NON-SYMBIOTIC NITROGEN FIXATION IN TROPICAL SOILS [1]

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Abstract

The actual knowledge of non-symbiotic nitrogen fixation in tropical soils is compared with the situation of temperate regions where a contribution of economical importance to the N-economy of these soils has been proved only under exceptional circumstances.

The main differences of tropical soils seem to be the presence of at least six non-symbiotic nitrogen-fixing bacteria which have not been found in temperate soils and highly stimulating effects of certain grasses, the association of sugar cane with *Beijerinckia* spp. and that of *Paspalum notatum* with *Azotobacter paspali* being the most remarkable cases.

INTRODUCTION

The problem of non-symbiotic nitrogen fixation has been studied by innumerous scientists since the description of *Azotobacter chroococcum* and *Azotobacter agilis* by Beijerinck in 1901. These studies seem appropriate because the proof of nitrogen fixation in amounts of economical importance could explain (1) the lack of plant response to nitrogenous fertilizer, (2) the recuperation of soils left under fallow, and (3) the origin of the large reserves of nitrogen in soils where legumes cannot be the main source of this nutrient.

The innumerable papers dealing with *Azotobacter* in temperate regions do not show more than 10 to 15 kg of N/ha/year fixed by this organism and even these quantities were dependent on available carbon sources (Henzell & Norris 1962). The same authors mention as main limiting factors of *Azotobacter* distribution in soil: low pH, lack of carbon sources and the presence of nitrate which stimulates other microorganisms in the soil. These organisms then compete with *Azotobacter* for energetic material.

The available carbon source requirement for non-symbiotic nitrogen-fixing bacteria in soil, could provide a solution to the problem where these organisms dominate the microbial equilibrium in the rhizosphere.

In this case, the plant could furnish energy material for nitrogen fixation. That this is possible becomes apparent in the legume symbiosis where all energy sources for nitrogen fixation are furnished by the plant. As the biochemical mechanism of symbiotic and of non-symbiotic nitrogen fixation are very similar, there is no reason why there could not exist a loose association of non-symbiotic nitrogen-fixing bacteria which develop along the roots, and use the carbon substances excreted by the plant to fix nitrogen which could then be directly or indirectly (after mineralization) available to the plant. If one continues comparing this type of association with the legume symbiosis it becomes evident that such a relationship is only possible if there is a close interchange between plant and bacteria and if the bacteria in question find good growth conditions in the rhizosphere, permitting them to compete successfully with the great bulk of other soil microorganisms.

The search for such an association between higher plants and the conventional *Azotobacter* species led again to innumerable papers which have been reviewed by Allison (1947) and recently by Macura (1966) and Rovira (1965). Peas, maize, tomatoes, oats and wheat were observed to stimulate *Azotobacter* in their rhizosphere, but only under certain conditions. Observations that older plants with roots already in decomposition stimulate *Azotobacter* while younger pants do not and that the rhizosphere within a certain distance from the root seems to be more appropriate for *Azotobacter* growth than the root surface proper, also indicate the non-existence of a close and specific relationship between *Azotobacter* chroococcum and *A. agilis* and higher plants. There might be casual associations which lead to a depres-

sion of the great bulk of soil microorganisms in favor of *Azotobacter* due to a deficiency in mineral nitrogen in the rhizosphere. The main difficulty seems to be the inability of *Azotobacter* to compete with other microorganisms for energetic material.

Summarizing we can conclude that the economic importance of non-symbiotic nitrogen-fixing bacteria under temperate climatic conditions is doubtful for the following reasons:

1) *Azotobacter chroococcum* and *A. agilis* were seldom found to occur that abundantly in soil that they were able to compete successfully with other soil microorganisms for carbon substances;

2) acid soils strongly restrict the development of these organisms;

3) optimal growth temperatures for these organisms occur during a limited period of the year;

4) no constant, close relationship between these two *Azotobacter* species and higher plants has as yet been demonstrated.

If one compares this situation with that found under tropical conditions, it will be noted that few of these arguments remain. It is the purpose of this paper to emphasize the differences that seem to exist in the situation of non-symbiotic nitrogen fixation under tropical conditions and which suggests an immense field of research that is certainly worthwhile undertaking.

The well-established contribution of blue-green algae to the N-economy of rice fields will not be considered in this paper.

NON-SYMBIOTIC NITROGEN-FIXING BACTERIA WHICH SEEM RESTRICTED TO TROPICAL ENVIRONMENT

There have been described and accepted six species of nitrogen-fixing bacteria which have not been found in soils of temperate regions. They will be referred to here only in respect to their characteristics as related to the purpose of this paper.

Beijerinckia indica

This organism was isolated in 1959 by Starkey and De from Indian soils, by Becking (1961a) from African, South American, South European and North Australian soils and by Döbereiner (1959a) from soils all over central and north Brazil. The occurrence of *Beijerinckia* in tropical and subtropical soils of the world is summarized in Table I.

TABLE 1. *Distribution of* Azotobacter *and* Beijerinckia *in temperate, sub-tropical and tropical soils (Data from Becking (1961), Tchan (1953) and Döbereiner (1959)*

Region	N.º of samples	Beijerinckia positive %	Azotobacter positive %
Temperate	110	0	28
Europe	30	0	17
Australia (South)			
Sub-tropical			
Europe (South)	45	4	98
Australia	3	0	—
Africa (North)	8	0	75
Africa (South)	40	38	13
America (Florida)	3	0	100
South America (Brazil)	19	21	10
Asia (Japan)	8	63	0
Tropical			
Africa (Central)	53	57	21
Asia (South)	43	53	37
Australia (North)	45	35	15
South America	191	56	1*

It is remarkable that in addition to the the three negative samples reported by Becking (1961a), there does not seem to be one report in the literature on the occurrence of *Beijerinckia* in the U.S. or Central America.

Beijeirinckia indica was found to be tolerant to soil pH from 4.9 to 7.4 (Becking 1961a) and was found to grow well in culture medium with pH 2.9 to 8.4. It is tolerant to much higher aluminium and manganese concentrations than *Azotobacter* (Becking 1961b) and its optimal temperature for growth in culture medium was found to be 20 to 37°C (Becking 1961b). Nitrogen fixation of *Beijerinckia indica* reached 17.2 mg N/g sucrose and 31.2 mg N/g glycerol (Döbereiner, unpublished data).

Interesting observations were reported by Dommergues and Mutaftschief (1965) who demonstrated an increase in nitrogen fixation when *B. indica* was cultivated in mixed cultures with *Lipomyces starkeyi*, a yeast which is frequently found associated with *Beijerinckia* in tropical soils.

Beijerinckia fluminensis

This organism was described by Döbereiner and Ruschel in 1958. It was found, although less abundant than *B. indica*, in tropical soils from four Brazilian States (Döbereiner & Ruschel 1958) and in the Congo (Dommergues 1965). Its tolerance to acid culture medium is almost equal to that of *B. indica* (pH 3.5 to 9.2) and the pH range of the soils it was isolated from, was 4.3 to 5.2. Its optimal growth temperature in culture medium was shown to be 26 to 33°C. The amount of nitrogen fixed reached 12.9 mg N/g sucrose (Döbereiner & Ruschel 1958).

As in the case of *B. indica* this organism showed increased N-fixation in mixed culture with *Lipomyces starkeyi* (Dommergues & Mutaftschief 1965).

Beijerinckia derxii

This species was described by Tchan (1957) and his description was later confirmed by Hilger in 1965. It was isolated from Australian soils (Tchan 1957), from Indian and Central African soils (Hilger 1965) and from Brazilian soils (Ruschel, unpublished data). It seems more sensitive to pH changes, pH 5.8 to 7.4, being the limits for good growth (Hilger 1965), while efficient nitrogen fixation took place only at pH 6.7 (Tchan 1957). Maximal nitrogen fixation was 13.5 mg N/g glucose (Tchan 1957).

Derxia gummosa

This organism was described in 1960 by Jensen *et al.* It was isolated from a West Belgal soil. Recently it also was isolated from 20 different samples of rhizosphere soil of pasture grasses collected in Rio de Janeiro State (Döbereiner, unpublished data). Colonies of the organism on silica-gel plates were seldom found to occur in large numbers. It is however, a peculiarity of the bacteria that only a few develop on N-free media in plates inoculated with steaks of thousands of organisms (Jensen *et al.* 1960). Some growth substances or vitamins might be essential for colony growth. When soil containing high numbers of *Derxia* is inoculated into silica-gel plates, probably only a small fraction of the bacteria is able to start a colony. For this reason, actual numbers of this bacteria might be many times higher than is estimated by plate counting. Once the colonies start growing they develop to immense size (up to 3 cm in diameter and 0.5 cm in heighth).

Derxia was found in soils with pH ranging from 5.0 to 6.2. In culture medium growth occurred between pH 5.5 and 9.0 (Jensen *et al.* 1960), and nitrogen fixation took place between pH 5.7 and 8.9. Efficiency in nitrogen fixation of this organism reached 25 mg N/g glucose (Jensen *et al.* 1960). Optimal temperatures were 25 to 37°C, the latter being better for isolation than 34°C.

Derxia indica

This species was recently described by Roy and Sen (1962). It was isolated from partially rotted plants in India. Optimal temperature for growth was 33-34°C and the pH range for growth in culture medium was 4 to 9. Efficiency in nitrogen fixation reached 30 mg N/g glucose.

Azotobacter paspali

This species was very recently isolated from the rhizosphere of *Paspalum notatum* and *Paspalum plicatum* (Döbereiner 1966) where it occurs in large numbers. Since the organism has not been found in soil without *P. notatum* (Bahia grass) or in the rhizosphere of other plants and since *Paspalum* is a grass which only occurs under subtropical and tropical conditions it is probable that the organism will not be found in temperate regions. *Azotobacter paspali* is sensitive to low pH as are the other species of this genus, pH 5.7 to 8.4 being the limits for growth in liquid culture medium (Döbereiner 1966). Recent observations showed even a much narrower pH range when only small amounts of inoculum are used (pH 6.3 to 6.8) (Machado & Döbereiner, unpublished data).

The organism was found however, in great numbers in the rhizosphere of *P. notatum* grown in soils with a pH between 4.9 and 7.8. This was explained by a buffering effect of the plant roots.

As for *Derxia*, higher temperatures are optimal for isolation of this organism than for the other *Azotobacter* species (Table 2). Nitrogen fixation of *Azotobacter paspali* in culture medium reached 30.4 mg N/g sucrose.

TABLE 2. *Effect of incubation temperature on the development of colonies of* Azotobacter paspali *and* Derxia *sp. on silica-gel plates with calcium citrate as carbon source (Döbereiner 1966)*[a]

Temp.	Species	Incubation time	
		4 days	5 days
29°C	*A. paspali*	50	140
	Derxia sp.	30	30
34°C	*A. paspali*	250	>1000
	Derxia sp.	30	30
37°C	*A. paspali*	600	>1000
	Derxia sp.	50	60

[a] Data represent no. of microcolonies/1 g of root surface soil from *Paspalum notatum*.

ASSOCIATION OF NON-SYMBIOTIC NITROGEN-FIXING BACTERIA WITH TROPICAL PLANTS

Stimulating effects of higher plants on non-symbiotic nitrogen-fixing bacteria have been reported in tropical environment since 1959. Sugar cane, rice and pasture grasses seem to be the most important ones, all of them belonging the family *Gramineae*.

Sugar cane

During the studies of *Beijerinckia* occurrence in Brazilian soils (Döbereiner 1959a,b), it became evident that most soils in sugar cane for more than one year showed an abundant population of this organism (Table 3). The stimulating effect of sugar cane on *Beijerinckia* development was explained by the excretion of substances rich in sucrose which is the preferred carbohydrate for this organism. Substances excreted by sugar cane leaves and stems and washed into the soil by the rain stimulated *Beijerinckia* development. Plants grown in pots protected from rain also stimulated *Beijerinckia* growth (Döbereiner & Alvahydo 1959). Under field conditions, in a sandy soil, cropping with sugar cane increased the number of microcolonies of *Beijerinckia*/g of soil from 20 to more than 1,000 in the root-surface soil and to 500 in the rhizosphere soil (Döbereiner 1961). This stimulation of *Beijerinckia* in the rhizosphere of sugar cane was accompanied by a reduction in the number of bacteria which require aminoacids, actinomyces and fungi indicating a dislocation of the microbial equilibrium in favor of *Beijerinckia* (Table 4).

TABLE 3. *Effect of sugar cane crop on the occurrence of Azotobacter and Beijerinckia in soil (Döbereiner 1959b)*

Nº of samples analysed	No sugar cane	Sugar cane
	124	131
Azotobacter positive	10	9
N.º of microcol./g (mean of pos. samples)	66 ± 19	37 ± 17
Beijerinckia positive	77	125
N.º of microcol./g (mean of pos. samples)	67 ± 14	331 ± 110

TABLE 4. *Changes in microbial equilibrium in the rhizosphere of sugar cane, in the field. Data represent relative increase in relation to check samples taken between rows. Means of 8 replicates (Döbereiner 1961)*

Months after planting	Microorganism	Ratio rhizosph./check	Ratio root surface/check
2	*Beijerinckia*	4.7	—
12	»	22.7	17.2
18	»	22.3	55.8
18	Bacteria in egg albumin agar	0.4	0.2
18	Actinomyces	1.0	0.1
18	fungi	0.7	0.7

Rice

It was also observed that rice under field conditions stimulated *Beijerinckia* development although not to the effect as sugar cane. Results of such an experiment are summarized in Table 5.

TABLE 5. *Effect of rice on Beijerinckia development in the field (n.º of microcolonies/1 g of soil; means of 6 replicates) (Döbereiner & Ruschel 1962)*

	Beginning of veg. cycle	Blooming	Ripening
Between rows	195	505	777
Rhizosphere	101	736	738
Root Surface	282	770	1250

In a greenhouse experiment with inoculation of rice with *Beijerinckia*, the plant effect became more pronounced (Table 6). Here it became apparent that inoculation of the seeds (that means the introduction of small amounts of *Beijerinckia* into the soil) brought about a marked change in the rhizosphere equilibrium, stimulating *Beijerinckia* and reducing the number of amino-acid requiring microorganisms.

TABLE 6. *Effect of inoculation of rice seeds with Berijerinckia on its establishment in the rhizosphere. Means of 4 replicates. (Döbereiner & Ruschel 1961)*

	Inoculated		Non inoculated	
	rhizosph.	root surf.	rhizosph.	root surf.
Beijerinckia, n.º microcol./1 g	86	357	6	7
Bacteria in egg albumin agar×1000	272	184	418	364

Forage grasses

The occurrence of *Beijerinckia* and *Azotobacter* in the rhizosphere of *Hiparrhenia rufa*, *Panicum maximum*, and *Paspalum notatum* were studied by Ruschel and Britto (1966), and that of *Digitaria decumbens*, *Panicum purpurescens*, *Cynodon dactylon*, *Setaria sphacelata* and *Melinis multifloris* by Ruschel and Döbereiner (1965). With the exception of *Melinis multifloris* which constantly reduced *Beijerinckia* occurrence, all other species showed stimulating effects on this bacteria. *Azotobacter* was found to be extremely abundant in the rhizosphere of *Paspalum notatum* (Ruschel & Britto 1966).

Paspalum notatum

Recently it has been shown that the association of *Azotobacter* with *Paspalum notatum* seems to be a specific one. A new *Azotobacter* (*Azotobacter paspali*) was characterized which occured in practically all samples from the rhizosphere of *Paspalum notatum* (Bahia grass) and always in extremely high numbers (Döbereiner 1966). Table 7 shows that *A. paspali* occurred on only 2 out of 7 *Paspalum* species and on no other plant, including 27 species of *Gramineae*, 8 legume species, and a number of other unidentified plants. It occurred in much higher numbers at the root-surface soil than in samples including the whole rhizosphere. Root-surface soils from other plants grown inbetween the *Paspalum* sward contained the organism but it was less abundant there than in the corresponding rhizosphere samples (Machado & Döbereiner, unpublished data).

The dependence of *A. paspali* growth in soil on a higher plant indicates a close relationship which brings a completely new approach to the question of non-symbiotic nitrogen-fixing bacteria in tropical soils. Furthermore it might lead to speculations on evolution hypothesis of non-symbiotic nitrogen fixer's to the symbiotic relationship of the legumes.

TABLE 7. *Occurrence of nitrogen-fixing bacteria in the root-surface soil of pasture plants.* (*Döbereiner 1966*)

Plant	N.° of samples	Positive samples %		
		A. chroococcum A. agilis	A. paspali	Derxia sp.
Paspalum notatum	76	17	98	15
Paspalum plicatum	3	33	66	0
Paspalum spp.[a]	27	7	0	0
Other grasses[b]	63	26	0	0
Legumes[c]	12	16	0	8
Other plants	10	40	0	20

[a] *P. convexum, P. vaginatum, P. clandestinum, P. coloatum, P. maritimum, P. erianthum.*
[b] More than 23 different species of *Gramineae*.
[c] More than 8 species of *Leguminosae*.

NITROGEN FIXATION IN TROPICAL SOILS

The evaluation of the amounts of nitrogen fixed bacteria-plant systems is awaiting further research. The final proof of atmospheric nitrogen being fixed by non-symbiotic organisms and then being absorbed by the plant should arise from experiments with N^{15} which, to the best of our knowledge, have not been carried out with any of the described sys-

tems. However, there are some experimental data which indirectly suggest nitrogen fixation in the rhizosphere of subtropical or tropical plants. Rouquerol (1963) and McRae and Castro (1967) observed significant increases of nitrogen in rice soils which were atributed to heterothrophic non-symbiotic bacteria. In a greenhouse experiment Döbereiner and Ruschel (1962) demonstrated nitrogen fixation in rice plants inoculated with one *Beijerinckia* strain while other strains of this organism did not affect plant growth (Table 8). Unfortunately, in this experiment algae were not eliminated and there is still the possibility of certain *Beijerinckia* strains having stimulated algae growth which then contributed to nitrogen fixation.

Recently inoculation with *Beijerinckia*, in sand cultures, in the greenhouse, resulted in significant increases of the total nitrogen content of elephant grass (*Pennisetum purpureum*) (Souto & Döbereiner 1967).

TABLE 8. *Effect of inoculation of rice with* Beijerinckia *on rice yield. Means of 4 replicates.* (*Döbereiner & Ruschel 1961*)

Beijerinckia	Straw g/pot	Grain g/pot
Strain A	23.3	6.2
Strain B	15.6	2.7
Strain C	15.5	3.8
Strain D	15.0	3.2
Check (no inoc.)	18.4	4.0
Check (strain C + Nitrogen)	30.1	6.0

CONCLUSIONS

Summarizing we can now focalize the following distinct features of tropical soil-plant systems as related to non-symbiotic nitrogen fixation:

1) there occur at least six species of non-symbiotic nitrogen-fixing bacteria which have not been found in temperate climate soils. All of these species were shown to be able to fix between 12 and 30 mg of N/g of carbon source;

2) all of these species have been found to occur in acid soils;

3) all *Beijerinckia* species require, for optimal growth, temperatures in the mesophylic range like the classical *Azotobacter* species. *Azotobacter paspali* and *Derxia* spp. seem to need higher temperatures for optimal development (33 to 37°C). In tropical regions optimal temperatures for these organisms occur practically during the whole year;

4) stimulating effects of higher plants on three of these species have been demonstrated, the association of *Beijerinckia indica* with sugar cane and of *Azotobacter paspali* with *Paspalum notatum* being the most remarkable cases.

It can be easily recognized that these points eliminate, for tropical soils, most of the arguments against the economic importance of non-symbiotic nitrogen fixation in temperate soils.

However, the importance of non-symbiotic nitrogen fixation in tropical soils must still be demonstrated and is therefore a research area of considerable importance.

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FIXAÇÃO ASSIMBIÓTICA DE NITROGÊNIO ATMOSFÉRICO EM SOLOS TROPICAIS

Sinopse

No presente trabalho são focalizadas diferenças entre solos tropicais e solos de regiões temperadas em relação à fixação assimbiótica do nitrogênio atmosférico. Enquanto nestes últimos uma fixação de importância econômica foi raramente demonstrada, sugerem-se estudos mais aprimorados do problema em solos tropicais, uma vez que diferenças fundamentais foram encontradas. As principais são as seguintes:

1) Ocorrem pelo menos seis espécies de bactérias assimbióticas fixadoras de nitrogênio que não foram encontradas em solos temperados. Tôdas estas espécies foram demonstradas de fixar entre 12 e 30 mg de N/g de glucose e de apresentar elevada tolerância a solos ácidos.

2) Temperaturas ótimas para o desenvolvimento dêstes organismos ocorrem pràticamente durante o ano todo.

3) Efeitos altamente estimulantes de plantas superiores sôbre três dêstes organismos foram demonstrados, destacando-se o efeito da cana-de-açúcar sôbre *Beijerinckia* spp. e o de *Paspalum notatum* sôbre *Azotobacter paspali*.