

BIOLOGICAL NITROGEN FIXATION

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Summary

Biological Nitrogen Fixation estimate the amount of fixed nitrogen and to select the most effective rhizobial strain x plant genotype combination. The ¹⁵N techniques are currently the most accurate method to measure the nitrogen fixed in a given system. The elite strains will be then used for the inoculum production.

However, whatever the elite strains are, the quality control of the inoculum must be performed before any use to maximize the BNF process

1. Introduction

Nitrogen is an essential element for plant growth and development and a key issue of agriculture. Most studies indicate that nitrogen fertilizers contribute to resolving the challenge the world is facing, feeding the human population. The Green Revolution was accompanied by an enormous increase in the application of nitrogen fertilizer. There is however a high heterogeneity of its distribution throughout the world: some areas subjected to pollution whereas others to depleted soil, decreased crop production, and other consequences of inadequate supply.

Biological Nitrogen Fixation (BNF) is known to be a key to sustain agriculture and to reduce soil fertility decline. Research on microorganisms and plants able to fix nitrogen contributes largely to the production of biofertilizers. Thus it is important to ensure that BNF research and development will take into account the needs of farmers in the developing countries mainly.

UNESCO has already addressed this challenge through the Microbial Resources Centre (MIRCEN) initiative. Two BNF MIRCENs were thus established in East (Kenya) and West (Senegal) Africa and focused on the diffusion of rhizobium technology including isolation, identification, collection, maintenance and distribution of rhizobial cultures and inoculants for leguminous crops. Considerable experience in the network approach has been gained through these MIRCENs, which functioned as the bases of projects in BNF technology for East and West Africa.

Below are described briefly some key issues of life supporting system related to basics and applied BNF technology emphasized to rhizobial bacteria (Table 1).

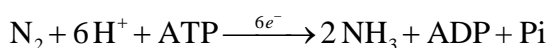
<i>Genres</i>	<i>Espèces</i>	<i>Plantes hôtes</i>	<i>Références</i>
	R. leguminosarum		Jordan, 1984
	biovar viciae	<i>Pisum, vicia, Lathyrus, Lens</i>	Jordan, 1984
	biovar trifolii	Trifolium	Jordan, 1984
	biovar phaseoli	Phaseolus vulgaris L.	Jordan, 1984
Rhizobium	R. galegae	Galega orientalis	Lindstršm et al, 1989
(croissance rapide)	R. tropici	P. vulgaris L., Leucaena	Martinez-Romero et al, 1991
	R. etli	Phaseolus vulgaris	Segovia et al, 1993
	R. hainanensis	<i>Lég. des régions arides et salées</i>	Chen et al.,1994a;Chen et al.,1997

	R. gallicum	Phaseolus vulgaris L.	<i>Amarger et al., 1997 *</i>
	R. giardinii	Phaseolus vulgaris L.	<i>Amarger et al., 1997 *</i>
	R. mongolense	Medicago ruthenica	<i>Van Berkum et al., 1998</i>
	R huautlense	Sesbania herbaceae	<i>Wang et al., 1998</i>
	M. loti	Lotus	<i>Jarvis et al., 1982</i>
	M. huakuii	Astragalus sinicus	<i>Chen et al., 1991</i>
Mesorhizobium	M. ciceri	Cicer arietinum	<i>Nour et al., 1994</i>
(croissance	M. hainanensis	Sev. arid reg. pl. sp.	<i>Chen et al., 1994</i>
Intermédiaire)	M. tianshanense	13 tropical pl. sp.	<i>Chen et al., 1995</i>
	M. mediterraneum	Cicer arietinum	<i>Nour et al., 1995</i>
	M. plurifarum	Acacia, Prosopis, Leucaena, Chamaescrista	<i>de Lajudie et al., 1994</i>
	M. amorphae	Amorpha fruticosa	<i>Wang et al., 1999</i>
	S. meliloti (a, b)	Medicago, Melilotus,	<i>Jordan, 1984 ; Eardly et al., 1990</i>
		Trigonella	<i>de Lajudie et al., 1994</i>
Sinorhizobium	S. mediceae	Medicago	<i>Rome et al., 1996</i>
(croissance	S. fredii	Glycine max, G. soja	<i>Chen et al., 1988 ; de Lajudie et al., 1994</i>
rapide)			
	S. saheli	Sesbania sp.	<i>de Lajudie et al., 1994</i>
	S. terangae		
	biovar acaciae	Acacia	<i>de Lajudie et al., 1994; Lortet et al., 1996</i>
	biovar sesbania,	Sesbania,	<i>de Lajudie et al., 1994; Lortet et al., 1996</i>
	S .xinjiangense	Glycine max, G	<i>Chen et al., 1988 ; de Lajudie et al., 1994</i>
	S. kostiense	Acacia, Prosopis	<i>Nick et al., 1999</i>
	S. arboris	Acacia, Prosopis	<i>Nick et al., 1999</i>
Allorhizobium	undicola	Neptunia natans	<i>de Lajudie et al., 1998</i>
(croissance			
rapide)			
Azorhizobium	A. caulnodans	Sesbania rostrata	<i>Dreyfus et al., 1988</i>
(croissance	A. sp.	Sesbania rostrata	<i>Rinaudo et al., 1991</i>
rapide)			
	B. japonicum	Glycine max, G. soja	<i>Jordan, 1982</i>
Bradyrhizobium	B. sp.	Vigna, Lupinus, Mimosa, Acacia,	<i>Jordan, 1982 ; Dupuy, 1994</i>
(croissance		Aeschynomene	<i>Alazard, 1985 ; Young, 1991</i>
lente)	B. elkanii	Glycine max	<i>Kuykendall et al., 1992</i>
	B. liaoningensis	Glycine max, G. soja	<i>Xu et al., 1995</i>

Table 1: Taxonomic evolution of nitrogen fixing bacteria of the family of *Rhizobiaceae* [from Yattara 2000]

2. Nodulation: From the Infection Process to the Functioning of the Nitrogenase

The production of nitrogen fertilizer by industrial fixation generates large quantities of carbon dioxide, contributing to earth warming. The natural process of BNF offers an economic means of reducing environmental problems and improving the internal resources. It is a process that allows microorganisms to convert atmospheric nitrogen (N₂) to ammonia (NH₃) assimilable by associated plants.



Different types of associations are listed in Table 2.

Types of association	Microorganisms	Host plants
Symbiotic	Bacteria (ex. <i>Rhizobium</i>) ^a Actinomycetes (ex. <i>Frankia</i>) Cyanobacteria (ex. <i>Anabaena azollaea</i>)	Legumes Actinorhiza Fern
Non symbiotic	Bacteria (ex. <i>Azotobacter</i> , <i>Azospirillum</i>)	Cereals
Free living systems	Bacteria (ex. <i>Thiobacillus</i> , <i>Clostridium</i>)	

Table 2. Different types of nitrogen fixing systems ^a : *Rhizobium*-leguminous plants is the most studied symbiotic association. There are six main genus of rhizobia : *Allorhizobium* (fast growing), *Azorhizobium* (fast growing) *Bradyrhizobium* (slow growing), *Mesorhizobium* (intermediate growing), *Rhizobium* (fast growing), *Sinorhizobium* (fast growing).

2.1. Nodule Formation

Leguminous plants and rhizobia communicate through the gene expression by reciprocally transmitting signals for the activation of the symbiotic genes in two partners. A type of phenolic called flavonoids, are released by host roots plants into the rhizosphere. Flavonoids act as a chemo-attractant for the bacteria to the plant roots, and eventually colonies of rhizobia attach to the root hairs. Flavonoid signal activates expression of nodulation (nod) genes.

In the rhizobial strains there are numerous nodulation genes including the nod genes nodABC and nodD (Figure 1). On the surface of the rhizobial bacteria the flavonoids are recognized by a expressed nodD protein. Then nodD binds to a promoter DNA sequence, and thereby activates transcription of nod genes of the operons. A group of nod genes encode enzymes synthesise the rhizobial nodulation signal, Nod factor (Figure 2), which triggers development of the root nodules by the plant. The plant roots

recognise Nod factor, through binding to to a surface protein receptor at the sub apical root tip. Perception of Nod factor induces a development inside the root, producing pronounced curling of the root hairs entrapping the rhizobia which establish infection of root. Bacteria gain access to plants cell membrane. The plasma membrane invaginates to form novel infection structure known as the infection thread, a tubular structure that extends from the root hair tip to the lower cells of the root cortex.

Rhizobia enter the infection threads in which they actively multiply. At the same time the underlying root cortex cells are quickly proliferated to constitute the nodule primordia. The infection threads branch out into cells of the nodule primordia. The rhizobia are finally released into the nodule cell and enveloped in a membrane derived from the host cell plasma membrane. At this stage, rhizobial bacteria become bacteroids able to fix nitrogen.

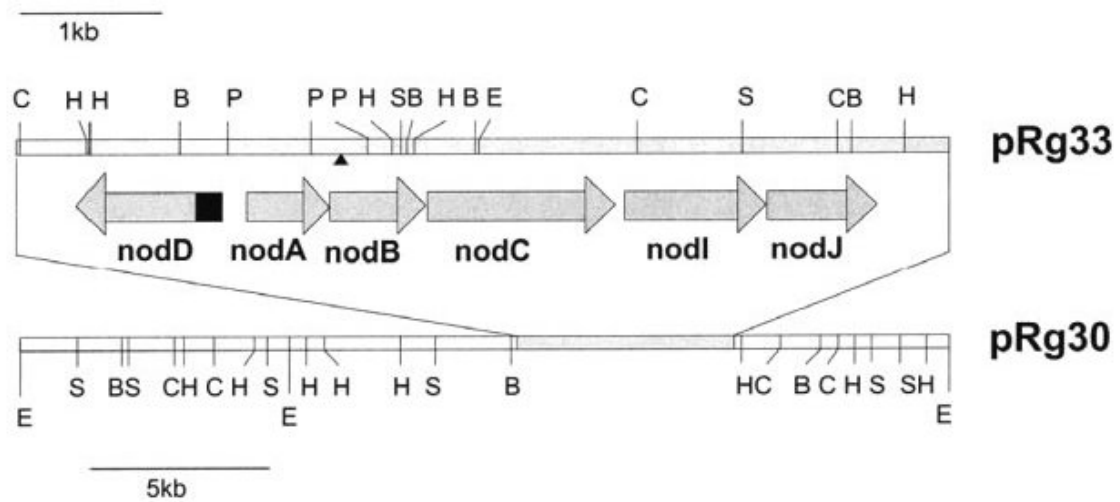


Figure 1. Restriction map of the common *nod* gene region of *Rhizobium galegae* HAMBI 1174. The cosmid clone pRg30 carries the six open reading frames homologous to *nodDABCIJ* genes subcloned in pRg33. ▲ indicates the site of the Tn5 insertion in pRg33. The black square indicates the *nod*-box sequence. Restriction enzymes used were as follows:

E = *EcoRI*; B = *BamHI*; C = *ClaI*; H = *HindIII*; P = *PstI*; S = *SalI*.

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Bibliography

Amarger N., Mariotti A. and Mariotti F. (1977). Essai d'estimation du taux d'azote fixé symbiotiquement chez le lupin par le traçage isotopique naturel (^{15}N). *Comptes Rendus de l'Académie des Sciences, Série D* 284 2179-2182. [This indicates an equation to estimate the nitrogen fixed by lupinus using the ^{15}N natural abundance].

Bardin R., Domenach A.M. and Chalamet A. (1977). Rapports isotopiques naturels de l'azote II. Application à la mesure de la fixation symbiotique de l'azote in situ. *Revue d'Ecologie et de Biologie du Sol* 14, 395-402. [in this work the ^{15}N natural abundance technique was used to estimate the fixed nitrogen].

Bliss, F.A.(1993). Breeding common bean for improved biological nitrogen fixation. *Plant and Soil* 152(1), 71-79. [Breeding procedure that allows maximum genetic gain for N_2 fixation may be used for screening common bean varieties].

Brockwell J. and Bottomley P.J. (1995). Recent advances in inoculant technology and prospects for the future. *Soil Biology and Biochemistry* 27, 683-697. [This is a review of the rhizobial inoculant technology emphasised on the importance of several limitations in the use of the inoculants].

Brockwell J., Herridge D.F., Morthorpe I.J. and Roughley R.J. (1988). Numerical effects of Rhizobium population on legume symbiosis. *Nitrogen fixation in legumes n Mediterranean agriculture* (ed. D.P. Beck and L.A. Materon), 179-193. ICARDA. [This is a report on relationships between rhizobial inoculum rates and nodulation and nitrogen content for soybean in field trials].

Broeshart H. (1974) ^{15}N tracer techniques for the determination of active root distribution and nitrogen uptake by sugarbeets. *International Proceedings Symposium on Nitrogen and Sugarbeet*, 121-124. International Institute For Sugarbeet Research, Brussels. [This is a description of a procedure to determine to estimate the portion of nitrogen uptaken by sugarbeet using ^{15}N technique].

Burris R.H. and Miller C.E. (1941). Application of ^{15}N to the study of biological nitrogen fixation. *Science* 93, 114-115. [This describes the earliest application of $^{15}\text{N}_2$ in N-fixation studies for estimating the amount of nitrogen fixed field condition].

Catroux G., Rivellin C. and Hartmann A. (1996). Practical aspects of legume inoculation : inoculum quality and inoculation efficacy. Consequences for the soil microflora. *Seminario microorganismos utiles para la agricultura y la forestacion*. Acts 1996 mayo 10-22.La Pampa. (eds F. Laich, N. Gonzales and H. Echeverria), 108- 125. INTRA/CERBAS, Balcarce, Argentina. [This is a description of the quality control of rhizobial inoculant, specially the number of rhizobia delivered per seed and for the presence of contaminants.].

Fried M. and Broeshart H. (1975). An independent measurement of the amount of nitrogen fixed by a legume crop. *Plant and Soil* 47, 707-711. [The amount of nitrogen fixed by a legume crop growing under normal field conditions is estimated using simultaneous determinations of the " A_{N} " values by the legume and a non-nodulating crop].

Fried M. and Dean L.A. (1952). A concept concerning the measurement of available soil nutrients. *Soil Science* 73, 263-271. [This work reports the equation for estimating the amount of fixed nitrogen when A-value method is used].

Fried M. and Middelboe V. (1977). Measurement of amount of nitrogen fixed by legume crop. *Plant and Soil* 47,713-715. [This work reports the equation for estimating the amount of fixed nitrogen when ^{15}N isotope dilution technique is used].

Hansen A.P., Rerkasem B., Lordkaew S. and Martin P. (1993). Xylem solute technique to measure N_2 fixation by *Phaseolus vulgaris* L.: Calibration and sources of error. *Plant Soil* 150, 223–231. [This is a use of the ^{15}N dilution technique to evaluate the relationship between the proportion of N derived from N_2 fixation and relative abundance of ureides in xylem sap for common bean (*Phaseolus vulgaris*) during vegetative and reproductive development].

Hardarson G (1990). *Use of nuclear techniques in studies of soil-plant relationships*. 223 pp. IAEA Training Course Series. No. 2. Vienna. [This issue described the different ^{15}N methods to estimate the nitrogen fixed by plants with many examples].

Hardy R.W.F. Burns R.C. and Holsten R.D. (1973). Applications of the acetylene-ethylene assay for measurement of nitrogen fixation *Soil Biology and Biochemistry* 5, 47-81. [The many uses of the acetylene-ethylene assay for investigations of the biochemistry of nitrogenase of N₂-fixing organisms, are reported.].

Herridge D.F., Holland J.F. (1993). Low nodulation and N₂ fixation limits yield of pigeon pea on alkaline vertisols of northern N.S.W.: effect of iron, rhizobia and plant genotype. *Australian Journal of Agricultural Research* 44, 137–149. [This is a description of selection of pigeon pea genotypes growing in vertisols of northern New South Wales by criteria symbiotic characteristics of nodulation and N₂ fixation].

Herridge D.F. and Peoples M.B.. (1990). Ureide assay for measuring nitrogen fixation by nodulated soybean calibrated by ¹⁵N methods. *Plant Physiology* 93, 495-503. [This reports experiments to quantify the relationships between the relative abundance of ureide-N in root-bleeding sap of nodulated soybean (*Glycine max*) and the proportion of plant N derived from nitrogen fixation].

Ireland J.A. and Vincent J.M. (1968). A quantitative study of competition for nodule formation. *Ninth International Congress of Soil Science Transactions*, Vol. 2, 85–93. Adelaide, Australia. [This reports some criteria for rhizobia to be competitive for nodulation].

McAuliffe C., Chamblee H., Huribe-Arango and Woodhouse W.W. (1958). Influence of inorganic nitrogen on nitrogen fixation by legumes as revealed by ¹⁵N. *Agronomy Journal* 50, 334-337. [This a report on use of N¹⁵-labeled fertilizer to determine the ratio of absorption of fixed vs. applied nitrogen by two legumes, alfalfa and clover].

Obaton M., and Rollier M. (1970). L'inoculation du soja : influence de la qualité de l'inoculum sur le rendement en grain et la richesse en protéine de la récolte. *Compte Rendu de l'Académie Agricole Française* 1174-1189. [This reports an increase in soybean yield in a field trial when the inoculation (*Bradyrhizobium japonicum*) rate per seed increased].

Peoples M.B., Faizah A.W., Rerkasem B. and Herridge D.F. (1989). Methods for evaluating nitrogen fixation by nodulated legumes in the fields. *ACIAR Monograph* No. 11, vii +76 p. [This describes simple and reliable methods for measuring nitrogen fixation].

Roche P., Maillet F., Plazanet C., Debelle F., Ferro M., Truchet G., Promé J.C. and Denarié J. (1996). The common *nodABC* genes of *Rhizobium meliloti* are host range determinants. *Proceedings of National Academy of Sciences* 93, 15305-15310. [This demonstrates that allelic variation of the common *nodABC* genes is a genetic mechanism that plays an important role in signaling variation and in the control of host range].

Schollhorn R. and Burris R.H.(1966). Study of intermediates in nitrogen fixation. *Feds Proc Fedn Am Socs exp Biol* 25, 710. [This is a knowledge that the nitrogen-fixing enzyme complex, nitrogenase, catalyses various reductions as well as the formation of ammonia from dinitrogen].

Suominen L., Roos C., Lortet G., Paulin L. and Lindström (2001). Identification and structure of the *Rhizobium galegae* common nodulation genes : evidence for the horizontal gene transfer. *Molecular Biology Evolutionary* 18, 907-916. [Phylogenetic analysis is used to examine the evolutionary relationships of the nodulation genes to the bacterial and the host plant genomes].

Vincent J.M. (1970) A manual for the practical study of the root nodule bacteria. IBP Handbook No. 15. Oxford, Blackwell Scientific Publications. Oxford, England. 164 p. [This handbook describes basic methods used in the study the root-nodule bacteria].

Yattara II (2000) Etude de la diversité et de l'écologie des bactéries rhizosphériques de *Dolichos lablab* Linn en vue de l'optimisation de sa croissance en zone sahélienne d'Afrique de l'Ouest. *Doctorat de Spécialité en Microbiologie* Université du Mali, Bamako. 112 p. [This is summary of classification of *Rhizobium* genus].

Biographical Sketches

Mamadou Gueye was born in 1950 in Dakar, Senegal, studied microbiology at the University of Lyon, France [1975-1982]. He studied for his doctorate at University of Lyon [1979-1982] on microbial ecology. After receiving his doctorate, he worked in the scope of biological nitrogen fixation (BNF) and

took up an appointment with Institut de Recherché pour le Développement (IRD, formerly ORSTOM) in Dakar [1979 – 1983] and Institut Senegalais de Recherches Agricoles (ISRA) in Senegal in 1983 for building up the West Africa MIRCEN. He participated and conducted numerous BNF training courses in Africa. His research area was BNF technology [*Rhizobium* inoculum production and quality control] and management of fixed nitrogen in cropping systems. He has participated as author or coauthor in numerous scientific publications in international journals. He also is member of editorial board of national and international journals. He is a member of various scientific companies, the Senegalese National Academy of Sciences and Technology (ANSTS) mainly since June 2003.

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