plant height and total biomass production (r = 0.03).

With *Leucaena*, the results were in almost total contrast. There were negative correlations between plant height, total stem length or leaf number and total N with the following coeficients of correlation : -0.85 (significant at P = 0.05), -0.69 and -0.76 respectively. Also, correlations between exchangeable Mg and plant height, total stem length or leaf number (r = -0.55, -0.41 and -0.53 respectively) were negative but not significantly. There was however a significant correlation between plant height and exchangeable K (r = 0.81). Plant height was also significantly correlated with total biomass production (r = 0.87) and leaf number (r = 0.63). Plant height seemed to be governed by the soil K/N ratio. The relationship was best described by a quadratic equation, and 98.9% of the variation in *Leucaena* plant height could be decribed by the equation $Y = 4.88 + 117.37X - 14.94X^2$, where Y = plant height in cm and X = K/N ratio in the soil (Fig.1), with K expressed in meq/100 g soil and N in percent.

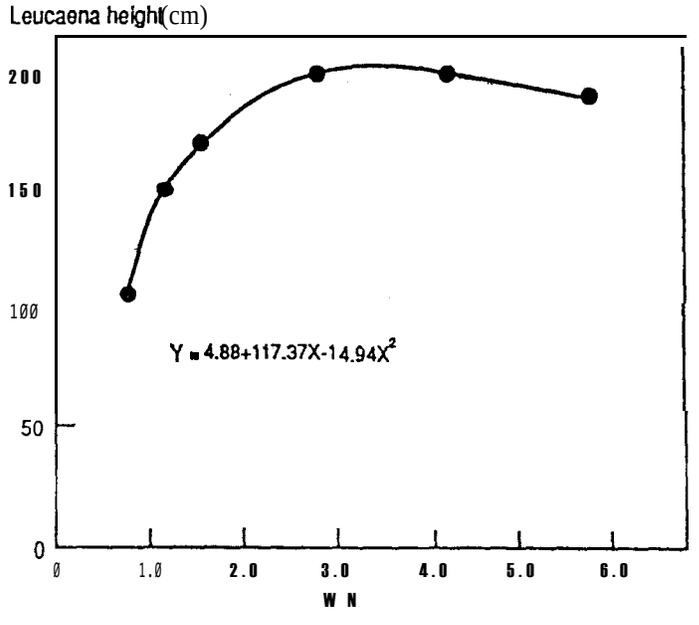
Figure 1: Relationship between *Leucaena* height and K/N ratio of the soil

Discussion

In spite of the deep rooting habit of *Cassia* and *Leucaena*, it was interesting to note some significant correlations with topsoil properties. The results highlight the very different response to N of the two species. Growth of the non-nodulated *Cassia* was significantly correlated with topsoil N and clearly limited by this element, so that plant biomass production was very low compared with that of Leucaena. The results further suggest that *Cassia* has a high requirement for P and exchangeable bases. This would explain the negative correlation with K because of the antagonism between K and Mg. Our results concour with observations by foresters that *Cassia siamea grows* well only in fertile soils, a fact which limits its use in re-aforestation programs (Anonymous, 1981).

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In contrast, all parameters 'of *Leucaena* growth showed negative correlations with soil N, an indication that this woody species relied for its N supply on biological N_2 fixation, a process inhibited by increasing levels of soil N. Apparently the N supplied in the experimental soils enabled *Leucaena* the plant to make rapid and prolific growth compared with *Cassia.*

Leucaena growth was also shown to be influenced by available K in the soil (r = 0.81 and 0.53 for plant height and total biomass respectively). These relatively high correlations were likely due to the rather high soil Mg levels. There was a negative correlation though non-significant between plant height and exchangeable Mg (r = -0.55). As K controls meristem growth, fast growing trees may be expected to have high K requirements. This has been demontrated for *Leucaena* which can respond to up to 800 ppm K and also appears to be rather inefficient at extracting the element from the soil (Schmidt and Saefuddin 1987). The K requirement of a plant is increased by an abundant N supply (Mengel and Kirby 1987). Thus the K requirements of N_2-fixing trees are likely more pronounced than for non-N_2-fixing trees. Indeed numerous studies have demonstrated that K is required in greater quantities than any other soil mineral for total N accumulation in legumes. It has also been establish that K-stimulated increases in legume dry matter production are accompanied by increases in N_2 fiition when no fertilizer N is used. This is believed to be caused by K-induced increases in photosynthesis, the transport of photosynthates to the root nodules and increased root growth (Duke and Collins, 1985). Fifty six percent of the variation in height of a related N_2-fixing woody species *Albizia falcataria* over 28 different sites has been reported to be determined by soil K levels (Delmacio 1987). Soil K levels of unfertilized soils were positively correlated with nodule productivity of *the* N_2-fixing *Inga jinicuil in* Mexico (Van Kessel and Roskoski, 1981). Relatively low levels of K fertilization stimulated N_2 fixation by this tree (Van Kessel and Roskoski, 1983). The critical K level for maximum growth and N_2 fixation of leguminous trees appears to be much higher than that required by cereals and needs to be further investigated.

In this experiment, low soil N levels promote N_2 fixation and thus plant growth. However, this condition increases plant K demand, and plant growth in turn becomes limited by the soil supplied K. There appears to be an optimum K/N ratio (Fig.1).

It can be concluded that while *Cassia* is dependent on general soil fertility, particularly N availability for adequate growth, nodulated *Leucaena* can grow well on poor soils, if supplied with enough K and possibly P and Zn, which were adequately supplied in this experiment. The relative growth of the two species at any one place will depend on site factors. *Cassia* is likely to compete with interplanted food crops for N, but *Leucaena* may not compete for K with cereals because legumes are usually less efficient than cereals at extracting K from soils.

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MESURE **DE LA FIXATION** D'AZOTE

Evaluation of biological nitrogen fixation in plants

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Abstract

Measuring the contribution of atmospheric nitrogen (N_2) to the growth of plants is an important step in understanding factors that influence the process, and for increasing N_2 fixation in various systems. Although the ^{15}N techniques generally appear to be superior to the other methods of measuring N_2 fixed, the facilities are not available in many laboratories, and like all the others, it has its weaknesses as well ; under some situations, or depending on the level of accuracy required. some of the non-isotopic methods, many of which are less expensive, may be equally or more suitable. The situations under which methods often regarded as inferior give *near accurate results* have seldom been adequately highlighted. In this paper therefore, the most frequently used methods, total nitrogen difference (TND), acetylene reduction, xylem exudate analysis and the ^{15}N techniques have been examined with this in mind. It is concluded that as simple and inexpensive as it is, the TND approach can under many circumstances, give satisfactory estimates of N_2 fixed. The acetylene reduction assay, on the other hand, has several drawbacks and cannot be used to obtain quantitative measures of N_2 fixed. However. it should at least be very useful in the initial screening for wide differences in N_2 fixation among genotypes and for distinguishing between absolute non-fixers and potential N_2 fixers.

Introduction

Increases in the contribution of nitrogen (N_2) fixation to crop and tree growth, and to high productivity of many agricultural soils are attainable. It is however necessary to know the current levels of N_2 fixed, so that any improvements and the conditions necessary to achieve them can be ascertained. Several methods already exist, and have been used to measure N_2 fixed in many crops and ecosystems. It is certain from all the reviews on these methods that each has some disadvantages (Knowles, 1981 ; Chalk, 1985 ; Danso, 1985 ; Vose and Victoria, 1986). Judging from the diversity of N_2 fixing crops and cropping systems, the prospects of an ideal method for all situations are slim. We are therefore compelled to make the best use of existing methodologies, insofar as we are aware of their limitations, and to use the most appropriate for each particular situation. This requires a thorough understanding of the strengths and weaknesses of each methodology. Methods have unfortunately been on many occasions adjudged as the most or least appropriate, without due regard for the situation. In this paper therefore, the greatest strengths and weaknesses of the most commonly used methods for measuring N_2 fixation will be examined, and as much as possible, the situation(s) under which each is best suited will be assessed.

Total Nitrogen Difference (TND) Method

This is one of the oldest and simplest methods, and has provided us with many estimates of N_2 fixed, upon which several useful management practices have been devised. In its simplest form, the TND method is based on the difference between the total N contents of plants or ecosystem in which N_2 is fixed and those that do not derive N from N_2 fixation. The method in essence is based on an assumption that both the N_2 fixing and non-fixing control plants absorb equal amounts of soil N for growth (Rennie and Rennie, 1983). This assumption may not however hold under most situations, as this would require that the different plants, in addition to being similar in root morphology and in several physiological attributes, should absorb their N from similar depths and horizon. This assumption is therefore often the greatest limitation of the TND method (Danso, 1985). In addition, where N_2 fixed in the whole ecosystem rather than only in plants is being measured, the inability to recover completely all the different forms of N using the usual Kjeldahl digestion procedures may result in erroneous estimates. For measuring N_2 fixation in an ecosystem using the TND approach, it is also difficult to quantify accurately all the many inputs and outputs of N into the ecosystem ; the errors in these TND estimates therefore tend to be high, and can be very serious when N_2 fixation is low (because the analytical errors in the determination of total soil N alone may sometimes be greater or close to the measured inputs from N_2 fixation). To overcome this, especially in N-rich soils, N_2 fixed is usually allowed to accumulate for a few to several years before measurement. In this way, the TND method tends not to be only tedious, but also requires long durations.

Despite the stated limitations, the TND method has often provided estimates that are not significantly different from those obtained using more sophisticated and expensive techniques (e.g., Ham, 1978 ; Phillips et *al.,* 1983). These similarities therefore justify a close examination of the strengths of the TND method, and when it is most suitable. As suggested by Danso (1988), and shown in the study of Paterson and LaRue (1983)

and Rennie (1984). the TND method reliably estimates of N_2 fixed in plants grown in soils or systems in which the initial N content is low. Under such circumstances, almost all the N would naturally be expected to be derived from N_2 fixation, with soil N contributing only negligible amounts. Several such situations exist, e.g., in areas consisting of sand dunes, systems undergoing primary colonization, and in many semi-arid areas. It is therefore not surprising that the values of N_2 fixed in *Casuarina* stands on the sand dunes in Cape Verde (Dommergues, 1963) are still quoted universally, and Gauthier et *al.* (1985) in their N_2 fixation studies on a sandy soil in Senegal frequently found no significant differences between TND estimates of N_2 fixed and those obtained using the more expensive and more sophisticated (both mathematically and analytically) ^{15}N methodologies. The situation could, however, be different even where the soil N level is low, depending on how much N_2 is fixed; when this is low, the estimates made could like when soil N is high, be highly erroneous. In this case, the otherwise small errors in the estimates of soil N uptake could be high, relative to N_2 fixed, and this would account for many of the negative estimates of N_2 fixed that have been reported using TND method (e.g. Patterson and LaRue, 1983 ; Rennie, 1984). All the same, it may not be worth bothering much about the magnitude of such inaccurate estimates since in my opinion, the real fact is, that the negative data only indicate that even if any N_2 was fixed, it was so low that it could be regarded as negligible and not worth taking note of.

The chances of very serious errors in TND estimates of N_2 fixed are certainly high when inorganic N is high in the soil, unless the control non-N, fixing crop and the N_2-fixing crop absorb closely similar amounts of soil N. As reported by Rennie (1984), this ideal situation is attained when the fertilizer utilization efficiencies (FUE) of the fixing and control plants are equal, and this would have to be ascertained using ^{15}N fertilizers. However when the FUEs are unequal, fairly accurate estimates of N_2 fixed may sometimes be obtained. As shown by Hardarson et *al.* (1988), errors in N_2 fixation estimates become smaller and less important at higher N_2 fixation rates, and although this principle was demonstrated using only the ^{15}N isotope dilution technique, the rationale should hold for all the other methods. Thus, for some forage legumes, many of which have been reported to derive around 80 to 90% of their N from N_2 fixation (e.g., Henzell et al.. 1968 ; Hardarson et al, 1988), it is likely that any erroneous estimate of N_2 fixed as a result of the inaccurate assessment of soil N uptake could be minimal, in contrast to several grain legumes, such as many commonbean (*Phasseolus vulgaris*) *varieties* that are known to be poor N_2 fixers.

Acetylene Reduction Technique Assay (ARA)

This methodology, used originally for N_2 fixation studies in pure microbial cultures (Dilworth, 1966), and which was later developed for measuring N_2 fixation in greenhouse and field-grown crops (Hardy et *al.*, 1968), is a paradoxical case of a method which has, at one stage or another, been used in almost every laboratory for measuring N_2 fixation, and which a little over a decade ago, stimulated an unprecedented interest in N_2 fixation studies, but which today, as shown in many reviews, is almost at the point of becoming "the" unacceptable or obsolete way of measuring N_2 fixation (e.g. Witty and Minchin, 1988).

The early, fast and wide acceptance of the ARA technique for assessing N_2 fixation was due to its being a simple, rapid and highly sensitive method (Hardy et al., 1968), and a reasonably lower instrumentational cost. Also, since ethylene is stable, the final product could thus be stored (allowing large samples to be collected at a time) and analysed when most convenient.

Although the early and popular acceptance of the ARA technique, for measuring N_2 fixation was almost universal, many of its drawbacks soon became apparent, and various attempts were made to overcome or minimize their effects. For example, several reports called attention to the instantaneous nature'of ARA assays, and the doubtful validity in extrapolating these short-term assays to cover N_2 fixed over whole growing seasons (involving large diurnal, daily and sometimes seasonal variations in rates of N_2 fixation) (Rennie et al., 1978). Attempts to reduce this problem have- included making several sequential assays rather than a single or few measurements (e.g. Hardy et *al.*, 1968). Because nitrogenase activity declines significantly in detached nodules, roots with attached nodules more frequently replaced the use of detached nodules. Also, studies showed that the digging of roots (especially in field-grown plants) resulted in unavoidable nodule losses, and the subsequent preparation of root samples for ARA assays, such as washing of roots (e.g. Carter and Sheaffer, 1983) or the drying of nodules in the field or after sampliig, adversely affected the diffusion of gases, such as oxygen and acetylene into the nodules (Minchin et al., 1983 ; Weisz *et al.*, 1985 ; Durand *et al.*, 1987). Also, it was noticed that the levels of acetylene used sometimes inhibited the process being measured (Witty and Minchin, 1988), while nodulated root segments exhibited a lower ARA than intact plants (Wych and Rains, 1978). Efforts made to solve or minimize these problems included conducting nondestructive *in situ* assays on intact plants in the field or greenhouse rather than on decapitated roots (Wych and Rains, 1978 ; Pfeifer *et al.*, 1983), and the use of a flow-through gaz system (Minchin *et al.*, 1983 ; Fellows *et al.*, 1987) which allowed a low and non-toxic concentration of acetylene to be continuously passed over the N_2-fixing system. None of these modifications was still able to overcome the need for artificial enclosures for the plants, and it was also often necessary to correct for the endogenous production of ethylene from soils.

Furthermore, it became known early that the validity of the factor of 3 originally proposed for converting C_2H_4 produced into values of N_2 fixed (Hardy *et al.*, 1968) was not valid for all crops and systems (Rennie *et al.*, 1978 ; Hudd *et al.*, 1980). This discrepancy was largely attributed to all the electrons becoming available to nitrogenase in the ARA assay, whereas Hudd *et al.* (1980), for example, reported that only about half the electron supply was used to fix N_2 in field bean (*Vicia faba*), resulting in a true conversion ratio of 5.75 rather than 3. This value was also not universally acceptable, since experimentally determined ratios have been reported to differ widely among different N_2 fixing plants and systems, with some of the reported ratios ranging from 1.5 to 8.4 (Bergersen, 1970 ; Hardy *et* al., 1973 ; Rermie *et al.*, 1978), and perhaps greater. Rennie et *al.* (1978) therefore recommended the experimental determination of the ratios to use under each situation using $^{15}N_2$ assays for calibration of the ARA technique. However, $^{15}N_2$ gas is expensive, and this approach would require determining the correct ratio to use for each study, as for the same crop, the electron flux (which the ARA technique essentially measures, rather than direct N_2 fixation) may differ under different experimental conditions or assay periods (Witty and Minchin, 1988). It was therefore not surprising that conversion of ARA assays into kg N/ha of N_2 fixed became less popular,

and was more often expressed as ethylene produced (either in nM or μM) per plant or nodule, and per unit time. The shift allowed seemingly valid relative comparisons of treatment effects to be made, by avoiding the need to use controversial conversion ratios. This approach held much promise for comparing N_2 fixation abilities (where the actual amounts of N_2 fixed were not essential or critical) until later evidence suggested that insofar as electron flux could be influenced differently by different treatments, such relative comparisons were of questionable validity (Witty and Minchin, 1988).

For those still dedicated to using the ARA technique, it seems that it may soon become difficult for their results to be accepted, unless the method can somehow or the other be imporved beyond its present capabilities. These adherents may however argue that, despite all these stated problems, numerous ARA estimates of N_2 fixation, statistically similar or highly correlated to values obtained by other "acceptable" methods abound in the literature (e-g. Hardy, *et* al., 1968 ; Paterson and LaRue, 1983). These results lead us to question whether all the methods used in these cases gave wrong results or that the limitation of ARA have in reality been exaggerated. These are valid questions for all scientists interested in N_2 fixation studies. It would be necessary to closely examine how often ARA estimates have deviated significantly from those obtained by other methods ; this would be particularly importaut in studies to rank N_2 fixation capabilites, for which the ARA technique at one stage held much promise. If these deviations are found to be only occasional, a case may have been established for re-examining when the ARA technique could be used to obtain valid results, and what precautions are necessary. The method at least holds much promise for identifying potential N_2 fixing crops in novel communities, and for grouping plants into broad categories in terms of N_2 fixation ability. It is also currently the only method that has consistently been used to establish that a plant intended to be used as a reference crop (in the ^{15}N methodology, and incidentally often the method of choice now) is truly a non-N_2 fixer (e.g. Fried *et al.*, 1983).

Relative Ureide Production

The xylem sap of N_2 fixing plants contains N compounds originating not only from soil N uptake by roots, but in addition, contains N of nodule origin, through the process of atmospheric N_2 fixation. In some N_2-fixing tropical legumes, differences in the N compounds carried by the xylem sap have been found. This is influenced largely by whether the N is of soil or nodule-origin (Pate *et al.,* 1980 ; Herridge. 1982a) ; the soil derived N is exported largely as free NO,- and amide amino acids (e.g. glutamine and asparagine), while N_2 fixation results largely in the formation and export of ureides (e.g. allantoin and allautoic acid). The relative composition of ureides versus NO,- + amides in these cases therefore serves as a useful index of the sources of the plant's N. The composition of the xylem sap changes progressively from one dominated by ureides to one composed largely of NO,- and amino compounds as N_2 fixation declines (Herridge, 1982a).

The assay of ureides, contrary to what many expect, is not that sophisticated, and involves only a simple colorimetric assay. However, like most colorimetric assays, it needs to be calibrated, and both the ARA and ^{15}N techniques have been used for this purpose (Pate *et* al., 1980 ; Heniedge, 1982a).

The initial handicaps of the methodology included (i) obtaining a representative exudate, since the ureide content of the plant was not. uniform in all organs (Herridge,

1982a), (ii) obtaining a sizeable volume of xylem exudate, a serious problem in dry soil (Herridge, 1982a). Both of these problems have been solved by using a vacuum-extraction method which involves the whole plant (Herridge, 1984) rather than any single plant part, or by root-bleeding (Herridge, 1982b). The problem of instantaneous assays is also being reduced by increasing the frequency of sampling. There are however still very few quantitative estimates of N_2 fixed based on this methodology, and it is yet to be established conclusively that an estimate obtained at any particular time could be used to make a reliable comparison among different treatments or varieties and crops, especially if they differ in maturity times, or the time courses for N_2 fixation are vastly different. This method was initially believed to hold much promise for measuring N_2 fixation in trees, which by their often massive size did not lend themselves easily to many of the methods available. This would have allowed the use of branches for example, to estimate % N_2 fixed. However, only a very small proportion of trees studied have so far been shown to produce ureides (D. Herridge, pers. comm.). The recent data of Kesses *et al* (1988) cast some doubts on the general applicability of this method for trees. From their study, they concluded that the presence of ureides, *per se* was not a reliable indicator of N_2-fixing ability. Moreover, the relative abundance of ureides in most of the species tested was too low to use as a presumptive test for, or as a means of, estimating N_2 fixation. Thus for both N_2-fixing crops and trees, this method's utility is at best limited to only those that produce ureides, and indeed, only a very small fraction of all plants that significantly contribute N_2 fixed into the ecosystem.

Nitrogen-15 Techniques

Use of $^{15}N_2$ Gas

This is the most direct method for measuring N_2 fixation in any crop or system (Fried *et al.*, 1983). Enriching the whole atmosphere in a field or greenhouse with $^{15}N_2$ is however unrealistic. The use of this technique has therefore had to be limited to incubation chambers and for very short incubation periods. The cost of $^{15}N_2$ gas is also high (Hudd *et al.*, 1980) thus making its frequent use unattractive. This method is therefore more suitable in biochemical and physiological studies of N_2 fixation than in field or greenhouse measurements of N_2 fixation.

Nitrogen-15 enriched soil

Although there are a few variations in the use of soil enriched with ^{15}N to estimate N_2 fixed, the rationale behind all of them is the same, i.e. that for a given soil situation and for a given growth period, the accumulation of atmospheric N_2 by a N_2-fixing plant results in a lower $^{15}N/^{14}N$ ratio in its tissues than that in a reference non-N_2 fixing crop. This arises from the higher $^{15}N/^{14}N$ ratio of the soil-derived N becoming diluted by the non-^{15}N enriched symbiotically fixed N_2. Because both the $^{15}N/^{14}N$ ratio of the ^{15}N-enriched soil and the amounts absorbed are changing with time (Fried *et al.*, 1983), it is not possible to accurately assess the time-integrated ratio in which $^{15}N/^{14}N$ was absorbed by the fixing plant throughout growth through instantaneous $^{15}N/^{14}N$ assays on soil-extracted N. It is therefore assumed that the $^{15}N/^{14}N$ in a non-fixing crop which absorbs all its N from soil only, is a reflection of the ^{15}N isotopic composition of soil-derived N in the N_2-fixing crop, once it is established that it absorbed N from the same soil zone and in a similar pattern as the fixing crop (Fried *et al.*, 1983; Witty, 1983).

The general advantages shared by all the ^{15}N soil-labelled techniques include obtaining integrated estimates of N_2 fixed, field applicability and the usually higher precision obatined, compared to other methods (Hardarson *et al.*, 1984 ; Rennie, 1984 ; Danso, 1986 ; Danso *et al.*, 1988). The following are the main variations of the ^{15}N techniques :

a) Natural ^{15}N abundance - Although for many common purposes, the $^{15}N/^{14}N$ ratio in air is assumed to be constant (approximately 0.3663%), deviations from this value have been found in many soils (Rennie *et al.*, 1976 ; Amarger *et al.*, 1979). These deviations, although often small, are within the detection limits of analytically sophisticated mass spectrometers (Rennie *et al.*, 1976). However, because these ^{15}N difference, are very small, they are expressed in units (i.e. one δ unit is 0.0001). Variations in the $^{15}N/^{14}N$ ratios in soil have been attributed to the isotopic discrimination between ^{14}N and ^{15}N in some soil pedogenic processes, especially in denitrification, in which ^{14}N is preferentially utilized, resulting in a soil containing relatively more ^{15}N than atmospheric N_2. Consequently, a non-N_2 fixing crop which does not derive N from atmospheric N_2 fixation (of a lower $^{15}N/^{14}N$ ratio) accumulates N of a slightly higher $^{15}N/^{14}N$ ratio than that of a N_2-fixing crop grown on the same soil.

There is considerable disagreement on whether this method is most useful for obtaining qualitative estimates of N_2 fixed (Edmeades and Goh, 1979 ; Rennie, 1986) or whether it can be relied upon to give quantitative estimates as well (Bergersen, 1988). The controversy arises from the fact that the differences in $^{15}N/^{14}N$ ratios being measured are usually so small that some consider that $\delta^{15}N$ values obtained are significantly biased by such factors as isotopic discrimination, and by otherwise normal minute analytical errors in N ratios determinations (Edmeades and Goh, 1979), which relative to the smallness of the δvalues, would eventually translate into big errors in the values of N_2 fixed. To take care of isotope discrimination, the $\delta^{15}N$ in soil sampled by on non-fixing plant (dependent solely on soil N) is used to correct for the $\delta^{15}N$ in the fixing plant, while the $\delta^{15}N$ samples by a fixing plant completely dependent on N_2 (i.e. grown hydroponically in N-free nutrient medium) is used to correct for the $\delta^{15}N$ in the non-fixer or reference crop. The equation used is :

$$\%\, N_2 \text{ fixed} = \left(1 - \frac{\delta^{15}N \text{ non fixer} - \delta^{15}N \text{ fixer}}{\delta^{15}N \text{ non fixer} - \delta^{15}N \text{ fixer on N-free medium}} \right) \times 100$$

It is of interest that groups, largely in Australia, have estimated similar values of N_2 fixed using either the natural ^{15}N abundance technique or soils into which ^{15}N fertilizers were incorporated to give higher ^{15}N enrichments, less influenced by isotopic discrimination and perhaps analytical errors (Bergersen, 1988). The ^{15}N natural abundance approach certainly requires greater sophistication in equipment and analytical capabilities. The high cost in at least the initial outlay has contributed to this technique being outside the reach of many laboratories (Danso, 1988). For most laboratories in Africa, for example, the use of this technique is definitely out of the question, unless a collaborating laboratory in an advanced laboratory accepts the responsibility to analyse the samples, a "symbiosis" that is becoming more frequent, and one that needs to be exploited for mutual benefits. The ^{15}N natural abundance method is of immense use in screening for

potential N_2 fixing plants in complex natural communities, such as in those containing trees, some, because their nodules occur deep in the soil, or may be detached during uprooting, might falsely appear to be non-nodulating. However, the validity in using this approach to obtain quantitative estimates of N_2 fixed in perennial N_2 systems is questionable. With time, root and litter turnover under the N_2-fixing and non-fixing plants could give rise to significant differences in ^{15}N isotopic composition in the soil under the two rhizospheres that of the N_2 fixing crop would tend to be of a lower $^{15}N/^{14}N$ ratio than the reference crop. The magnitude of this error would among others be influenced by the level of N_2 fixation, the duration of growth, and the extent to which roots intermingle, as discussed by Danso *et al.* (1988) and Labandera *et al.* (1988). These effects have not received much attention, and merit being studies, as well as what effects the retranslocation of unharvested N of in the fixing and non-fixing crops (which may differ widely in ^{15}N enrichments) during regrowth, would have on N_2 fixation estimates in perennials (Danso *et al.*, 1988; Labandera *et al.*, 1988).

b) Addition of ^{15}N enriched fertilizers - The addition of ^{15}N enriched fertilizers to soil results in greater differences in the ^{15}N enrichment between soil-derived N and atmospheric N_2 than those between the ^{15}N enrichments encountered in natural soils and the air. Their are two slightly different approaches in using this method, frequently referred to as the isotope dilution and the A-value approaches (Danso, 1988), although this distinction is difficult to justify fully.

(i) The isotope ^{15}N dilution approach Like the natural abundance technique, both N_2-fixing and non-fixing plants are grown on the same soil, of similar N content and ^{15}N enrichment. Both crops are therefore sampling a similar $^{15}N/^{14}N$ ratio, and the following equations are used for the N_2 fixation estimations :

$$\% N_2 \text{ fixed} = 100 \left(1 - \frac{\% \ ^{15}N \text{ atom excess (fc)}}{\% \ ^{15}N \text{ atom excess (nfc)}} \right) \qquad \text{(i)}$$

$$\text{and total } N_2 \text{ fixed} = \frac{\% N_2 \text{ fixed}}{100} \times \text{total N (fc)} \qquad \text{(ii)}$$

where fc and nfc are the fixing and non-fixing reference crops, respectively.

The greatest difficulty reported with both this approach and the A-value approach is that of ensuring that the $^{15}N/^{14}N$ ratio in the reference crop is a true reflection of the integrated $^{15}N/^{14}N$ ratio absorbed by the fixing crop from soil (Chalk, 1985 ; Duque *et al.*, 1985 ; Jensen, 1986 ; Duc *et al.*, 1988). The difficulty arises largely because the $^{15}N/^{14}N$ ratio in a soil into which ^{15}N fertilizer is incorporated often declines sharply with time, causing significant changes in the temporal patterns of the ^{15}N enrichments absorbed by the different crops from soil. When these changes are not well-matched, this results in highly dessimilar assessments of the ^{15}N enrichments of soil-derived N (Fried *et al.*, 1983; Witty, 1983 ; Danso, 1985). Procedures to adopt to ascertain the selection of a satisfactory reference crop have been discussed (Fried *et al.*, 1983; Witty, 1983), although it has not always been easy to verify that these requirements were rigidly met. However, as discussed by Danso *et al.* (1986) and Hardarson *et al.* (1988), the need to rigidly

meet all these requirements has sometimes been exaggerated. Under situations where it is essential to ensure that a strictly suitable reference crop is selected, or where problems are anticipated unless this is done, practices that result in less rapid decline in $^{15}N/^{14}N$ ratios in soil (the major source of problems) have been attempted. One of these is by incorporating ^{15}N-enriched slow-release fertilizer formulations into soil (Witty, 1983 ; Witty and Ritz, 1984). The amendment of soil with organic matter labelled with ^{15}N (Fried *et al.*, 1983 ; Witty and Ritz, 1984), ensuring the slow release of added ^{15}N by the simultaneous addition of highly carbonaceous materials (Legg and Sloger, 1975 ; Wagner and Zapata, 1982), or nitrification inhibitors (Rennie, 1986 ; Rennie *et al.*, 1988) have all been used to give a less rapid decline of the $^{15}N/^{14}N$ ratio in soil. Frequent small additions of ^{15}N enriched inorganic fertilizers have also been found to result in reduced rate of ^{15}N decline with time (Boddey and Victoria, 1986), as is the use of residual ^{15}N from previous ^{15}N fertilizer applications (Kohl and Shearer, 1981 ; Poth *et al.*, 1986). Although these procedures that result in less drastic declines in $^{15}N/^{14}N$ ratios of soil are becoming increasingly popular, they are not without limitations (Chalk, 1985). The seriousness of the consequences or praticality of some of these procedures have unfortunately not been adequately examined or highlighted. For example, the wide-scale adoption of ^{15}N-labelled slow release formulations for N_2 fixation studies is at present not practical for many laboratories, as they are not yet available commercially, and the procedures used for laboratory manufacture may sometimes be dangerous (Witty and Ritz, 1984). Getting the small amounts required for a few experiments made by competent manufacturers from my experience is also unreasonably costly and hardly affordable by most laboratories, especially in the developing countries. It is however possible that in future, increased demand could stimulate their large-scale manufacture, and hopefully, decreased costs. It is, however, doubtful that these reduced prices will ever rival those of the conventional ^{15}N-labelled fertilizers. The use of ^{15}N-labelled organic matter has interestingly, frequently resulted in substantially higher estimates of % N_2 fixed for crops like soybean (e.g. Legg and Sloger, 1975), compared to the values in the neighbourhood of 50% often found using other methods (Rennie *et al.*, 1982). The higher N_2 fixation almost certainly arises from the artificially induced higher dependence of the crop on fixed N_2, once much of the available soil N has been used up in decomposing these highly carbonaceous materials often added in unrealistically high amounts. To lessen these undesirable effects, it may be necessary to add materials of lower C/N ratios (although these may not maintain as stable and high $^{15}N/^{14}N$ ratios) and of higher ^{15}N enrichment (so that lower amounts can be added). There may in all cases, however, be a need for a prolonged waiting period (the length of which would vary depending on the local environment). This is to ensure a reasonable equilibration of the added ^{15}N with soil N, and is bound to cause delays in setting up N_2 fixation experiments, unless the incorporation was done long ahead of when the experiment should be set up. Another potential difficulty, that of ensuring that the ^{15}N from the organic matter is uniformly distributed in the soil has not been addresed at all. This may not be as easy as when inorganic forms are added, and needs to be examined relative to the other methods. Also, organic matter addition may alter the quality and quantity of the microbial flora and fauna, aspects that may influence N_2 fixed, and the effects of which have also not been adequately studied. Any of these effects could however be lessened when the $^{15}N/^{14}N$ ratio of the added materiel is close to that in the soil. Fried *et al.* (1983) have in addition pointed out that with this approach, supplemental N addition is not feasible. Thus, where soil N levels are too low, or become depleted during growth

(especially for long-duration crops and perennials), the reference crop could be physiologically affected in growth (affecting the accuracy of the N_2 fixation estimates), or may even die. The search for a perfect solution is therefore far from complete, although for the moment, the ^{15}N techniques continue to be quite superior to most other available ones (Phillips and Bennett, 1978 ; Ruschel *et al.*, 1982 ; Hardarson *et al.*, 1984).

(ii) The A-value approach The A-value concept (Fried and Dean, 1952) was originally elucidated for assessing the amounts of nutrient elements in soil available for plant growth. It assumes that the soils capacity (referred to as A_{soil}) to furnish a particular nutrient element remains unchanged even when fertilized with different rates of that element. Fried and Broeshart (1975) further extended this concept for the estimation of N_2 fixed in plants. This has made it possible to apply higher doses of N to the reference crop (to ensure good growth) than the fixing crop (in order not to inhibit N_2 fixation). The various controversies with this approach have ranged from the lack of a constant A-value in soil fertilized with different amounts of ^{15}N labelled fertilizer (Broadbent, 1970) to the estimates of % N_2 fixed by the A-value method (in contrast to those of the isotope dilution approach) being yield-dependent (Chalk, 1985). The latter notion has however been shown by Danso (1986) to be incorrect, and Aleksic *et al.* (1968) have shown that the A-values of soils into which different amounts of ^{15}N-labelled fertilizer were added remained constant. The likelihood is however strong that the more divergent the rates of N used, the greater the chances that pedogenic processes and other effects would affect soil and the added N differently, and therefore in amounts not directly proportional to the differences in rates of N applied. This would then cause errors in the estimates of A values and thus N_2 fixed. For these reasons, it is always best to add minimum N rates that would only ensure optimum growth rather than maximum growth of the reference crop.

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Influence du déficit hydrique sur la fixation symbiotique de l'azote atmosphérique chez le soja

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Résumé

L'effet du déficit hydrique sur l'activité nitrogénase est étudié chez le soja par application de mannitol. Ce travail a été réalisé en serre en culture hydroponique. sur deux variétés de soja présentant, dans les essais agronomiques, des sensibilités différentes à la sécheresse : Kingsoy résistante et Hodgson sensible. Sur ces plantes de soja en symbiose avec *une souche de Bradyrhizobium japonicum*, sont mesures l'activité fixatrice d'azote et l'effet de pressions partielles d'oxygène croissantes, sous différentes intensités de contrainte osmotique : - 0,2, -0,4, -0.6 et -0.8 MPa. De fortes pressions partielles d'oxygène permettent de lever sensiblement, mais pas totalement, l'inhibition de la fixation due à la contrainte hydrique. L'influence de la contrainte osmotique sur la nitrogénase varie suivant la teneur en nitrate du milieu de culture. Ces résultats suggèrent que le déficit hydrique limite l'alimentation des bactéroïdes en oxygène mais également affecte la quantité de pouvoir réducteur et la quantité d'enzyme active présente dans les nodosités pour la fixation de l'azote. La fixation de l'azote est affect& différemment chez les deux cultivars : elle est moins déprimée chez Hodgson que chez Kinsoy, apparemment parce que Hodgson possède une réserve énergétique plus importante dans ses nodosités.

Introduction

Dans les conditions de la culture du soja en plein champ chez les plantes inoculées avec *Bradyrhizobium juponicum,* la fixation symbiotique de l'azote atmosphérique fléchit rapidement, pouvant même s'annuler lorsque le déficit hydrique est sévére. (Obaton et *al.,* 1982 ; Bouniols et *al.,* 1982 ; Wéry *et al.,* 1986).

La baisse de l'activité nitrogénase par le déficit hydrique est observée par la méthode de l'activité réductrice de l'acétylène (ARA) (Pankhurst et Sprent, 1975 ; Sheehy et *al.,* 1983 ; Benett et Albrecht, 1984) et par la méthode ^{15}N (Sinclair et Goudriaan, 1981). Alors que certains auteurs considèrent que la diminution de l'activité nitrogénase est due à l'inhibition de la photosynthbe (Huang *et al.,* 1973 ; Sprent, 1976), Finn et Brun (1982) concluent que la nitrogénase est plus sensible au déficit hydrique qu'à la photosynthèse. Cette interprétation est soutenue par Bennet et Albrecht (1984) qui ont montré que la fixation de l'azote atmosphérique est étroitement corrélée au-potentiel hydrique des nodules, ce dernier étant plus sensible au déficit hydrique que le potentiel hydrique foliaire et la résistance stomatique.

Selon Weisz *et al.* (1985), l'inhibition de l'activité nitrogénase par le déficit hydrique est détectable au champ trois jours seulement après l'installation de la contrainte hydrique et avant qu'il y ait une différence pour la surface foliaire, Ie poids des plantes ou des nodules et le nombre de nodosités entre plantes stressées et plantes témoins.

Pankhurst et Sprent (1975) notent qu'un accroissement de la pression partielle d'oxygène (pO_2) lors d'un déficit hydrique, augmente la fixation de l'azote mesurée par l'activité réductrice d'acétylène (ARA) et la respiration des nodules. Ce résultat obtenu sur nodules excisés nous a conduit à réaliser des expériences similaires sur plantes entières pour essayer de mieux comprendre ce phénomène, tout en perturbant au minimum la physiologie des plantes.

Matériels et méthodes

Matériel biologique et culture des plantes

Deux variétés de soja (*Glycine* max L. Merrill) sont cultivées : Hodgson du groupe 1 demi-précoce, sensible à la sécheresse, et Kingsoy du groupe II demi-tardif, retenue pour sa bonne tolérance à la sécheresse (Bouniols, comm. pers.).

Les graines de soja, après désinfection à lhypochlorite de calcium (30 g l^{-1} pendant 30 mn), sont mises à germer à l'obscurité durant 3 jours à 28°C sur vermiculite stérile. Les plantules de soja sont placées sur des plaques de polystyrène préalablement percées pour laisser croître la partie racinaire. L'ensemble est ensuite placé sur des bacs de 40 litres (60 cm x 40 cm) contenant la solution nutritive aérée dont le pH est maintenu à 6,5. Les plantules de soja sont inoculées dès la mise en place avec la souche de *Bradyrhizobium japonicum* G 49 (SB 16, IARI, Inde). Les cultures sont conduites en serre éclairée pendant 16 h (éclairement additionnel pendant l'hiver, 100 W m^{-2} par des lampes Philipps), à une température variant entre 15 et 35°C. Pendant la phase obscure de 8 heures. la température varie entre 15 et 20°C. L'humidité relative, augmentée grâce à l'action d'un brumisateur, est supérieure à 70% et varie suivant les saisons.

La solution nutritive contient : KH_2PO_4 : 025 µM ; $CaCl_2$: 33 µM ; K_2SO_4 : 1,25 µM ; $MgSO_4$, $7H_20$: 2.05 µM ; H_3BO_3 : 4µM ; $MnSO_4$: 6,6 µM ; $ZnSO_4$,$7H_2O$: 1,55 µM ; $CuSO4,5H_2O$: 1,56 µM ; Na_2MoO_4, $2H_2O$: 0,12 µM et du fer sous forme d'EDTA (0,015 g l^{-1} de produit). Pendant les 2 premières semaines, l'azote de complément est apporte sous forme d'urée (1 mM). Lorsque la nodnlation est installée, les plantes ne reçoivent aucune autre source d'azote que la fixation sauf dans les expériences avec nitrate où l'apport d'urée est remplace par des doses variables de NO,-. Le milieu de culture est renouvelé toutes les semaines, et régulièrement réajusté avec de l'eau distillée.

Intensité et mode d'application du déficit hydrique

La contrainte hydrique est obtenue par abaissement du potentiel osmotique de la solution par apport de mannitol pendant 24 h. Le mannitol est peu ou pas absorbe par les cellules (Heath, 1977) et ne présente pas de phytotoxicité. Pour éviter la multiplication des microorganismes dans la solution nutritive, un antibiotique, la tétracycline, est apporte (10 mg l^{-1}) avec le mannitol. Quatre traitements sont réalisés : -0.2 MPa (0,074 M) : déficit faible ; -0,4 MPa (0,153 M) : déficit moyen ; -0,6 MPa (0,233 M) : déficit fort mais non destructif ; -0,8 MPa (0,312 4 M) : déficit très fort et plus ou moins destructif suivant les conditions climatiques.

Mesures des échanges gazeux

Les différentes mesures effectuées dans ce travail, sont faites à partir du quarantième jour de culture ; les plantes sont alors en pleine floraison, stade le plus sensible à la sécheresse (Bouniols *et al.* 1982). Les mesures de l'activité nitrogénase sont réalisées par la technique de réduction de l'acétylène en éthylène (Hardy *et al.*, 1968) appliquée à des racines nodulées en place sur la plante. L'incubation est faite en flacon sérum de 500 ou 1000 ml, ce qui permet de réaliser des mesures non destructives de l'activité nitrogénase. L'étanchéité au niveau de la tige est assurée par un joint de coton imbibe de Rhodorsil RTV 1502 (Rhône-Poulenc). Les incubations en présence de C_2H_2 sont réalisées soit en système ferme, soit en continu avec balayage du milieu racinaire par un mélange gazeux contenant 10% C_2H_2.

Mesures en système fermé - Le protocole appliqué est celui décrit par Kalia et Drevon (1985). La partie racinaire de la plante est hermétiquement enfermée dans le flacon sérum, avec 10% (v/v) de solution nutrive pour maintenir la turgescence et l'activid des organes. La nitrogénase est saturée par une concentration en acétylène de 10%. La quantité d'éthylène accumulée dans ce compartiment mcinaire est évaluée par des prélévements d'échantillons de l'atmosphère interne qui sont ensuite analysés à l'aide d'un chromatographe Girdel 30, muni d'un détecteur à ionisation de flamme (colonne de Porapak T: 1,5 m de long et 3,2 mm de diamètre, température du four : 80°C, gaz vecteur azote à 30 ml mn-'). Les mesures d'activité nitrogénase (ARA) obtenues dans ces conditions sont plus élevées que celles obtenues sur les racines séparées car l'excision entraîne une baisse de 50 à 80% de l'activité enzymatique (Ralston et Imsande, 1982). Toutefois, en système fermé, la réduction d'acétylène ne reste constante que pendant environ 60 mn (Kalia et Drevon, 1985). Les mesures doivent donc être effectuées rapidement

Mesures en système continu - Le principe de mesure de l'activité de la nitrogénase est le même qu'en système ferme, mais cette technique a l'avantage de permettre le contrôle du mélange gazeux qui balaie en permanence l'atmosphère du système racinaire. Ainsi, grâce à des débitmètres Tylan, le flacon est balayé par un melange gazeux à débit constant et contenant de l'acétylène (10%), de l'oxygène et de l'azote avec la possibilité de moduler les concentrations respectives de ces deux gaz. Le système comprend le flacon sérum qui porte la plante, les débitmètres connectés aux différentes bouteilles de gaz et un tuyau de sortie du milieu d'incubation qui est connecté au chromatographe et au catharomètre à conductivité thermique. Le catharomètre permet de doser les quantités d'oxygène et d'azote contenues dans les échantillons. Les deux appareils sont munis chacun d'une vanne d'injection automatique qui fonctionne alternativement à la fréquence programmée. Un autre débitmètre à bulles de savon, relié à la sortie du catharomètre, permet de vérifier le débit du mélange gazeux. Il est ainsi possible de suivre l'évolution de l'activité nitrogénase en fonction de la pression d'oxygène (Drevon et *al.*, 1988). Le dispositif permet de réaliser quatre incubations simultanément Des prélèvements à la seringue d'échantillons de l'atmosphère racinaire permettent de mesurer le dégagement de CO, par des dosages avec un IRGA ADC, 225 MK3.

Résultats

Influence de la contrainte osmotique sur l'ARA à 21% 0,

Les données présentées à la Fig.1A montrent que chez Kingsoy, l'ARA diminue dès les premières heures qui suivent l'application du mannitol dans le compartiment racinaire. Cette inhibition de l'activité nitrogénase est d'autant plus forte que l'intensité du déficit osmotique est élevée. Après 24 h en présence de mannitol, l'ARA exprimée en pourcent de celle du témoin n'ayant pas reçu de mannitol, n'est plus que de 72, 63, 52 et 29% respectivement pour une pression osmotique de -0,2, -0,4, -0,6 et -0.8 MPa. Après avoir levé la contrainte osmotique, I'ARA continue à décliner pendant au moins 4 jours chez les plantes ayant été soumises à la contrainte osmotique de -0,8 MPa. Bar contre, chez les plantes soumises à -0,4 et -0,6 MPa, ce déclin d'activité cesse apparemment après 2 jours, et chez celles soumises à une contrainte de -0,2 MPa la stabilisation de SARA s'effectue après environ 24 h. Chez ces demieres, l'ARA augmente même significativement durant le troisième jour.

Chez la variété Hodgson (Fig.1B), l'ARA est apparemment moins affectée que chez la variété Kingsoy. En effet, après 24 h de contrainte osmotique, I'ARA exprimée en pourcent du témoin n'est que de 82 à 62% pour les trois traitements. Il n'y aurait pas de différence significative de l'inhibition de l'activité nitrogénase chez cette variété pour des concentrations de mannitol correspondant à -0,4 et -O,6 MPa. Apres retrait du mannitol, la baisse de la fixation se prolonge encore pendant environ 3 jours pour les plantes ayant subi les traitements à -0,4 et -0,6 MPa alors que la stabilisation de I'ARA est plus rapide à -0,2 MPa comme pour la variété Kingsoy. Ainsi, 4 jours après la fii de la contrainte osmotique, les plantes précédemment soumises à -0,2 MPa retrouvent 84 % de leur activité fixatrice d'azote, contre 52 et 40 pour -0,4 et -0,6 MPa respectivement.

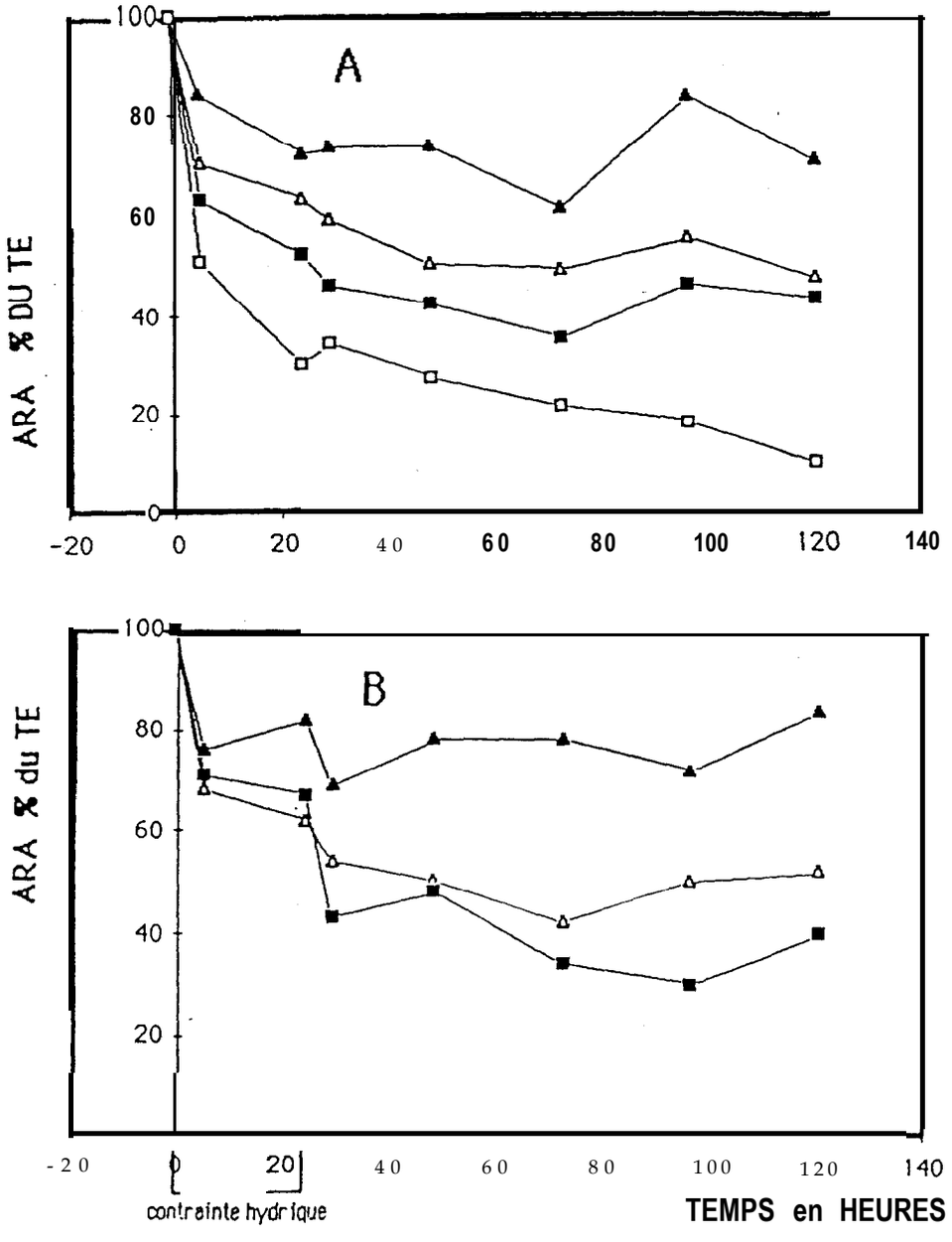


Figure 1 : Influence du déficit hydrique sur la fixation symbiotique de l'azote mesurée par la méthode ARA, chez les variétés de Soja KINGSOY (A) et HODGSON (B). Les résultats sont exprimés en pourcentage du témoin. Chaque point est la moyenne de 5 répétitions. Le traitement au mannitol dure 24 heures. Les mesures sont faites 1 heure avant le traitement, 5 h et 24 h après. Les mesures suivantes sont effectuées au retour à un régime hydrique non limitant. 2 bars A , 4 bars ▲ , 6 bars ● 8 bars □

Influence du déficit hydrique sur la réponse de l'ARA à la pO_2

Les données de la Fig.2 montrent qu'après environ 800 mn (environ 13 heures) d'application d'une contrainte osmotique à l'aide de mannitol, l'ARA devient à peu près stable. Cette stabilité relative permet donc d'étudier l'influence de pO_2 croissantes appliquées progressivement pendant plusieurs heures.

Chez Kingsoy, sur les plantes témoins n'ayant pas subi de contrainte osmotique, l'ARA augmente jusqu'à 30% d'oxygène puis baisse pour des pO_2 supérieures (Fig.3A). En présence d'une contrainte osmotique de -0,2 et -0.4 MPa, l'ARA maximale (ARA

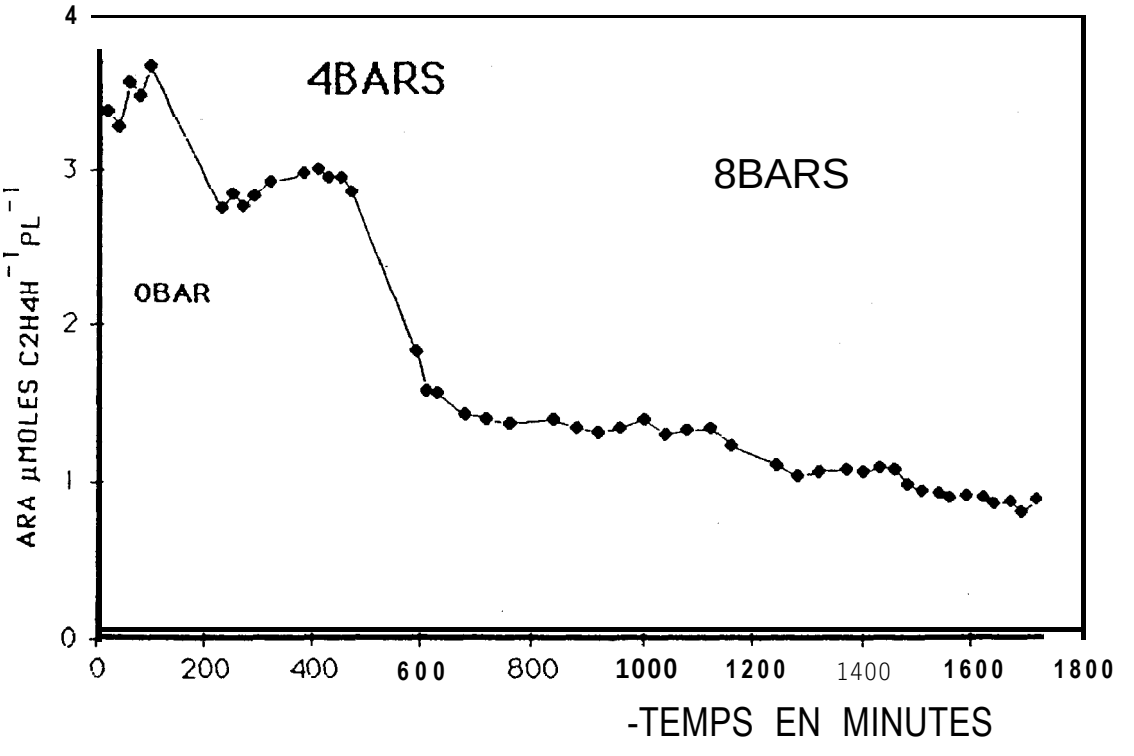


Figure 2 : Cinétique de l'ARA chez le soja variété HODGSON, en fonction de l'intensité de la contrainte osmotique (appliquée à l'aide de mannitol) : 0,4 , et 8 bars. Les mesures sont faites eu continu à 20% d'oxygène. Chaque point est la moyenue de 5 à 8 répétitions.

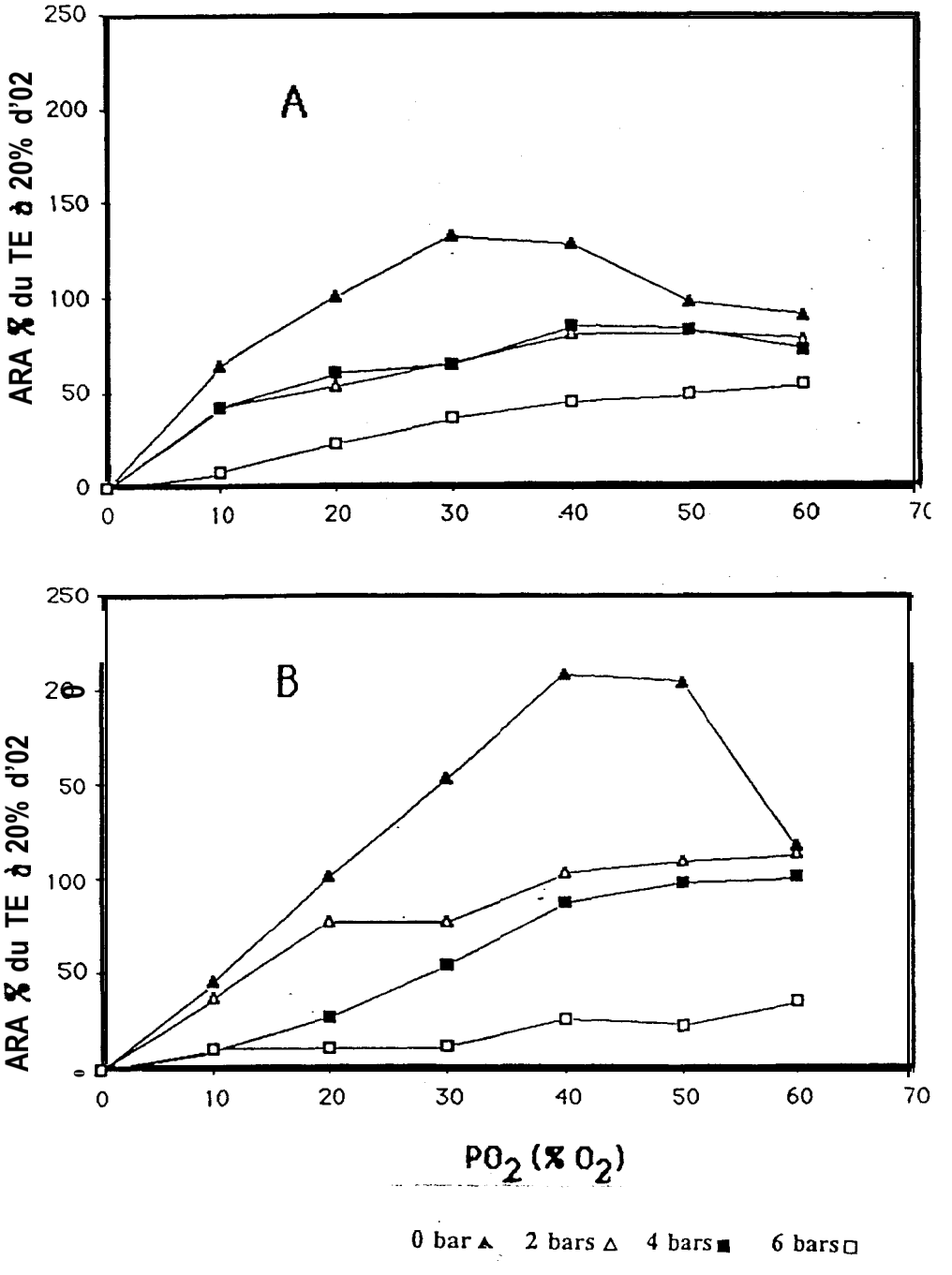


Figure 3 : Influence de différentes intensités de contrainte osmotique sur la réponse de l'ARA à la pO_2 (en continu) chez deux variétés de soja KINGSOY (A) et HODGSON (B). Les résultats sont exprimés en pourcentage du témoin à 20% d'oxygène. Chaque point est la moyenne de 8 à 10 mesures sur une plante. L'expérience est répétée deux fois.

correspondant à la pO_2 optimale) est atteinte pour une pO_2 plus élevée, voisine de 40% et supérieure à 60% pour -0,6 MPa. La pO_2 optimale est donc damant plus élevée que la contrainte hydrique est plus sévère. Toutefois l'augmentation de la pO_2 ne permet pas d'obtenir une ARA maximale comparable à celle du témoin.

Chez la variété Hodgson, en l'absence de contrainte osmotique, la pO_2 optimale observée dans la Fig.3B est plus élevée que chez Kingsoy. En effet l'ARA est multipliée par 2 lorsque la pO_2 augmente de 20 à 40% d'oxygène ; elle baisse au delà de SO% d'oxygène. De même que chez Kingsoy, l'ARA maximale en présence d'une contrainte osmotique est observée pour des pO_2 d'autant plus élevées que la concentration en mannitol est plus forte. L'ARA à 60% 0, est alors d'environ 80 à 90% de celle du témoin n'ayant pas subi une contrainte osmotique pour les traitements -0,2 et -0,4 MPa ; mais pour un déficit hydrique sévère (-0,6 MPa) l'effet de la pO_2 est beaucoup plus faible (environ 25% de l'ARA du témoin pour une pO_2 de 60% 0_2).

Influence du déficit hydrique sur la respiration nodulaire

En système fermé chez Kingsoy, la respiration du système racinaire nodule est influencée par l'intensité de la contrainte hydrique (Fig. 4A). Pendant le déficit hydrique, la respiration diminue d'autant plus que la contrainte osmotique est plus intense. Au retour à un régime hydrique non limitant, la respiration reprend légèrement, surtout pour la faible intensité, mais ne retrouve pas le niveau du témoin. Hodgson se comporte de la même façon que Kingsoy, mais est légèrement plus affecte que celui-ci, surtout pour le traitement -0,6 MPa (Fig.4B). Après 24 h. de contrainte osmotique, la respiration chez Kingsoy est de 95, 70 et 67% du témoin contre 88, 62 et 55% chez Hodgson respectivement pour -0,2, -0,4 et -0,6 MPa.

Dans les expériences de réponse à l'oxygène en continu, aussi bien chez Kingsoy que chez Hodgson, le dégagement de CO, augmente avec la pO_2 au moins jusqu'à 60% 0_2. Contrairement à l'ARA, il n'y a pas Ctablissement d'un plateau et la respiration semble être limitée par la pO_2 quelle que soit la variété ou l'intensité du déficit hydrique (Fig.5). Ces mésures de l'émission de CO, réalisées simultanément à celles d'ARA permettent d'établir une relation linéaire entre ces deux activités (Witty *et al.*, 1983). La pente de la droite de régression ainsi obtenue correspond à la quantité de CO, émise par molécule de C_2H_2 réduite. L'ordonnée à l'origine représente la respiration des tissus racinaire et nodulaire qui n'est pas associée à la fixation. Les données présentées dans le Tableau 1 ne révèlent aucune différence significative entre les traitements pour ces deux paramètres, excepté pour la contrainte de -0,2 MPa. Les résultats obtenus chez Hogdson (données non présentées) sont similaires. Ainsi la contrainte osmotique de -0,2 MPa augmenterait le coût énergétique de la fixation puisque le rapport vitesse d'émission de CO_2/ARA est voisin de 10 µmol CO_2.µmol^{-1} C_2H_4 pour -0,2 MPa contre environ 5 chez le témoin. Cette observation mériterait d'être vérifiée dans des travaux ultérieurs.

Influence de l'azote minéral sur l'inhibition par le déficit hydrique

L'action du nitrate est dépressive sur la nitrogénase : en effet la fixation est faible chez les plantes avec nitrate et sans déficit hydrique comparées à celles soumises au même traitement maïs sans nitrate (Tableau 2). Après 24 h en présence de mannitol, les plantes sans nitrate maintiennent une activité de plus 60% du témoin contre 12% chez les plantes

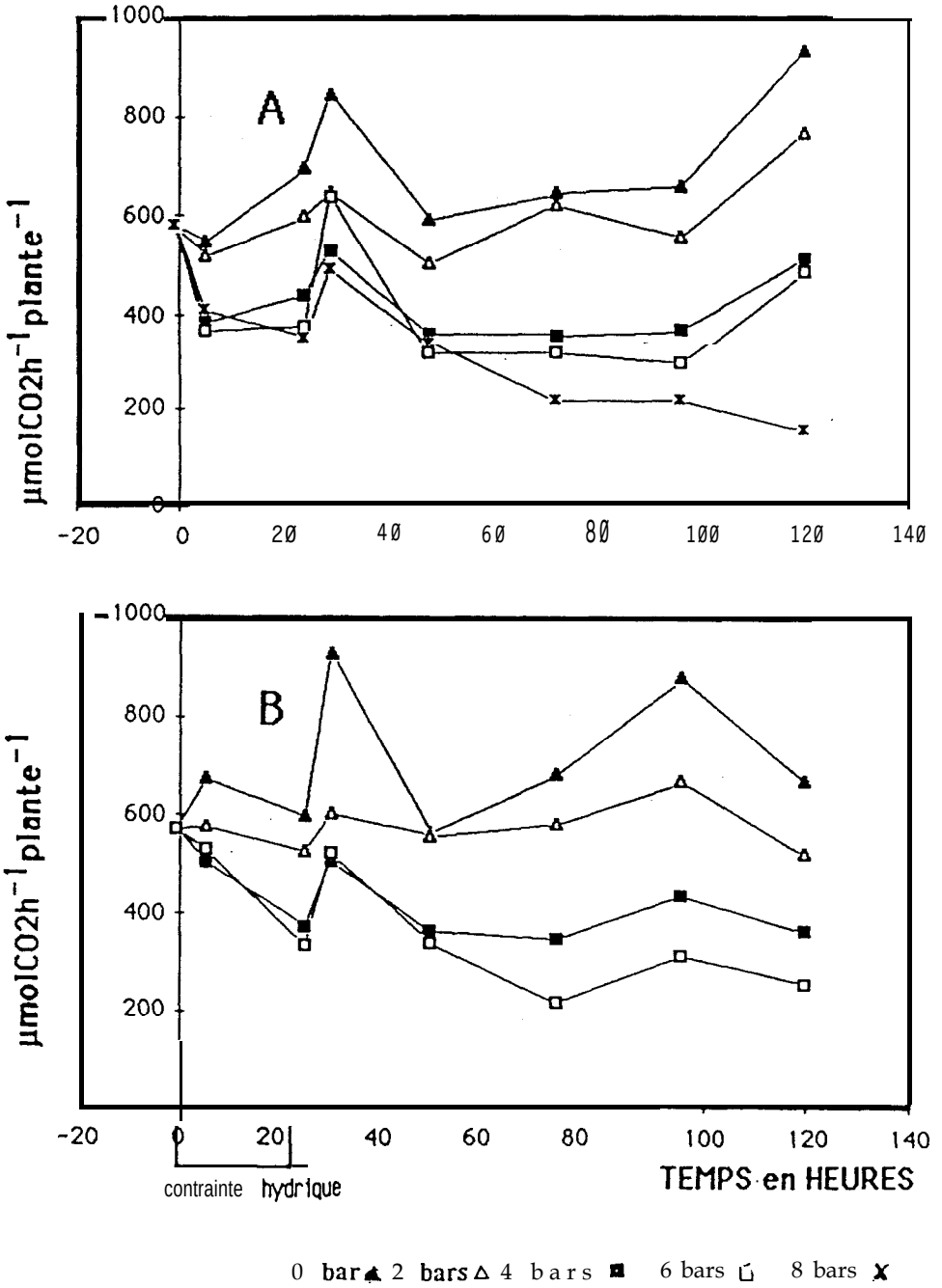


Figure 4 : Influence de la contrainte osmotique sur la respiration des racines nodulées. Les mesures sont effectuées simultanément aux mesures d'ARA en système fermé, chez les variétés de soja KINGSOY (A) et HODGSON (B). Le traitement au mannitol dure 24 heures. Les mesures sont faites 1 heure avant le traitement, 5h et 24h après. Les mesures suivantes sont effectuées après retour à un régime hydrique non limitant.

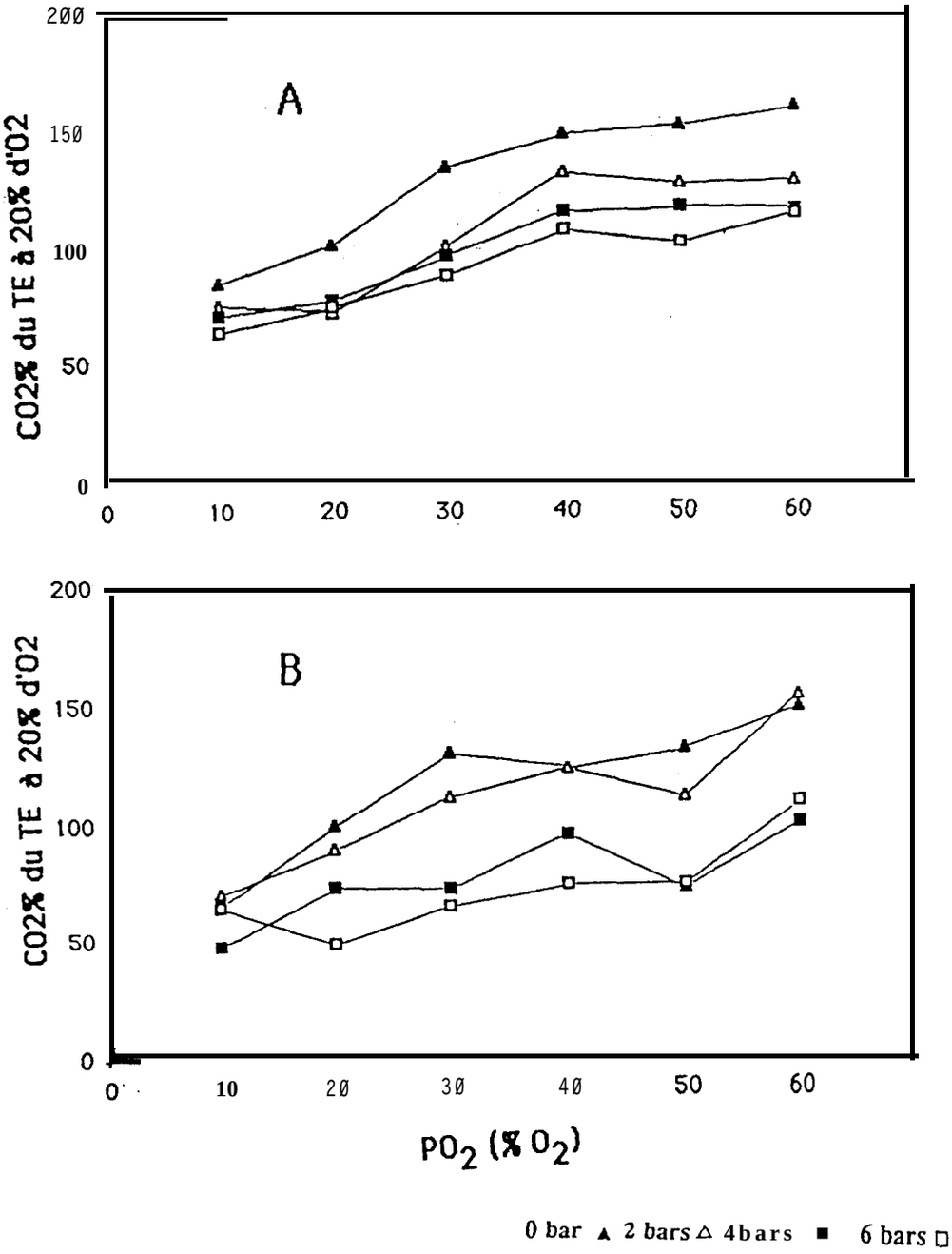


Figure 5 : Influence de la contrainte osmotique sur la respiration des racines nodulées. Les mesures sont effectuées simultanément aux mesures d'ARA en système fermé, chez les variétés de soja KINGSOY (A) et HODGSON (B). Les résultats sont exprimés en pourcentage du témoin à 20% d'oxygène. Chaque point est la moyenne de 8 à 10 mesures sur une plante. L'expérience est répétée deux fois.

Tableau 1 : Influence du déficit hydrique sur la respiration du système racinaire nodulé et sur le coût énergétique de la fixation de l'azote (ARA) chez le soja, variété Kingsoy. Les mesures d'ARA sont faites en système continu. La respiration basale correspond à l'ordonnée à l'origine de la droite de régression simple entre l'ARA et la respiration, et le coût énergétique est la pente de cette droite.

Intensité de la contrainte osmotique en MPa	Respiration basale $\mu mol\ CO_2\ h^{-1}\ pl^{-1}$	Coût énergétique de nitrogénase $\mu mol\ CO_2\ umol^{-1}\ C_2H_4$
Ø	255 ± 68	5,5 ± 1,0
-0,2	146 ± 58	9,4 ± 2,1
-0,4	224 ± 89	4,3 ± 2,6
-0,6	234 t 44	6,1 ± 2,1

Tableau 2 : Effets cumulés du nitrate et du déficit hydrique sur la fixation symbiotique de l'azote (ARA) du soja, variété Hodgson ; intensité de la contrainte osmotique -0,4 MPa, pendant 24 h. Le point -1 correspond aux mesures faites une heure avant application du mannitol, et le point 120h à celles réalisées 96h soit 4 jours après suppression de la contrainte osmotique.

Heures après stress	ARA : umoles $C_2H_4 h^{-1}$ Plante^{-1}			
	Sans déficit hydrique		Avec déficit hydrique	
	$-NO_3^-$	$+NO_3^-$	$-NO_3^-$	$+NO_3^-$
-1	52,0	11,0	52,0	11,0
+5	62,5	13,2	42,4	1,2
+24	59,7	25,0	37,1	3,0
+120	86,4	10,8	45,4	4,0

cultivées avec 3 mM de nitrate. L'inhibition de la fixation par le nitrate et le déficit hydrique sont donc approximativement cumulatives.

A la suppression de la contrainte hydrique, les valeurs de l'ARA sont très faibles chez les plantes avec nitrate, mais augmenteut assez rapidement. Chez les plantes sans nitrate, l'inhibition de la nitrogénase n'est que partiellement levée.

Discussion et conclusion

D'après les travaux initiaux de Sprent (1976), la contrainte osmotique inhibe la fixation symbiotique de l'azote en limitant la quantité d'oxygène disponible dans les nodosités pour la synthèse de l'ATP qui est indispensable au fonctionnement de la nitrogénase. Les résultats obtenus dans ce travail sur nodosités intactes en place sur la plante sont en accord avec cette hypothèse. En effet l'inhibition de l'ARA par la comrainte osmotique, due à l'apport du mannitol dans le milieu racinaire à 20% d'oxigène, est levée partiellement par l'augmentation de la pO_2 externe. Le déplacement de la pO_2 optimale vers des valeurs d'autant plus élevées que la concentration de mannitol est plus forte, ainsi que les observations sur la respiration nodulaire, renforcent cette interprétation et suggèrent que la diffusion de l'oxygène est perturbée par la contrainte osmotique. Cependant, la manipulation de la pO_2 externe ne permet pas de lever totalement l'inhibition. En effet les valeurs d'ARA maximale en présence de la contrainte osmotique sont inférieures à celles observées snr les plantes témoin ne recevant pas de mannitol. La limitation de l'ARA par l'oxygène ne serait donc pas le seul mécanisme implique dans l'inhibition de la fixation par une comrainte osmotique.

L'ARA maximale est vraisemblablement déterminée par le flux de pouvoir réducteur aux bactéroïdes (Heckmann et Drevon, 1988) et par la quantité d'enzyme active. L'irréversibilité de l'inhibition pour des pressions osmotiques inférieures à -0.2 MPa suggère qu'une fraction des protéines indispensables à la fixation a été détruite durant les 24 h d'incubation en présence du mannitol. Ceci est apparemment confirmé par l'observation de la Fig.2 : l'inhibition de l'ARA durant les premières heures d'incubation en présence de mannitol serait due à un ralentissement de la diffusion de 0, tandis que la baisse plus lente d'activité nitrgénase observée entre 14 et 20 h s'expliquerait par une détérioration du tissu nodulaire, due elle-même à un manque prolonge d'oxygène ou encore à un manque d'énergie. Il est toutefois peu probable que œ manque d'énergie au niveau nodulaire provienne d'une diminution de la photosynthèse sous l'effet de la contrainte osmotique car une diminution de la réduction foliaire de CO, par limitation du flux de photons (Schweitzer et Harper, 1980) ou une stimulation par la pCO_2 aérienne (Finn et Brun, 1982) n'affectent pas l'ARA avant au moins 24 h. Il est par contre possible que la contrainte osmotique ait ralenti le transfert des photosynthats aux nodosités, œ qui d'après Sloger *et al.*, (1975), diminue l'ARA à court terme, vraisemblablement en limitant la quantité de pouvoir réducteur disponible pour le fonctionnement des bactéroïdes. Afin de vérifier ces hypothèses, ii conviendrait de tester l'effet à plus court terme du mannitol sur l'inhibition de la fixation et la réversibilité de cette inhibition, et de mesurer l'activité ex-planta de bactéroïdes prélevés à des moments successifs de la cidtique d'inhibition.

La présence de nitrate dans la solution nutritive pourrait, par son accumulation vacuolaire, diminuer l'inhibition en permettant à la plante et aux nodosités de maintenir leur turgescence. Les résultats présentés dans le Tableau 1 ne soutiennent pas cette hy-

pothbse puisque le pourcentage d'inhibition de l'ARA par une contrainte osmotique de -0,4 MPa est supérieure en présence (88%) qu'en absence (38%) de NO,-. L'inhibition plus forte en présence de cet ion serait due à son effet inhibiteur propre qui augmenterait la résistance corticale à la diffusion de 0, dans les nodosités (Minchin *et* ai., 1986) et diminuerait la quantité de pouvoir réducteur disponible pour les bactéroïdes (Heckmann et Drevon, 1987). Ceci pourrait également être dû à une accumulation dans la nodosité de nitrite issu de la réduction du nitrate (Rigaud, 1976 ; Streeter, 1982) d'autant plus que cette accumulation de nitrite est plus forte en condition d'aliientation limitante de 0, (Heckmann et Drevon, 1987).

La comparaison des denx cultivars en l'absence de perturbation osmotique, suggère que Hogdson aurait d'avantage de réserves glucidiques facilement mobilisables que Kingsoy puisque sa pO_2 optimale pour l'ARA est plus élevée. Ceci pourrait expliquer la meilleure réversibilité de l'inhibition chez Hogdson et soutiendrait l'hypothèse d'un rôle des réserves glucidiques dans le maintien de l'intégrité des nodosités en présence d'une contrainte osmotique de moins de 24 h.

Du point de vue agronomique, cette étude entraîne deux remarques :

(i) Il est reconnu que l'apport de nitrate à une culture permet à celle-ci de mieux résister à la sécheresse, car cet anion joue le rôle d'osmoticnm dans la vacuole et permet à la plante de rester turgescente. Cependant, cet anion diminue également la fixation et son rôle dépressif s'ajoute à celui de la contrainte hydrique. Dans ce cas, le soja perd son intérêt comme plante fixatrice. Il serait utile de faire la même étude sur les légumineuses très résistantes à la sècheresse, telles celles des zones désertiques.

(ii) Le cultivar de soja Kingsoy, dont le rendement est moins diminue que celui d'Hodgson par une irrigation insuffisante (Paul, com. pers.), présente cependant une fixation d'azote très sensible au déficit hydrique. Cette apparente contradiction est sans doute due au fait qu'un déficit hydrique de plusieurs jours ne provoque pas de perturbations physiologiques profondes chez Kinsoy du fait que la régulation stomatique est efficace chez cette variété (Sall, 1984). Par contre, œ déficit hydrique déprime plus vite et plus fortement son activité nitrogénase, cette variété possédant des nodosités ayant une plus faible réserve énergétique que celles d'Hodgson.

Il y aurait donc sans doute intérêt à rechercher des variétés peu perturbées par le déficit hydrique, à la fois pour le rendement et la fixation de l'azote, ou additionner œs deux critères par croisement.

Ce travail préliminaire qui demande confiiation, notamment en poursuivant la même recherche sur d'autres cultivars de soja ou même sur d'autres espèces de légumineuses, montre l'intérêt d'une étude physiologique sur les différentes étapes de l'élaboration du rendement pour optimiser la production d'une culture.

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Effect of ^{15}N-labelled mineral-N and strains of *Bradyrhizobium* on biological nitrogen fixation and N-partitioning in cowpea (*Vigna unguiculata*) (L.) Walp.

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Abstract

^{15}N-labelling technique was used to study the effects of different strains of *Bradyrhizobium* and two doses of mineral-N on the fate of biologically fixed and fertilizer derived N on the partitioning of N in cowpea. Cowpea plants treated with 40 mg N plant^{-1} at planting had greater N-content and fixed more N_2 than plants receiving 240 mg N plant? in split applications. Plants treated with the lower dose of mineral-N contained more N in all parts except the pods and roots as compared to the. higher mineral N treatment. The high dose of mineral-N inhibited BNF of cowpea, but N accumulation in the pods was not reduced For this treatment 52-58% total N fixed and 32-58% of fertilizer-N absorbed were accumulated in the pods. The nitrogen content of nodules *was influenced by the strain* of *Bradyrhizobium. The strain* of *Bradyrhizobium also* influenced the partitioning of N derived from mineral fertilizer among plant parts.

Introduction

Cowpea is an important crop for tropical developing countries due to its high protein content. Scope for improving cowpea yield by applications of nitrogen fertilizer is limited due to high costs of fertilizer nitrogen, especially in developing countries. Great attention must, therefore, be paid to inoculation of cowpea with efficient strains of *Bradyrhizobium.*

Many studies have been done concerning the effects of combined N on the physiology of the *Rhizobium*/legume symbiosis and the partitioning of fertilizer-N in plants during reproductive development (Summerfield et *al.,* 1977 ; Eaglesham et al., 1983 ; Douglas and Weaver, 1986 ; Weaver et al., 1987)

Many authors recommended the use of low doses of N to stimulate nodule growth and N_2 fixation (Pate and Dart, 1961 ; Dart and Willon, 1970 ; Subba Rao et *al.,* 1974 ; Eaglesham *et* al., 1983, Weaver et al., 1987). However, the determination of starter-N level is complex and may depend on many factors, such as the form of the N-compound, strain of *Rhizobium,* season, temperature, time of application, plant cultivar, etc...

Some experimental data indicated that applied N at a level lower than 360 mg N/plant may have synergistic effects on N_2 fixation by vigorously growing cowpea and soybean (Broeshart, 1974, Eaglesham *et al.,* 1983). Morever, Weaver et *al.* (1987), demonstrated with glasshouse experiments that the addition of 60 mg plant-' mineral-N (compared with no addition) to cowpea changed the ranking of strains for effectiveness, influenced the total N content on shoots and whole plant and N concentration in leaves and pods.

Partitioning of N during reproductive development of grain legume is largely a genetic trait of the plant (Minchin *et al.,* 1978) ; some authors found a *Rhiwbium* strain effect on N partitioning in reproductive soybean plant parts (Morris and Weaver, 1983 ; Neves *et al.,* 1985).

In the previous research, rhizobial strains influenced N partitioning in plants, only when they differed in effectiveness. The present work utilized four rhizobial strains of equal effectiveness to determine their influence on N partitioning in cowpea in a long-term greenhouse experiment under two doses of mineral-N.

Materials and Methods

Plant and bacteria

Cowpea (*Vigna unguiculata*) (L.) Walp. "Bush Purple Hull") seeds were pregerminated and planted in plastic pots of expanded vermiculite (Weaver, 1975). Four pregerminated seeds were planted in each pot and were inoculated at'planting with 1 x 10^7 cell l^{-1} per *seed* of *Bradyrhizobium* (strains BDC 22, 6A, 24B from Texas A & M University culture collection isolated from Barbados soils and strain 32Hl from Nitragin Co., Inc.).

One week after planting, the seedlings were thinned to one per pot. Plants were watered with a N-free nutrient solution (Evans et *al.,* 1972). every 1 to 2 days and excess nutrient solution drained from holes at the bottom into containers placed under the pots (Eaglesham et *al.,* 1983).

Mineral-N application

There were 10 treatments, five (four inoculated and one uninoculated) supplied with 40 mg N/pot of ^{15}N-labelled KNO_3 (1.2786 atom % ^{15}N excess) and five (four inoculated and one uninoculated) supplied with 240 mg N/pot of ^{15}N-labelled KNO_3 (0.4783 atom % ^{15}N excess). The low dose was applied as a single application 1 week after planting, and the higher dose was applied in split application 1.4, 5, 6 and 7 weeks after planting at rate of 40, 40, 80, 40 and 40 mg N/pot respectively at each time. Also an uninoculated treatment without N was included as a control to determine the quantity of N coming from the plant support medium. These uninoculated plants without mineral-N displayed early N-deficiency symptoms and yielded a maximum of 6.5 mg N/plant. Each treatment was replicated four times and treatments were arranged in a completely randomized design.

Harvesting and Nitrogen analysis

Plants were harvested at maturity (when 3-4 pods on each plant were completely purple). Plants were separated into leaves, stems, peduncles, pods, roots and nodules and oven dried (65-70°C) for at least 72 h before dry weights were determined. Samples of leaves, stems, peduncles, pods and roots were ground and N analysed by kjeldhal method as described by Nelson and Sommers (1973) but was modified to include NO, nitrogen.

^{15}N Determination

^{15}N content was determined by the method described by Hauck (1982) using a Micromass 602-D mass spectrometer. From the atom % ^{15}N excess in sample and atom % ^{15}N excess of mineral-N applied, the fraction of N in the plant sample derived from fertilizer was determined by the formula :

$$\% \text{ N derived from fertilizer} = \frac{\text{Atom } \%^{15}\text{N excess in plant part}}{\text{Atom } \%\ ^{15}\text{N excess in fertilizer}} \times 100$$

Results and Discussion

Performance of rhizobial strains was not significantly different within each N treatment with respect to total N and dry matter contained in various plant parts except for nodules. The main effects are presented in Table 1. Starter N (40 mg plant $^{-1}$) resulted in more dry matter being partitioned into nodules and less into roots as compared to the split application of 240 mg N plant $^{-1}$ (Table 1). However, application of only starter N resulted in more total N being contained in leaves, stems, peduncles and nodules (Table 1). Whole plant dry matter was not significantly different ($p = 0.05$) for the two N treatments (28.5 vs 29.8 g plant-‘) but total N content was higher for the starter N treatment (624 vs. 596 mg plant $^{-1}$) as indicated by the F test for significance.

Table 1 : The influence of starter N and continous N on dry weight and total N contained in various plant parts.

Nitrogen treatment	Leaves	Stems	Peduncles	Pods	nodules	roots
	dry weight g plant^{-1}					
Starter	5.93	5.96	3.37	10.1	0.49	2.59
Continuous	5.71	6.35	3.50	10.8	0.33	3.05
LSD 05	0.50	0.72	0.28	0.93	0.054*	0.35*
	Total N mg plant^{-1}					
Starter	158	45.0	39.5	328	30.1	24.7
Continuous	131	35.9	28.2	341	20.8	29.4
LSD 05	14*	5.9*	5.2*	28	4.1*	4.6*

*Indicates a significant difference due to treatment.

The dry weight of nodules was reduced by the continuous N treatment but within N treatments rhizobial strains performed similarly (Table 2). Rhizobial strain bad a significant influence on the N content of the nodules (Table 2). Nodules induced by strains 22 and 32Hl contained substantially more N than nodules induced by the other two strains. The effect of strain was less pronounced when more mineral N was provided. The N content of the nodules was a significant portion of the whole plant N and for the starter N treatment, the nodules contained more N than the roots (Table 1). The level of N provided in the continuous N treatment not only reduced nodulation (Tables 1 and 2) but dinitrogen fixation (Table 3) even though the percentage of total N in the plant, derived from the fertilizer was only 15%. Eaglesham et *al.* (1983) reported a significant decrease in N_2 fixation when plant uptake of NO, provided more than 20% of the total N in the plant The quantity of biologically fixed N contained in plant parts other than roots was reduced by the continuous N treatment (Table 3) but the quantity of fertilizer N in all plants was greatly increased.

Table 2 : Total dry weight and nitrogen content of nodules on cowpea as influenced by strain of rhizobia and mineral nitrogen.

Strain	Dry weight		Total N	
	Starter	Continuous	Starter	Continuous
	g plant^{-1}		mg plant^{-1}	
22	0.490	0.347	35.3	25.9
248	0.484	0.325	24.7	16.8
32Hl	0.535	0.340	40.2	24.7
6A	0.445	0.320	20.4	15.9
$LSD_{0.05}$	0.107		8.24	

Table 3 : The influence of starter N and continuous N on partitioning of biologically fixed N (BNF) and fertilizer N into various plant parts.

Plant part	BNF		Fertilizer N	
	Starter	Continuous	Starter	Continuous
	mg N plant^{-1}			
Leaf	154.Ø (12.8)+	113.Ø	2.63	17.5
Stem	44.2 (5.27)	31.Ø	Ø.62	5.31
'Peduncle	39.2 (4.75)	23.7	Ø.32	4.55
Pod	326.Ø (22.9)	291 .0	2.45	5Ø.1
Root	23.5 (3.77)	23.Ø	1.15	6.32
Nodule	3Ø.2 (5.83)	2Ø.8	Ø.36	Ø.63
Total	617.1 (27.7)	5Ø2.5	7.53	84.41

+ The number in parenthesis is the LSD Ø.Ø5 for making comparisons on the effect of mineral N treatments on the quantity of biologically fixed N in the particular plant part. A test of significance was not made for fertilizer N comparisons.

Fertilizer N treatments influenced the relative partitioning of fertilizer N into various plant parts (Table 4). Distribution of starter N was largely into vegetative organs probably because it was depleted from the plant growth medium relatively soon whereas for the other N treatment the split applications made the N available throughout the growth period and half of the fertilizer N was contained in the pods (Table 4). The largest percentage of the biologically fixed N was contained in the pods regardless of the fertilizer N treatment In the case of the split application N treatment, the same proportion of the fertilizer N and biologically fixed N in tbe whole plant partitioned into the pods. Eaglesham et al. (1977) reported similar results for cowpea continuously provided with NO,. It appears that the plant did not have a preference for the source of N for pods when both sources of N were available throughout plant development. However, the data in Table 4 are averages across four strains of rhizobia. The nodules only received 3% of their N from mineral N in contrast to the roots which received 24% (calculated from Table 3). Eaglesham et al. (197'7) also reported that nodules on plants fertilized with NO,- received 96% of their N from dinitrogen but roots received only 86% of their N from dinitrogen and concluded that nodules were largely self-sufficient. Nineteen days after plants were pulsed with ^{15}N labelled NO,- the roots contained 19% of the NO, that was taken up and the nodules only contained 2% (Douglas and Weaver, 1986).

Table 4 : Distribution of biologically fixed N (BNF) and fertilizer N (FN) in plant parts of cowpea as a percentage of the total plant N derived from each source.

Nitrogen. treatment	Plant part											
	Leaves		Stems		Peduncles		Pods		Roots		Nodules	
	BNF	FN	BNF	FN	BNF	FN	BNF	FN	BNF	FN	BNF	FN
Starter	25.1	35.2	7.3	8.3	6.3	4.3	53	32	3.8	15	4.8	4.9
Continuous	22.7	21.2	6.2	6.3	4.7	5.5	58	58	4.6	7.6	4.0	0.4
$LSD_{.05}$	2.3	'3.5	0.85	1.6	0.76	0.87	3.0	5.0	0.66	1.7	0.60	0.71

The strain of rhizobia did not influence uptake or distribution of starter N in the plant (data not shown) but rhizobial strain influenced distribution of N from the continuous N treatment (Table 5). The actual quantity of fertilizer N contained in various plant parts was only significantly different for leaves, stems, and nodules. The coefficient of variation was quite large for the reproductive plant parts and although large differences occurred for pods they were not statist&ally significant. However, the data provide quite strong evidence that the strain of rhizobia influenced the relative distribution of fertilizer N into the pods (Table 5). For two of the strains approximately 52% of the fertilizer N was

Table 5 : The influence of rhizobial strain on the quantity and percentage distribution of continuous N into plant parts of cowpea

	Plant part						
Strain	Leaves	Stems	Peduncles	Pods	Roots	Nodules	Total
	mg N plant^{-1}						
6A	15.7	4.98	4.19	51.4	4.76	Ø.41	83.4
22	18.5	3.83	3.79	64.6	6.2Ø	Ø.47	97.4
24B	13.8	5.ØØ	4.51	36.Ø	6.87	Ø.61	66.8
32H1	22.1	7.42	5.72	48.3	7.46	1.Ø4	92.Ø
LSD Ø.Ø5	4.4*	1.86*	2.21	23.9	2.96	0.24*	3Ø.8
	% distribution						
6A	19.7	6.32	5.15	62.5	5.85	Ø.53	
22	2Ø.1	3.9Ø	3.95	65.2	6.45	Ø.48	
24B	2Ø.8	7.5Ø	6.62	53.7	9.88	Ø.93	
32H1	24.4	8.1Ø	6.13	52.1	8.1Ø	1.13	
LSD Ø.Ø5	5.6Ø	1.94*	1.76*	9.5*	2.18*	0.28*	

* indicates significant difference by F-test (P = Ø.Ø5) between strains.

partitioned into the pods but for the other two strains approximately 63% of fertilizer N was partitioned into the pods. These results indicate that the strain of rhizobia influenced partitioning of fertilizer N in cowpea as has been reported for pigeon pea (Hernandez et al., 1987). However, the data do not confii that cowpea strains influence uptake of mineral N as reported by El Hassan and Focht (1986) but different strains of rhizobia were utilized in our experiments and El Hassan and Focht (1986) grew their plants in soil rather than a defined medium.

Evidence is accumulating that the strain of rhizobia may have an effect on nitrogen partitioning in grain legumes. Our results indicated that four strains of rhizobia of equal effectiveness based on total dinitrogen accumulated produced similar pod yields but the relative distribution of fertilizer N partitioned into pods was different (Table 5). The strains also influence the quantity of N contained in nodules (Table 2). Depending on the mineral N treatment the quantity of N in nodules was more or less than that contained in roots (Table 1) which accounted for approximately 5% of the total plant N. Averaged across strains of rhizobii the plant partitioned N from dinitrogen and continuously supplied mineral N into pods in the same relative proportions (Table 4). This agrees with the data of Eaglesham *et al* (1977) in which 66% of the N from each N source was partitioned into cowpea fruit. Thus, there was no evidence that the plant preferentially utilized biologically fixed N over mineral N for pod development or that the rhizobial strain influenced partitioning of biologically fixed N into pods.

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Quantification of the contribution of BNF to field grown plants : the use of the ^{15}N isotope dilution technique - problems and some solutions

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Summary

When the ^{15}N isotope dilution technique is used to estimate the contribution of biological N_2 fixation (BNF) to plants, the assumption is made that both the "N_2-fixing" (test) and control crops remove N from the soil with the same ^{15}N enrichment. The most practical method of labelling the soil with ^{15}N is to apply soluble labelled fertilizer but this produces in the soil mineral N a rapid fall in ^{15}N enrichment with time. Hence plants with different uptake patterns can acquire soil-N with different ^{15}N enrichments. While it is recommended that control crops with the same uptake patterns as the test crops are used, this requirement is exceedingly difficult to test for, and in each study uptake patterns will vary with different edaphic and environmental conditions. In these circumstances the use of several control crops in conjunction with slow release forms of ^{15}N can be a successful strategy for the reliable quantification of BNF. In a soil with a stable ^{15}N label any control crop may be used or even dispensed with altogether. These problems and possible solutions are discussed with examples of the quantification of BNF in both grain legumes and grasses.

Introduction

In recent years the ^{15}N isotope dilution technique has been widely used for the quantification of the contribution plant-associated biological nitrogen fixation (BNF) to both nodulated legumes and other crops. The acetylene reduction technique (Hardy *et* al., 1968) has made a great contribution to knowledge in BNF research, but its use is severely limited for the definitive quantification of contributions of N_2 fixation to higher plants. The limitations of the application of this technique to the quantification of BNF associated with nodulated legumes have been thoroughly discussed in a very recent paper by Witty and Minchin (1988), and the problems in its use to quantify possible N_2 fixation associated with grasses and cereals have also been reviewed recently (Boddey, 1987). While techniques using total N measurements can be very 'valuable and should not be discounted (Weber, 1966 ; Bell and Nutman, 1971 ; Nelson and Weaver, 1980 ; App *et al.*, 1980, 1986 ; Lima et al., 1987), they are often of limited value in field experiments unless very long term (Jenkinson, 1977, 1982).

It may be thought that the cost of using the stable isotope ^{15}N is very high, but a few hundred dollars (US) can buy sufficient of the labelled salts to perform one or two field experiments. While the instruments to analyse ^{15}N enrichment are very expensive (a mass spectrometer is likely to cost upwards from US$ 100,000, the less sensitive emmission spectrometer approximately 30 to 50% of this), commercial laboratories in the US and elsewhere will perform analyses for $10 to $20 per sample or even less if many hundreds of analyses are required. However, if it is essential for the aims of the project in question that the contribution of BNF to a crop be quantified, then the isotope dilution technique can provide the answer if used with great care.

The principle of the ^{15}N isotope dilution technique

The ^{15}N technique requires that "N_2 fixing" crop under study (the test crop) is grown in a soil enriched with ^{15}N along with a control ("non-N_2-fixing") crop. The ^{15}N enrichment of the plant material of the control crop is regarded as representing the enrichment of the plant-available (mineral) nitrogen in the soil. Hence if the test crop has a lower enrichment than this it is concluded that unlabelled nitrogen from the air was incorporated into the tissue of the test crop via N_2 fixation. It is apparent that the underlying assumption in the use of this technique is that both the control and test crops remove nitrogen from the soil with the same ^{15}N enrichment.

Assuming this to be the case the calculation of the proportion of nitrogen in the test crop derived from BNF (%NF) can be calculated from the equation :

$$\%NF = 100 \left(1 - \frac{\text{Atom \% } ^{15}N \text{ excess in test crop}}{\text{Atom \% } ^{15}N \text{ excess in control crop}} \right) \quad \text{....Eqn 1.}$$

It may be pointed out that the technique quantifies the contribution of N_2 fixation specifically associated with the test crop, and that any input of unlabelled N derived from rainfall, pollution or from BNF associated with cyanobacteria or other sources, if equally available to both test and control crops, will not be detected by this technique. For the acetylene reduction or total N balance techniques this may not be true.

Enrichment of the soil with ^{15}N

It is inevitable that the test and control crops will remove N from the soil with the same ^{15}N enrichment if the soil is labelled uniformly in area and depth, and is stable with time. In this case any crop which certainly has no associated BNF will serve as a valid control for any test crop. This approach has been used by several authors to quantify BNF associated with both legumes and grasses (Kohl and Shearer, 1981; Broadbent *et al.*, 1982 ; Rennie et al., 1982 ; Butler and Ladd, 1985 ; Miranda and Boddey, 1987). In fact the control crop in this case may be dispensed with altogether and the ^{15}N enrichment of the N derived from the soil determined directly from measurements on the soil mineral N, (Chalk *et* al., 1983).

The most practical and usual method for enrichment -of a soil with ^{15}N is by the application of a solution of labelled nitrogen fertilizer (e.g. urea, ammonium sulphate or potassium nitrate) to the surface of the soil. If the fertilizer is uniformly distributed over the area of soil to be planted then lateral variations in ^{15}N enrichment should not occur. Injection of labelled fertilizer at points within the planted area such as that performed by Rennie and Thomas (1987) are not to be recommanded as differences in lateral root distribution between test and control crops may cause differences in the ^{15}N enrichment of the N derived from the soil.

The application of soluble fertilizer to the surface of the soil does result in considerable gradients with depth (Ladd et al., 1981 ; Ledgard et al., 198.5). Boddey and Victoria (1986) found that soil amended with repeated (small) applications of labelled ammonium sulphate showed mean enrichments of mineralizable nitrogen of 0.140. 0.069, 0,048 and 0.030 atom % ^{15}N excess in four successive 11 cm layers. They also found evidence to suggest that this caused plants with different vertical root distributions to obtain different ^{15}N enrichments in the soil-derived N.

However, the most important problem associated with the use of soluble labelled fertilizers is that immediately after application there is a sharp decline in ^{15}N enrichment in the mineral N in the soil, due to continuing mineralization of unlabelled soil organic N into a mineral pool continuously depleted by plant uptake, leaching and other losses (Fried *et* al., 1983 ; Witty, 1983). This is illustrated by recent data from a study by our group performed near Brasialia (Boddey, R.M., Urquiaga, S., Suhet, A., Peres, J.R. and Neves M.C.P. in preparation). In one treatment of this experiment, inoculated soybean was grown with four different control crops (a non-nodulating genotype of soybean, sorghum, sunflower and rice) in soil amended with 10 kg N ha^{-1} of ^{15}N-labelled potassium nitrate (10.0 atom % ^{15}N excess) at planting. The data show a continuous decrease in ^{15}N enrichment in the plant aerial tissue due to the fall in the enrichment of soil mineral N (Fig. I).

The reason for the initial increase in ^{15}N enrichment of the soybean treatments is that at the early harvests the unlabelled nitrogen derived from the large N-rich soybean seeds constitutes a large proportion of the N in the young plants. This contribution of unlabelled N decreases as it becomes diluted with labelled N from the soil and the ^{15}N enrichment of the plants then increases to reflect that accumulated from the soil. This effect is not significant for the sorghum and sunflower as the seeds are smaller and of lower N content. It should be noted that if a small seeded cereal plant (e.g. wheat, barley, sorghum etc) is used as a control for soybean or other large-seeded legumes, then at early harvests the contribution of unlabelled seed N may be confused with that derived from BNF.

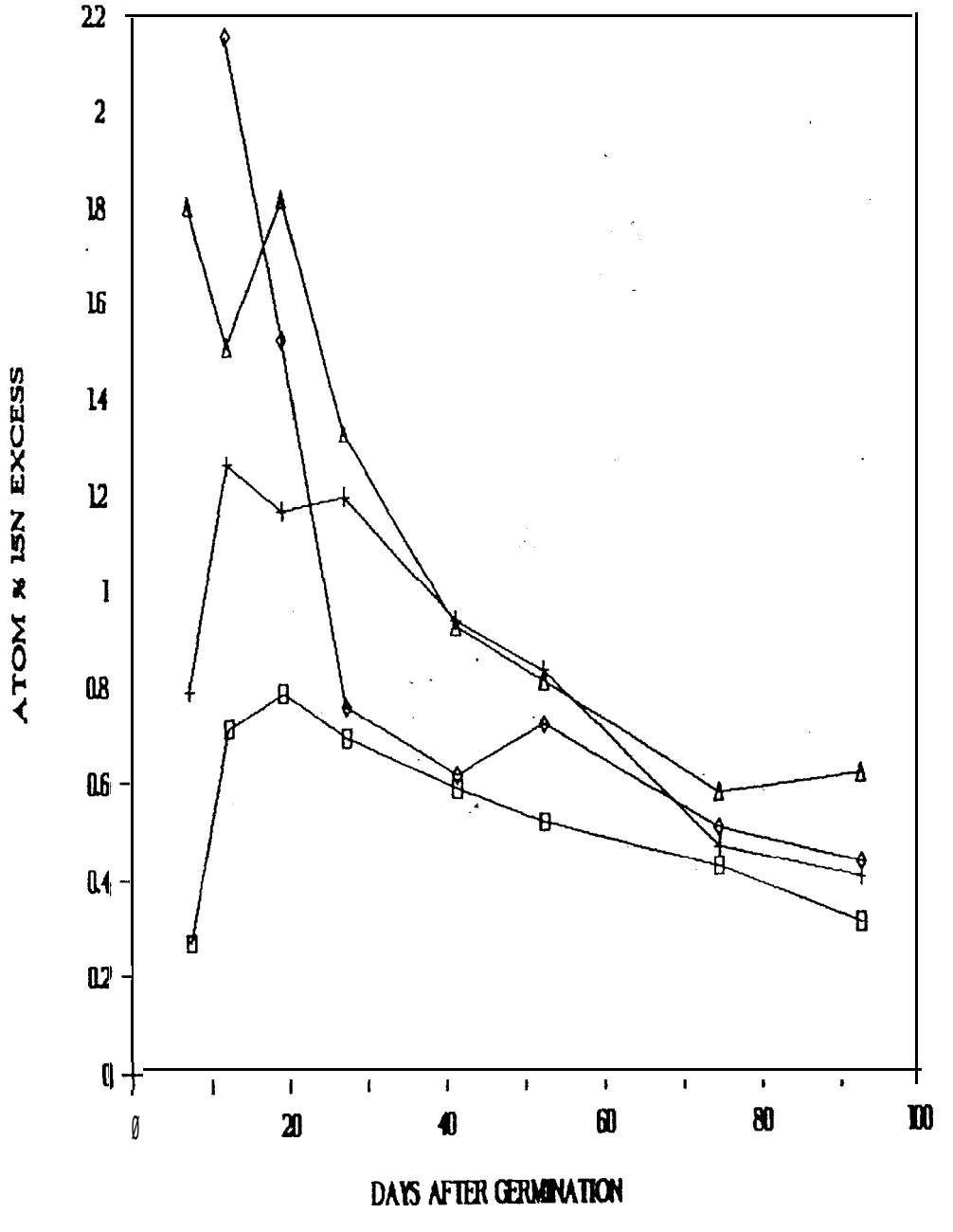


Figure 1 : ^{15}N enrichment of nodulated soybean (⊡), non-nodulating soybean (✚), sorghum (◇) and sunflower (△) grown in a soil amended at planting with soluble ^{15}N labelled ammonium sulphate (10 kg N ha^{-1}, 10.0 atom % ^{15}N).

As the patterns of soil nitrogen uptake in this study were different (Fig. 2) this resulted in the different control plants accumulating nitrogen from the soil with different ^{15}N enrichments. This problem has been discussed by many authors and is particularly well illustrated by the data reported by Witty (1983).

Most isotope dilution studies have been performed using single additions of soluble labelled fertilizer in conjunction with the use of a single control crop (see reviews of Chalk, 1985 and Danso, 1988). As the data displayed above, and that of Witty, illustrate, in such studies it is dangerous to assume that different crops will accumulate N from the soil with the same ^{15}N enrichment, and in many of these studies the different ^{15}N enrichment observed in the nodulated legume may have been partly due to differences in N uptake patterns introducing an error of unknown magnitude to the resulting BNF estimates.

It has been suggested that certain control crops are appropriate for specific legume crops for use with this technique to quantify BNF (Rennie 1986). There is little evidence from the sources cited by Rennie that these control crops were necessarily "appropriate" (i.e. accumulated N with the same ^{15}N enrichment as that derived from the soil by the legume). Furthermore, as edaphic and environmental conditions will cause differential changes in soil N uptake patterns, even if a control proved to be "appropriate" in one experiment there is no guarantee that this would be true for other experiments on the same legume at different sites, seasons or years.

Slow release forms of labelled nitrogen.

As Witty (1983) showed, if the decrease in ^{15}N enrichment of soil mineral N is slowed down by applying a slow release form labelled N fertilizer then the differences in enrichment between different controls are attenuated. In our recent study near Brasilia a further treatment was included in the experiment where the same quantity of ^{15}N labelled potassium nitrate (10 kg N ha^{-1}) was mixed with sugar to give a C:N ratio of 10:1 and added 7 days before planting. The patterns of uptake of soil N were not altered significantly by the addition of sugar to immobilize the mineral nitrogen (Fig. 3) but the rate of temporal decline in ^{15}N enrichment was attenuated (Fig. 4) resulting in a narrower range of enrichments of the four different control crops (Table 1).

Many different techniques have been utilized to slow down the decline in the ^{15}N enrichment of soil mineral N with time. Some workers have mixed various carbon substrates (sucrose, glucose, cellulose, straw etc.) with labelled N fertilizer to immobilize the mineral N (Legg and Sloger, 1975 ; Broadbent et al.,1982 ; Wagner and Zapata, 1982 ; Talbott et al.,1982 ; Giller and Witty, 1987). Another similar technique adopted by some has been to incorporate ^{15}N-labelled plant material (especially prepared or resulting from other studies) into the soil (Henzell et al.,1968 ; Boddey et al.,1984).

In a recent study on 10 cultivars of sugar cane, ^{15}N-labelled ammonium sulphate and potassium nitrate were composted with bagasse and filter cake from a sugar cane mill to form a labelled organic matter which was mixed with soil to fill a concrete tank (20 x 6 m) to a depth of 60 cm (Urquiaga et al.,1989). There was still a dramatic fall in ^{15}N enrichment of the soil mineral N with time (Fig. 5) although it should be emphasized that the measurements were made over a period of almost 2 years. Data for the ^{15}N enrichment of the sugar cane cultivars and the control crop (*Brachiaria radicans*) at the first complete harvest (250 days after emergence) suggested that there were considerable BNF contri-

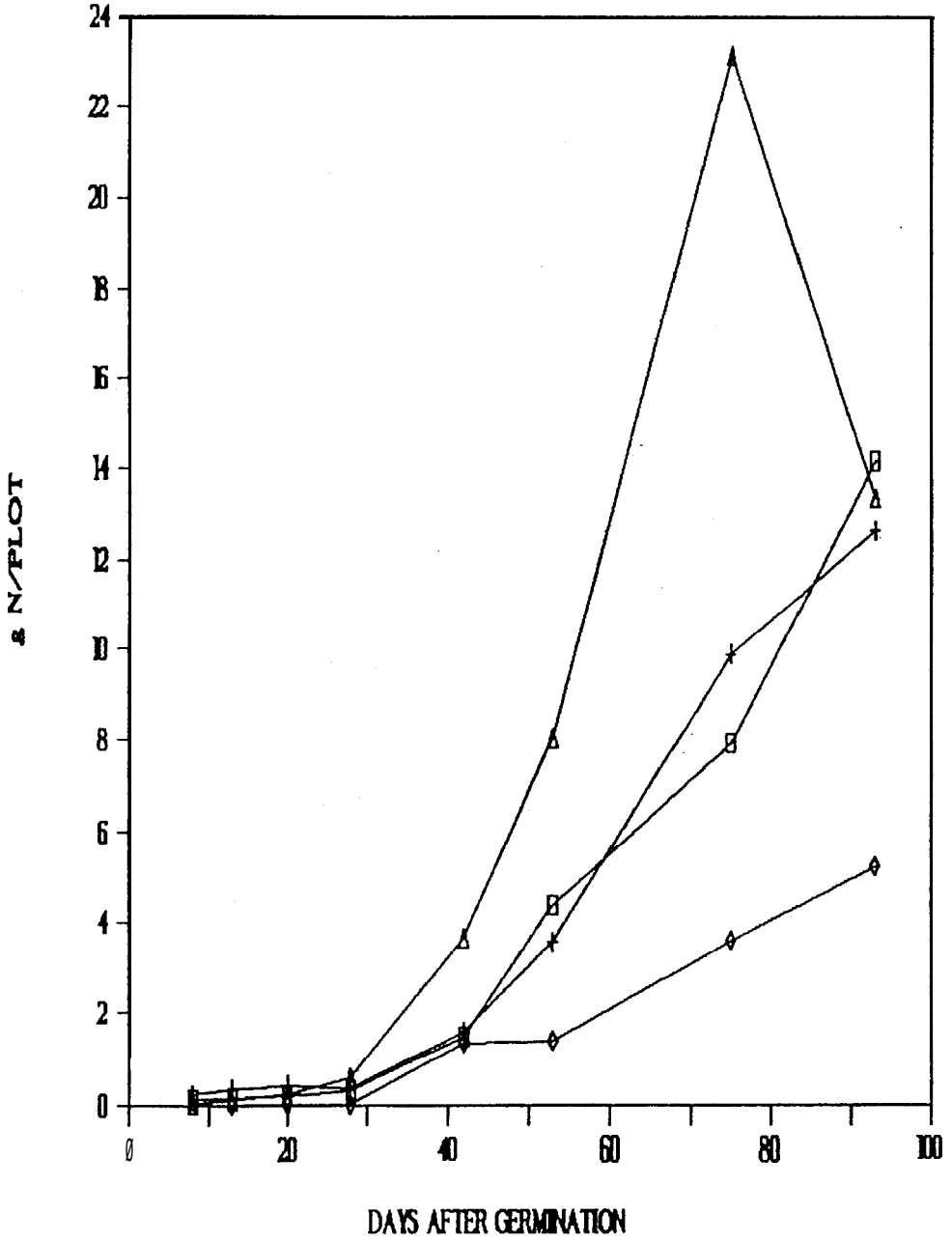


Figure 2 : Total N accumulation of nodulated soybean (□), non-nodulating soybean (+), sorghum (◇) and sunflower (△) grown in a soil amended at planting with soluble ^{15}N labelled ammonium sulphate (10 kg N ha^{-1}, 10.0 atom % ^{15}N).

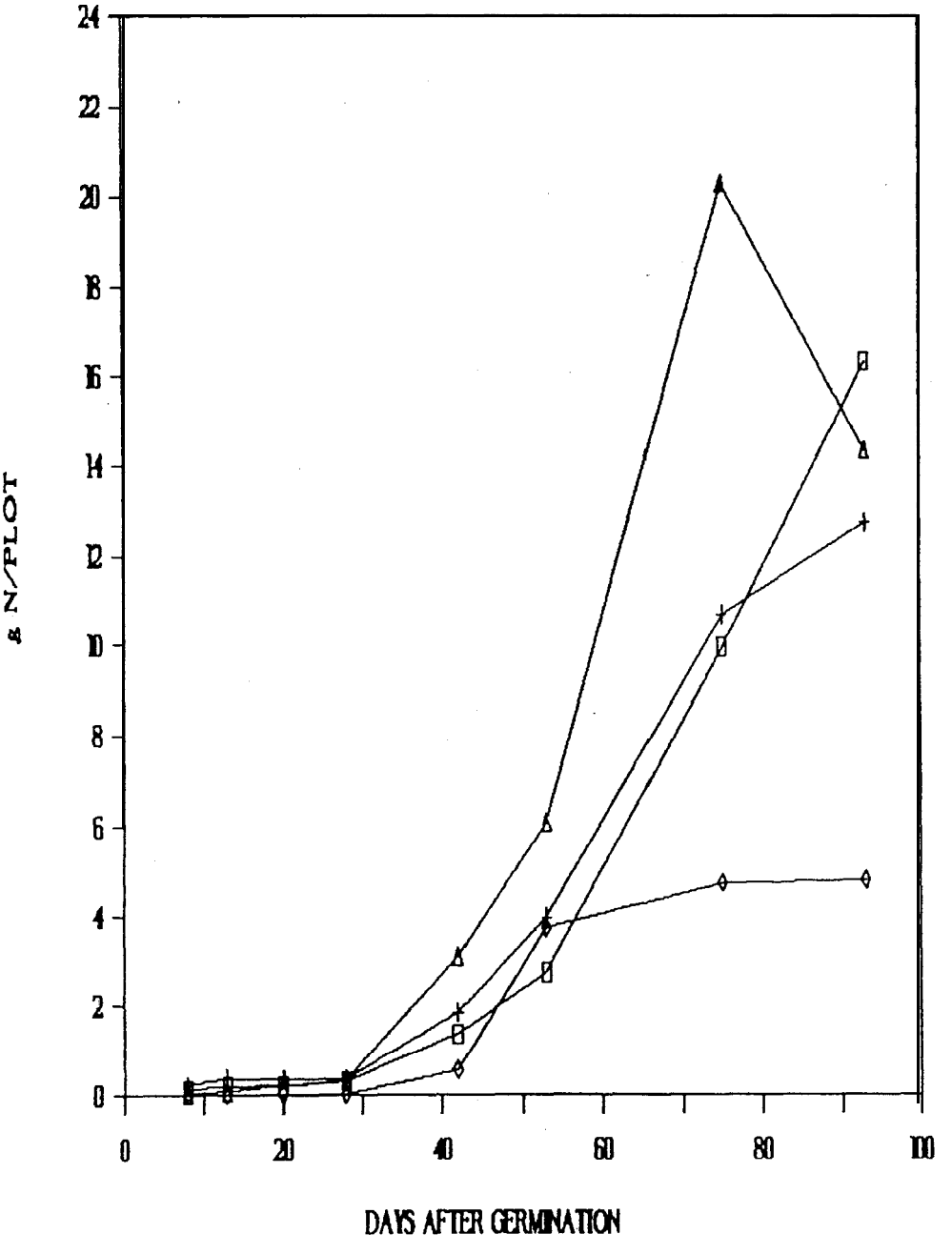


Figure 3 : Total N accumulation of nodulated soybean (□), non-nodulating soybean (+), sorghum (◇) and sunflower (△) grown in a soil amended 7 days before planting with ^{15}N labelled ammonium sulphate (10 kg N ha-', 10.0 atom % ^{15}N) mixed with sugar to give a C:N ratio of 10:1.

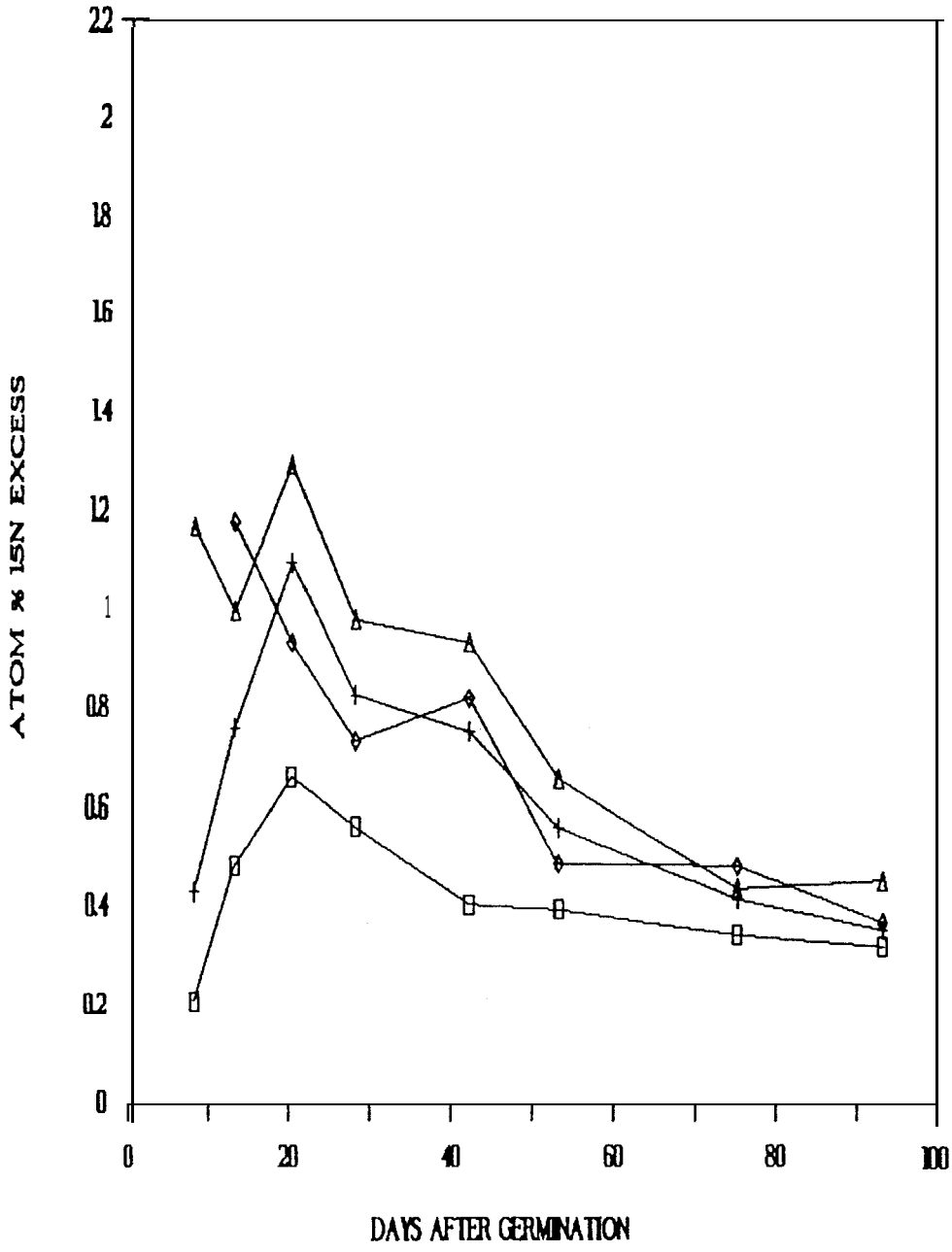


Figure 4 : ^{15}N enrichment of nodulated soybean (⊡), non-nodulating soybean (+), sorghum (◇) and sunflower (△) grown in a soil amended 7 days before planting with ^{15}N labelled ammonium sulphate (10 kg N ha^{-1}, 10.0 atom % ^{15}N) mixed with sugar to give a C:N ratio of 10:l.

Table 1. ^{15}N enrichment of nodulated soybean and 4 control crops grown in soil amended with ^{15}N-labelled fertilizer, with and without addition of sucrose. Means of 4 replicates.

	ATOM % 15N EXCESS	
CROPS	SOLUBLE FERTILIZER	FERTILIZER + SUGAR
Non-Nod Soybean	Ø.4Ø39	Ø.3538
Sorghum	Ø.4367	Ø.3679
Sunflower	Ø.62Ø9	Ø.4522
Rice	Ø.5Ø44	Ø.4355
Nodulated soybean	Ø.3131	Ø.32Ø8
Honest significant Difference (P=0.05)*	Ø.2Ø83	Ø.1925

* Tukey test, Coefficient of variation 2Ø.5%

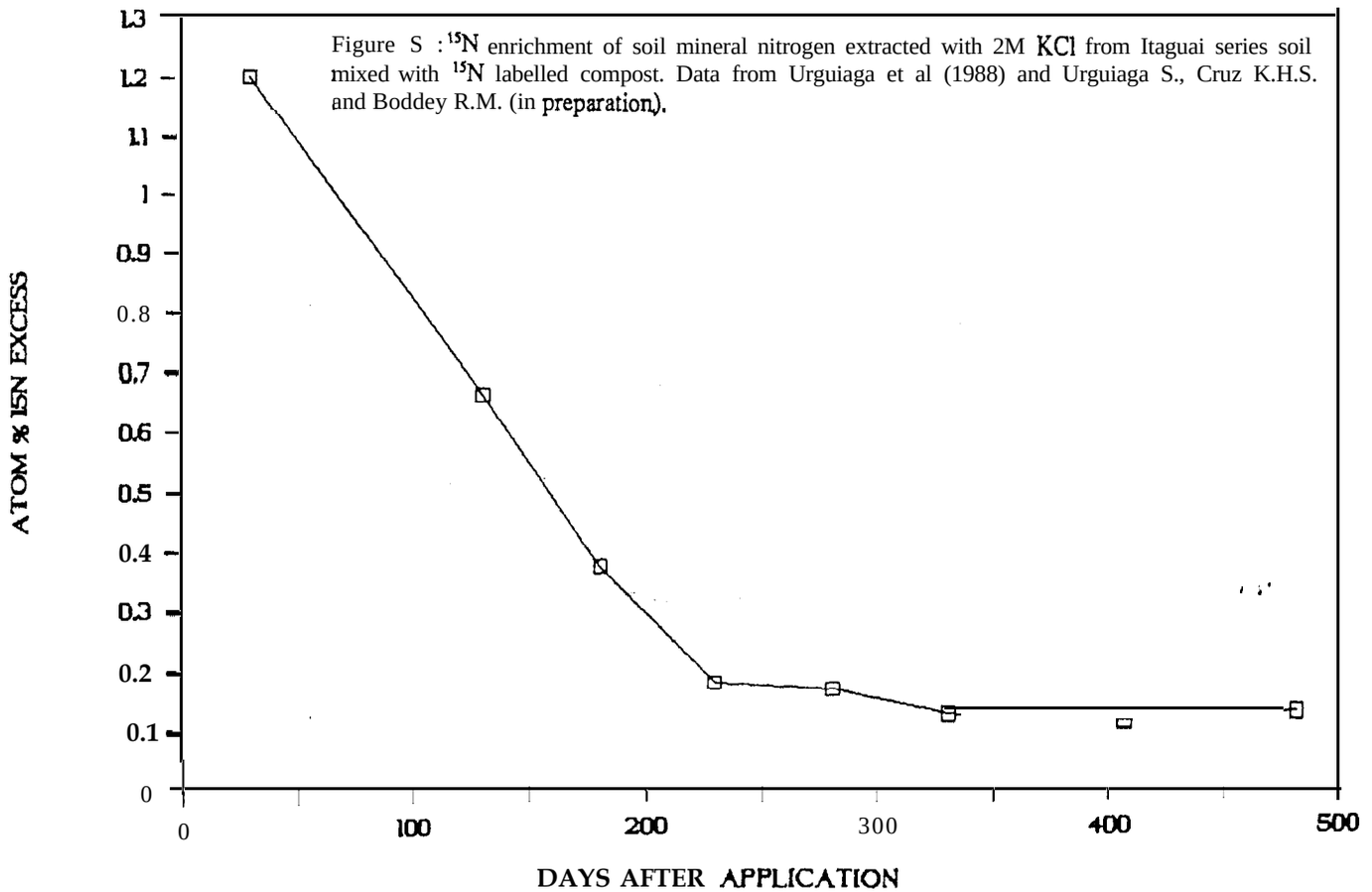


Figure S : ^{15}N enrichment of soil mineral nitrogen extracted with 2M KCl from Itaguai series soil mixed with ^{15}N labelled compost. Data from Urguiaga et al (1988) and Urguiaga S., Cruz K.H.S. and Boddey R.M. (in preparation).

butions to several of the sugar cane cuhivars, notably Krakatau and SP 70-1143, but because of the large fall in the ^{15}N enrichment in the soil mineral N during this period the estimates could not be regarded as accurate (Table 2). However, as subsequent to this harvest there was very little further drop in the enrichment of the soil mineral N, the estimates of the BNF contribution to the ratoon crop can be regarded as being much more reliable.

From the above discussion it is apparent that in order to accurately quantify plant associated BNF using the ^{15}N isotope dilution technique it is desirable that slow release forms of labelled nitrogen are used. Nearly all of the slow release forms of N when incorporated into the soil immobilize soil mineral N and hence change soil N availability to the plants, and/or cause other changes in soil fertility. It is important therefore to examine the objectives of the study in question to decide whether this will negate them. In our work performed on grasses and sugar cane the interest has been in determining under optimum conditions whether these plants are able to obtain significant contributions from plant associated BNF and in the comparisons of cultivars with regard to this trait. In this case the immobilization of nitrogen by the addition of slow release forms of labelled N does not adversely affect the objectives of the experiment This may be true for many studies with legumes, but there will be circumstances where it is necessary that the soil N availability is not significantly affected by application of the isotope dilution technique.

One form of slow release labelled N which would probably only marginally affect soil N availability is that based on the gypsum pellets described by Witty (1983). However, more recent data suggests that release of N from such pellets is not much slower than soluble N (Giller and Witty, 1987 ; Boddey et al.,1989). One other solution that may be possible is the addition of small quantities of soluble labelled N at regular (e.g. weekly) intervals. This thechnique has been adopted by many authors (Vallis et *al.*,1967 ; Goh et *al.*, 1978 ; Boddey et al., 1983, 1984 ; Ledgard et *al.*, 1985) but as Rennie (1985) has pointed out this technique has its dangers if sequential harvest of plants are to be made and soil N availability is high. If in this case the test and control crops remove different quantities of N from the soil then the residual mineral N in the soil at the subsequent harvest will be different. Further addition of labelled N to the soil will then result in a different initial enrichment of mineral nitrogen for the two crops at the start of the next growth period. Experience to date suggests that the use of small frequent additions of labelled fertilizer may be the best technique of labelling the soil if soil N availability is not to be radically altered (Boddey et *al.*,1983, 1984 ; Ledgard *et al.*, 1985b) but further, more detailed, studies on this subject are required.

A strategy for the field application of the isotope dilution technique

Unless the ^{15}N enrichment of the soil mineral N is known to be reasonably stable with time it is advisable that more than one control crop should be used within the same experiment. This strategy was recently recommended following experiments performed by our group at two different sites in Brazil (Boddey et *al.*,1989). In both experiments the objective was to quantify BNF contributions to inoculated and non-inoculated soybean, cowpea and groundnut using three different control crops (a non-nodulating genotype of soybean, sorghum and rice).

At the first site (Rio de Janeiro) the soil was enriched with ^{15}N by addition of labelled gypsum pellets (Witty, 1983). The data showed that there were considerable and

Table 2 : Estimates of the contribution of biological nitrogen fixation (BNF) to 10 sugar cane varieties

Variety/ species	1st HARVEST 250 DAE[1]				2nd HARVEST 380 DAE[1]			
	Total N accumulation (kg.ha^{-1})	^{15}N enrichment (ATM % ^{15}N exc.)	Estimate of the contribution of BNF2 %	(kg.ha^{-1})	Total N Accumulation (kg.ha^{-1})	^{15}N Enrichment (ATM % ^{15}N exc.)	Estimate of the contribution of BNF2 %	(kg N.ha^{-1})
CB 47-89	265 ab	0.316 bc	41.9	111	209 bc	0.108 bc	19	41
CB 45-3	257 ab	0.305 bcd	44.1	110	258 b	0.120 ab	10	27
NA 56-79	246 ab	0.313 bc	42.9	106	188 bcd	0.126 ab	6	11
IAC 52-150	271 ab	0.300 bc	46.2	125	173 bcd	0.115 abc	14	24
SP 70-1143	245 ab	0.261 bc	52.3	128	216 b	0.109 bc	19	40
SP 71-799	244 ab	0.298 bc	46.1	112	200 bc	0.109 bc	19	37
SP 79-2312	202 abc	0.344 bc	41.7	84	277 b	0.127 ab	5	14
Chunnee	152 c	0.349 b	37.2	56	119 bcd	0,124 ab	7	9
Caiana	49 d	0.309 bc	42.2	21	26 d	0.123 ab	8	2
Krakatau	294 a	0.255 c	55.6	163	452 a	0.097 c	28	125
B. radicans	189 bc	0.546 a			43 cd	0.134 a		
Coefficient of variation (%)	27.0	11.4			35.6	7.6		

[1]Days after emergence

2Values calculated using the isotope dilution technique using B. radicans as control.

significant ($P < 0.05$) differences between the ^{15}N enrichments of the three different control crops but all were far higher in ^{15}N enrichment than any of the nodulated legumes which indicated that the legumes were all obtaining considerable contributions from BNF (Table 3). For each nodulated legume, three estimates of the BNF contribution could be made both by using the total N difference method (total N in nodulated legume - total N in control crop) and the isotope dilution technique and these estimates are displayed in Fig 6. It is apparent that the three different estimates of BNF for each nodulated legume calculated from the total N difference are more diverse than the three estimates derived from the isotope dilution technique. Furthermore, the estimates for cowpea are negative and hence obviously erroneous. This was due to the fact that the cowpea grew very poorly due to fungal disease and accumulated very little N from the soil. These data show that, at least in this case, the isotope dilution technique yielded far better results than the total difference N technique. However, the date show that despite the considerable differences in ^{15}N enrichment between the different control crops the estimates of BNF contributions fall within a fairly narrow range. The probability that the true estimate of the BNF contribution lies within this range increases with increasing numbers and diversity of control crops.

At the second site (Brasilia) ^{15}N-labelled compost (Urquiaga et al.,1989) was used to label the soil. At this site there were smaller differences between the enrichments of the different control crops suggesting that this form of slow release N was superior to the gypsum pellets. This resulted in relatively small differences between the isotope dilution estimates of BNF contributions (Fig. 7) and leads us to recommend this technique of combining slow release fertilizer (or perhaps repeated small additions of soluble fertilizer) with the use of several (3 or more) control crops. The advantage of using several control crops of a diverse nature is that it gives the experimenter an idea of the range of possible ^{15}N enrichments that crops of different growth habits are able to obtain from a soil with a non-uniform ^{15}N enrichment.

Aknowledgements

Financial support for many of our recent studies on the quantification of biological nitrogen fixation has been provided by the Financiadora de Estudos e Projetos (FINEP) of Brazil, and by the U.S. National Academy of Sciences, National Research Council through a grant from the U.S. Agency for International Development.

Table 3 : final grain yield, and dry matter nitrogen and ^{15}N(1) recovery at mid-pod fill stage of 3 control crops and inoculated and non-inoculated soybeans, cowpea and groundnuts. Experiment 1, Riz de Janeiro. Means of 4 replicates.

Treatment	Dry matter accumulation(2) g.m-2	Total N(2) g.m^{-2}	% 15N excess	15N recovery mg 15N excess m-2	Grain(3) yield kg ha-1
Soybean inoc'd(4) 29 w	621	20.6a(5)	0.0134e(5)	2.56b(5)	
Soybean inoc'd(4)	638	20.3a	0.0104e	2.15bc	
Soybean non inoc'd(4)	589	18.8ab	0.0125e	2.32b	
Cowpea inoc'd	202	3.6de	0.0289cd	1.02cd	304
Cowpea non-inoc'd	144	2.5e	0.0315cd	0.78d	334
Groundnut inoc'd	669	16.3ab	0.0143e	2.14bc	1710
Groundnut non-inoc'd	593	14.7b	0.0219de	3.29b	2322
Sorghum	1142	9.7c	0.0574a	5.08a	
Soybean, non-nodulating	408	7.7cd	0.0398bc	3.04b	
Rice	443	5.2de	0.0484ab	2.67b	
Coefficient of Variation %	18.3	21.4	35.5	32.2	

1. 15N added as slow release gypsum pellets (Witty, 1983) at 2.15 kg labelled N/ha
2. Mid-pod fill stage, 110 days after planting, Harvested area = 1 m2/plot.
3. Nature grain stage, 117 and 141 days after planting for cowpea and groundnut, respectively. Harvested area 2 m2/plot.
4. Grain yield lost due to weevil attack.
5. Means followed by the same letter are not significantly different at P = 0.05 (Duncan).

Figure 6 : Total N difference (▨) and isotope dilution (⊓) estimates of the contribution of N_2 fixation to inoculated and non-inoculated soybean, cowpea and groundnut using 3 different control crops ; sorghum (S), non-nodulating soybean (Y) and rice (R). Experiment 1, Rio de Janeiro. Means of 4 replicates. Data from Boddey et al (1989).

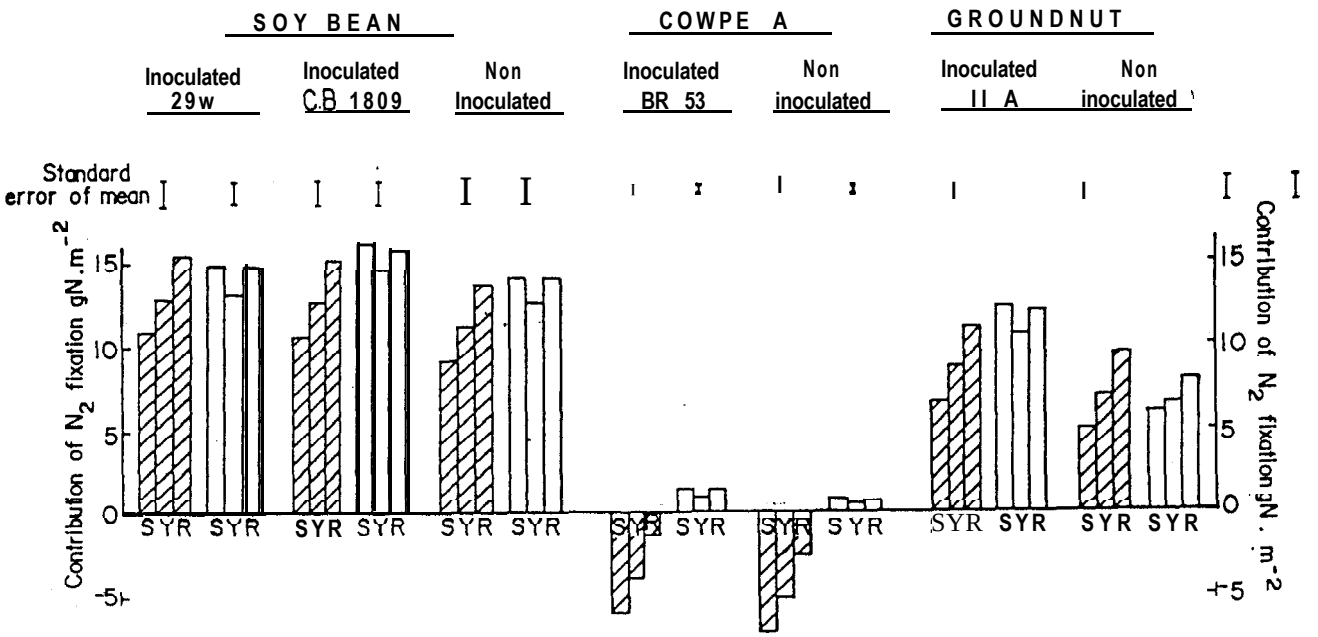
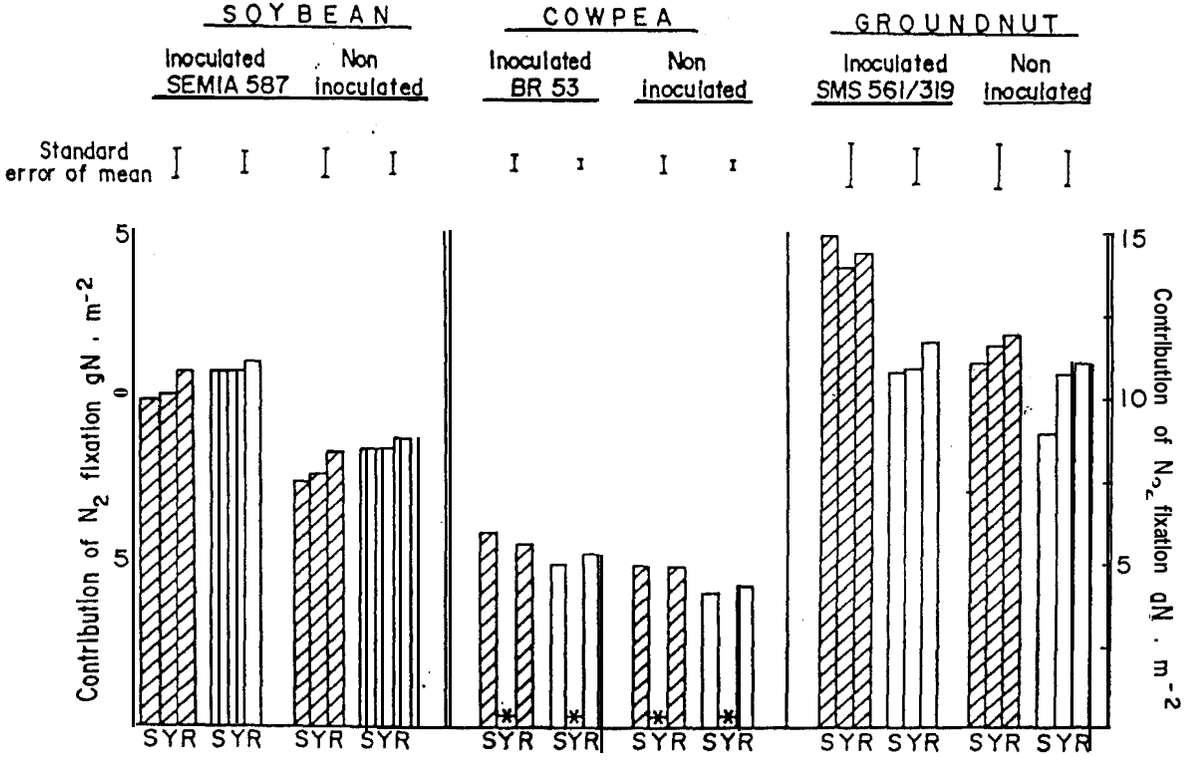


Figure 7 : Total N difference (▨) and isotope dilution (⊓) estima f the ntribu on of N_2 fixation to inoculated and non-inoculated soybean, cowpea and ground: using differ t control crops ; sorghum (S), non-nodulating soybean (Y) and rice (R). Exper .ts 2. and Brasilia. Means of 4 replicates. * Data not available. Data from Boddey et al (198



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Utilisation de la méthode ^{15}N pour estimer la fixation symbiotique de l'azote chez les plantes herbacées et ligneuses.

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Résumé

La méthode de mesure de la fixation symbiotique de l'azote basée sur les variations isotopiques naturelles de l'azote est un outil privilégié pour des études sur le terrain et en *conditions naturelles.* En effet, elle ne nécessite aucune intervention de la part de l'expérimentateur et n'entraine pas de perturbation du système étudié. Cet article veut mettre en évidence les exigences, d&e&nation de l'identité isotopique de l'azote fix6 et assimilé, et les contraintes de cette méthode, en particulier au niveau de l'échantillonnage, tant pour les plantes annuelles que pour les arbres. Les quelques exemples d'application donnés illustrent les grandes possibilités de cette méthode qui permet, en particulier, d'aborder l'étude des systèmes ligneux.

Introduction

L'estimation de la fixation symbiotique d'azote est une &ape essentielle dans toute étude des systèmes fixateurs. En effet, aussi bien au niveau globaliste, pour connaître les rentrées d'azote dans un écosystème et pour les optimiser, qu'au niveau réductionniste pour tester des cultivars ou des souches microbiennes sélectionnées ou transform&s,

la quantification s'impose. Aussi est-il nécessaire d'avoir des outils de mesures fiables à sa disposition.

Une nouvelle generation de spectrométres de masse à double introduction et double collection dune très grande sensibilité, a permis de penser qu'il Ctait possible d'utiliser les differences isotopiques namrelles de l'azote entre l'air et le sol pour estimer, chez les plantes fixatrices, la part d'azote venant de l'une et l'autre source (Delwiche and Steyn, 1970 ; Rennie et al., 1976).

Mais si les variations isotopiques naturelles de l'azote ont été mises en evidence dès 1939 par Schoenheimer et Rittenberg, variations qui, d'après Hoering (1955), auraient leurs explications dans les processus biologiques, leur utilisation darts la comprehension et la quantification des processus biologiques a été plus difficilement admise. Edwards (1975) trouve ces variations trop faibles pour Ctre exploitables. Les résultats de Kohl et al., 1971 sur la quantité de nitrate polluant la nappe phréatique calculée à partir des variations isotopiques naturelles ont soulevé une vive polémique (Hauck *et* al., 1972 dans "a questionable approach"). Bremner (1977) s'étonne, que les critiques formulées n'aient pas découragé les recherches à ce sujet et pour lui, même si ces variations existent, elles ne peuvent donner que des informations qualitatives.

Pourtant, depuis 1977, la méthode de mesure de la fixation symbiotique basée sur les variations naturelles des abondances isotopiques de l'azote s'est élaborée (Amarger *et* al., 1977, Bardin et al., 1977, Kohl *et* al., 1980). Si cette méthode présente certaines contraintes et limites, son champ &application reste très vaste et inclut même les arbres fixateurs d'azote. Sa spécificité reside essentiellement dans le fait qu'elle n'implique aucune intervention de l'expérimentateur et peut donc être appliquée sur les systèmes naturels sans les modifier ni les perburber.

Principe

Rappelons que l'abondance isotopique d'un compose azoté est le pourcentage du nombre d'atome ^{15}N par rapport au nombre total d'atomes d'azote. L'azote atmosphérique montre une composition isotopique très stable qui est estimée à 0,3663 ± 0,0002% ^{15}N (Mariotti 1984) et celle des composes azotés varie entre 0,3640 et 0.37% ^{15}N.

Ces variations sont liées à l'existence de fractionnements isotopiques. On peut défmir le fractionnement comme &ant la repartition différente des isotopes d'un element contenu darts le produit initial et final au cours d'une reaction chimique ou biologique. Les liaisons établies par l'isotope léger seraient plus aisémment brisées que celles établies par l'isotope lourd et les molécules contenant l'isotope léger réagiraient plus vite que celles contenant l'isotope lourd. Chaque processus biologique est une suite de plusieurs reactions et la teneur isotopique finale est une résultante de l'ensemble des fractionnements.

Le principe de la méthode de mesure de la fixation symbiotique basée sur les variations isotopiques nature&s de l'azote est simple : une plante fixatrice est aliment& par deux sources d'azote, l'azote moléculaire de l'air et l'azote minéral du sol. Si ces deux sources ont une abondance isotopique différente, il peut être possible, comme on le fait après un marquage, de connaître par un simple calcul de dilution isotopique, le pourcentage d'azote venant de chacune de ces deux sources. Tout consiste donc à determiner la valeur isotopique de l'azote venant du sol et celle venant de l'air.

Valeur isotopique de l'azote fixé

Même si on sait que lair a une valeur isotopique constante (Mariotti, 1984). celle-ci peut être modifiée lorsque l'azote moléculaire est métabolisé par une plante. Cette modification très faible, négligeable lorsqu'on utilise le marquage, peut entraîner, si on n'en tient pas compte, une grossière erreur dans l'estimation du taux de fixation pouvant aller jusqu'à donner des valeurs aberrantes. Cette méthode nécessite donc la determination de l'abondance isotopique de l'azote provenant de la fixation. Cette determination est faite à partir dune plante fixatrice poussant sur un milieu dépourvu d'azote et qui ne dispose que de l'air comme source d'azote.

Dans la pratique, la méthode utilise les valeurs isotopiques des parties aériennes des plantes fixatrices car souvent les racines sont difficilement accessibles. Dans ce cas, il est nécessaire de tenir compte également du fractionnement isotopique possible de l'azote se produisant à l'intérieur même de la plante. On a pu voir en effet, que les nodosités du soja et du robinier s'emichissaient en ^{15}N pendant que la partie aérienne s'appauvrissait en ^{15}N (Reinero et al., 1983, Domenach, 1985). Ce fractionnement lit à la fixation peut être different suivant l'espèce de la plante hôte (Mariotti *et al.*, 1980, Steele *et al.*, 1983) ou de la souche microbienne (Yoneyama *et* al., 1984). Si on a pu montrer que, si dans le genre *Alnus*, l'espèce *(A. incana* et *A. glutinosa)* et le type de souches de *Frankia* influencent peu la composition isotopique de l'azote fixé (Dommenach *et* al., 1988), la *valeur* trouvée ($\delta^{15}N$ = *-2)* est différente de celle du soja ($\delta^{15}N$ = -3). 11 est donc nécessaire de determiner pour chaque système fixateur étudié, le fractionnement isotopique se produisant à l'intérieur de la plante avant de pouvoir appliquer cette méthode. Ce fractionnement évolue au cours du temps au moins durant les premiers stades de développement de la plante (Shearer et *al.*, 1980 ; Fig. 1) puis semble se stabiliser. En effet, les feuilles d'aulnes âgés de 5-6 ans transplantés sur un milieu sans azote ont une valeur isotopique identique à celle de jeunes plantes de 8 mois (Domenach *et al.*, 1988). Aussi il est nécessaire de laisser pousser les plantes sur milieu sans azote pendant plusieurs mois avant de pouvoir d&z-miner la valeur isotopique de l'azote fixé.

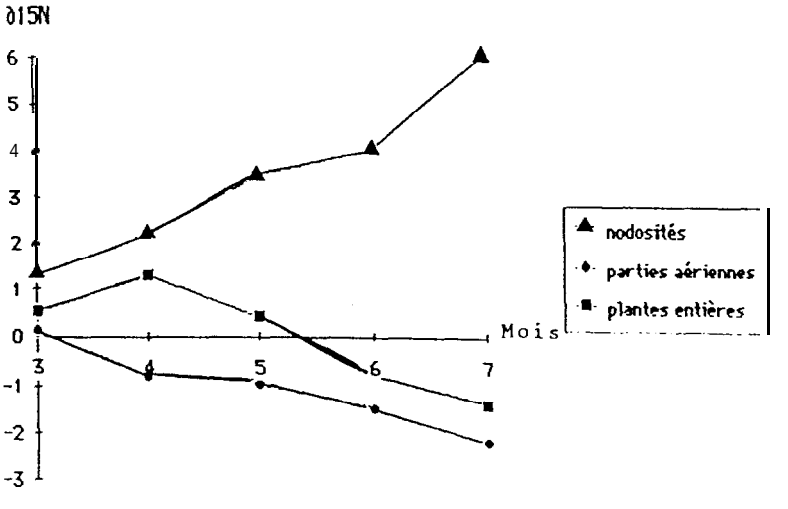


Figure 1: Evolution en fonction du temps du $\delta^{15}N$ des nodosités, parties aériennes et plantes entières de *Robinia pseudoacacia* poussant sur milieu sans azote (d'après Domenach, 1985).

Les valeurs trouvées de ce fractionnement varient d'un laboratoire à un autre et semblent en partie likes aux procedures de preparation des échantillons et au type d'appareil utilisé (Shearer and Kohl, 1986). Cette difficult& vient également du fait qu'il n'existe pas de gaz standard dont on connait la valeur absolue. Ces variations d'un appareil à un autre ne semblent pas génantes pour la mesure du taux de fixation si l'ensemble des mesures se rapportant à une experience sont faites avec la même méthode analytique et sur le même appareil. Ceci impose à chaque laboratoire de déterminer précisément la valeur de ses paramètres et de ne pas utiliser pour ses propres plantes les valeurs données par la littérature.

Devoir déterminer les fractionnements isotopiques liés à la fixation de chaque système symbiotique est une contrainte propre à cette méthode qui vient de l'exploitation de faibles variations isotopiques qui sont de l'ordre de 10^{-4} (une unité δ). En respectant cette contrainte la méthode peut être généralisable à presque tous les systèmes symbiotiques fixateurs.

Valeur isotopique de l'azote assimilé

Mesurer directement la valeur isotopique de l'azote du sol s'avère difficile dans le choix de la forme à mesurer (organique ou minérale). On sait en effet que le passage d'une forme à une autre provoque un fractionnement isotopique. De plus, la mesure des différentes formes d'azote du sol à un instant donné ne permet pas d'intégrer la valeur isotopique de l'azote assimilé par la plante durant toute sa vie. Enfin, un fractionnement isotopique peut parfois se produire au moment de l'assimilation et modifier l'abondance isotopique de l'azote provenant du sol (Mariotti et al., 1980). Aussi comme dans la méthode de marquage du sol de Fried et Middleboe (1977), on appréhende la valeur isotopique de l'azote assimilé par le biais dune plante non fixatrice poussant dans les mêmes conditions que l'on appelle plante de référence ou plante témoin. Lorsque ies études sont faites sur des systèmes annuels, cette plante témoin sera choisie en fonction de l'enracinement et de la période de croissance comparables aux plantes fixatrices étudiées. Dans les écosystèmes naturels on chosira les planks témoins ayant un développement equivalent à celui de la plante fixatrice. Toutefois, le problème de la plante témoin est moins crucial dans cette méthode que dans celle du marquage du sol (Rennie, 1982), car ii n'existe pas d'effets lies au déclin prononcé de l'emichissement du sol avec le temps, aucun engrais marqué n'ayant été rajouté. L'évolution de la valeur isotopique naturelle de l'azote d'un sol est lente car elle résulte de l'ensemble des processus liés à la formation de ce sol et la valeur isotopique mesurée de l'azote assimilé est peu différente d'une année sur l'autre. Dans les articles (Domenach and Corn-tan, 1984 ; Domenach and Kurdali, sous presse), on peut se rendre compte que les mesures faites sur une aulnaie montrent la grande stabilité de la valeur isotopique de l'azote assimilé et que le même champ de culture en donne la même valeur sur deux ans d'expériences.

Cheng *et al.* (1964) et Karamanos and Rennie (1980) ont mis en evidence une variation isotopique de l'azote en fonction de la profondeur du sol, aussi il est important que la plante non fixatrice ait un système racinaire exploitant la m&me zone de sol. Ceci est surtout vrai pour les écosystèmes naturels install6 sur des sols très évolués qui peuvent presenter ces variations. En système agricole, grace à la pratique des labours, la valeur isotopique de l'azote assimilé s'avère beaucoup plus homogène (Selles et *al.*, 1986). Ainsi, nous avons pu montrer, que si un soja génétiquement non nodulant représentait la plante de reference la plus satisfaisante pour une culture de soja, le sorgho pouvait également

remplir ce rôle (Domenach and Corman, 1984), comme le ray-grass peut l'être pour le trèfle (Bergersen and Turner, 1983) ou la luzerne (Ledgard et al., 1985). Les plantes fixatrices ne possèdent pas toutes une isoligne génétiquement non nodulante, aussi la possibilité d'utiliser une graminée comme plante de référence permet d'appliquer la méthode à ces dites plantes fixatrices et donc globalement permet une grande utilisation de la méthode. Ceci donne Cgalement la possibilité de choisir une plante non fixatrice bien adaptée à la region. Dans un système naturel, les plantes de reference sont imposées par le milieu. Elles seront donc des arbres si on veut estimer la fixation d'arbres symbiotiques. Ces arbres non fixateurs peuvent avoir des valeurs relativement homogènes (Fig. 2) ou comme

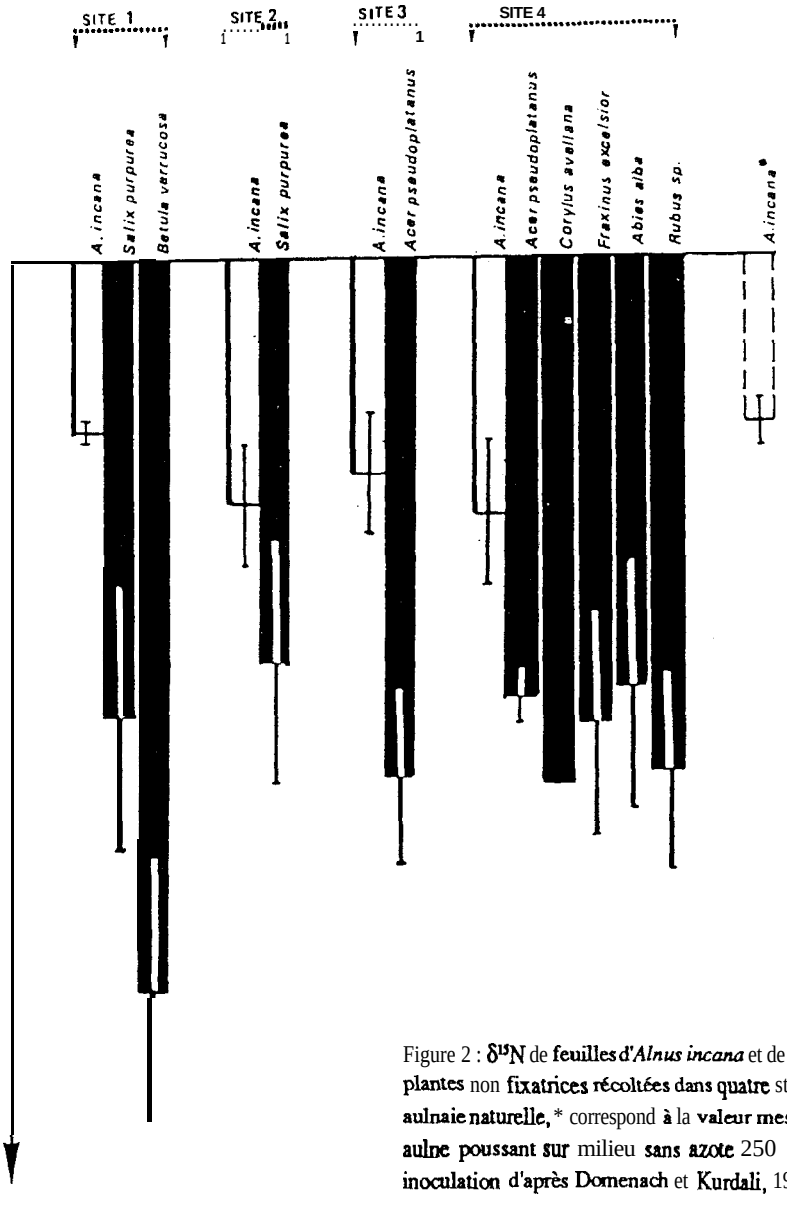


Figure 2 : $\delta^{15}N$ de feuilles d'*Alnus incana* et de différentes plantes non fixatrices récoltées dans quatre stations d'une aulnaie naturelle, * correspond à la valeur mesurée sur un aulne poussant sur milieu sans azote 250 jours après inoculation d'après Domenach et Kurdali, 1989).

le montrent Shearer et *al.* (1983), pour le desert de Sonoran, des valeurs assez hétérogènes (Fig. 3). 11 est alors nécessaire de prélever le maximum de plantes non fixatrices afin de rendre compte de l'hétérogénéité isotopique du milieu et de collecter les échantillons par paire. Chaque paire sera formée dune plante fixatrice et dune non-fixatrice les plus proches afin d'optimiser la similarité isotopique de l'azote que chacune de ces plantes assimile (Shearer and Kohl, 1986).

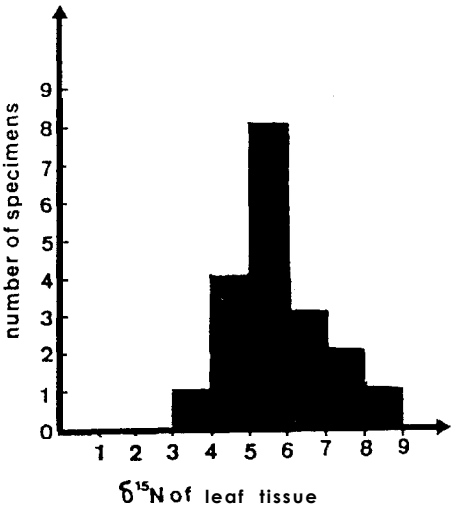


Figure 3 : Répartition du $\delta^{15}N$ des plantes non fixatrices du désert de Sonoran (d'après Shearer et al, 1983).

La méthode basée sur les variations isotopiques naturelles de l'azote nécessite l'existence dune difference entre la valeur isotopique de la plante témoin et celle de l'azote fixé. La precision depend directement de cette difference. Cette exigence marque une limite dans l'application de la méthode. La valeur de cette difference est fonction du sol et échappe à notre contrôle. Aussi, suivant les conditions du milieu et sans que nous puissions le savoir à l'avance, le taux de fixation ne pourra pas être mesuré ou au contraire, aura une valeur très precise. La précision des mesures peut se situer entre 5 et 25%. Les sols agricoles foumissent en général une valeur isotopique de l'azote assimilé largement plus haute que celle de l'azote fixé. Par contre, en sol de forêt tempérée, l'azote assimilé a une valeur isotopique souvent basse et même négative venant de l'importance de l'immobilisation comme le montre le modèle de Shearer et *al.* (1974). Cette valeur est alors peu différente de celle de l'azote fixé et entraine une estimation plus grossière de la fixation et parfois m&me entraine l'impossibilité d'utiliser cette méthode.

La valeur isotopique de la plante à étudier est une combinaison des valeurs isotopiques des dew sources azotées précédemment déterminées, l'azote assimilé et l'azote fixé.

Le problème se pose alors de savoir comment realiser l'échantillonnage de ces plantes. Les résultats de Reinero *et al.* (1983) et de Yoneyama *et al.* (1986) ont montré une grande hétérogénéité dans les valeurs isotopiques trouvées pour les différents organes d'un même soja au champ. Si on considère uniquement les feuilles de sojas nodules et

de sojas non nodules poussant dans les mêmes conditions (Fig. 4), on remarque une grande hétérogénéité liée à la position des feuilles (F1 correspondant aux feuilles les plus basses, F15, aux feuilles les plus hautes) et liées à leur stade phénologique (les valeurs isotopiques des mêmes feuilles sont différentes à la formation des gousses et à la maturation des graines). Sachant que des sojas poussant sur milieu sans azote ont un $\delta^{15}N$ très homogène (Mariotti, 1982), les variations observées dans la Fig. 4A sont likes à l'assimilation (fractionnement isotopique lors de l'assimilation ou evolution dans le temps du $\delta^{15}N$ de l'azote assimilé ?). On peut penser que la complete pertubation des valeurs mesurées à maturation des graines (Fig. 4B) proviendrait de la remobilisation de l'azote au profit des graines. Cette remobilisation affecterait plus spécialement l'azote fixé. En effet, si

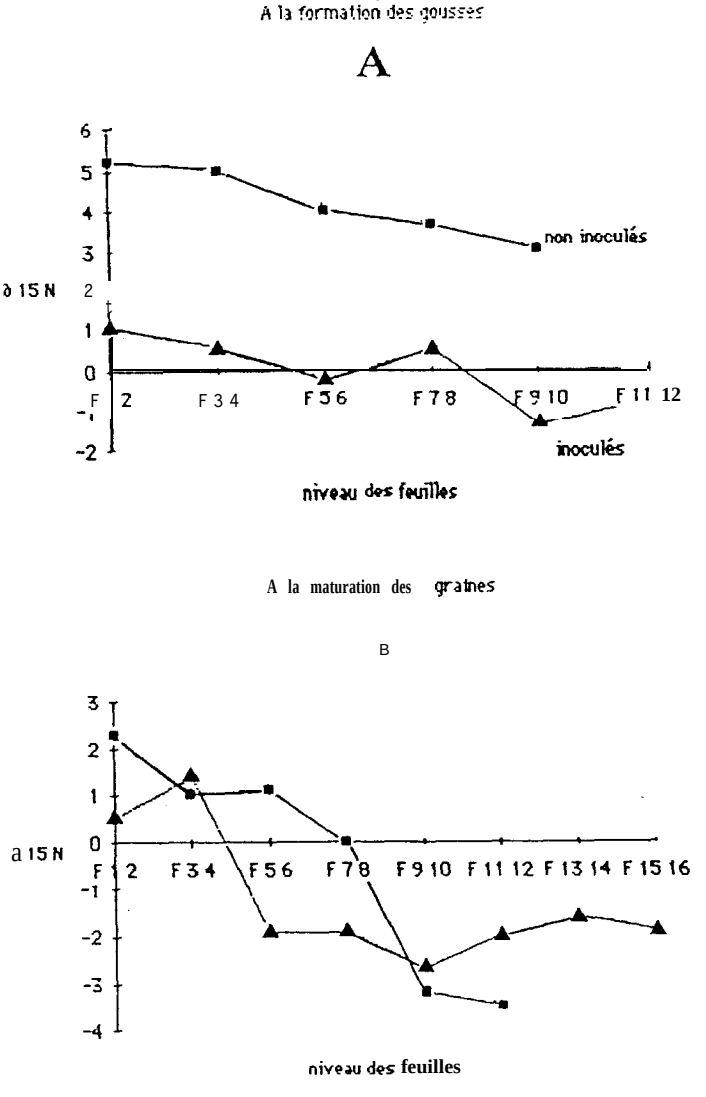


Figure 4 : $\delta^{15}N$ de feuilles de soja (Hodgson) inoculés et non inoculés en fonction de leur position sur la plante (F1 correspond aux feuilles les plus basses), à deux stades phénologiques.

on considère la valeur isotopique de la partie aérienne entière, la difference persiste entre les sojas nodulés et non-nodules alors que les valeurs isotopiques de leurs feuilles deviennent semblables. Cette remobilisation préférentielle de l'azote fixé a d'ailleurs été mise en évidence grace au $^{15}N_2$ par Warembourg et al. (1984.)

Ces réultats montrent que, s'il existe une variation isotopique de l'azote assimilé, celle-ci n'est pas génante dans la mesure où elle est prise en compte aussi bien par la plante fixatrice que par la plante non-fixatrice. De plus, ces résultats mettent en évidence l'importance de l'échentillonge dans l'application de la méthode basée sur les abondances isotopiques naturelles. En effet, pour connaître le taux de fixation d'une plante fixatrice herbacée, il est necessaire de prélever la partie aérienne entière de ces plantes : le fait de mesurer exclusivement les graines entraine une surestimation du taux d'azote provenant de la fixation et la mesure des feuilles pourrait entrainer, suivant la période de prélèvement, une sous estimation de ce taux.

Pour estimer le taux de fixation d'un arbre, il parait difficile d'analyser toute la partie aérienne de cet arbre. Les feuilles, production annuelle de ces arbres apparaissent comme le matériel de choix qui peut Cue utilise pour estimer le taux de fixation d'une période vegetative de cet arbre. Ainsi, si on choisit les feuilles, on se réfère à la production végétale de l'année alors que si on choisit le tronc ou les tiges, le phénomène d'accumulation interfere sur le résultat. La valeur isotopique de l'azote mesurée sur le tronc devrait représenter l'historique de la nutrition azotée de l'arbre alors que la valeur isotopique de l'azote des feuilles représente celle de l'année. Mais dans ces feuilles, une certaine quantité d'azote a été remobilisée à partir des reserves situées darts les racines (Domenach et Kurdali, 1989) : pour des aulnes agés de 5-6 ans les r&serves représentent encore 10% de l'azote des feuilles en fin de période végétative (Fig. 5). Cet azote remobilisé constitue avec l'air et le sol, une troisième source de nutrition dont il faut tenir compte si on veut estimer le taux d'azote fixé dans ces feuilles. Ce distingo perd de son importance si l'échantillon est constitué des dernières feuilles form&es, mais on risque ainsi de n'avoir qu'une

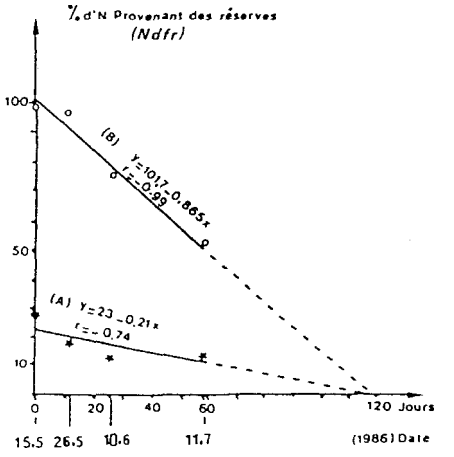


Figure 5 : Pourcentage d'azote provenant des réserves des racines dans les feuilles d'*Alnus glutinosa* et de *Populus alba* âgés de 5-6 ans et poussant en conditions naturelles durant toute la période de croissance. (d'après Domenach et Kurdali, 1989).

vue partielle de ce qui s'est passé dans la saison. Le même problème se pose dans la mesure de l'activitt fixatrice des légumineuses herbacées pérennes dont les parties aériennes sont composées de 20% de l'azote venant des réserves après 40 jours de repousse (Lescure et Chalamet, 1985).

Sur des aulnes en milieu naturel, les feuilles peuvent présenter une valeur isotopique relativement homogène ou au contraire, hétérogéne comme le montre la Figd. Les variations peuvent être le signe de l'existence de périodes de fixation et d'assimilation plus ou moins intenses. Il est donc important, si on veut avoir une estimation globale de l'azote fixé, de prélever, autant que possible, les feuilles sur l'ensemble de la hauteur. Les arbres constituent un type de matériel biologique malaisé à étudier, mais quelles que soient les difficult& d'application de la méthode basée sur les variations isotopiques naturelles, il convient de noter que c'est la seule utilisable sur les arbres en conditions naturelles aujourd'hui.

Méthode de calcul du pourcentage de fixation (%Ndfa)

La base de la méthode est simple et se résume à une application du principe de la dilution isotopique. Le calcul est le même pour les plantes herbacées ou ligneuses.

$$\%\text{Ndfa} = \frac{\delta^{15}\text{N pl. non fixatrice} - \delta^{15}\text{N pl. fixatrice}}{\delta^{15}\text{N pl. non fiiatrice} - \delta^{15}\text{N pl. fixatrice sur milieu sans N}} \times 100$$

Cette équation est de la forme : %Ndfa = (X-Y) / (X-C). L'écart-type peut être dond par la formule utilisée par **Shearer** and Kohl (1986).

$$A\,\%\,Ndfa \pm \left| \frac{(Y-C)}{(C-X)^2} \right| A \times \left| \frac{(\Delta Y)}{(C-X)} + - \right| + \left| \frac{(X-Y)}{(C-X)^2} \right| AC$$

Comme il n'est pas nécessaire de rajouter une source d'azote comme on le fait dans la méthode de marquage du sol, on n'utilise pas le concept de la valeur "A" ce qui supprime les problèmes liés à cette valeur. Toutefois lorsque l'on introduit des engrais, la méthode isotopique des abondances naturelles reste applicable mais peut poser quelques probèmes. En effet, les engrais, &ant fabriqués à partir de l'azote de l'air, ont souvent un $\delta^{15}N$ très bas ce qui a pour conséquence de diminuer la valeur isotopique de l'azote assimilé. Il en résulte une moins bonne précision dans la mesure de fixation. Cet inconvénient peut être pallié par le controle du rapport isotopique de l'engrais utilisé et si nécessaire, la modification de ce rapport par un infine apport de produit marqué.

Comparaison des méthodes marquage du sol et abondance isotopique naturelle

La possibilité de quantifier la fixation par la seule connaissance des variations isotopiques naturelles entre une plante fixatrice et une plante non fixatrice est confirmée par la comparaison des résultats obtenus avec ceux que donne la méthode de marquage du sol sur des plantes similaires.

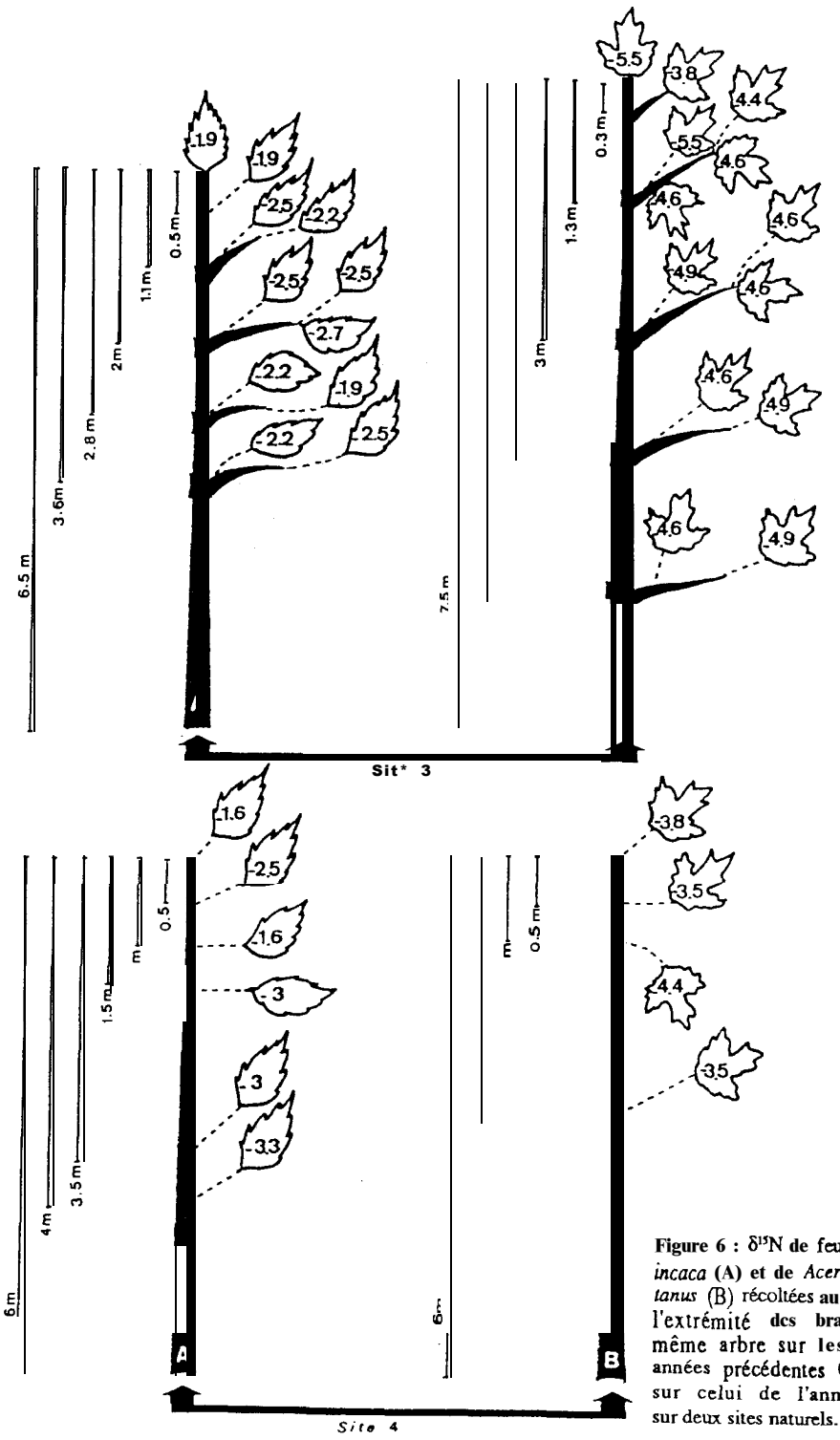


Figure 6 : $\delta^{15}N$ de feuilles d'*Alnus incaca* (A) et de *Acer pseudoplatanus* (B) récoltées au départ et à l'extrémité des branches d'un même arbre sur les bois des années précédentes (——) et sur celui de l'année (------) sur deux sites naturels.

Ces deux méthodes sont en effet comparables car tomes deux sont indépendantes des quantités d'azote assimilées, utilisent une plante de reference, le concept de la dilution isotopique, et donnent une valeur globale de la fixation. Seul le principe de base change : dans un marquage, on admet que les microorganismes et les plantes ne peuvent pas faire la difference entre ^{15}N et ^{14}N lorsqu'ils métabolisent l'azote alors que c'est sur l'existence de cette reconnaissance des deux isotopes par les microorganismes qu'est basée la méthode $\delta^{15}N$. Les deux principes ne sont pas contradictoires et I'un ou l'autre s'applique suivant l'échelle de precision dans laquelle on se place, les variations isotopiques naturelles n'étant sensibles qu'à 10^{-4}% ^{15}N. Cette comparaison de-s deux méthodes est essentielle pour établir la validité de la méthode basée sur les variations isotopiques naturelles même si peu de publications (Bergersen and Turner, 1983 ; Domenach et Chalamet, 1979; Ledgard et al., 1985) en font &at.

Le Tableau 1 montre que les deux méthodes donnent des résultats équivalents aussi bien sur les plantes herbacées que ligneuses justifiant l'emploi des variations isotopiques naturelles pour mesurer la fixation symbiotique de l'azote.

Si l'utilisation de la méthode $\delta^{15}N$ présente quelques limites et contraintes que nous avons soulignées tout au long de cet article, elle offre également beaucoup de possibilités. Comme la méthode de marquage du sol, elle fait partie des méthodes globales intégrant

Tableau 1 - Comparaison de deux méthodes d'estimation du taux de fixation symbiotique : marquage du sol et variations isotopiques naturelles de l'azote.

		% FIXATION	
TRAITEMENTS	plantes	marqué	non marqué
NH_4^+	Soja	60	68
		56	60
	Tréfle	42	42
NO_3^-	Soja	72	69
	Aulne	98	100
N-organique			
	Tréfle	45	47

Domenach et Chalamet 1979, Domenach and Kurdali 1989.

la fixation de toute la vie de la plante et permet ainsi d'avoir une estimation chiffrée de la contribution de la fixation dans un système donné. Elle est également indépendante de la production et n'utilise pas l'hypothèse d'égalité des quantités d'azote assimilé entre une plante fixatrice et une plante non fixatrice. Mais contrairement à la méthode de Fried and Middleboe (1977), cette méthode n'utilise pas de produits marqds. Cette différence implique de nombrenx avantages :

(i) il est possible de realiser des mesures sur l'ensemble du système étudié et non plus de se contenter de microparcelles comme on le fait avec le marquage, ce qui donne une meilleure image de la fixation réelle, d'un champ par exemple ;

(ii) tous les problèmes liés à l'hétérogenéité du marquage due à la difficult6 d'une application homogene de l'engrais disparaissent ;

(iii) le choix de la plante témoin est plus large car le rapport isotopique de l'azote du sol est homogene et ne varie que très lentement au cours du temps, différemment du déclin très rapide du $\%^{15}N$ dun sol après un marqnage ;

(iv) enfin et surtout, elle n'implique aucune intervention de l'expérimentateur et peut donc être appliquée sur les écosystèmes naturels sans les perturber ni les modifier. Cette méthode apparait ainsi la mieux adaptée à la mesure d'activité fixatrice des arbres sur qui par le fait même de leur taille, la méthode à l'acétylène ou les méthodes de marquage semblent délicates à apphquer.

La grande difficult6 tenant à la méthode vient des valeurs isotopiques de l'azote assimilé souvent basses en forêt dont la consequence est de n'avoir qu'une estimation grossière de la fixation.

Champs d'application

Si on peut disposer d'un spectromètre de masse, l'emploi de cette méthode in situ est très aisé puisque la seule intervention de l'expérimentateur consiste à prélever des échantillons au moment opportun. Son champ d'application est très vaste et quelques exemples peuvent rendre compte des nombreuses possibilités qu'elle offre :

❶ Cette méthode pourrait permettre, à la manière de Virginia and Delwiche (1982), de réaliser des criblages de plantes peu connues dans des milieux vierges. En effet, ces auteurs, en réalisant des mesures isotopiques systématiques sur les plantes présumées non fixatrices, ont pu mettre en evidence qu'une rosacée, ***Chamaebatia foliolosa***, était en fait fixatrice, ce qui, par la suite, a pu Cue confirmé.

❷ Estimation des entrées d'azote dans un système agronomique ou naturel. Des estimations de taux de fixation de plusieurs légumineuses cultivées dans différentes regions de France (Tableau 2) montrent la plus ou moins bonne adaptation du pouvoir fixateur de ces plantes au climat, ce qui ne signifie pas que les dites plantes n'aient pas un bon rendement m&me si leur taux de fixation est bas.

Tableau 2 Estimation des rentrées d'azote dans différents systèmes par le biais de la fixation symbiotique.

	% d'azote fixé			
Stations	Aulne*	Soja	Luzerne	Trèfle
Ornon	98			
Montpellier		*40-75*		
Dijon		10-20	*58*	*56*

* Apport de litière : 700kg N ha^{-1}

(d'après Bardin et al 1977, Domenach and Corman 1984, Domenach and Kurdali 1988).

On pourrait ainsi préciser, pour les différentes espèces de plantes, les zones géographiques où se situe I'optimum de leur capacité fixatrice. Un exemple de la très grande adaptation au milieu et au climat est donné par des aulnes, arbres à actinorhizes dont la repartition des espèces depend naturellement des conditions de milieux. Par exemple en France, *Alnus incana* est localise en montage et *Alnus glutinosa*, au bord des rivières. Les mesures faites sur ces arbres croissant dans leur milieu naturel, soit pour *A. incana*, poussant en altitude sur un support constitué par des moraines et pratiquement dépourvu d'azote, soit pour *A. glutinosa* poussant en plaine sur un sol riche en azote, montrent la très grande capacité de ces arbres à fixer l'azote de l'air (Tableau 3). On comprend mieux ainsi le rôle pionnier que ces arbres fixateurs peuvent avoir dans la constitution d'un sol et l'intérêt de boisements mixtes permettant le développement d'essences plus nobles.

❸ Optimisation des entrees d'azote par la fixation symbiotique d'azote.

(i) par la meilleure connaissance de l'impact des facteurs de l'environnement sur cette activité. Un exemple est donné par Etude de l'influence du stress hydrique sur la fixation du soja (Obaton *et* al., 1982 ; Domenach and Corman, 1985). Cette estimation est faite sur des cultures en champ ayant supporté des stress hydriques durant l'optimum de leur cycle nitrate-réductase (stress A.N.R., arrêt de l'irrigation de mi-juin à mi-juillet) ou nitrogénase (stress A.R.A., arrêt de l'irrigation au mois d'août) ou les deux (stress A.N.R. + A.R.A., arrêt de l'irrigation de la mi-juin à

Tableau 3 - Estimation du taux d'azote de fixation de deux espèces d'aulnes dans leur milieu naturel, Alnus glutinosa (arbre de plaines, de bord de rivière), Alnus incana (arbre de montagne, poussant sur des moraines),

Espèces	pH	% C organique	% N total	% $CaCO_3$	% N fixé
A glutinosa	7.1	2.7	Ø.33	Ø	95±13
A incana	8.3	Ø.5	Ø.Ø6	64	98±20

la fm aofit). Les mesures obtenues à partir de la méthode des abondances isotopiques naturelles montrent la grande sensibilité de la fixation au manque d'eau alors que l'assimilation est moins touchée par ces stress (Pig.7, Tableau 4).

(ii) par la recherche du couple symbiotique le plus efficace et le mieux adapté aux conditions de milieu Sélection de la souche microbienne : s'il est aid de tester des souches au laboratoire, il est difficile de préjuger de leur capacité en conditions naturelles de culture. Pour connaître les modifications que ces souches peuvent apporter dans la nutrition azotée de leur plante hôte, la mesure de fixation s'impose (Tableau 5). La méthode à l'acétylène per-met de classer les souches en fonction de leur activité nitrogénasique mais ne peut pas donner une estimation quantitative de l'azote fixé et surestime le pouvoir fixateur des souches comme la G49 qui présente une faible capacité hydrogénasique.

(iii) sélection de la plante hôte : par la connaissance du rendement et des capacités fixatrices de différents cultivars de sojas (Tableau 6), ou d'aulnes de différentes origines (Fig.8), il est possible de choisir, pour un sol donné ou une region donnée, la plante hôte qui offrira le meilleur compromis qui tient compte à la fois du rendement et des capacités de fixation de I'azote.

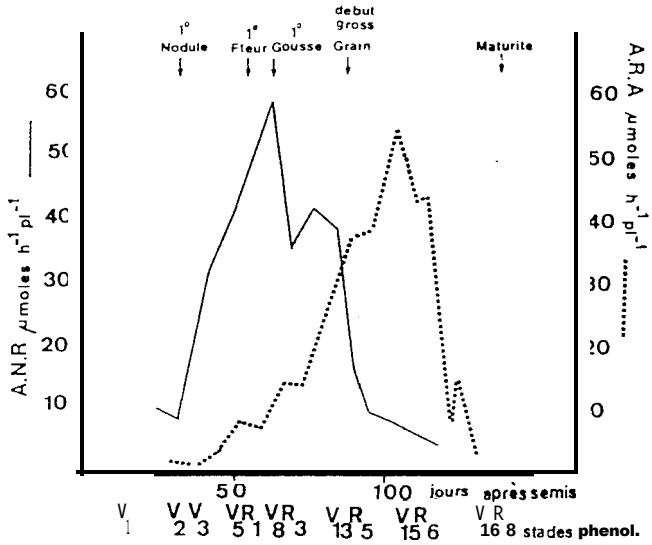


Figure 7 : Activités nitrate réductase (A.N.R) et réductrice de l'acétylène (A.R.A) chez le soja (Hodgson) au cours du cycle végétatif à l'évapotranspiration maximale (E.T.M) (d'après Obaton *et Al.*, 1982).

Tableau 4 - Influence du stress hydrique provoqué lot-s de l'optimum de l'activite nitrogenasique (stress A.R.A.) et de l'activité de la nitrate-réductase (stress A.N.R.) sur la fixation et l'assimilation de l'azote.

Conditions hydriques	% N fixé	Quantités assimilées ($mg.pl^{-1}$)
M.E.T.	41	750
Stress A.N.R.	25	540
Stress A.R.A.	16	491
Stress A.N.R.+ A.R.A.	0	410

M.E.T. : Maximum d'evapo-transpiration. (Domenach and Corman 1985)

Tableau 5 - Soja (hodgson) poussant sur sol et inocules avec différentes souches de Bradyrhizobium japonicum.

Souches	% N fixé	QN fixé mg/plante	ARA. nm./pl./h.	$\delta^{15}N$ nodosites
G1	25	149	202	2.5
GA	36	293	1105	5.2
GMB1	81	1140	1614	8.7
G49	69	1736	2374	9
G3S	87	1948	1777	9
G2sp	77	1990	2317	9.8

Avec la collaboration de J.C. Cleyet-Marrel, INRA, Montpellier, laboratoire des Symbiotes des Racines.

Tableau 6 - % d'azote fixé par différents cultivars de sojas inoculés avec la même souche (G3 Sp.)

Variétés	% N FIXE E.T.M.	Quantité N fixé par plante (mg)
-tardives		
106-17	93	1120
Amsoy	75	857
A100	70	831
-précoces		
Hodgson	57	579
Dobrudza	70	258
20-18-82	59	230

Avec la collaboration de M. Obaton, INRA, Montpellier, Laboratoire des symbiotes des racines.

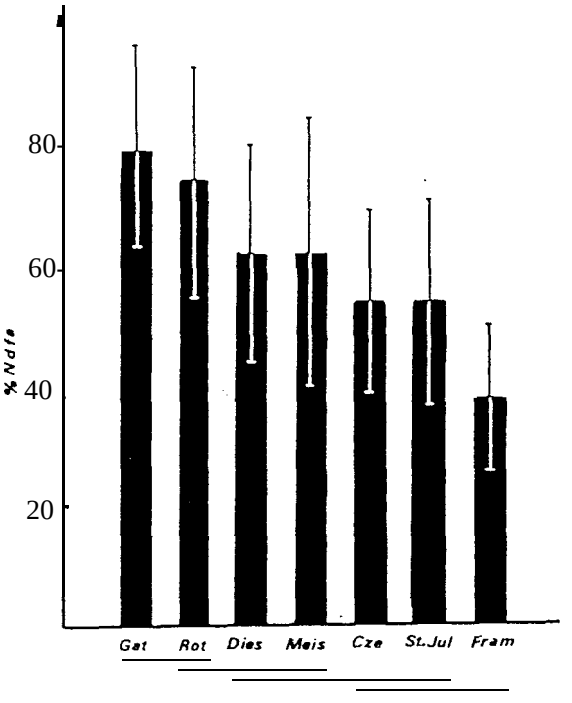


Figure 8 : Pourcentage d'azote fixé dam les feuilles d'*Alnus glutinosa* de différentes origines récoltées dans une plantation d'arbres âgés de 5 ans.

Conclusion

Quoique spectaculaires, ces résultats sont obtenus pour la plus grande part, à partir d'essais réalisés en pays tempérés. Pour connaître toutes les potentialités de la méthode d'estimation de la fixation symbiotique d'azote basée sur les variations isotopiques naturelles, il serait nécessaire de la tester sous d'autres types de climats (tropical, semi-désertique...). Egalement, peu de travaux utilisant cette méthode ont été réalisés sur les écosystèmes naturels et il est difficile de se rendre compte des limites que le milieu peut imposer (en particulier sur la valeur isotopique de l'azote assimilé à partir du sol).

Si les travaux qui seront effectués dans le futur confirment et complètent les larges possibilités de cette méthode, celle-ci pourrait permettre une meilleure connaissance des écosystèmes naturels, et être particulièrement utilisée en foresterie, domaine dans lequel les méthodes classiques ne peuvent ou que difficilement être appliquées.

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Nitrogen fixation in tropical trees : Estimations based on ^{15}N techniques

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Abstract

Because of the importance of trees in agroforestry. there is a crucial need to assess the magnitude of N_2 fixed by them, directly in the field The long-term evaluation of the N_2-fixing potential and actual amounts of N_2 fixed in a given tree raise problems in perennial plants esp[illegible] duration of [illegible] in obtaining reference crops over several seasons. For young or small trees, ^{15}N procedures similar to those adopted in gram or pastnre legumes would be expected to give equally satisfactory results. The greatest problems with N_2 fixation estimations using ^{15}N will occur in mature trees, due to their perennial nature and massive sizes, leading to logistic and sampling difficulties or differences in $^{15}N/^{14}N$ ratio of soil due to diierences in nitrogen turnover processes that occur under the fixing and reference crops with time. The influence of these effects may differ depending on how the ^{15}N is applied. An examination of which of the existing ^{15}N procedures, e.g. isotope dilution, A-value or the natural ^{15}N abundance and what method of ^{15}N application should be adopted under different situations is therefore urgently needed. In the paper we have considered the strategies for obtaining representative sampling (as against the whole destructive plant sampling) that would closely reflect the overall ^{15}N enrichment in the whole plant, and the selection of the appropriate reference tree. The advantage in using the ^{15}N labelled plant samples for additional agronomic studies besides N_2 fixation, e.g. contribution of tree litter to plant N uptake and soil organic matter are briefly discussed.

Introduction

In recent years the potential of leguminous and actinorhizal trees has aroused considerable interest., especially as regards to their socioeconomic importance and increased use as firewood, paper pulp, forage and timber (NAS, 1979 ; Dommergues, 1982 ; Torrey, 1982). These nitrogen fixing trees (NFTs) contribute to restoration and/or maintenance of soil fertility (Kang, 1985) and are very important in sound tropical soil management for sustained agricultural productivity, and controlling soil erosion and degradation.

Until recently however, few studies have been conducted on the examination of root nodules (Allen and Allen, 1981) and the magnitude of atmospheric N_2 fixed by different *Rhizobium* or *Frankia* strains, key factors in the successful growth of NFTs in nitrogen deficient soils. To maximize the contribution of this natural and inexpensive source of N in agroforestry systems, reliable methods are needed for quantifying biologically N_2 fixed in trees.

Various methods, such as the total nitrogen difference (Comet *et al.*, 1985 ; Gauthier *et* al., 1985 ; Sanginga *et al.*, 1985, 1986 ; Ndoye and Dreyfus, 1988), acetylene reduction assay (Roskoski, 1981; Högberg and Kvarnström, 1982 ; Lulandala and Hall, 1988) nodule number and mass (Roskoski, 1981 ; Högberg and Kvamstrom, 1982) and ureides production (Kessel *et* al., 1987 ; Sanginga *et* al., unpublished data) have been used to assess N_2 fixed by NFTs. Most of these techniques, analyzed in several critical reviews (Fried *et al.*, 1983 ; Rennie and Rennie, 1983 ; Danso, 1985, 1986 ; Dommergues, 1987), are based on indirect criteria, and are either qualitative or cannot distinguish between the various sources of nitrogen in a fixing plant.

Nitrogen-15 techniques, that involve the application of ^{15}N labelled materials to soil have been suggested in many studies to be the most reliable method for quantifying N_2-fixed (Legg and Sloger, 1975 ; Rennie, 1982 ; Vose *et* al., 1982 ; Rennie and Kemp, 1983 ; Hardarson *et* al., 1984 ; Danso, 1985 ; Danso *et al.*, 1986). These ^{15}N techniques are particularly useful as unlike many others, they provide an integrated measurement of the atmospheric N_2 fixed by field-grown crops during the experimental period and can discriminate among the various N sources available to the crop.

The ^{15}N substratum labelling techniques were first used to estimate N_2 fixed in pasture and gram legumes. They have however not been used much in tree N_2 fixation studies. Experience gamed from grain and pasture legumes would therefore be valuable for extending this technique to trees.

This paper examines critically the advantages and difficulties reported for the various techniques involving the application of ^{15}N-labelled fertilizers to soil, and the reliability of the method based on the differences in the natural abundance of ^{15}N between soil and atmospheric N_2 to measure N_2 fixed and how these could be used in devising suitable procedures for tree N_2 fixation assessment Some estimates of N_2 fixed in various NFTs under greenhouse and field conditions using these methods will be also reported.

Basis of the ^{15}N methods in NFT studies

There are two main approaches of the ^{15}N techniques for assessing N_2-fixation : the natural abundance of ^{15}N and the W-labelled fertilizers. The ^{15}N natural abundance is based on the small differences in ^{15}N abundance which frequently occur in nature between atmospheric N_2 and soil N, in contrast to ^{15}N substratum labelling techniques where the

larger differences in ^{15}N between the soil N and atmospheric N_2 are induced by applying ^{15}N labelled materials.

Both approaches are however in essence, based on the same principle, i.e. the higher $^{15}N/^{14}N$ ratio absorbed from soil N by the N_2-fixing plant decreases during fixation by incorporation of the lower ^{15}N enrichment of atmospheric N_2 ; the more N_2 that is fixed, the greater this dilution. Since it is not possible to assess the integrated $^{15}N/^{14}N$ ratio of the soil N over a growing season by chemical extraction a non-fixing reference plant is used for this purpose.

Several articles have reviewed the concepts, equations and procedures used in applying the ^{15}N methods for measuring nitrogen fixation of grain and pasture legumes (Shearer et al., 1978 ; Knowles, 1981 ; Fried et al., 1983 ; Rennie and Rennie, 1983 ; Danso, 1985, 1986 ; Dommergues, 1987 ; Hardarson *et* al. 1987). The researcher must be aware that there are however as yet no generally accepted procedures which can be claimed to be valid under all conditions. NFTs present specific problems which deserve special attention.

Factors affecting the validity and precision of N_2 fixation estimation by trees using ^{15}N-labelled materials

Reference tree

The accurate determination of the actual amounts of N_2 fixed in the field is crucial only in some instances, such as in N-balance studies in agro-ecological systems i.e. agroforestry, or in comparing N_2 fixed in different seasons, years and environments. In these cases the reference crop constitutes the main potential source of error in the ^{15}N technique. The criteria used in the selection of suitable reference crops have been discussed by Fried *et al.*, (1983) and Danso (1985, 1986). However, according to Danso (1986) and Danso *et* al. (1986) the need for the precise quantification of N_2 fixed may not be that compelling in studies to simply compare treatment effects or rank plant genotypes or *Rhizobium/Frankia* strains for N_2 fixing abilities.

Uninoculated N_2-fixing legume or actinorhizal trees have often been used as reference crops (Table 1). Since no indigenous rhizobia or *Frankia* were present in the experimental soil the uninoculated nitrogen fixing trees were found to be suitable reference crops (Comet *et* al., 1985 ; Gauthier *et al.*, 1985 ; Sanginga *et* al., 1986 ; Sougoufara et al., 1988). However, care must be taken to thoroughly examine roots of such uninoculated trees to ensure that they are not nodulated or run an acetylene reduction test to confirm the absence of nitrogenase activity. Cross-contamination of uninoculated control from inoculated treatments has been observed in our pot experiment involving NFTs (unpublished data) and has also been reported by others (Sougoufara *et al.*, 1988). Using such controls, N_2 fixation measured by the isotope dilution and the difference methods were underestimated, (Sanginga *et* al., unpublished data). It may be difficult to avoid cross-contamination in the field and even in the greenhouse unless special precautions are taken (Bromfield and Ayanaba, 1981). If possible, uninoculated control plants should be compared with other potential reference crops such as known non nitrogen-fixing trees. The non-fixing trees, however, have to fulfill the conditions outlined by Fried et al. (1983) and Danso (1986) to be an appropriate reference crop. The validity of such selections can be established by comparing the isotopic composition of nitrogen with that of non-nodulating isolines (Fried *et* al., 1983). Since non-nodulating tree genotypes have not

yet been identified, further search for non-nodulating fixing trees in natural populations or their development (e.g., through mutation induction) are most valuable for the ^{15}N methodology and attempts have commenced. A simple procedure can be planting of several hundreds of *Casuarina* and *Allocasuarina* photosynthetic branches in a sandbath irrigated with heavy suspensions of *Frankia*. After a period of growth, the roots are examined for the absence of nodules. The identified potential non-nodulating isolines can be vegetatively propagated and again re-inoculated with *Frankia to ensure* that this trait is stable. Screening fixing trees for host and strain specificity could also provide suitable controls in some situations.

Table 1 : Reference plants for estimating N_2 fixation using ^{15}N techniques

Authors	Type of experiment	Fixing plant	Reference plant
GAUTHIER et al. (1985)	Field	Casuarina equisetifolia (I)	Casuarina equisetifolia (U)
CORNET et al. (1985)	Field	Acacia holosericea (I)	Acacia holosericea (U)
SANGINGA (1985)	Field	Leucaena leucocephala	Leucaena leucocephala
ZAHARAH et al. (1986)	Field	Leucaena leucocephala (I)	Setaria ancepts (U)
NDOYE and DREYFUS (1988)	Pot 1m3	Sesbania rostrata (I) S. sesban (I)	Sesbania rostrata (U) S. sesban (U)
SOUGOUFARA et al. (1988)	Pot (15,50,1000 l)	C. equisetifolia (I)	C. equisetifolia (U)
SANGINGA et al. (unpublished)	Pot (5 kg)	C. equisetifolia C. cunninghamiana Allocasuarina	Allocasuarina
SANGINGA et al. (1987. 1988)	Pot (5 kg)	Leucaena, Gliricidia Acacia albida	Eucalyptus Cassia siamea

I: Inoculated
U: Uninoculated

Genetic variation in ^{15}N fixation

Large plant-to-plant variation in nodulation and N_2 fixation have been reported for NFTs (Gauthier *et* al., 1985 ; Sanginga *et* al., 1988b; Sougoufara et al., 1987). The genetic heterogeneity of NFTs has hardly received the attention it deserves. Danso *et al.* (in preparation) indicated that this genetic variation may contribute to the poor precision in the estimates of N_2 fixation using the ^{15}N methodology. Duhoux and Dommergues *(1985)* observed that average percentage N_2 fixed based on tree sire, varied from 16 (in the smallest trees) to 70 (in the biggest-sized trees). In such cases, it is essential to select well-matched reference crops for each plant to lessen errors. Where plants can be grown by vegetative propagation, this variation could certainly be reduced, and this could be practical with many NFTs.

Labelling techniques

There are several practical questions on the techniques of applying the labelled ^{15}N materials which need to be addressed before designing ^{15}N experiments to measure N_2 fixed. These include the N rate of application and ^{15}N enrichment, chemical and physical form and time of application. There is evidence that labelling techniques can have a significant effect on estimates of N_2 fixed. These techniques, described in several reviews (Chalk, 1985 ; Danso, 1985, 1986), have mainly been used for grain and pastures legumes. The limited data available for NFTs indicate that much more work still needs to be carried out to develop adequate techniques for measuring N_2 fixation by NFTs.

(i) Type and rate of ^{15}N labelled fertilizer

The chemical form of the labelled fertilizer is important because of its influence on N_2 fixation. This is due to the difference in solubility, uptake by the reference and fixing crops, relative movement and biological transformations in the soil. The ^{15}N fertilizers used so far for NFTs have been in the form of NH_4, i.e. ammonium sulphate (Comet *et* al., 1985 ; Gauthier et *al.,* 1985 ; Zaharah et *al.* 1986 ; Ndoye and Dreyfus, 1988 ; Sanginga et *al.,* 1988a) although no specific reasons can yet be adduced for this preference. Lower cost of the ammonium sulphate, less interference with fixations and lower potential losses of N may be one of the reasons. Only recently, Sougoufara et al. (unpublished data) used urea but there is a high risk for significant N losses.

The amount of ^{15}N applied for labelling a tree will depend upon the N rate and the ^{15}N enrichment of the labelled material utilized. The N rates and ^{15}N enrichment used for grain and forage legumes can be readily adopted for small tree plants grown in the greenhouse or in field conditions.

If isotope-aided experiments are performed with large-sized trees, the amount of N already present in tree may constitute an extra-dilution factor of the ^{15}N applied. Under such conditions, it is highly advisable to conduct a preliminary experiment with few trees, to ascertain several questions, e.g. total N and its partitioning among tree organs, labelling techniques, as well as sampling procedures.

Single application rates of 2g N/m2, or 0.5 g N/tree enriched with 10 atom % N have been used to NFTs (Comet et al., 1985 ; Gauthier et al., 1985 ; Ndoye and Dreyfus, 1987). Sanginga et al. (1988) in a single application used twice this rate. Application rates will however vary for different NFTs, type of studies, locations and soil characteristics, and ^{15}N measuring equipment. As a general guide, the ^{15}N rate must be enough not to significantly interfere with N_2 fixation in NFTs (Danso, 1986), and also capable

of being detected within the sensitivity range of the ^{15}N measuring equipment. For an emission spectrometer assay, a higher ^{15}N level is needed for detection than by a mass spectrometer.

In most studies, the isotope dilution method, which involves the application of the some amount of N-15 labelled material to both the reference and fixing NFTs has been adopted (Table 2). Comet et al. (1985) and Gauthier *et al.* (1985) compared the "A value" with the isotope dilution method for estimating N_2 fixed by *Acacia holosericea* and *Casuarina equisetifolia* grown in a N-deficient sandy soil. To prevent N-deficiency in the reference crop so as to ensure comparable satisfactory growth as the N_2-fixing plant, a higher rate of N, 100 kg N/ha was added to the plots of the non-fixing reference crops, while a lower rate, 20 kg N/ha that would not significantly inhibit N_2 fixation was applied to the fixing crops. These authors found that the "A value" technique gave slightly lower estimates of N_2 fixation than the isotope dilution and the difference method. Although the "A value" expression is not dependent on fertilizer N addition rates and fertilizer recovery by both fixing and non-fixing plants, errors can be introduced with factors affecting differentially the applied fertilizer, e.g. losses due to leaching, denitrification, N turnover, etc.

Table 2 : Labelling techniques for N_2 fixation studies using ^{15}N methods

Authors	Type of experiment	Form	N Addition rate	Atom % ^{15}N excess	Comparison other methods
GAUTHIER et al. 11985)	Field	$(NH_4)_2SO_4$	20(I,U) 100(U)	10.5(I,U) 1.91 U)	Difference N A-Value
CORNET et al. (1985)	Field	$(NH_4)_2SO_4$	20(I,U)	10 (I,U)	Difference
SANGINGA (1985)	Field	$(NH_4)_2SO_4$	40(I,U)	10 (I,U)	Difference
ZAHARAH et al. (1986)	Field	$(NH_4)_2SO_4$	20(I,R)	10 (I,R)	-
NDOYE and DREYFUS (1987)	Pot	$(NH_4)_2SO_4$	20(I,U)	10 (I,U)	Difference
SOUGOUFARA et al. (1988)	Pot 115.50. 1000 1)	Urea	20(I,U) 100 (U)	9.19(I,U) 1.89 (U)	Difference N A-Value
SANGINGA et al. (1987, 1988)	Pot (5 kg)	KNO_3	20(I,U) 100(U)	10 (I,U) 2 (U)	Difference N A-Value
SANGINGA et al. (1987, 1988)	Pot (5 kg)	KNO_3	20 (I) 20,100(U)	10 (I,U) 2 (U)	Difference N A-Value

(ii) Methods, frequency and time of ^{15}N application

Several workers have reported that the decline in the soil $^{15}N/^{14}N$ ratio with time influences the accuracy of N_2-fixation estimates ; the lower the rate of decline, the less serious the errors associated with any mismatch between the N uptake by reference and fixing crops (Fried et al., 1983 ; Witty, 1983). This could be very important in field experiments involving NFTs because of the long period of growth. In this case a single initial application of labelled fertilizer may prove to be inadequate for measuring N_2-fixation over a long period. Multiple additions of small concentrations of highly labelled fertilizer to the soil, use of slow-release N fertilizers and ^{15}N-labelled organic matter offer great promise in N_2-fixation with NFTs, since the $^{15}N/^{14}N$ ratio would remain relatively stable due to the rather slow release of a small but fairly constant amount of N with time. The greatest problem with these approaches however is that when applied N rates are lower than needed to support a non-fixing crop over several seasons, the reference crop may grow poorly or even die.

The ^{15}N fertilizer has been applied to NFTs at seeding (Comet et al., 1985) or soon after germination (Sanginga et al., 1988) or at transplantation of the 3.5 month-old plants (Gauthier et *al.,* 1985). However, it has been proved by several workers that early growth of many NFTs is slow and that nodulation and N_2-fixation is delayed sometimes for several months (Reddell et *al.,* 1986 ; Sanginga et *al.,* 1988a). It is therefore advisable to delay ^{15}N application until both fixing and reference crops are well-matched in growth and probably in N uptake.

^{15}N labelled fertilizer has generally been applied in solution, although a few investigators (Sanginga et *al.*, 1988a) have broadcasted $(NH_4)_2SO_4$ on field plots and then incorporated it into the soil with water additions. Several other methods of ^{15}N application have been tried for grain legumes (Fried *et al.*, 1983 ; Chalk, 1985). They have been reported to give similar results and are thus all satisfactory ways of adding ^{15}N to soil for N_2-fixation studies. Methods of application and their influence on N_2-fixation estimate by NFTs have not been investigated yet. In some cases the soil injection may provide a satisfactory approach to apply the ^{15}N labelled fertilizer to similar root depths of both fixing and non-fixing trees. The choice of one over the other for NFTs will be dictated largely by the homogeneity of application, necessity of diluting the labelled materials and the objectives of the experimental work.

Cropping systems and nitrogen cycling

The integration of trees, especially NFTs, into stable agroforestry systems can contribute significantly towards restoring and maintaining soil fertility, combating erosion and desertification and in providing fuelwood. The potential of such trees has already been shown in alley farming systems in the humid tropics (Kang et al., 1981, 1985). In this system in which rows of NFTs alternate with several rows of food crops, the NFTs are periodically lopped, the foliage is used as green manure for food crops or as forage for animals and the stems provide fuel. However much of scientific background required for the development of agroforestry technology is still in its initial stage and almost nothing is known about the magnitude of factors that influence N_2 fixation and N cycling processes in this system.

The cropping systems could also affect N_2 fixation as shown in grain and pasture legumes (Danso *et* al., 1986) by influencing the suitability of the reference crop and therefore the validity of the estimates made.

Variations in N_2 fixing activity with the age of trees, or interference by different processes such as litter fall and its decomposition, and the redistribution of nitrogen in the different compartments of the tree/soil system may present some difficulties for measuring N_2-fixation using ^{15}N isotopic methods. Significant amounts of N_2 fixed by NFTs are undoubtedly finally incorporated into the soil, but no attempts have been made so far to quantify their magnitude and significance on soil N pool. This could be very important in agroforestry systems such as in alley cropping where NFTs and non-NFTs are grown in alternate rows. The soil nutrients under these contrasting plants have been reported to mineralize at different rates due to differences in litter quality (Swift, 1984, 1985). NFTs, because they contain more N than non-NFTs tend to contribute more N to the soil. This may lead to conditions where the fixing and non-fixing crops are not absorbing soil N from the same isotopic composition ($^{15}N/^{14}N$ ratio) during the growing period and thus invalidate one of the most important assumptions of N_2 fixation estimates (Chalk, 1985). These factors can significantly affect the application of the ^{15}N methodology. The periodic collection and an estimation of the N in fallen leaves in a given area could be used to assess the error of measurement caused by N in falling and decomposing litter.

Transfer of N_2 fixed by NFTs to associated reference non-NFTs could be another source of error for N_2-fixation estimates (Fried *et* al., 1983 ; Chalk, 1985 ; Danso *et al.*, 1986).

Sampling of plant material

Witty (1983) emphasized that N_2 fixation estimates based on isotope dilution are not estimates of the amount of N_2 fixed by a crop, but rather estimates of the amount of fixed N contained in the harvested portion of the crop. The ^{15}N enrichment in different plant parts of crops grown on ^{15}N enriched soils frequently differ (Fried *et* al., 1983 ;Rennie *et* al., 1978). Measurements of N_2-fixation, based on only one plant part are therefore not adequate because the ^{15}N enrichment of one plant part is not representative for the whole plant. This difference in enrichment among plant parts has been cited as a problem in the "N-isotope technique for estimating N_2-fixed (Heichel *et al.,* 1984 ; Chalk, 1985). According to Danso *et al.*,(1986) and Fried *et al.*,(1983), errors due to differences in ^{15}N enrichment can be minimized by sampling each of these plant parts with fairly uniform N-isotopic composition separately for N and ^{15}N determinations and using theweighted atom % ^{15}N as a basis for the N_2 fixation estimate for the whole plant (Danso et *al.*, 1986). This seems to be a standard procedure adopted in most N_2 fixation studies that use ^{15}N and is feasible for tree seedlings and young NFTs. Problems may occur when sampling big trees, because of their high above and below-ground biomass. In contrast to grain legumes, NFTs have significant proportions of their total biomass and N content below ground (Young 1985). In this case, quantification of N_2 fixed that disregards roots, nodules and crowns would result in serious errors and the proportion of N_2 fixed may be largely underestimated. In addition, for many perenniel legumes the N stored in these underground structures and unharvested portions of foliage may, after decomposition exert a significant effect on the dilution of the soil $^{15}N/^{14}N$ ratio. This is a practical problem

which may not be easy to resolve, given the difficulty in recovering all roots and litter.

As suggested by Danso (1986), it is advisable to estimate this stored N and make a correction for its effect on the isotopic composition of N at harvest, An examination of procedures for obtaining a representative sampling (as against whole plant sampling) that would closely reflect the overall ^{15}N enrichment of the whole plant is urgently needed.

In alley cropping and most the agroforestry systems however ^{15}N assay in leaf samples appears to be the practical method of sampling. The validity of the technique would be based on the assumption that any defined type of leaf sample would probably provide the same answer with regard to N_2 fixation of the whole plant. Leaves of similar age and morphological position should be sampled from the tree at various intervals after application of ^{15}N. The method of sampling however, should be established after a series of preliminary experiments. Another approach may involve the forester methods of using formula to estimate the standing biomass of trees. In this case, it should be possible sample a few trees to estimate small portions of different organs of the tree for ^{15}N enrichment and using the formula to estimate total N_2 fixed.

Sampling of NFTs constitutes the greatest problem in N_2-fixation estimation and ways to overcome this needs to be seriously addressed. These sampling procedure problems are further confounded by the practicality to use single trees usually as experimental units to cut down cost, a practice that introduces high variability in the estimates.

N_2 fixation estimates by some NFT using ^{15}N isotope techniques

The isotope dilution method has predominantly been used to evaluate N_2 fixation by a few trees in the field. Estimates of N_2-fixed of some NFTs by different methods used are summarized in Table 3.

The natural ^{15}N abundance based on the study of small differences between the natural abundance of ^{15}N in non-fixing and fixing trees has been used for tree by few workers (Khol and Shearer, 1981 ; Domenach, 1987). One of the first studies using this method was *carried* out on *Prosopis* in the Sonoran desert. The natural ^{15}N abundance in the tree was significantly lower than in the soil, indicating that it had fixed about 40 to 60% of its nitrogen though no nodules were found. *Prosopis was* presumed to develop nodules on deep roots which are not normally harvested (Virginia *et al.,* 1981). More recently, Högberg (Pers. comm.) reported in a pilot study of N_2-fixing potential of legume trees in a mixed tropical woodland in Tanzania that the N:P ratio and the ^{15}N natural abundance appeared to be more reliable indicators on N_2-fixation than the N content alone. However, its application is limited by the availability of a high precision mass spectrometer and special sample preparation procedures (Amarger *et al.*, 1979; Domenach *et* al., 1979). Other problems include the natural variations in atom % ^{15}N in soil with depth (Steele *et al.*, 1981) the spatial variability in the field (Shearer *et al.,* 1978), and the soil enrichment in ^{15}N during litter decomposition and subsequent plant ^{15}N uptake (Turner *et* al., 1983 ; Domenach, 1987). This could be important in agroforestry systems such as alley cropping where NFTs are sequentially cut and used as N source after decomposition (Kang *et al.,* 1985).

The feasibility of using ^{15}N-depleted material for assessing N_2 fixation of leguminous trees has only been investigated once (Kessel and Nakao, 1986). Significantdifferences in atom % ^{15}N and percentage N derived from N_2 fixation were measured in *Albizzia*

Table 3 : N_2 fixation estimates (Proportion and actual amounts of N2 fixed) by NFT's

Authors	Fixing plant	N2 fixation estimates					
		% Nitrogen derived from Atmosph.			N2 fixed from Atmosphere (g/pL)		
		Difference	Isotope dilution	A-value	Difference	Isotope dilution	A-Value
GAUTHIER et al. (1985)	C. equisetifolla	49	55	3 9	3.07	3.27	2.31
CORNET et al. (1985)	A. holosericea	29	33	32	1.12	1.35	1.26
SANGINGA et al. (1985)	L. leucocephala	39	40	ND	98*	134*	ND
ZAHARAH et al. (1986)	L. leucocephala	ND	78	ND	ND	230*	ND
NDOYE and DREYFUS (1987)	S. rostrata S. sesban	35 18	36 18	ND ND	0.59 0.12	0.62 0.13	ND ND
SOUGOUFARA et al. (1989)	C. equisetifolia	62	45	46	3.8	2.5	2.8
SANGINGA et al. (unpublished)	Casuarina, Allocasuarina	NO	30-80	30-80	ND	NO	ND
SANGINGA et al. (unpublished)	L. leucocephala A. albida Gliricidia sepium	ND ND ND	6-52 14-44 19-78	37-72 14-37 28-79	ND ND ND	4-37 3-9 13-40	21-47 3-11 28-52

ND = not determined
* = kg/ha/yr

lebbeck and leucaena indicating the possibility of ^{15}N depleted ammonium sulphate for measuring N_2-fixation and N-allocation in NFTs. However. difficulties such as those described for the natural abundance methods may limit the usefulness of this method for measuring N_2-fixed by trees.

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CONCLUSIONS ET RECOMMANDATIONS

Les recherches sur la fixation biologique de N_2 et leur impact présent et futur sur la production végétale et forestière en Afrique

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La troisième Conférence de l'AABNF qui s'est tenue à Dakar du 7 au 12 novembre 1988 revêtait, au moins autant que les précédentes, une importance considérable pour le développement de l'agriculture africaine. Les experts sont en effet unanimes à reconnaître que l'Afrique n'a que très faiblement bénéficié de la Révolution Verte en raison notamment des caractéristiques particulièrement defavorables de beaucoup de ses sols et de certains de ses climats. C'est seulement en recourant à un matériel végétal robuste et frugal, notamment en ce qui concerne l'azote, que l'on pourra rattraper le retard accumulé par rapport à d'autres continents. Il est évident que l'optimisation de la fixation de l'azote constitue une voie prometteuse mais encore insuffisamment exploitée pour atteindre cet objectif.

Dans cette note finale, on tentera de souligner les domaines dans lesquels les efforts entrepris devront être poursuivis et intensifiés. Ces domaines concernent plus particulièrement la maîtrise de l'inoculation avec les microorganismes fixateurs d'azote, l'étude qualitative des effets de l'inoculation et l'évaluation de la fixation de l'azote, les stratégies d'amélioration des partenaires des symbioses fixatrices d'azote et l'intégration des systèmes fixateurs de N_2 dans les systèmes culturaux.

Outre ces recommandations qui portent sur les programmes de recherche fondamentale ou appliquée, on indiquera les grandes lignes de la politique à suivre pour mener à bien ces programmes de recherche et assurer le transfert des résultats à l'application.

Maîtrise de l'inoculation

Identification des caractéristiques des sols

Il est absolument nécessaire que, contrairement à ce que l'on observe trop souvent, l'on fasse précéder les essais d'inoculation d'une étude systématique des sols. Cette étude doit consister essentiellement à déterminer les niveaux de populations de *Rhizobium* (ou *Frankia*) présents dans les sols que l'on utilisera. Compte tenu de l'état actuel de nos connaissances et, sauf cas particulier, si le nombre de *Rhizobium* compétents dépasse le seuil de 10^2 à 10^3 par g de sol, il ne faut pas s'attendre à une réponse significative. de l'inoculation. Cette règle relative aux *Rhizobium* ou *Frankia* s'applique Cgalement aux champignons mycorhiziens et Cventuellement aux bactéries fixatrices d'azote en association. Il importe de mettre au point des méthodes relativement simples et ne nécessitant aucun appareillage particulier pour déterminer approximativement ce potentiel infectieux des sols.

L'analyse chimique des sols effectuée conjointement devra consister à déterminer le pH et si possible en analyser le complexe absorbant, évaluer les teneurs en C, N, P (assimilable), K et Cventuellement permettre l'identification de carences en certains éléments.

Sélection des souches

Cette sélection sera effectuée à partir des collections de souches, notamment celles des MIRCENs.

Parmi les critères dont il sera nécessaire de tenir compte, le critère de spécificité devra être considéré avec attention. Il est désormais admis par beaucoup d'experts qu'il vaut mieux développer des souches spécifiques (c'est-Mire à spectre d'hôte étroit) que des souches à large spectre.

Conditionnement des inoculums

La tourbe est encoure le support le plus souvent utilise, mais d'autres supports méritent encore d'être essayés notamment dans tous les pays où il est difficile de se procurer la tourbe. L'usage des inoculums matriciels - résultant de l'inclusion des microorganismes dans une matrice de polymère - devrait être développé à l'avenir.

Survie des inoculums

Les inoculants doivent conserver leur pouvoir infectieux le plus longtemps possible au cours du stockage et même après l'application dans le sol ou sur les graines. Il est souhaitable qu'une méthode simple puisse permettre, dans un proche avenir, de vérifier la survie des bactéries dans l'inoculum immédiatement avant l'application de celui-ci au champ.

Pour répondre à la question de savoir si un inoculum doit être appliqué plusieurs années de suite, il est nécessaire que l'on mesure, dans différents sols et sous différentes conditions climatiques, le taux de survie des bactéries après la ou les premieres applications d'inoculum.

Etude qualitative des effets de l'inoculation et évaluation de la fixation de N_2

Effets spécifiques autre que la fixation de N_2

Il est certain que l'inoculation d'une plante avec *Rhizobium (ou Frankia)* peut modifier la distribution de l'azote dans la plante, cet effet étant mesuré par l'indice de récolte ; il peut y avoir aussi une modification du développement des parties aériennes par rapport aux racines, cet effet étant mesure par le rapport parties aériennes (shoots) / racines. On sait aussi que les ***Rhizobium*** peuvent, dans certains cas particuliers, réduire l'incidence de l'attaque des pathogènes.

Il apparaît nécessaire de développer les recherches de nature phénoménologique sur ces effets afin de pouvoir en entreprendre ensuite une étude mécanistique, ce qui implique le développement de recherches sur la physiologie des symbioses considérées.

Evaluation de l'azote fixé par voie biologique dans les écosystèmes

Il convient de souligner ici qu'avant de choisir la méthode d'estimation à utiliser (notamment s'il s'agit dune méthode isotopique qui peut être relativement coûteuse) il est indispensable de définir les objectifs précis de l'expérimentation. C'est ainsi que la sélection de clones d'une plante donnée en fonction de son pouvoir fixateur de N_2 ne requiert pas obligatoirement le recours à la méthode isotopique.

Il faut être toujours conscient (i) du fait qu'aucune méthode n'est parfaite, (ii) de la nécessité de mettre en place une ou plusieurs plantes témoins et (iii) de la nécessité d'apporter le plus grand soin au marquage du sol (lorsque l'on utilise la méthode de dilution isotopique).

Les problèmes spécifiques aux arbres ont été évoqués à plusieurs reprises au cours de la conférence. On ne les énumérera pas ici, mais il apparaît nécessaire d'attirer l'attention sur ces problèmes dont certains risquent de passer inaperçus.

Il est bien connu que l'extrapolation au champ d'essais effectues en pot est très risquée. Toutefois, les résultats rapportes suggèrent que la culture de plantes dans des bacs de très grandes dimensions (tanks) pourrait, dans une certaine mesure, se rapprocher de la culture en plein champ. Il est donc recommandé de faire appel autant que possible à ces dispositifs expérimentaux.

Stratégies d'amélioration des partenaires des symbioses fixatrices de N_2

Ces stratégies sont bien connues dans leur principe ; elles portent à la foi sur la plante hôte et sur le microsymbiote. Jusqu'à présent, elles ont été fondées essentiellement sur l'emploi de méthodes conventionnelles qui consistent à exploiter la variabilité naturelle des populations microbiennes ou végétales. Mais grâce au développement récent de la biologie moléculaire, on peut envisager avec optimisme la mise au point de nouvelles techniques d'amélioration des deux partenaires de la symbiose.

Dans le cas des arbres fixateurs de N,, la stratégie fondée l'exploitation de la variabilité intraspécifique des plantes hôtes et la multiplication végétative des individus les plus performants devrait permettre, dans un très proche avenir, de mettre à la disposition des agriculteurs et forestiers un matériel végétal à haut rendement et doté d'un potentiel fixateur de N_2 considérablement accru.

Intégration des plantes fixatrices de N_2 dans les systèmes culturaux

Jusqu'à présent, il est certain que, sauf rares exceptions, peu d'efforts ont été consacrés à l'étude de l'intégration des plantes fixatrices de N_2 dans les systèmes culturaux qu'il s'agisse de cultures mixtes (multiple cropping), cultures alternées (intercropping), ou cultures séquentielles (sequential cropping), ou de systèmes agroforestiers. Il apparaît indispensable : (i) de chercher à identifier et à quantifier les interactions entre les cultures composant les systèmes et les microorganismes du sol notamment ceux qui interviennent dans le cycle de l'azote ; (ii) d'évaluer avec précision les économies d'azote que l'intégration des plantes fixatrices de N_2 permet d'obtenir dans les différents systèmes culturaux ; (iii) de mettre au point les méthodes d'aménagement des différents systèmes de façon à tirer le profit maximum des apports d'azote provenant de la fixation et de façon à assurer au mieux la conservation et la régénération des sols.

Ces différentes investigations se heurteront à des difficultés qu'il faudra tenter de surmonter, ces difficultés étant liées notamment (i) à l'évaluation de la fixation biologique de N,, (ii) à l'évaluation des transferts d'azote des plantes fixatrices aux plantes non fixatrices de N_2, (iii) aux problèmes inhérents aux recherches sur le terrain, recherches qui doivent être conduites dans des conditions simulant aussi parfaitement que possible celles de la ferme.

Politique de formation, de collaboration et de vulgarisation

A l'unanimité, les séminaristes sont arrivés à la conclusion que l'effort de formation entrepris devait être amplifie, étant entendu que cette formation devrait être continue.

Une attention particulière a été accordée à la conception des programmes établis en collaboration dans le sens Nord-Sud et Sud-Sud, ces programmes devant idéalement présenter l'un et/ou l'autre des caractères suivants, c'est-à-dire être : (i) focalises sur des thèmes bien précis ; (ii) limités à des zones géographiques présentant de grandes similarités sur le plan écologique ; (iii) fondés sur l'exploitation des découvertes récentes ouvrant la voie à des applications pratiques importantes (ex. découvertes des susbtances élicites des gènes nod excrétées par la plante hôte) ; (iv) considérés comme prioritaires dans une région donnée.

La vulgarisation des résultats obtenus au niveau du laboratoire ou de la station de recherche devrait faire l'objet d'un effort tout particulier. Il s'agit là dune activité différente de celle du chercheur de laboratoire ou de station et à laquelle des chercheurs, ingénieurs et techniciens spécialisés devraient être affectes. Dans le cadre de cet effort de vulgarisation, il est souhaité que l'on quantifie, partout où ce sera possible, les bénéfices qui pourront découler, pour le paysan, des nouvelles technologies mises à sa disposition.

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Maximiser la fixation Biologique de l'Azote (FBA) est une des voies prometteuses pour développer l'agriculture et la foresterie en Afrique dans le contexte général de l'économie de l'azote.

La 3è conférennce de l'Association Africaine pour la Fixation Biologique de l'Azote (AABNF) qui s'est tenue à Dakar du 7 au 12 Novembre 1988 a particulièrement souligné les domaines dans lesquels les efforts entrepris doivent être poursuivis et intensifiés. Cette 3è conférence comprenait les sessions suivantes :

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