Gluconacetobacter diazotrophicus (syn. *Acetobacter diazotrophicus*), a promising diazotrophic endophyte in tropics

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Endophytes are plant-associated prokaryotes that form association with their host plants by colonizing the internal tissues, which has made them valuable for agriculture as a tool in improving crop performance. Although the interaction between endophytes and host plants has not been fully understood, many bacterial species are reported to promote plant growth and the mechanism attributed includes nitrogen fixation, production of growth-promoting substances and increased resistance to pathogens and parasites. They have been reported from numerous plant species including sugarcane. *Gluconacetobacter diazotrophicus* (syn. *Acetobacter diazotrophicus*) – sugarcane association represents a model system for monocot-diazotrophic associations. This allows experimentation to answer questions pertaining to their establishment, colonization process, biological nitrogen fixation, growth promotion, etc. The main objective of this review is to summarize the recent works on this bacterium with special emphasis on its interaction with sugarcane. The topics being covered range from the sources of *G. diazotrophicus*, its classification and characteristics, genetic analysis, in planta colonization and detection, inoculation experiments with suggestions for future research.

THE element nitrogen is highly abundant in the earth's atmosphere and is a major component of dietary proteins (as incorporated in amino acids). However, the availability of fixed N is the most significant yield-limiting factor in many agricultural production systems. Plant growth is directly influenced by the availability of reduced N, leading to the long accepted practice of manuring, fertilizer application, or rotational crop practices[1]. Nitrogenous chemicals account for as much as 30% of the total fertilizers needed for agricultural crops. With the increasing cost of chemical fertilizers and concern about environmental pollution, the role of biological nitrogen fixation (BNF) in supplying plants with needed N, which can make agriculture more productive and sustainable without harming the environment, has to be harnessed efficiently. As BNF is not restricted to legumes only, for sustainable agriculture it becomes necessary to increase the amount of biologically fixed N in non-legume crops also. Significant nitrogen input into the global nitrogen cycle has been reported through the *Azolla–Anabaena* symbiosis[2]; nitrogen fixation by free living cyanobacteria, *Azotobacter*, *Azospirillum*, *Acetobacter*, *Herbaspirillum* or *Pseudomonas* species[3,4]; and the *Frankia*–non-legume nodulation[5], etc.

Studies indicate that rhizosphere, roots, stems and leaves of even healthy plants harbour diverse microbial communities that include N fixing bacteria[6–9], and part of the N accumulated by non-leguminous plants was proved to have been fixed by the root-zone bacteria[10]. A special term, diazotrophic associative symbiosis, was suggested to describe the process of N fixation in the plant root-zone[11]. However, the interaction studies between N fixing bacteria and non-leguminous plants began only three decades ago[12].

Gluconacetobacter diazotrophicus

A variety of diazotrophic bacteria have been isolated from rhizosphere (*Beijerinckia*) and roots (*Azospirillum*, *Bacillus*, *Klebsiella*, *Enterobacter*, *Erwinia*) of sugarcane

Table 1. *G. diazotrophicus* – sources

Source	Part
Sugarcane	Root, root hair, stem, leaf
Cameroon grass	Root, stem
Sweet potato	Root, stem tuber
Coffee	Root, rhizosphere, stem
Ragi	Root, rhizosphere, stem
Tea	Root
Pineapple	Fruit
Mango	Fruit
Banana	Rhizosphere
Others – mealy bugs, VAM spores	Internal environment

From refs 16–18, 23, 69.

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plant. In 1988, Cavalcante and Dobereiner[4] reported an acid-tolerant N-fixing bacterium, *Acetobacter diazotrophicus*, associated with sugarcane which contributed abundant N to sugarcane crop[6], with a capability to excrete almost half of the fixed N in a form that is potentially available to plants[13].

A. diazotrophicus has also been isolated from other plants, viz. cameroon grass (*Pennisetum purpureum*), sweet potato (*Ipomoea batatas*), coffee (*Coffea arabica*), tea, banana, ragi, rice and pineapple[14–19] (Table 1) and even from insects that infest sugarcane[20–22]. In the absence of plants, it has also been reported to associate with VAM fungal resting spores[23]. Even though basically an endophyte, it has also been isolated/detected from rhizosphere soils[14,16,24,25]. This is attributed to the presence of root hairs, small root pieces and vesicular-arbuscular mycorrhizal spores[23,26] in rhizosphere soil samples inoculated into the culture medium. A report by Jimenez-Salgado and his co-workers[16] suggested that the presence of this bacterium in the rhizosphere soil of coffee plants, may be due to the high organic matter present in those soils compared to the sugarcane soils, where the sugarcane is burned-off virtually eliminating all organic matter originating from senescent and trash leaves. It is further suggested that the soil organic matter could protect this bacterium against soil physiological factors. Further study to know the relationship between its survival in relation to the organic matter content is required to confirm this hypothesis.

Classification and characteristics

Recently, Acetobacteraceae has been reported to include various genera, viz. *Acetobacter*, *Gluconobacter*, *Gluconacetobacter* and *Acidomonas*[27]. Subsequently, based on the 16s rRNA sequence analysis, *A. diazotrophicus* has been corrected as *G. diazotrophicus*[28]; recently two more N-fixing species have been added to the list, viz. *G. johannae* and *G. azotocaptans*[29] (Table 2).

G. diazotrophicus is a Gram-negative, acid-tolerant, obligate aerobe and the cells are straight rods with rounded ends (0.7–0.9 μm by 1–2 μm). The cells can be seen under microscope as single, pair or chain-like structures without endospores. This bacterium grows on high sucrose concentration (10% sucrose) and very low pH (3.0) and has the ability to fix N under microaerophilic conditions[4,30–32]. The carbon source that best supports growth is sucrose at 10% and the bacterium prefers to grow even at high concentrations of sucrose (30%; Table 3). Since sucrose cannot be transported or respired by *G. diazotrophicus*, it grows by secreting an extracellular enzyme, levansucrase[33], that can hydrolyse sucrose into

Table 2. Phenotypic characteristics of isolates of diazotrophic *Gluconacetobacter* species*

Determining characteristics	*G. diazotrophicus*	*G. johannae*	*G. azotocaptans*
Gram reaction	–	–	–
Oxidase	–	–	–
Catalase	+	+	+
Dark-brown colonies on potato agar (10% sugar)	+	–	–
Growth on:*			
D-Galactose	+	±	+
D-Xylose	+	–	+/–
D-Raffinose	+/+	–	+
D-Arabinose	±/–	–	+
Melibiose	±	±/–	±
Maltose	+/+	±/–	+/–
Mannose	–	–	+
D-Sorbitol	+/±	+/±	+
Glycerol	±/–	–	+
D-Mannitol	±/–	–	+
Ethanol	+	+/±	±
Butanol	±	–	–
Growth on L-amino acids in the presence of sorbitol as carbon source:			
L-Cysteine	–	+	+
L-Glutamic acid	–	+	+
L-Proline	–	–	+
L-Tryptophan	+	–	+|
Growth with 30% sucrose and glucose	+	+	+
Nitrogenase activity	+	+	+

Regardless of the presence or absence of growth factors from yeast extract.
+, Good growth; ±, Slight growth; –, No growth.
*From ref. 29.

Table 3. Nitrogenase activity of *G. diazotrophicus* with different concentrations of sucrose and yeast extract 100 mg l^{-1}, *n* moles of C_2H_4 h^{-1} $vial^{-1}$ after 72 h

Sucrose concentration (%)	Isolate code				
	TR1	MR4	Mg	T8	LMG
	222	408	157	648	155
20	184	161	166	423	95
30	141	128	183	113	56
40	8	30	13	56	29
50	0	0	0	0	0

0, No nitrogenase activity; Mean (SD), $n = 4$ replications; From ref. 39.

fructose and glucose[31,34]. Some other good sources of carbon include gluconate, glucose, fructose, mannitol, arabinose, meso-inositol, i-inositol, sorbitol, glycerol, galactose, jaggery and sodium gluconate[4,14,15,35,36]. Amino acids such as glutamate, serine, alanine and histidine can be efficiently used as carbon and N sources[37] by *G. diazotrophicus*. However, cellobiose, starch, meso-erythritol and methanol (1%) did not favour its growth[14]. None of the common organic acids, viz. succinate and other dicarboxylic acids supported the growth of *G. diazotrophicus*[38], except 2-keto gluconic acid[20], which has its presence in sugarcane plants. It was reported that the bacteria utilize it as a carbon source and it favours higher N fixation[39]. The optimum pH for growth was reported to be 5.5 (ref. 32), although pH for rates of respiration was reported to vary considerably according to C sources[40]. Given such characteristics, search for novel *G. diazotrophicus* strains which could multiply both in sugars (sucrose, glucose, etc.) and organic acids (malate, citrate, etc.) as energy sources, that are present abundantly in root environment as exudates, will aid the biofertilizer research.

G. diazotrophicus has been recognized as an aerotolerant diazotroph[32] in which oxygen is instrumental for the generation of large quantities of ATP required for N fixation. The extracellular oxidation to gluconate plays a major role in the first step of glucose metabolism by *G. diazotrophicus*[32,41]. A pyrroloquinoline quinone (PQQ)-linked glucose dehydrogenase carries out the periplasmic conversion of glucose to gluconate[41,42]. Recently Luna *et al.*[43] showed that the biomass yield of *G. diazotrophicus* was 30% less in N-fixing conditions, than in non N-fixing conditions when gluconate was used as the carbon source. It was also reported to grow profusely under high aeration with increasing diazotrophic activity, an indication of the key role played by two components of the respiratory system, glucose dehydrogenase and cytochrome *ba*, during aerobic diazotrophy in *G. diazotrophicus*[44].

In addition, it does not inhibit the effect of nitrate on N fixation to a certain extent, which is an added advantage and encourages the use of *G. diazotrophicus* as biofertilizer even in environments applied with nitrate fertilizers[24]. This phenomenon is attributed to the absence of nitrate reductase in *G. diazotrophicus*[32]. Reis and Dobereiner[45] observed that nitrogenase of this organism is protected against inhibition by oxygen at high sucrose concentration (10%), but is much more sensitive to inhibition at 1% of sugar. Nitrate was also found to influence nitrogenase activity in *G. diazotrophicus* at higher concentrations[6,14,24] and complete repression was reported upon NH_4Cl complementation. Recently, we have isolated a novel acetobacteria from wetland rice that could tolerate up to 150 mM nitrate in the culture medium, which will be very useful in inoculation experiments as biofertilizer in N unlimited conditions[46]. Most of *G. dizotrophicus* isolates were found to be tolerant to streptomycin than tetracycline and rifampicin[15,24], and a recent report indicates the tolerance of *G. diazotrophicus* to various antibiotics, viz. ampicillin, erythromycin and roxithromycin[47].

It has also been established that *G. diazotrophicus* exhibits antagonistic potential against *Colletrotrichum falcatum*, a causal organism of redrot in sugarcane[48]. This was attributed to the ability of *G. diazotrophicus* to ferment sugars and reduce pH of the medium to below 3.0 (ref. 4). The acid production by *G. diazotrophicus* has been an added value, where it could also solubilize insoluble phosphates in broth assays[49]. As *G. diazotrophicus* is an endophyte, how far the P solubilization inside the cells will benefit a plant system needs to be addressed and is open for further studies.

Genetic analysis

The order and arrangement of *nif* and associated genes on chromosomes (or sometimes plasmids) vary tremendously among diazotrophs[50]. The 16s rDNA-based phylogenetic analysis shows that *Gluconacetobacter* clusters with the genera *Rhodopila*, *Acidomonas*, *Acidiphilium* and *Gluconobacter*[51–53]. Sevilla *et al.*[54] reported the similarity between *nif*HDK of *G. diazotrophicus* (Table 4) and other N-fixing bacteria, particularly with diazotrophic members of plant-associating α-subgroup of Proteobacteria. The similarity was in the ranges of 91% *Nif*H (*R. leguminosarum bv phaseoli*), 91 and 89% of *Nif*D (*Herbaspirillum seropedicae* and *Azospirillum brasilense*) and 76% *Nif*K (*Bradyrhizobium japonicum*). Franke *et al.*[55] determined the *nif*H sequence of *G. diazo-*

Table 4. Features of G. *diazotrophicus nif*HDK genes and gene products

Gene	Coding region	G + C content	Total amino acid	Predicted MW (Da)
Nif H	448–1344	64	298	31,873
Nif D	1409–2905	59	498	55,896
Nif K	2970–4505	60	511	57,236

From ref. 54.

trophicus and found it to cluster with *A. brasilense*, *Rhodobacter capsulatus* and *Rhodospirillum rubrum*, and to be similar to *A. brasilense* in pairwise sequence similarity analysis. The overall arrangement of genes in the *nif/fix* cluster of *G. diazotrophicus* also seems to be more like that of *A. brasilense*[56,57]. Lee *et al*.[58] reported *nif/fix* and associated gene cluster of *G. diazotrophicus*, as a unique one, representing the largest assembly of contiguous genes so far characterized in any of the diazotrophs. The *nif*A and *nif*B gene products were reported to be similar to the NifA and NifB proteins of *Azorhizobium caulinodans* and *R. capsulatus* respectively. The *Nif*A activity in *G. diazotrophicus* is reported to be inhibited by oxygen as in the case of other proteobacterial α-groups of diazotrophs[59]. A conspicuous absence is the *nif*Y in *G. diazotrophicus*. In addition, *Mod*D (molybdenum transport) and *Mcp*A (chemotaxis response) genes were also found, where their influence on N-fixing ability or plant colonization has not yet been determined. Moreover, *G. diazotrophicus* and *A. brasilense* are the only two diazotrophs so far characterized that have an *Mcp*A-like gene associated with *nif/fix* genes[60]. The similarity shared between *A. brasilense* and *G. diazotrophicus* in harbouring *Mcp*A protein responsible for chemotactic responses of specific genes, could be due to lateral transfer from one to another in common monocot-associated ancestral species[60]. The similarity showed by *Nif*D gene product of *G. diazotrophicus* with *Herbaspirillum seropedicae*, another known endophyte of sugarcane, suggests that there is a likelihood of a bacterial gene transfer among diazotrophs in the plant vascular system. *G. diazotrophicus* was also reported to harbour plasmids ranging from 2 to 170 kb (ref. 21). However, their functions are yet to be identified, and most of the fundamental characters are reported to be plasmid-encoded[61].

The genetic diversity of *G. dizotrophicus* from various environments seems to be less[16,21,62] and estimated diversity through RAPD analysis was more among *G. diazotrophicus* isolates than the diversity on the basis of morphological and biochemical characters[63]. The SDS–PAGE and MLEE (multilocus enzyme electrophoresis) analysis[20,22,31] also showed only slight differences among *G. diazotrophicus* strains, an indication of genotypic differences[64]. Recently, the existence of genetically distinct *G. diazotrophicus* strains in sugarcane cultivars of Louisiana was reported based on genomic fingerprinting studies[65]. Tapia-Hernandez *et al.*[19], based on their detection studies of *G. diazotrophicus* in pineapple, hypothize that (i) only some genetically related groups of *G. diazotrophicus* or its ancestor have acquired the capability of colonizing plants by themselves or with the aid of the vectors such as insects or fungi, and (ii) only in the recent period some selected genotypes would have found their way into different plant taxa. However, it is essential to perform comparative genetic analysis of different *G. diazotrophicus* isolates obtained from different hosts and regions to confirm this hypothesis.

Colonization *in planta*

Unlike legume symbiosis, specialized structures similar to nodules or other gross morphological changes, are not found in grasses colonized by diazotrophic bacteria. Association of *G. diazotrophicus* with sugarcane represents a new kind of symbiosis between a diazotroph and a monocot[60]. Considering the association of *G. diazotrophicus* with sugarcane, it is termed as 'obligate endophytes'[66], since it cannot be isolated from root-free soils and can only be isolated from plants, fungi, insects, etc. However, based on the uniqueness of the association, Reinhold-Hurek and Hurek[67] proposed the term 'opportunistic endophytes', as they do not colonize living plant cytoplasm or host cells or form any organelle as evidenced in arbuscular mycorrhizal fungi or rhizobia[9,68].

The microscopic observation and culture studies have confirmed its endophytic nature by its occurrence in relatively high numbers (10^6–10^7 cells g^{-1} fresh wt)[24,69] in surface-sterilized stems, leaves and roots of sugarcane[15,35,70]. It has been found to colonize sugarcane tissues that are devoid of dissolved carbon compounds, such as root and stem xylem vessels[71]. It was also observed to colonize intercellular apoplastic stem spaces[35] that constitute the sucrose niche and phloem sieve tubes[72] that translocate sucrose. The cells were found in microcolonies inside the stems with clumping[73] and randomly distributed on the plant surface in an apolar orientation forming a monolayer covering roots and leaves with lateral root junction[74].

The entry of bacteria starts from the points of emergence of lateral roots, where bacterial cells have been detected between the cell layers of the lateral root and the cortex of the main root[67]. It is also reported that *G. diazotrophicus* can use the cracks caused by the formation of lateral root junctions, the loose root-cap cells[15,54] and the tear wound sites created during separation of the plants before inoculation[72], as points of entry into the root apoplast. This kind of invasion has been reported to occur in other diazotrophic bacterial symbiosis also[9,67]; as this bacterium could not survive outside the plant tissue environment, it was also reported to transfer to subsequent crops through setts used in the vegetative propagation of sugarcane. *G. diazotrophicus* was also occasionally seen entering leaves via damaged stomata and subsequently colonizing sub-stomatal cavities and inter-cellular spaces[75]. It was reported that though there was clear evidence for internal colonization of the stems especially in vascular tissue and *G. diazotrophicus* was seen accumulating the lateral root junctions and colonizing the damaged epidermal cells, it does not penetrate beyond the root epidermis. Some other reports indicate the presence

of *G. diazotrophicus* even in the heat-treated setts of sugarcane[74], raising the possibility of transmission of *G. diazotrophicus* through generations. It is also possible that sugarcane plants are infected with *G. diazotrophicus* through various modes, viz. root materials, trash and infection by mealybugs that carry *G. diazotrophicus*[76]. A peculiar inference is that *G. diazotrophicus* has not been isolated from non-rhizosphere soil or from other weed species found in cane fields[14,76,77]. Sevilla *et al.*[54] observed that *Nif*$^-$ mutant of *G. diazotrophicus* colonized sugarcane plants to the same extent as that of wild strains.

Detection of *G. diazotrophicus in planta*

Electron microscopic studies have confirmed the presence of *G. dizotrophicus* in the apoplast and xylem vessels of sugarcane[14,35,54,71]. Use of *lac*Z (*b*-galactosidase encoding gene)-tagged *G. diazotrophicus* resulted in identifying the decline in population within days of inoculation and in areas that receive high N fertilization[72,78]. Sevilla *et al.*[54] as part of the colonization study, used three different marker genes – *uid*A, *gfp*, and *cob*A, and were successful in getting positive results on using *uid*A (GUS)-marked strains. Another indirect identification of the presence of bacteria in plant tissues is PCR, which warrants killing the tissues for DNA extraction. But the molecular-level identification provides a useful tool to identify the possible association. The PCR detection assay specific for *G. diazotrophicus* from plant tissues grown in field was reported by Kirchhof *et al.*[79]. Sievers *et al.*[51] developed a specific PCR method based on the amplification of specific 16S rRNA gene-fragment for the detection of different strains of *G. diazotrophicus*. Recently, Muthukumarasamy *et al.*[80] observed ammonium ions inhibiting the culturability of *G. diazotrophicus* inside the sugarcane plants, though it is present in high numbers (10^6–10^7 cells g^{-1} fresh tissue), through PCR detection using specific primers (Figure 1). Further, it was observed that high levels of N especially in the form of ammonium ions induce morphological changes in *G. diazotrophicus* cells resulting in long pleomorphic cells (Figure 2).

Inoculation experiments

The close association between a plant and an endophyte may provide suitable conditions for nutrient transfer between the bacteria and their host, than the association between predominantly rhizosphere bacteria and plants[81]. It was reported that up to 80% of the plant nitrogen in certain sugarcane varieties has been derived as a result of BNF[6]. Application of *G. diazotrophicus* to sugarcane has been proved beneficial where the plant height[54],

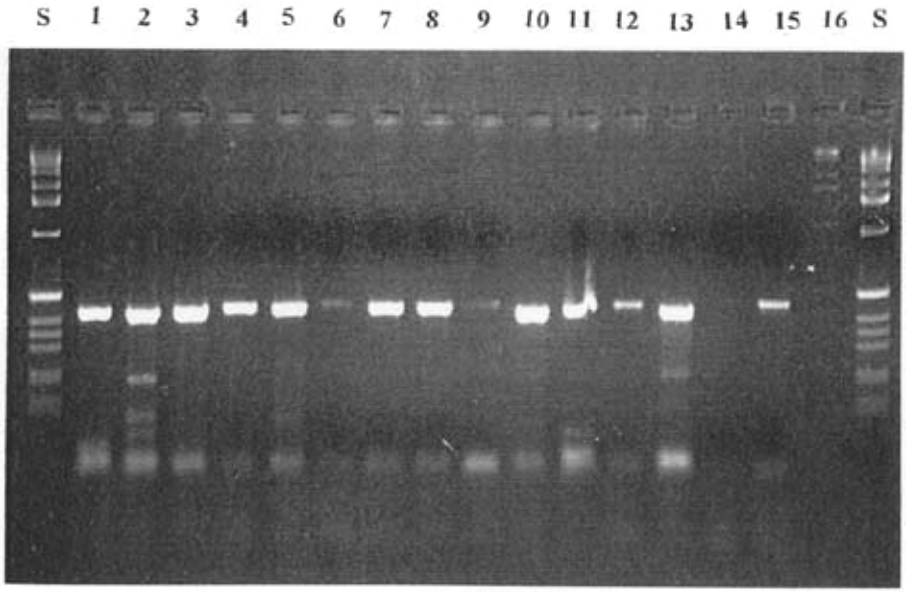

Figure 1. PCR products with primers AC/DI specific for *G. diazotrophicus* after 15 d of inoculation. Lane S, 1 kb ladder; lanes 1–3, Root, lower half and upper half portion of the variety Co 8021; lanes 4–6, Root, lower half and upper half portion of the variety Co 86249; lanes 7–9, Root, lower half and upper half portion of the variety Co 86010; lanes 10–12, Root, lower half and upper half portion of the variety of Co 86032; lanes 13–15, Root, lower half and upper half portion of the variety Co 87025; lane 16, Uninoculated control (whole plant).

nitrogenase activity, and yield[24] of the inoculated plants were higher than the control. Laboratory experiments on inoculation of rice with *G. diazotrophicus* have proved beneficial, where the inoculated seedlings grew taller than the uninoculated ones[56]. The maintenance of endophytic, diazotrophic populations by plants could be advantageous for growth in soils with low fixed N. However, it requires one to prove Koch's postulates through experimental evidence[67].

Plant inoculation studies revealed the abundant population of *G. diazotrophicus* in the artificially inoculated sugarcane plantlets, reflecting through enhanced ARA[82], supporting the suggestion of James *et al.*[71] that direct inoculation of *G. diazotrophicus* is possible. Inoculation of *G. diazotrophicus* was reported to enhance leaf N, biomass and yield[36]. Field trials conducted in sugarcane system revealed the usefulness of *G. diazotrophicus* with other diazotrophs, which have contributed to the yield equal to that of control (275 kg N ha^{-1}). Mixed inoculation of VAM spores and *G. dizaotrophicus* also proved beneficial in improving the yield of different sugarcane varieties. The yield was also not reduced even under 50–100% reduction from the recommended dose of chemical N compared to the control, attributing the role of inoculated *G. diazotrophicus* in N contribution[36]. It has been reported that inoculation of micropropagated sugarcane seedlings would make the plants not only grow faster, but also ensure efficient N-fixing plants in fields[83]. However, a recent report[75] negates these findings and concludes that the availability of this bacterium inside the micropropagated plants was relatively less and would not contribute significantly to any N-fixation by the plants.

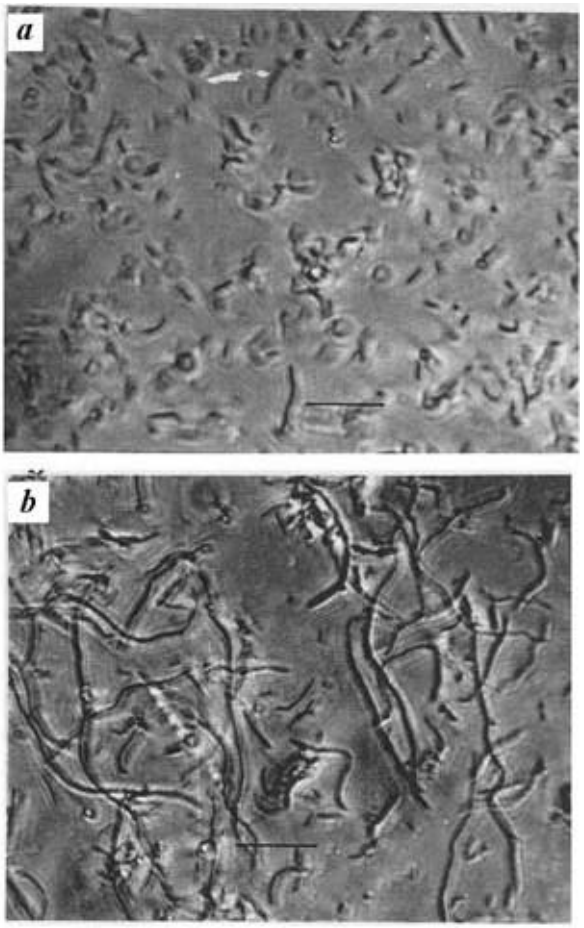

Figure 2 *a* and *b*. Phase contrast photomicrograph of *G. diazotrophicus* cells grown with high levels of N sources (25 mM of ammmonium nitrate) in LGI P semi-solid media after 15 days of growth. Bar = 10 μM.

Some reports indicate that N-fixation by *G. diazotrophicus* was suppressed in the presence of excess fixed N[45,84]. The inoculation experiments conducted by Fuentez-Ramirez *et al.*[70] indicated that low fertilizer N (120 kg N ha^{-1})-applied sugarcane fields contained more *G. diazotrophicus* than high fertilizer N-applied fields. The ^{15}N incorporation experiments, using sterile sugarcane plants, have also demonstrated the potential for N-fixation in *G. diazotrophicus*–sugarcane interaction[54]. Use of mutant strains (carrying *nif*D::kan interposan mutation that prevents N-fixation entirely) in plant experiments proved the participation of *G. diazotrophicus* in N-fixation[56]. The influence of environmental factors, viz. hydric stress and seasonal variation on the colonization and N-fixation by *G. diazotrophicus* was also addressed[84].

It is an established fact that the growth hormones, viz. auxins, cytokinins, gibberellins play a role in enhancing the growth of grasses associated with diazotrophs[85]. Apart from N-fixation, *G. diazotrophicus* is also reported to benefit sugarcane through production of plant growth-promoting factors[60,72]. Reports on the production of IAA apart from N-fixation[70,86] (Figure 3) and gibberellins[87] in the culture flasks support these claims. However, their role in sugarcane–*G. diazotrophicus* association remains to be established.

Conclusion and future prospects

Plants infected by endophytes are known to be chemically protected against other factors, viz. herbivore consumption[88–90]. However, the influence of microbe–plant associations on multitrophic interaction remains largely unexplored. The hidden microbial symbionts are also reported to have community-wide impacts on the pattern and strength of resource–consumer interactions[91]. These inconspicuous mutualistic associations were also reported to exert a regulatory force in many ecosystems[91].

Recent studies on association of *G. diazotrophicus* with rice seedlings seem to be encouraging, where a substantial increase in growth of rice plants (cv. Ponni) was recorded[46]. These observations suggest that *G. diazotrophicus* may play a crucial role, not only in sugarcane, but in growth promotion of other plants also. However, it

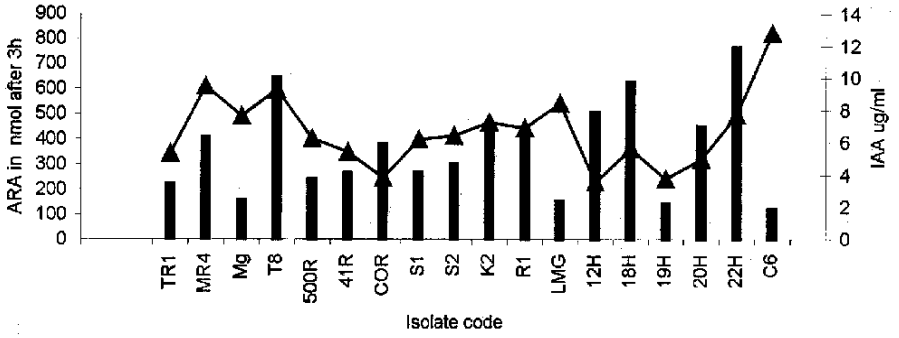

Figure 3. IAA production and nitrogenase activity of *G. diazotrophicus* and *Herbaspirillum* spp. obtained from different sugarcane varieties (in liquid medium supplemented with tryptophan 100 µg ml^{-1} and ammonium chloride 100 mg 1^{-1}).

requires further studies to prove whether *G. diazotrophicus* is solely responsible in N nutrition and plant-growth promotion, as various other diazotrophs have also been isolated from these crop plants.

It has been observed that co-inoculation of *G. diazotrophicus* and *Herbaspirillum* sp. could enhance the colonization of both these bacteria in micropropagated sugarcane seedlings[92]. A molecular marker based study will provide further evidence.

Revival of this bacterium is difficult after a longer period in artificial media with high concentration of ammonium salts and in N-deficient media[81]. Though the exact reasons are not known till date, several authors present many views. Ammonium concentration in the culture media plays a determinant role and affects the respiratory components of *G. diazotrophicus* such as glucose dehydrogenase, *c*-type cytochromes and alternative oxidases *ba* and *bd*[44]. In addition, high percentage of pleomorphic cells are induced at high concentrations of ammonium salts, with an increase in cell size coupled with increased generation time[93], and a poor revival was observed during subculturing[81]. In this context, the role of NH_4 ions on the culturability of *G. diazotrophicus* in field samples needs to be studied further.

As *G. diazotrophicus* utilizes higher concentrations of sucrose and glucose, the role of this bacterium inside sugarcane plants at maturity phase and during commercial sugar extraction needs to be studied in detail. Also the role of acetic acid produced by this bacterium on the physiology of sugarcane needs to be examined.

G. diazotrophicus population was reported to be between 10^6 and 10^7 cells/g fresh tissue[75,80]. In such cases, the cells can be observed under a microscope by just crushing the plant samples, as done in the case of *Rhizobium* nodules[9]. There are no reports to prove this fact through microscopic observations. Also it needs an in-depth study to substantiate the exact contribution of these diazotrophs in plant growth, as it was suggested that the differences in the rates of BNF found in sugarcane genotypes were not caused by the differences in the presence or the number of N-fixing bacterial species[83].

As *G. diazotrophicus* is reported to possess antifungal properties, it is worthier to identify potential genes of action and clone them for further studies. We hope that an intensive tritrophic level interaction study keeping sugarcane as a model would yield better results in future for the effective control of diseases.

Future areas of research include (1) identification of the exact role (nitrogen enrichment, growth-hormone production, pathogen resistance) of endophytes in sugarcane *in planta* and identification of genes, if any, responsible for the role; (2) genetic improvement for disease control and their expression; (3) identification of host range, including varieties and (4) identification of compatibility among heterologous strains in sugarcane environment and host range capability among sugarcane cultivars and strains.

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