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Review article

History and New Perspectives of Diazotrophs in Association With Non-Leguminous Plants

JOHANNA DÖBEREINER
EMBRAPA-CNPBS, Seropédica, 23851, Rio de Janeiro, Brazil
Tel. 55 (21) 682-1500, Fax 55 (21) 682-1230

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Abstract

The first observations indicating interactions, later called rhizosphere associations, of diazotrophs with non-leguminous plants were those of a selective enrichment of *Beijerinckia* spp. in the rhizosphere of sugar cane and the specific association of one ecotype of *Paspalum notatum* with *Azotobacter paspali*. The identification of the various *Azospirillum* species, all of which were found to form some kind of association with non-leguminous plants, expanded hopes for N_2 fixation in cereals and grasses. Even though azospirilla were found to infect cereal roots, multiply within xylem vessels and translocate to aerial parts, they are considered soil microorganisms. More ambiguous seems to be the habitat of the less well-known diazotroph *Herbaspirillum seropedicae* which originally was isolated from roots and stems of several cereals. Very recent results indicate that this diazotroph is closely related to sugar cane pathogens known as *Pseudomonas rubrisubalbicans*, a group of taxonomically misplaced *Pseudomonas* spp. DNA/rRNA hybridizations indicate them to be two species in the genus *Herbaspirillum*. Both species show aerotactic growth in N-free semisolid medium and high nitrogenase activity as well as slight plant pathogenicity. Another new diazotroph, *A. diazotrophicus*, is also considered a plant endophyte in sugar cane stems and leaves. These endophytic bacteria have not been isolated from soil and occur predominantly in plants propagated vegetatively. They have been also isolated from spores of VA mycorrhizal fungi and could be introduced into sugar cane, sorghum and sweet potatoes via VAM infection by such spores. These recent findings open up entirely new ways of approaching the expansion of N_2 fixation to cereals and grasses. That this is possible, has been shown in recent ^{15}N and N balance studies on sugar cane, where more than 150 kg $N \cdot ha^{-1} \cdot yr^{-1}$ were obtained from N_2 fixation.

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1. Introduction

At the second Bayreuth Meeting of this Series in 1984, we celebrated ten years of *Azospirillum* with only three species known and the *Azospirillum* rhizosphere association, as it is usually still dominated, was considered the principle alternative to the legume *Rhizobium* symbiosis, for cereals and other grasses. Since then however, besides two new *Azospirillum* species, two plant endophytic diazotrophs have been described which, together with reports of large numbers of azospirilla in aerial parts of cereals and several other plants, lead to substantial changes in the concept of plant diazotroph associations, which will be the subject of this paper.

The plant is the prime source of nutrients for microorganisms either in soil, providing them indirectly from exudates or dead tissues, or directly when microorganisms colonize the interior of plant roots or other organs. It is understandable that microorganisms should have developed various ways of interacting with plants in order to gain access to the nutrients provided by them. The elucidation of the mechanisms and especially the identification of the responsible microorganisms in such associations is still in its infancy. It is considerably more advanced with pathogenic associations than with those of cereals or grasses with diazotrophs. Various bacteria have been isolated from roots or from the rhizosphere which were described as diazotrophic but later were not confirmed as such (Hill and Postgate, 1969). More recently, several new actively N_2-fixing microaerobic bacteria have been identified and their association with cereals and grasses demonstrated (Döbereiner and Pedrosa, 1987).

Since the rediscovery, in 1975 (Döbereiner and Day), of *Spirillum lipoferum* as a very common, root associated diazotroph, almost all basic research and most inoculation experiments have used one strain of *Azospirillum brasilense*, Sp7 (or Cd which is a red mutant of the same strain) and many conclusions based on results with this strain have been widely generalized. Few results are available on experiments with other *A. brasilense* or *A. lipoferum* strains and none on the new endophytic diazotrophs. No unequivocal proof of N_2 fixation in economically important amounts due to inoculation with diazotrophs is as yet available. But several data obtained from experiments without inoculation give clear evidence that N_2 fixation associated with Gramineae can be significant. A typical example is that of rice reported by App et al. (1984) where total N analyses in long term fertility plots at two sites in the Philippines, performed before and after 17 and 24 crops, yielded positive balances of 103 and 79 kg N/ha per year. Estimates over shorter periods were obtained by $^{15}N_2$ incorporation or ^{15}N dilution methods. Substantial, although very variable amounts

of N_2 fixation have been demonstrated with these methods in rice (Eskew et al., 1981), sorghum (Giller et al., 1984), forage grasses (De-Polli et al., 1976) and sugar cane (Lima et al., 1987). Substantial N_2 fixation in the order of more than 150 kg N/ha·yr (superior to 60% of the total N incorporation) has now been shown in ^{15}N dilution and N balance experiments with sugar cane (Boddey et al., 1991). In order to exploit all these naturally occurring associations and improve them, the responsible diazotrophs must be identified and mechanisms of their interactions with plants be better understood.

In this paper we will summarise and discuss the most important characteristics of several new diazotrophs which associate with cereals and sugar cane.

2. New Root Associated Diazotrophs

Semi-solid N-free media, besides for azospirilla, were the key for the isolation of a number of additional root-associated diazotrophs indicating that the localization of such bacteria is at sites where O_2 is limiting. A comparison of the most important microaerobic diazotrophs is given in Table 1.

The two new *Azospirillum* spp., *A. halopraeferans* (Reinhold et al., 1987) and *A. irakense* (Khammas et al., 1989) seem more similar to *A. amazonense* than to the two classical species even though their salt and temperature tolerances are very distinct characteristics of adaptation to their environment. *A. halopraeferans* so far has been found only in association with Kallar grass (*Leptochloa fusca*) an extremely vigorous forage grass used in Pakistan for the recuperation of saline, alkaline soils. Attempts to find this bacteria in the semi-arid Northeast of Brazil failed although the *A. lipoferum* and *A. brasilense* strains isolated from various grass roots in this region showed significantly higher pH and temperature tolerance than the type strains (Reinhold et al., 1988). The more recently described *A. irakense* was found in the rhizosphere and in roots of paddy rice in Iraq (Khammas et al., 1989). The description of this new species was based on physiological and genetic characteristics of 7 isolates, all from the Diwaniyah region in Iraq. Although cell form and N_2 dependent growth in NFb medium are similar to that of other azospirilla, colonies on NFb agar with 20 mg yeast extract per litre are translucent, glistening and very small. While *A. halopraeferans* needs 0.25% NaCl for optimum N_2 dependent growth, *A. irakense* is not salt dependent even though both organisms are tolerant to more than 1% NaCl. Both new species were confirmed in DNA/DNA homology experiments.

Table 1. Comparison of microaerobically N_2 fixing bacteria which occur in association with plant roots (Tarrand et al., 1978; Baldani et al., 1986; Magalhães et al., 1983; Cavalcante and Döbereiner, 1988; Gillis et al., 1989; Reinhold et al., 1987; Khammas et al., 1989).

	Azospirillum brasilense	*A. amazonense*	*A. halopraeferans*	*A. irakense*	*Herbaspirillum seropedicae*	*H. rubrisubalbicans*	*Acetobacter diazotrophicus*
Growth under air	+	+	+	+	+	+	+
Microaerobic N_2-fixation	+	+	+	+	+	+	+
Aerotaxis	+	+	+	+	+	+	+
N_2 fixation unaffected by 10 mM NO_3^-	–	–	–	–	–	–	+
Use of sucrose	–	+	–	+	–	–	+
Optimum pH	6.7–7.0	5.8–6.6	6.8–8.0	6.5–7.5	5.3–8.0	6.0–8.0	3.8–5.5
Growth at 41°C	–	–	+	–	+	+	–
Isolated from surface steril. roots	+	+	–	+	+	+	+
Isolated from stems	+	+	–	–	+	+	+

3. Endophytic Diazotrophs

A smaller acid tolerant diazotroph was found to predominate in maize roots in Brazilian Savanna soils (cerrados) and was later isolated from washed and surface sterilized roots and stems of maize, rice and sorghum in Rio de Janeiro. It was initially identified as a new *Azospirillum* species but later RNA/RNA hybridization studies showed it to be a new genus, named *Herbaspirillum seropedicae* (Baldani et al., 1986) (Fig. 1). The organism has bipolar flagella, and

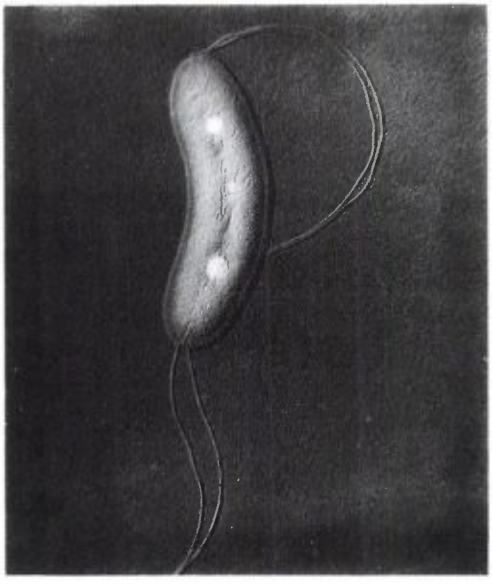

Figure 1. **Scanning electron micrograph of *Herbaspirillum seropedicae* (10,000×).**

N_2 fixation is more O_2 and pH tolerant than that of azospirilla. Nitrogenase activity of this organism and of *A. amazonense* is only partially inhibited by 10 mM NH_4^+ during several hours, while that of *A. brasilense* and *A. lipoferum* is completely inhibited within minutes (Hartmann et al., 1986, Fu et al., 1988). Clear-cut differences between *Azospirillum* spp. and *Herbaspirillum* were also confirmed by their membrane protein patterns in SDS-PAGE where this last species showed a characteristic major band at 37.5 Kdaltons (Dianese et al., 1989). This organism can not be isolated from soil (recent unpublished observation) and therefore must be considered a plant endophyte.

Very surprising results of DNA/rRNA and DNA/DNA hybridization confirming computer assisted analysis of auxonographic tests showed that the generically misnamed "*Pseudomonas rubrisubalbicans*", causing mottled strips

disease in sugar cane must be included in the genus *Herbaspirillum* (Gillis et al., 1991).

Pimentel et al. (1991) showed that the type strain of "*P. rubrisubalbicans*" isolated from sugar cane in the USA as well as 5 additional strains of this species, all isolated from sugar cane in Reunion, Jamaica and Mauritius, respectively, were found to grow abundantly in N-free semi-solid medium, showing growth pattern and nitrogenase activity rates as high, or higher, than *H. seropedicae* (Table 2). All other *Pseudomonas* spp. found so far to fix N_2, as for example *P. diazotrophicus* (Watanable et al., 1987), are unable to grow on N_2 as sole N source and ARA is less than 10% of that reported here.

In addition to N_2-fixation the "*P. rubrisubalbicans*" strains showed a number of physiological characteristics identical to those of *H. seropedicae*. Acid was formed from arabinose but only variable (among strains, independent of origin) weak acid production was observed from glucose, galactose and mannitol. No acid was formed from glycerol. All strains grew very well in semi-solid media with these carbon sources. There was N_2 dependent growth and ARA at 37°C but at 41°C, growth occurred only with yeast extract as N source (unpublished). The swarming on soft nutrient agar observed for *H. seropedicae* (Baldani et al., 1986) was also shown with all "*P. rubrisubalbicans*" strains

Table 2. Nitrogenase activity after 44 hr growth in N-free semi-solid medium and plant pathogenicity of "*P. rubrisubalbicans*" and *H. seropedicae* in *Sorghum* and *Pennisetum* (Pimentel et al., 1991)

Bacterial strain	nmoles C_2H_4/hr 5 ml[4]	Symptoms in *Sorghum*[2] & *Pennisetum*	Re-isolation[4]
"*P. rubrisubalbicans*"			
LMG 2286 (ATCC 19308)	385	+	+
LMG 1278	754	+	+
LMG 6415	95	+	+
LMG 6420	426	+	+
IBSBF[2] 175	34	+	+
IBSBF 198	266	0	+
H. seropedicae			
Z67 (ATCC 35892)	92	+	+
Z78 (ATCC 35893)	135	0	+
Z176	34	+	+
Uninoculated control	0	0	0

[1] Belgian culture collection (Gent).
[2] Brazilian culture collection (Inst. Biol. Seção de Bacteriologia Citopatológica).
[3] Variable intensity of mottled stripe disease (+) or no symptoms (0)
[4] In N-free semi-solid NFb medium.

tested. Tolerance to high sugar concentrations would be expected from isolates of sugar cane as also observed with *Acetobacter diazotrophicus* (Gillis et al., 1989). All strains of "*P. rubrisubalbicans*", as well as those of *H. seropedicae*, grow and fix N_2 with 10% sucrose even though this sugar is not being used (Pimentel et al., 1991).

When inoculated into leaves of Napier grass and sorghum by the traditional phytopathological methods, *H. seropedicae* caused similar symptoms as the "*P. rubrisubalbicans*" strains (Table 2). Both organisms were pathogenic to sorghum which reacted with symptoms of red stripes on leaves and stems. Symptoms on Napier grass were mottled stripes and water soaking on the inoculation point. The sugar cane cv. B 4362, the only Brazilian cultivar susceptible to mottled stripe disease, did not develop symptoms when inoculated with *H. seropedicae* while some of the "*P. rubrisubalbicans*" isolates from various origins produced the expected symptoms on this cane cultivar.

The inoculated bacteria could be reisolated from the leaves, 3 cm above the inoculation mark (Table 2) from all inoculated plants. All attempts to isolate these organisms from control plants failed except those from sugar cane in which all contained N_2-fixing *H. seropedicae* (until dilution 10^{-5}). *Herbaspirillum* spp. has then also been isolated in numbers of up to $10^6 \cdot g^{-1}$ from leaves and stems of sugar cane collected in various regions of Brazil and Uruguay (see paper Baldani et al. at this symposium).

Acetobacter diazotrophicus isolated from sugar cane (Cavalcante and Döbereiner, 1988; Gillis et al., 1989) is the most recently discovered diazotroph (Fig. 2). This most extraordinary diazotroph was originally isolated from semi-solid sugar cane juice inoculated with dilutions of sugar cane roots and stems which showed ARA up to dilutions 10^{-6} or 10^{-7}. Improved counting and isolation procedures were obtained in N-free mineral medium containing 10% cane sugar and 0.5% cane juice, acidified with acetic acid to pH 5.5. The bacterium is a small Gram-negative aerobic rod showing pellicle formation (Micro-aerobic chemotaxis) forming a thick surface pellicle after 5 days. Best growth occurs with high sucrose or glucose concentrations (10%) and strong acid production results in a final pH of 3.0 or below. Growth and N_2 fixation (more than 100 nmoles/h·ml) continues at this pH for several days (Stephan et al., 1988). Ethanol is used for growth and is oxidized to CO_2 and H_2O. Dark brown colonies form on potato agar with 10% sugar and dark orange colonies on N poor (0.005% yeast extract) mineral agar medium with 10% sugar and bromothymol blue. The bacterium posesses no nitate reductase and N_2 fixation is not affected by high levels (25 mM) of NO_3^-. Also NH_4^+ shows only partial inhibition of nitrogenase (Teixeira et al., 1987; Fu et al., 1988; Reis et

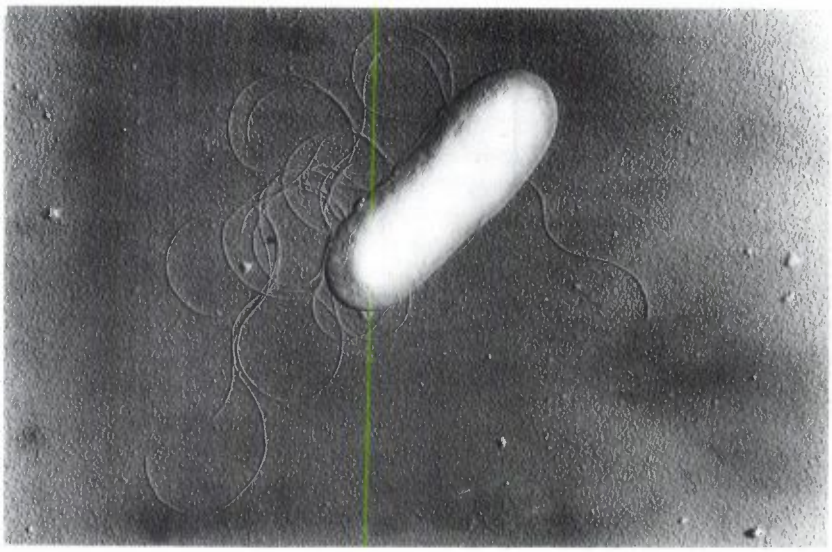

Figure 2. Scanning electron micrograph of *Acetobacter diazotrophicus* (9,800×).

al., 1990; Stephan et al., 1991) and $^{15}NH_4^-$ experiments showed that NH_4 inhibition of nitrogenase was further reduced by high sugar concentrations (See Reis and Döbereiner, this symposium). The incomplete inhibition of N_2 fixation by NH_4^- in these organisms as well as the lack of nitrate reductase cited above are of considerable ecological and agronomic importance because they may permit complementation of biological nitrogen fixation with N fertilizers.

Taxonomic studies based on DNA and rRNA analyses showed that the bacterium belongs to the *Acetobacter* rRNA cistron (Gillis et al., 1989) and there it is most similar to *A. liquefacieus.* This species, however, does not fix N_2, does not form pigmented colonies on potato media and shows several other physiological differences. DNA/DNA binding experiments confirmed it to be a new species (Gillis et al., 1989). The name originally proposed, (*Saccharobacter nitrocaptans*, (Cavalcante and Döbereiner, 1988) had to be changed to *Acetobacter diazotrophicus.*

The bacterium has been found in many sugar cane cultivars in several regions of Brazil and numbers were in the range (all per g fresh weight) of 10^3 and 10^5 in rhizosphere soil, 10^3 to 10^7 in washed roots, 10^3 to 10^5 in surface sterilized roots, 10^3 to 10^4 in basal and apical stems and 10^4 to 10^7 in sugar cane trash (Döbereiner et al., 1988). It was not found in soil between rows or roots from 12 different weed species growing there. It also was not found in grain or sugar sorghum but was isolated from a few samples of washed roots and aerial parts of *Pennisetum purpureum* cv. Cameroon and from sweet potato stems and

roots, another plant propagated vegetatively by cuttings (Paula et al., 1989; Reis and Döbereiner, 1991). When micro-propagated sterile plantlets were inoculated with *A. diazotrophicus* by immersion of the rootlets into a culture, before acclimatizing and transplanting into fumigated soil inoculated with vascular arbuscular mycorrhizae (VAM) pronounced effects on plant growth and N incorporation were observed (Table 3). Non-mycorrhizal plants grew very poorly.

Recent results of Paula et al. (1991) showed that spores of the VAM fungus *Glomus clarum* obtained from sweet potatoes grown with this fungus and with an enrichment culture of *Acetobacter diazotrophicus*, contained this bacterium besides several others, including a diazotrophic *Klebsiella* sp. *A. diazotrophicus* infected aerial parts of sweet potatoes only when inoculated together with VAM or within VAM spores. Micropropagated sugar cane seedlings inoculated with the same VAM spores containing diazotrophs grown in non-sterile soil confirmed highest infection of aerial parts after inoculation with VAM spores containing diazotrophs (Table 4). This treatment also increased VAM colonization and numbers of spores formed within roots. Similar effects were observed in sweet sorghum except that the bacteria were not translocated to the tops.

The possibility of infecting sugar cane seedlings with endophytic diazotrophs via VA mycorrhizae represents a unique possibility of introducing selected or genetically improved bacteria into plants which then are further propagated within the stem cuttings. Recent changes in the technology of sugar

Table 3. Effect of inoculation with *Acetobacter diazotrophicus* on yield and N incorporation of sweet potatoes grown from micropropagated sterile plantlets transfered into fumigated soil inoculated with VAM (means of 6 replicates) after Paula et al. (1989)

Treatment	N% roots	N% tops	Total N mg/2 plants	Tuber yield g/pot
20 ppm N				
Control	0.57c[2]	1.07b	225c	13.5c
A. diazotrophicus + *Klebsiella* sp.[1]	0.93a	1.81a	848a	32.8a
A. diazotrophicus Type strain Pal-3	0.95a	1.74a	710ab	25.8ab
60 ppm N				
Control	0.84b	1.83a	693b	17.7bc

[1] *A. diazotrophicus* and *Klebsiella* sp. isolated from sweet potato.
[2] value with different letters are statistically different at p = 0.05.

Table 4. Colonization of *A. diazotrophicus* of micropropagated sugar (c) and sugar Sorghum (s) seedlings in fumigated substrate (Means of 4 replicates). (Paula et al. (1991))

Inoculant	No. of *A. diazotrophicus* g^{-1} fresch wt $\times 10^5$					
	Washed Roots		Surface roots	Sterile	Plant	Top
	C	S	C	S	C	S
Control	0	0	0	0	0	0
Glomus clarum	0	0	0	0	0	0
A. diazotrophicus inoculated *in vitro*	17.0	0.06	1.0	0.03	1.0	0
Spores containing mixed diazotrophs[1]	12.5	0.20	5.3	0.25	27.0	0

[1] Enrichment culture in N free LGI medium (Cavalcante and Döbereiner, 1988) obtained from sweet potato root macerte.

cane plantations in Brazil's largest alcohol and sugar producing cooperative COPERSUCAR, envisage the plantation, in nurseries, of disease-free micropropagated seedlings which produce sugar cane for cuttings to be planted by the farmers for renewal of their plantations. The inoculation of such seedlings with selected diazotrophs and VA mycorrhizae will not only make seedlings grow faster, but will ensure disease-free highly efficient N_2 fixing sugar cane in the field. The use of VA mycorrhizae might also help to introduce such endophytic bacteria into seed plants such as sugar sorghum and with this permit extension of nitrogen fixation, to other sugar/starch accumulating crops, for biofuel production in cooler climates where sugar cane does not grow.

The occurrence of diazotrophs like *A. diazotrophicus* and *Herbaspirillum* spp. in leaves and stems in large numbers (there are more than 100 t of plant material containing 10% sugar) contrasts with the classical concept of rhizosphere associations, where only a small proportion of the root exudates is available for them. Considering laboratory efficiencies of 20 mg N/g sugar even at high sugar concentrations (2.5%) (Cojho et al., 1991) less than 30% of the total sugar produced by a sugar cane crop would be used up for N_2 fixation. Plants with the C_4 photosynthetic pathway, like sugar cane or sorghum however, are known to use less than 50% of their photosynthetic capacity (Machado, 1987) and therefore can well afford 30% to feed diazotrophs. Brazilian sugar cane cultivars have been bred for more than 50 years, with very low nitrogen fertilizer levels (less than 25% of the amount incorporated) and it is therefore not surprising that these cultivars unknowingly have been bred for nitrogen fixation.

The recent advances in N_2 fixation research in the tropics indicate many new possibilities for replacement of nitrogen fertilizers and increasing crop yields. Biotechnology will play a major role in speeding up progress and helping to solve problems which with traditional selection or adaptation methods would take much longer to be solved.

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