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Biotechnologies Using Dinitrogen Fixation as an Alternative to Traditional Agrochemicals (**)

1. Introduction

There are many possible alternatives for the development of agricultural systems which make full use of the potential of biological processes in the soil in order to replace as much as possible agrochemical inputs. These are especially predominant in the intensive, highly mechanised cropping systems of the industrialized countries but often are taken as examples in developing countries, especially on larger properties farmed by more educated people. These farmers are exposed to intensive advertising campaigns of multinational firms which tend to ignore the fundamental differences between temperate climate agriculture and the extensive much more diversified cropping systems of the tropics, and which are mainly interested in selling their products for profit.

At the other extreme, farmers in developing countries are exposed as much or even more than those in industrialized countries to excessive mostly demagogic theories propagated by certain ecologists who never analyzed critically properly planned experiments and therefore propose agricultural systems which usually are not viable, especially over longer periods of time.

Solid agricultural cropping systems must be based on scientific experiments with proper statistical designs, which produce reproducible results and must be economically viable. Very little such information is available, especially for tropical agriculture. The most promising systems are based on crop rotation or agroforestry systems such as those considered "traditional" before agrochemicals became "traditional", which can make maximal use of biological dinitrogen fixation (BNF)

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and of rock phosphates, and which also are the most logical approach to biological control of plant diseases and pests and in addition help soil conservation.

Scientific investigations of crop rotations, and even more so of agroforestry systems, require long-term experiments which are costly and not easy to perform, especially in tropical countries, where research funds are usually scarce. Such experiments need patience and much perseverance and hardly ever result in published papers, and there are not many scientists left to date who think in these terms.

Even though such systems attempt to recycle as much as possible of all nutrients, the minerals removed with the harvest have to be replaced in order to maintain soil fertility. The only two exceptions here are carbon and nitrogen, the two key elements of life. Natural vegetations like climax savannas and forests are closed systems where few elements are lost and where the energy used for respiration is obtained by photosynthesis, using sun energy. Once man interferes, other elements, mainly nitrogen, are rapidly lost through lixiviation. This usually results in rapid multiplication of legume annuals and trees which can help to restore the natural vegetation if other elements are sufficiently available.

In agricultural systems, leaching of soil nitrogen followed by the decomposition of soil organic matter is the main reason for declining soil fertility. Fortunately it is precisely this element for which most diversified alternatives exist to obtain it from the unlimited reserves of atmospheric dinitrogen. There are no eucarionts which can use atmospheric dinitrogen, and therefore the various symbiotic and associative systems of bacteria with higher plants represent the only major way of recycling nitrogen from the air. Biological dinitrogen fixation is therefore the most important single item within the various alternatives potentially viable for the replacement of agrochemicals. Tropical cropping systems, on the one hand, offer more favorable climatic conditions for biological processes during most of the year and also many more alternatives for the use of BNF, but on the other hand, due to the very same favorable climatic conditions, they favor soil erosion and loss of fertility.

It is the purpose of this lecture to summarize recent advances in BNF research in relation to tropical agriculture in order to point out the many already available possibilities and to focalize means to make better use of BNF in order to contribute to more conservative cropping systems. The Brazilian problems and solutions will be taken as examples because we know them best.

2. The Legume-*Rhizobium* Symbiosis

Even though there is an unlimited N_2 reserve in the atmosphere, this element is the major limiting factor of agricultural yields and represents, in developing countries, more than 70% of the fertilizer costs. Crop rotations, which include pulses and green manure legumes, can replace nitrogen fertilizers to a large extent, but such systems require well defined technologies. They are based on the exploitation of the highly sophisticated symbiosis of plants of the family Leguminosae which harbor in root nodules, more or less specific *Rhizobium* strains which are fed by the plant and in exchange furnish all the nitrogen necessary for optimal yields, in the form of combined N to the plant.

Grain legumes

One of the most successful examples of the impact of biotechnologies on the agriculture of a country is that of soybeans in Brazil. *Rhizobium* strain selection for Brazilian conditions was started for soybeans in 1940 (Freire, 1982). The Brazilian soybean cultivars, in contrast to the U.S. and Japan, were bred since the 1960's without nitrogen fertilizer and with highly efficient *Rhizobium* inoculants. As a result, this major export crop needs no N fertilizer and competes better in the world market. Brazil's highland edaphic savannas called "cerrados" comprising 180×10^6 ha are being rapidly taken into agriculture. Economically viable and highly productive farming systems must rely on crop rotations with legumes as their major nitrogen input, and soybeans are one of the major crops (4×10^6 ha in 1985). For more than 10 years the commercial soybean inoculants did not work in new lands until specifically adapted *Rhizobium* strains were found (Vargas and Suhet, 1980). These strains were found to be resistant to high levels of streptomycin, a characteristic later found to be a general feature of *Rhizobium* strains isolated from cerrado soils (Scotti *et al.*, 1980). Soils with similar problems occur in the Colombian llanos and also in newly cleared Amazon land planted to cowpeas (Döbereiner *et al.*, 1981a). The resistance to certain antibiotics, however, is not the only cause of better establishment of certain strains under adverse conditions. Tolerance to soil acidity problems (Munns and Franco, 1982) and saprophytic competence (Vidor and Miller, 1980; Peres and Vidor, 1980) play important roles.

Phaseolus beans, the major food basis of Brazilian people until 5 years ago, have not been bred for nitrogen fixation and therefore needed N fertilization for improved yields. A large cooperative research project has already yielded promising results as exemplified with the results in Table 1, where it is shown that cultivars and inoculants are already available which permit complete replacement of N fertilizer. These data stress the importance of plant breeding for N_2 fixation. As inoculation becomes a common practice, the physiological factors affecting nodule functioning and limiting seed production are of concern. Differences were found among *Rhizobium* strains in the efficiency of incorporation of the fixed N into seeds which were correlated ($r = 0.80^{**}$) with the N transported as ureide in the xylem sap (Hungria and Neves, 1984). Similar differences between *Rhizobium* strains were also observed in soybeans (Neves *et al.*, 1985).

Plants inoculated with selected strains transport practically all fixed N as ureides which is directly incorporated into grain proteins. Soybeans inoculated

TABLE 1 - Nitrogen fixation and yield in *Phaseolus vulgaris*.

Cultivar	Control		Fertilizer 100 kg N/ha		Inoculated		
	nodule wt (mg/pl)	grain yield (kg/ha)	nodule wt (mg/pl)	grain yield (kg/ha)	nodule wt (mg/pl)	grain yield (kg/ha)	N_2 fixed kg/ha[a]
Carioca	4	379	10	663	123	991	31.7
Negro Argel	46	494	22	620	155	883	18.4
Venezuela	3	378	5	601	39	438	3.6
Rio Tibagi	1	316	29	790	17	583	2.7

[a] As evaluated by $^{15}NO_3$ dilution.

[b] The interaction treatment x cultivar was significant for nodule weight ($p = 0.01$) and grain yields ($p = 0.05$).

with commercial inoculants transport the fixed N first into the leaves and produce 30% less. This places a new challenge on *Rhizobium* biotechnologies which will have to develop new ureide producing strains for the Cerrado regions.

Another important problem in the bean-*Rhizobium* symbiosis is excessive soil temperatures. Possibilities of selecting heat-tolerant strains are indicated in Table 2. In tropical forage legumes nodulation and N_2 fixation were observed to be even more tolerant to high temperatures (up to 40°C) (Lee and Döbereiner, 1982).

TABLE 2 - Selection of *Rhizobium phaseoli* strains for heat tolerance (Oliveira *et al.*, 1984).

Rhizobium strain	Temperature for *Rhizobium* growth (°C)	Nodule weight (mg/plant) Temperature for plant growth[a] Ambient	35°C
SEMIA 487	28	18.2	29.5
	35	21.9	34.7
SEMIA 4021	28	46.8	1.3
F 413	28	28.8	25.7
F 413 Mn	28	24.0	0.0
BR 292	35	24.0	35.5
SEMIA 4002	28	20.0	31.1
CO5	38	24.0	25.7

[a] Plants were grown in sterilized jars placed into waterbaths with either ambient temperature or 8 h/day at 35°C.

Legumes for green manure

A large variety of tropical legumes is available which can be planted in between crops either as intercropping or after the crop is harvested. Several of them can fix large amounts of dinitrogen and some assimilate phosphorus from rock phosphates which are unavailable for grain crops. The example in Table 3 shows that, e.g., *Stizolobium aterrimum* can obtain as much phosphorus from rock phosphate as from soluble phosphates. When such green manure cover plants are incorporated into the soil besides the organic matter, phosphorus and nitrogen are incorporated in slow release organic forms. These features will contribute substantially to make green manuring economically viable and a valuable constituent of crop rotations. An estimate of the economic returns of a crop rotation including such green manures in comparison with monocropping of maize is given in Table 4.

Forest legumes

Brazilian reforestation projects, until recently, did not consider one of the important characteristics of so many native legume trees: their ability to fix N_2. The most precious hardwood species and many native fast growing trees are legumes, but little is known about their capacity to nodulate or fix N_2. Surveys in the North Eastern dry regions (Vasconcelos and Almeida, 1979, 1980) in the Amazon rain forests (Bradley *et al.*, 1978; Magalhães *et al.*, 1982) and in South East Brazil (Faria *et al.*, 1984a,b) revealed many economically important N_2 fixing trees not known as such before (Table 5). Mesquite (*Prosopis juliflora*), called algaroba in Brazil, is being planted in large government projects in the North

TABLE 3 - Yield and mineral assimilation of two green manure legumes from rock phosphate (Silva *et al.*, 1985).

	Plant dry wt. (t/ha)	N kg/ha	P	K
Stizolobium aterrimum				
Thermophosphate	14.8	353	37.2	184
Rock phosphate[a]	14.0	318	35.8	164
Crotalaria juncea				
Thermophosphate	16.6	253	31.7	237
Rock phosphate[a]	8.4	151	15.7	86

[a] Phosphate from Patos de Minas.

TABLE 4 - Effect of crop rotation on maize yield (kg/ha) and profits of 5 years (F.F. Duque and G.G. Pessanha, in preparation).

	Maize yield 1982/83	Maize yield 1984/85	Profit all crops after 5 years (US$/ha)
Maize in monoculture	4480	1855	1178.51
Maize in crop rotation [a]	3696	2703	1869.35
With rock phosphate	4808	2671	1780.38
With rock phosphate and green manure [b]	5283	3023	1575.24

[a] The crop rotation from 1981 to 1985 was *Phaseolus* beans - maize - peanuts - cassava intercropped with cowpea - maize.
[b] Green manure was *Stizolobium*.

East dry regions and since 1982 is inoculated with commercially available inoculants developed by EMBRAPA.

The development of agroforestry systems which include in the crop rotation ten-year periods of legume forests which supply the farm with energy and emergency fodder during dry years and recover eroded soils, building up organic matter from the large amounts of protein-rich leaves which fall on the ground, seems another prospect as yet almost unexplored.

3. Cereals and Grasses

The extension of biological nitrogen fixation to the major cereals has been a major research challenge in the last two decades. Because plants as other

TABLE 5 - Nodulation of Brazilian forest legumes (Faria *et al.*, 1984a,b; Bradley *et al.*, 1978, 1980; Magalhães *et al.*, 1982; Vasconcelos and Almeida, 1979, 1980).

	Subfamilies			
	Mimosoideae	Papilionoideae	Caesalpinoideae	Total
Nº of species verified	60	75	72	207
Nº of species with nodules	51	53	9	113
Nº of species found for the first time with nodules	25	37	8	70
Nº of genera found for the first time with nodules	0	4	2	6
Nº of *Rhizobium* strains isolated	257	218	62	537

eucarionts cannot use molecular N_2, the most promising approach seems the search for more or less symbiotic associations of bacteria which are able to fix N_2 with cereals, which can be improved by modern technologies. The transference of N_2 fixation genes into plant cells seems a more pretentious alternative, which, if successful, could become the best solution. Unfortunately, progress in this field is very slow, while many new alternatives have become available during the last 15 years for improved already identified naturally occurring associations of cereals with N_2 fixing bacteria.

A typical result which leads to the conclusion that nitrogen fixation must occur under rice is that of App *et al.* (1980, 1984). In this study, nitrogen analyses of long term fertility plots in two sites of the Philippines were performed before and after 17 and 24 crops of paddy rice, yielding positive N balances of 103 and 79 kg N/ha per year respectively. Under temperate conditions, after 82 years of continuous wheat at the Rothamsted Broadbalk experiment, a positive N balance of 30 kg N/ha per year was estimated (Jenkinson and Rayner, 1977). Evaluations over shorter periods with forage grasses are in the same range (Jaiyebo and Moore, 1963; White *et al.*, 1945). More precise estimates over short-term periods can be obtained by the use of the isotope ^{15}N. There, either the incorporation of $^{15}N_2$ gas into plant material or soil, or the dilution by $^{14}N_2$ from the air of plants growing with ^{15}N labelled fertilizer has been used. Substantial although very variable amounts of N_2 fixation have been demonstrated with these methods in rice (Watanabe and Roger, 1984), sorghum (Wani *et al.*, 1984) and forage grasses (De Polli *et al.*, 1977; Boddey and Victoria, 1986). Very recent experiments with sugar cane, combining N balance and ^{15}N dilution measurements, have brought unequivocal proof of more than 50% of the plant nitrogen coming from the air (Lima *et al.*, 1986).

Now that it is known that amounts of nitrogen of economic interest can be fixed in association with cereals and other Gramineae, the understanding of the physiology is essential in order to start to manipulate and increase their efficiency. Many different N_2 fixing bacteria have been isolated from the rhizosphere and from roots of cereals, but only where plant-bacteria interactions exist can one speak of an association. Pathogenic plant-bacteria associations have been known for long, and there, effects of microorganisms are visible as damage to the plant tissue. Characteristic of these associations is the specificity that can be on strain or species level. Plant breeding for resistance to specific pathogens is one of the major objectives in agricultural research. Breeding for improved associations of plants with N_2 fixing bacteria will have to envisage opposite characteristics.

New approaches to the study of nitrogen fixation in the major cereals and grasses have been started in the last decade (Döbereiner and Day, 1975; Neyra and Döbereiner, 1977; Boddey and Döbereiner, 1984). Several new N_2 fixing bacteria have been described which associate with grasses and cereals. Besides *Azotobacter paspali* there are now three *Azospirillum* spp, one new *Bacillus* (*B. azotofixans*) and several ill defined *Pseudomonas* (Döbereiner, 1966; Tarrand *et al.*, 1978; Barraquio *et al.*, 1983; Magalhães *et al.*, 1983; Seldin *et al.*, 1984). A new acid tolerant bacterium was found to predominate in maize roots in cerrado soils and was initially classified as a fourth *Azospirillum* species (Baldani *et al.*, 1984). Later RNA/RNA hybridization studies showed it to be a new genus (E.C. Falk and N.R. Krieg personal communication) and it therefore was renamed *Herbaspirillum seropedicae* (Baldani *et al.*, 1986).

The mode of infection of cereal roots by N_2 fixing bacteria has not yet been identified, but root hair deformations with specific *Azospirillum brasilense* strains could be associated with plant responses to inoculation with the same strains (Patriquin *et al.*, 1983), and numbers of cells of these strains within roots correlated well with plant N increases (Baldani *et al.*, 1983). Infection of maize root xylem during the growth cycle of field grown plants followed similar patterns as N_2 fixation (Magalhães *et al.*, 1979). Establishment of inoculated *Azospirillum* strains in roots of field grown wheat and sorghum varied with strains. Root isolates became dominant within roots while the soil isolates seemed less competitive (Table 6). Similar results have been found for sorghum where the distribution of the inoculated strain in the root system was not at random but localized in the upper root system. Plant responses under field conditions to *Azospirillum* inoculation have been reported from many places (Okon, 1982; Subba Rao, 1981; Vlassak and Reynders, 1978) but, as expected there are large differences between strains (Freitas *et al.*, 1983; Baldani *et al.*, 1983). Although such plant responses were usually accompanied by increased N incorporation, especially into seeds, unequivocal proof of N_2 fixation has not been brought forward in *Azospirillum* inoculation experiments. Attempts to show $^{15}NO_3^-$ dilution in wheat experiments showed higher fertilizer recovery but no sign of N_2 fixation (Table 7). Two sorghum experiments gave similar results. Bacterial hormones which proportion enlargement of the root system and a sponge effect were suggested by Okon (1982). Enhanced NO_3^- reduction aided by the *Azospirillum* nitrate reductase is another possibility. The data in Table 8 strongly support this hypothesis in wheat.

TABLE 6 - Effect of inoculation with *Azospirillum brasilense* on its establishment and on N incorporation in field grown wheat (after Baldani *et al.*, 1986).

Inoculant strain	% establishment [a]		Total plant N kg/ha [b]	
	Rhizosph. soil	Within roots	15 kg N	60 kg N [c]
Control	1	5	57	69
Rhizosphere isolate (Cd)	61	11	56	66
Root isolate (Sp 245)	44	76	69	68

[a] % cultures identified as inoculated strain.

[b] Total N in straw and grain harvests.

[c] Fertilizer N applied per ha.

TABLE 7 - Nitrogen accumulation and N incorporation from $^{15}NO_3^-$ in grains of field grown wheat inoculated with various *Azospirillum brasilense* strains (Boddey *et al.*, 1986).

Inoculum	Total N mg/cylind.	% ^{15}N excess	^{15}N recovered mg/cylind.
Sp 107st[a]	1195	0,190	2,30
Sp 245[a]	1271	0,171	2,20
Sp 7	1276	0,159	2,00
Control	866	0,156	1.33
LSD (Tukey)	318	n.s.	0,67

[a] Isolated from surface sterilized wheat roots.

In plant genotype comparisons, however, N_2 fixation in the order of 10-40% of the total plant N incorporation has been shown by N balance studies (App *et al.*, 1980) and by $^{15}N_2$ incorporation (De-Polli *et al.*, 1977; Eskew *et al.*, 1981), in forage grasses (Fig. 1) (Boddey *et al.*, 1983a,b) and by balance and ^{15}N dilution in sugar cane (Table 9).

Although N_2-fixation in association with Gramineae is a very exciting field due to the importance of these plants for agriculture, it is improbable that complete replacement of N fertilizers will be possible because of the more primitive nature of these associations. Still it remains a major challenge to soil biologists and agronomists, and prospects for new break-throughs are good.

The recent confirmation of specificity, very similar to that observed with plant pathogens in experiments which show that it is possible to establish under

TABLE 8 - Establishment of inoculated *Azospirillum brasilense* and effect on N incorporation into grains of wheat (Boddey *et al.*, 1986).

	% isolates inoculated strains[a]		Total N in grain g/m^{-2}
	washed roots	surface ster. roots	
Sp 245 spec	100	67	1.24a
Sp 245 nr⁻ spec[b]	81	0	0.69cd
Sp 246 spec	94	27	1.03b
Sp 7 kan st	50	0	0.75c
Control	0	0	0.59d

[a] 8-16 single colony isolates were tested against the various antibiotics and control isolates against all antibiotics.

[b] Nitrate reductase negative mutant obtained by selection in chlorate agar, pH 8.0 with NO_3.

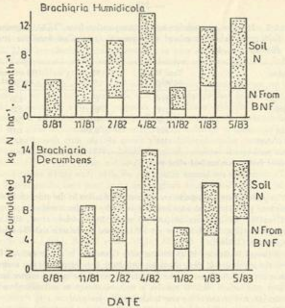

Fig. 1 - Seasonal variation of N accumulation by two *Brachiaria* spp. from nitrogen fixation and from soil. Values represent monthly means for the time intervals between the months stated. BNF was evaluated by $^{15}NO_3$ dilution with a nonfixing *Brachiaria* as control (*B. radicans*) (Boddey and Victoria, 1986).

TABLE 9 - Nitrogen fixation in sugar cane cultivars (g N per 450 litre bucket) (Lima *et al.*, 1987).

Cultivar	Initial soil N	Total plant N[a]	Final soil N	Balance	^{15}N atom % excess
CB 47-89	53.6	30.3a	46.8	+ 20.9a	0.0586
CB 47-355	49.5	13.5b	44.5	+ 5.9b	0.1015
IAC 52-150	52.7	11.6b	45.1	+ 1.3b	0.1097
NA 56-79	54.2	11.1b	45.8	0.0b	0.1047
No plant	51.2	—	44.1	− 9.6c	—

[a] Total plant N obtained in two harvests in 21 months.

field conditions selected or genetically manipulated *Azospirillum* strains (Baldani *et al.*, 1986) even in soils which contain 10^{4} to 10^{5} native *Azospirillum* cells per g, opens many possibilities to improve N_2 fixation in such associations. The two new acid-tolerant species, *A. amazonense* and *H. seropedicae*, have not even been tested yet as inoculants. Plant breeding programs will have to start with more primitive genotypes which have not been selected for response to high mineral fertilizer levels. There the plant breeder may encounter problems in breeding for resistance to plant pathogens and at the same time susceptibility to *Azospirillum* infection. On the other hand tolerance to acid soils with Al^{+++} toxicity seems to support enhanced N_2 fixation. In sorghum breeding programs, Al^{+++} tolerant selections have been found to produce more malic and transaconitic acids (Cambraia *et al.*, 1983) which chelate the toxic Al^{+++}. These two organic acids represent the preferred carbon source for *Azospirillum* and other N_2 fixing bacteria and Al^{+++} tolerant sorghum cultivars seem to enhance N_2 fixation (Christiansen-Weniger *et al.*, 1985).

The recent results indicating plant genotype differences in sugar cane may lead to entirely new concepts of the possibility to use sun energy through agriculture (Döbereiner *et al.*, 1981b). The success of the Brazilian alcohol program which exceeded all expectations ($0,2 \times 10^{-9}$ l of ethanol are now produced annually and 97% of all cars sold in 1985 run on 95% ethanol) is due to a relatively low N fertilizer input. In Hawaii, producing sugar cane ethanol on large scale is considered energetically un-economic (more energy is used than it yields) because of the high mechanization and fertilizer inputs into this crop. Sugar cane breeding programs which yield cultivars which obtain more than 100 kg of N from the air as estimated from the data in table 8 may even in industrialized countries become net energy yielders and may open to the world a new renewable energy source.

The use of the many new findings in all fields of N_2 fixation in agricultural systems will lead to more economical but still productive farming systems with reduced risks for the environment.

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REFERENCES

App A., Santiago R., Daez C., Menguito C., Ventura W., Tirol A., Po J., Watanabe I., Datta S.K. de and Roger P. (1984) - *Estimation of the nitrogen balance for irrigated rice and the contribution of phototrophic nitrogen fixation.* « Field Crops Research », 9, (1), 17-28.

App A.A., Watanabe I., Alexander M., Ventura W., Daez C., Santiago T. and Datta S.K. de (1980) - *Nonsymbiotic nitrogen fixation associates with the rice plant in flooded soils.* « Soil Science », *130* (5), 283-289.

Baldani J.I., Baldani V.L.D., Seldin L. and Döbereiner J. (1986) - *Characterization of* Herbaspirillum seropedicae *gen. nov., sp. nov., a root-associated nitrogen fixing-bacterium.* « Int. J. Syst. Bacteriol. », *36*, 86-93.

Baldani J.I., Baldani V.L.D., Sampaio M.J.A.M. and Döbereiner J. (1984) - *A fourth* Azospirillum *species from cereal roots.* « An. Acad. Brasil. Ciênc. », 56, 365.

Baldani V.L.D., Baldani J.I. and Döbereiner J. (1983) - *Effects of* Azospirillum *inoculation on root infection and nitrogen incorporation in wheat.* « Can. J. Microbiol. », 29, 924-929.

Barraquio W.L., Ladha J.K. and Watanabe I. (1983) - *Isolation and identification of N_2-fixing* Pseudomonas *associated with wetland rice.* « Can. J. Microbiol. », 29 (8), 867-873.

Boddey R.M., Baldani V.L.D., Baldani J.I. and Döbereiner J. (1986) - *Effect of inoculation of* Azospirillum *spp. on the nitrogen assimilation of field grown wheat.* « Plant & Soil », 95, 109-121.

Boddey R.M., Chalk P.M., Victoria R. and Matsui E. (1983a) - *The ^{15}N-isotope dilution technique applied to the estimation of biological nitrogen fixation associated with* Paspalum notatum *cv. batatais in the field.* « Soil Biol. Biochem. », *15* (1), 25-32.

Boddey R.M., Chalk P.M., Victoria R.L., Matsui E. and Döbereiner J. (1983b) - *The use of the ^{15}N isotope dilution technique to estimate the contribution of associated biological nitrogen fixation to the nitrogen nutrition of* Paspalum notatum *cv. batatais.* « Can. J. Microbiol. », 29 (8), 1036-1045.

Boddey R.M. and Döbereiner J. (1984) - *Nitrogen fixation associated with grasses and cereals.* In: *Current Developments in Biological Nitrogen Fixation*, Subba Rao, N.S (ed.), Oxford & IBH Publishing Co., New Delhi. 277-313.

Boddey R.M. and Victoria R.L. (1986) - *Estimation of biological nitrogen fixation associated with* Brachiaria *and* Paspalum *grasses using ^{15}N labelled organic matter and fertilizer.* « Plant & Soil », 90 265-292.

Bradley R.S., Oliveira L.A. de, Podestá J.A. de F. and John T.V. St. (1978) - *Fixação do nitrogênio associado com raízes em solos diferentes na floresta Amazonia-Central.* Anais do 3° Congresso Florestal Brasileiro, Manaus.

Bradley R.S., Oliveira L.A. de, Podestá J.A. de F. and John T.V. St. (1980) - *Nodulation of legumes, nitrogenase activity of roots and occurrence of nitrogen-fixing* Azospirillum *spp. in representative soils of Central Amazonia.* « Agro-Ecosystems », *6*, 249-266.

Cambraia J., Galvani F.R., Estevão M.M. and Sant'Anna R. (1983) - *Effects of aluminum on organic acid, sugar and amino acid composition of the root system of sorghum* (Sorghum bicolor L. Moench). « J. Pl. Nutr. », *6* (4), 313-322.

Christiansen-Weniger C., Boddey R.M. and Döbereiner J. (1985) - *Evaluation of nitrogen fixation in sorghum cultivars inoculated with different strains of* Azospirillum *spp.* In: Klingmiller, W., ed. Azospirillum III: Genetics, physiology, ecology. Berlin, Springer, Verlag. p. 180-188.

De Polli H., Matsui E., Döbereiner J. and Salati E. (1977) - *Confirmation of nitrogen fixation in two tropical grasses by* $^{15}N_2$ *incorporation.* « Soil Biol. Biochem », 9, 119-123.

Döbereiner J. (1966) - *Azotobacter paspali* sp. n., uma bacteria fixadora de nitrogenio na rizosféra de *Paspalum.* Pesq. agropec. brasil 1: 357-365.

Döbereiner J., Araujo Neto J.S. and Arkcoll D.B. (1981a) - *Energy alternatives from agriculture.* In: « Pontificiae Academiae Scientiarum Scripta Varia », 46, 431-458.

Döbereiner J. and Day J.M. (1975) - *Nitrogen fixation in the rhizosphere of tropical grasses.* In: *Nitrogen fixation by free-living microorganisms.* IBP 6, (Stewart, W.D.P. ed.), Cambridge University Press, 39-56.

Döbereiner J., Scotti M.R.M.M.L., Sá N.M.H. and Vargas M.A.T. (1981b) - *Resistance to streptomycin of* Rhizobium *isolates from cerrado and Amazon soils.* In: *Current Perspectives in Nitrogen Fixation* (A.H. Gibson & W.E. Newton, eds.) Australian Academy of Sciences. Canberra, 434.

Eskew D.L., Eaglesham A.R.J. and App A.A. (1981) - *Heterotrophic* $^{15}N_2$ *fixation and distribution of newly fixed nitrogen in a rice-flooded soil system.* « Plant Physiol. », 68 (1) 48-52.

Faria S.M. de, Franco A.A., Menandro M.S., Jesus R.M. de, Baitello J.B., Aguiar O.T. de and Döbereiner J. (1984a) - *Levantamento da nodulação de leguminosas nativas na região Sudeste do Brasil.* « Pesq. agropec. bras. », *19* (s/n), 143-153.

Faria S.M. de, Franco A.A., Jesus R.M. de, Menandro M.S., Baitello J.B., Mucci E.S.F., Döbereiner J. and Sprent J.I. (1984b) - *New nodulating legume trees from South-East Brazil.* « New Phytol. », *98*, 317-328.

Freire J.R.J. (1982) - *Research into the* Rhizobium/*Leguminosae symbiosis in Latin America.* « Plant and Soil », *67*, 227-239.

Freitas J.L.M. de, Rocha R.E.M. da, Pereira P.A.A. and Döbereiner J. (1983) - *Matéria orgânica e inoculação com* Azospirillum *na incorporação de N pelo milho.* « Pesq. agropec. bras. », *17* (10), 1423-1432.

Hungria M. and Neves M.C.P. (1984) - *Variação sazonal da fixação do* N_2 *translocação do nitrogênio fixado em* Phaseolus vulgaris *L.* XIX Congr. Bras. Ciê. do Solo, Coritiba-Pr. (Resumo nº 42).

Jaiyebo E.O. and Moore A.M. (1963) - *Soil nitrogen accretion under different covers in a tropical rain-forest environment.* « Nature Lond. », *197*, 317-318.

Jenkinson D.S. and Rayner J.H. (1977) - *The turnover of soil organic matter in some of the Rothamsted classical experiments.* « Soil Sc. », *123* (5), 298-305.

Lee K.K. and Döbereiner J. (1982) - *Effect of excessive temperatures on rhizobia growth nodulation and nitrogen fixing activity in symbiosis with siratro.* « Pesq. agropec. bras. », *17* (2), 181-184.

Lima E., Boddey R.M. and Döbereiner J. (1987) - *Quantification of biological nitrogen fixation associated with sugar cane using an* ^{15}N *aided nitrogen balance.* « Soil Biol. Biochem ». (In press).

Magalhães F.M.M., Baldani J.I., Souto S.M., Kuykendall J.R. and Döbereiner J. (1983) - *A new acid-tolerant* Azospirillum *species.* « An. Acad. brasil. Ciênc. », 55 (4), 417-430.

Magalhães F.M.M., Magalhães L.M.S., Oliveira L.A. de and Döbereiner J. (1982) - *Ocorrência de nodulação em leguminosas florestais de terra firme nativas da região de Manaus-Am.* « Acta Amazônica », *12* (3), 509-514.

Magalhães F.M.M., Patriquin D. and Döbereiner J. (1979) - *Infection of field grown maize with* Azospirillum *spp.* « Rev. Brasil. Biol. », *39* (3), 587-596.

Munns D.N. and Franco A.A. (1982) - *Soil constraints to legume production.* In: *Biological Nitrogen Fixation Technology for Tropical Agriculture* (P.H. Graham & S.C. Harris, eds.) CIAT, Cali-Colombia, 133-152.

Neves M.C.P., Didonet A.D., Duque F.F. and Döbereiner J. (1985) - Rhizobium *strain effects on nitrogen transport and distribution in soybeans.* « J. Exp. Bot. », *36*, 1179-1192.

Neyra C.A. and Döbereiner J. (1977) - *Nitrogen fixation in grasses.* « Adv. Agron. », *29*, 1-38.

Okon Yaacov. (1982) - Azospirillum: *Physiological properties, mode of association with roots and its application for benefit of cereal and forage grass crops.* « Israel J. Botany », *13* (1-4), 214-220.

Oliveira L.C.B. de, Franco A.A. and Döbereiner J. (1984) - *Estabilidade da infectividade, efetividade e capacidade de produzir pigmento de estirpes de* Rhizobium phaseoli *submetidas a altas temperaturas.* « Pesq. agropec. bras. » (no prelo).

Patriquin D.G., Döbereiner J. and Jain D.K. (1983) - *Sites and processes of association between diazotrophs and grasses.* « Can. J. Microbiol. », *29* (8), 900-915.

Peres J.R.R. and Vidor C. (1980) - *Relação entre concentração de células no inoculante e competição por sítios de infecção nodular entre estirpes de* Rhizobium japonicum *em soja.* « Rev. Bras. Ciê. Solo », 4, 139-143.

Scotti M.R.M.M.L., Sá N.M.H., Vargas M.A.T. and Döbereiner J. (1980) - *Requirement for streptomycin resistance of* Rhyzobium *for the nodulation of legumes in cerrado regions.* « An. Acad. Brasil. Ciênc. », *52* (3), 650-651.

Selden L., Van Elsas J.D. and Penido E.G.C. (1984) - Bacillus azotofixans *sp. nov., a new nitrogen fixing Bacillus species isolated from Brazilian soils and grass roots.* « An. Acad. Brasil. Ciênc. », *56*, 364-365.

Silva E.M.R. da, Almeida, D.L. de, Franco A.A. and Döbereiner J. (1985) - *Adubação verde no aproveitamento de fosfato em solo ácido.* « R. Bras. Ciê. Solo », *9* (1), 85-88.

Subba Rao N.S. (1981) - *Response of crops to* Azospirillum *inoculation in India.* In: *Associative N_2-fixation* (Vose, P.B. & Ruschel, A.P. eds.) CRC Press, Inc., Boca Raton, vol. I, 137-144.

Tarrand J.J., Krieg N.R. and Döbereiner J. (1978) - *A taxonomic study of the* Spirillum lipoferum *group, with descriptions of a new genus,* Azospirillum *gen. nov. and two species,* Azospirillum lipoferum *(Beijerinck) comb. nov. and* Azospirillum brasilense *sp. nov.* « Can. J. Microbiol. », *24* (8), 967-980.

Vargas M.A.T. and Suhet A.R. (1980) - *Efeito de níveis e tipos de inoculantes na soja (Glycine max) cultivada em um solo de cerrados.* « Pesq. agropec. Bras. », *15* (3) 343-347.

Vasconcelos J.I.P. and Almeida R.T. de (1979/1980) - *Fixação biológica de nitrogênio em plantas de interesse econômico do Nordeste.* Relatórios técnicos I, II e III. Fundação Cearense de Pesquisa e Cultura.

Vidor C. and Miller R.H. (1980) - *Relative saprophytic competence of* Rhizobium japonicum *strains in soils as determined by the quantitative fluorescent antibody technique (FA).* « Soil Biol. Biochem. », 12, 483-487.

Vlassak K. and Reynders L. (1978) - *Associative dinitrogen fixation in temperate regions.* In: *Isotopes in biological dinitrogen fixation.* International Atomic Energy Agency, 71-87. (Panel Proceedings Series).

Wani S.P., Upadhyaya M.N. and Dart P.J. (1984) - *An intact plant assay for estimating nitrogenase activity (C_2H_2 reduction) of sorghum and millet plants grown in pots.* « Plant and Soil », *82* (1), 15-29.

Watanabe I. and Roger P.A. (1984) - *Nitrogen fixation in wetland rice field.* In: *Current Developments in Biological Nitrogen Fixation* (Subba Rao, N.S. ed.). Oxford & IBH Publishing Co., New Delhi, 237-276.

White J.W., Holben F.J. and Richer A.C. (1945) - *Maintenance level of nitrogen and organic matter in grassland and cultivated soils over periods of 54 and 72 years.* « J. Amer. Soc. Agron. », 37, 21-31.