



Review article

Gluconacetobacter diazotrophicus: An overview

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Abstract: Amplification in agricultural practices to maximize the crop productivity had led to excessive exploitation of the technologies like application of agro-chemicals (fertilizers and pesticides) in agricultural fields. But these are not in favor of the sustainability of soil health and also posing threats to the soil fertility. During the last couple of decades, an economically feasible and ecologically sound alternative strategy to minimize this problem has been developed. In this strategy soil microorganisms have been exploited in agriculture for improving the soil health and enhancing productivity. Among various microbial species used, *Gluconacetobacter diazotrophicus* is very important. It has a long-standing history of bacterial-plant interrelationship as a symbiotic endophyte capable of fixing atmospheric nitrogen. Its association with sugarcane represents a model system for monocot-diazotrophic associations. Therefore it is necessary to collect information related to their establishment, colonization process, biological nitrogen fixation, growth promotion, etc. In this review, the association of *G. diazotrophicus* with sugarcane and other crop plants and with various hosts is discussed. Then the plant-growth-promoting traits identified in this bacterium, including N₂ fixation, phytohormone synthesis, P and Zn solubilization and biocontrol, are analysed.

Key words: *Gluconacetobacter diazotrophicus*, N₂ fixation, phytohormone synthesis, P and Zn solubilization, biocontrol

Introduction

Plant associated microorganisms can be associative or endophytic in nature. Term 'endophytic' is referred to those, which colonizes in the interior of the plant parts, viz, root, stem or seeds without causing any harmful effect on host plant (Hallmann *et al.*, 1997). These may promote plant growth in terms of increased germination rates, biomass, leaf area, chlorophyll content, nitrogen content, protein content, hydraulic activity, roots and shoot length, yield and tolerance to abiotic stresses like draught, flood, salinity etc. They can also promote plant growth directly through Biological Nitrogen Fixation (BNF), phytohormone production, phosphate solubilization, inhibition of ethylene biosynthesis in response to biotic or abiotic stress (induced systemic tolerance) etc., or indirectly through inducing resistance to pathogen (Bhattacharya and Jha, 2012). Application of these microorganisms for sustainable agriculture holds immense potential. *G. diazotrophicus*, since its discovery, has become a very interesting microorganism to study due to the characteristics overviewed in this article.

Gluconacetobacter diazotrophicus, an efficient nitrogen fixing diazotrophic bacterium is one among the endophytes studied as a model organism to evaluate the plant-bacterial interactions with non legumes (Cocking, 2003 and Cocking *et al.*, 2006). Although a number of diazotrophs have been isolated so far but *G. diazotrophicus* have conclusive proof for nitrogen fixation is available at present. *G. diazotrophicus* can grow in a wide range of conditions, such as low pH, high sucrose concentrations and high salt conditions. The bacterium is unique because it allows nitrogen fixation to remain active in nitrogen-fertilized fields, unlike soybean inoculating *Rhizobium* bacteria which shut down when the soil is high in nitrogen. Compared to *Rhizobium* bacteria *G. diazotrophicus* is found to have multiple entry sites, such as stems and leaves, whereas *Rhizobium* that colonize legumes are limited to root nodules alone. The purpose of this review is divided into two broad sections; the first part comprising natural occurrence of *G. diazotrophicus* in different hosts is discussed. In this section, *G. diazotrophicus* isolation or association with sugarcane was

elaborated. In addition, its occurrence in various crops, including coffee, pineapple, finger millet, rice, certain vegetables and other hosts like sugarcane mealybugs and arbuscular mycorrhiza (AM), was presented. The second major topic concerned is to appraise the multifaceted research conducted employing *G. diazotrophicus* to unravel various plant growth-promoting mechanisms. It comprises of N₂-fixation and its inoculation effects, production of phytohormones such as indole acetic acid (IAA) and gibberellic acid (GA), in vitro solubilization of plant macro and micronutrients like P and Zn and biocontrol of the phytopathogens *Colletotrichum falcatum*, *Xanthomonas albilineans* and the nematode *Meloidogyne incognita*.

General Characteristics

Gluconacetobacter diazotrophicus is a Gram-negative, acid tolerant, obligate aerobe and the cells are rod shaped with rounded ends (0.7–0.9 µm by 1–2 µm) having lateral or peritrichous flagella (Cavalcante and Dobreiner, 1988; Gillis *et al.*, 1989; Muthukumarasamy *et al.*, 2002). Cells produce brown water-soluble pigments on GYC medium (5% glucose, 1% yeast extract, 3% CaCO₃, 2.5% agar). Dark brown colonies are formed on potato agar supplemented with 10% sucrose, and dark orange colonies are formed on a nitrogen-poor medium containing bromo thymol blue (Cavalcante and Dobreiner, 1988). High sucrose concentrations (10%) are the best source of carbon for the bacterium's growth (Cavalcante and Dobreiner, 1988); James and Olivares, 1997; Chanway, 1998). It can grow even upto 30% sucrose and responsible enzyme is levansucrase (Hernandez *et al.*, 1995; Arrieta *et al.*, 1996; Martinez-Fleites *et al.*, 2005; Kesters *et al.*, 2006; Nakano and Fukaya, 2008) that hydrolyse sucrose into fructose and glucose. That enzyme was identified as a constitutive exoenzyme in 14 strains of *G. diazotrophicus* recovered from different host plants cultivated in diverse geographical regions (Hernández *et al.*, 2000). The enzyme, consisting of a single 60 kDa polypeptide coded by LsdA gene and targeted disruption of the LsdA gene in four representative strains abolished their ability to grow on sucrose, indicating that the endophytic species *G. diazotrophicus*

utilizes plant sucrose via levansucrase. Arrieta *et al.* (2004) reported that LsdA, unlike other extracellular levansucrases from Gram-negative bacteria, is transported to the periplasm by a signal peptide-dependent pathway. Extracellular glucose oxidation via a pyrroloquinoline quinone-linked glucose dehydrogenase (PQQ-GDH) is the main pathway for glucose metabolism in *G. diazotrophicus* (Alvarez and Martínez-Drets, 1995; Attwood *et al.*, 1991; Galar and Boiardi, 1995; Luna *et al.*, 2000, 2006; Crespo *et al.*, 2011). PQQ-GDH activity was observed to be constitutively present in all strains of *G. diazotrophicus* (Luna and Boiardi, 2008). However, low but significant hexokinase activities have been reported in this organism; moreover, a nicotinamide adenine dinucleotide-linked glucose dehydrogenase (NADGDH) was found to be actively synthesized in glucose containing cultures (Attwood *et al.*, 1991; Alvarez and Martínez-Drets, 1995). Therefore, two oxidative routes seem to be simultaneously expressed in *G. diazotrophicus*, one being intracellular by way of a NAD-GDH, and the other being periplasmic by way of a pyrrolo-quinoline-quinone-linked glucose dehydrogenase (PQQ-GDH). It was observed also that a PQQ-GDH was primarily responsible for the high rates of gluconic acid formation exhibited by *G. diazotrophicus* (Attwood *et al.*, 1991; Galar and Boiardi 1995). Studying the regulation of both enzymes, Luna *et al.* (2006) observed that *G. diazotrophicus* metabolizes glucose mainly by way of a PQQ-GDH, particularly under BNF and/or limited conditions. However, under glucose excess, a NAD-GDH is simultaneously expressed, and this enzyme would likely participate in glucose oxidation. Besides sucrose, bacterium can also grow on various other carbon compounds like glucose (forms 2-ketogluconic acid and 2,5-diketogluconic acid from glucose), sodium salts of gluconate, lactate, pyruvate and acetate but did not utilize sodium salts of succinate, malate, fumarate, ketoglutarate, tartarate, formate or sodium citrate as sole carbon sources all taken at 10, 20 and 30 mM (Stephan *et al.*, 1991). The optimal initial pH for growth was found to be 6.0 but it can grow even at very low pH (3.0). It has the ability to fix N under microaerophilic conditions. Reis *et al.* (1990) demonstrated higher O₂ tolerance of *G. diazotrophicus*, when grown in 10 percent sucrose, the bacteria showed acetylene reduction at optimum dissolved oxygen concentration that was at equilibrium with 0.2 KPa O₂ in atmosphere. The cells continued to reduce C₂H₂ upto 4.0 KPa. *G. diazotrophicus* can fix N₂ at a wide range of atmospheric pO₂ and can adapt to maintain nitrogenase activity in response to both long-term and short-term changes in atmospheric pO₂ (Pan and Vessey, 2001). *G. diazotrophicus* as a member of the acetic acid bacteria group, oxidize alcohol to acetic acid through two sequential reactions catalyzed by the alcohol dehydrogenase (ADH) and the aldehyde dehydrogenase, both enzymes are membrane-bound and oriented to the periplasmic space. ADH is a quinoxinoprotein carrying one pyrroloquinoline quinone moiety, one (2Fe:2S) cluster and four c-type cytochromes, as prosthetic groups.

Discovery and Taxonomy

G. diazotrophicus (Yamada *et al.*, 1997) (formerly *Acetobacter diazotrophicus*, Gills *et al.*, 1989) was originally isolated from sugarcane (Cavalcante and Dobereiner, 1988). The observed characters of the newly isolated bacterium which were similar to that of acetic acid group of bacteria and the fact that *Acetobacter* do not possess nitrogen-fixing ability led Cavalcante and Dobereiner (1988) to coin a new name as *Saccharobacter nitrocaptans* (meaning Saccharum-sugar, bacter-bacterium, nitrum-nitrogen, captans- to put in accessible form). Later

on, Gillis *et al.* (1989) proposed the name *Acetobacter diazotrophicus* on the basis of all the phenotypic, chemotaxonomic and genomic characters. They also compared seven strains of *A. diazotrophicus* isolated from roots, stems and rhizosphere with type strains of *Acetobacter* which served as controls. The protein electrophoretic prints of seven *A. diazotrophicus* strains were similar and the level of DNA binding was more than 84 per cent indicating that these new strains form a single DNA homology group. Later on based on the partial sequences of 16S ribosomal RNA and on the predominant type of ubiquinone produced, the subgenus of acetic acid bacteria that is *Gluconacetobacter* was elevated to the generic level (Yamada *et al.*, 1997, 1998; Franke *et al.*, 1999) and thus *A. diazotrophicus* is now known as *Gluconacetobacter diazotrophicus* (Yamada, 2000).

Isolation and Enumeration

The procedure to quantify populations of these bacteria utilises semi-solid N-free media inoculated with plant macerate after serial dilutions (Barraquio *et al.*, 1997; Cavalcante and Dobereiner, 1988; Olivares *et al.*, 1996; Reis *et al.*, 2001; Reis Júnior *et al.*, 2000) as their survival in the soil is very poor (Baldani *et al.*, 1997; Oliveira *et al.*, 2004). Reis *et al.* (1994) developed an efficient, defined, nitrogen-free semi solid medium containing 10 per cent cane sugar. The medium was found to be most selective for isolation of *G. diazotrophicus*. Enumeration has been achieved using the Most Probable Number (MPN) technique (Pochon *et al.*, 1962) based on positive scoring of semi-solid cultures where the characteristic pellicle formation is observed. Both the use of semi-specific media and the use of the MPN technique are fraught with difficulties and the numbers obtained almost certainly underestimate true population's size, as was indicated by the results of Li and MacRae (1992) for *Gluconacetobacter diazotrophicus*. In the utilisation of the MPN method it is assumed that the bacteria are released from adherence to the plant tissue and that no aggregates are formed so that the maceration step extracts contain all the bacteria present in the plant tissue and subsequent serial dilutions are based on a homogenous suspension of individual bacterial cells. It is further assumed that single cells of the target organisms can grow under these conditions and no other bacteria present in the suspension inhibit their growth in the semi-solid medium. The use of Enzyme Linked Immunosorbent Assay (ELISA) use of species-specific polyclonal antibodies with the indirect ELISA (enzyme-linked immunosorbent assay) can be an alternative which is rapid and specific to quantify these populations of bacteria. As was firstly used to detect viruses (Lommel *et al.*, 1982) and subsequently, amongst many other applications, has been applied to detect and count rhizobia (Olsen *et al.*, 1989; Schloter *et al.*, 1997; Tajini *et al.*, 2008). There have been several applications of this technique in studies of associative N₂-fixing bacteria (Barraquio *et al.*, 1986; Levanony *et al.*, 1987; Li and MacRae, 1992; Reis *et al.*, 2000; Reis Júnior *et al.*, 2004; Schloter *et al.*, 1996; Schloter *et al.*, 1994). The ELISA procedure has several advantages in comparison to the MPN technique. In this immunological method, the bacteria do not have to be completely dispersed into a suspension of individual cells, as is supposed to occur during the dilution procedure of MPN counts. The complete analysis takes less than 24 h and the result can be specific to the target bacteria if the sera are prepared in a suitable manner to achieve this objective. The extracted sample after fixation can be maintained in the refrigerator and analysed several times or the material adsorbed on the ELISA plate can be stored for a long period under the same conditions (Silva, 1999).

Classification and Differential Characteristics

Gluconacetobacter belongs to phylum *Proteobacteria* (comprising Gram negative bacteria) in section α -Proteobacteria, order Rhodospirillales and family *Acetobacteraceae* (Kerstens *et al.*, 2006). Currently, Genus *Gluconacetobacter* comprises 16 species in two phylogenetic groups: the *Gluconacetobacter liquefaciens* group and the *Gluconacetobacter xylinus* group. These two groups were discussed taxonomically (Yamada and Yukphan *et al.*, 2008). Recently, Ilse Cleenwerck *et al.* (2010), generated a phylogenetic tree using Multilocus sequence analysis (MLSA) of the three housekeeping genes confirmed the existence of two phylogenetic groups within the genus *Gluconacetobacter*. These groups clustered separately in trees constructed using concatenated sequences of these three genes, indicating that the genus *Gluconacetobacter* should not remain a single genus and should be split, as suggested previously. Among the various species, *Gluconacetobacter diazotrophicus* is more closely related with N_2 -fixing species *Gluconacetobacter azotocaptans*, *Gluconacetobacter johannae* and *Gluconacetobacter kombuchae*

Association with Sugarcane

Sugarcane is one of the major sugar producing plant species in the world. The stem of this plant, consist of apoplast (intercellular spaces) and symplast (vacuolar spaces). During the maturation of sugarcane, sucrose gets accumulate in developing internodes (Glasziou and Gaylor, 1972; Moore, 1995). Most interest of sugarcane stem knowledge has risen from the finding of Do'bereiner's group in Brazil (Baldani *et al.*, 1997) regarding the presence of nitrogen fixing endophytes in sugarcane stems. Endophytes in the apoplast may support plant growth in many instances by increasing resistance to biotic or abiotic stress factors, as well as by contributing directly to plant mineral nutrition (Sattelmacher, 2001). *G. diazotrophicus* is an obligatory aerobe with the ability to fix atmospheric nitrogen (Stephan *et al.*, 1991; Alvarez and Marty' nez-Drets, 1995) was isolated from sugarcane (Cavalcante and Dobereiner, 1988; Li and MacRae, 1991; Fuentes-Ramirez *et al.*, 1993; Caballero-Mellado *et al.*, 1995; Prabudoss and Stella, 2009). The bacterium's ability to provide its host (sugarcane) with large amounts of fixed nitrogen without the formation of nodules led to the recognition of its importance. Its endophytic nature was confirmed in Brazil by counts of this diazotroph in roots, stems, aerial parts and cane trash (Reis *et al.*, 1994). Numbers in all plant parts were between 10^3 and 10^6 cells/g fresh weight. High numbers (10^6 – 10^7 cfu/g fresh weight) were found also in sugarcane plants in India (Muthukumarasamy *et al.*, 1999). This diazotroph was also isolated from sugarcane trash and xylem sap, indicating latter translocation of the bacterium through the plant tissues in the xylem (Reis *et al.*, 1994). James *et al.* (1994) confer the presence of *G. diazotrophicus* on and within sugarcane plant tissues by immunogold labeling technique. They observed that the loose cells of the root cap at root tips were a site of entry of the organism into root tissues. Both at lateral root junctions and root tips, bacteria were also seen in enlarged, apparently intact, epidermal cells. Likewise, bacteria were present in xylem vessels at the base of the stem, without pathogenic reaction to the bacteria within the xylem (James *et al.*, 1994). Further evidence that the N_2 -fixing endophytic bacterium from the intercellular spaces of sugarcane stems is *G. diazotrophicus* was reported by Dong *et al.* (1995). The occurrence of this diazotroph was directly demonstrated in tissues of micropropagated sugarcane plants using a species specific oligonucleotide probe and PCR amplification (Kirchhof

et al., 1998). In commercial plantations, sugarcane is commonly propagated through stem cuttings (setts). Heat treatment method used to treat setts before planting to eliminate the plant pathogen *Clavibacter xyli* subsp. *xiii*, which causes ratoon stunting disease, is (50 °C for 30 min). Reis *et al.* (1994) observed that the number of *G. diazotrophicus* within the plant was not affected after heat treatment, confirming the endophytic habitat of this diazotroph and its propagation within the stem cuttings. It was also observed that when inoculating stem cuttings with *G. diazotrophicus*, the bacterial colonization stimulated the production and development of root hairs (Bellone *et al.*, 1997). Although the colonization of sugarcane by *G. diazotrophicus* and its endophytic nature was reported in different works (Cavalcante and Dobereiner, 1988; Dong *et al.*, 1995; James *et al.*, 1994; Kirchhof *et al.*, 1998; Reis *et al.*, 1994; Walsh *et al.*, 2006) observed in Australia that sugarcane- N_2 -fixing *G. diazotrophicus* association is not easily achievable, being primarily limited by a lack of infection. There is limited data on the endophyte-host molecular interactions. The sequence analysis of the cDNA and other libraries derived from messenger RNAs expressed in sugarcane when inoculated with *Gluconacetobacter diazotrophicus* revealed that genes for nitrogen assimilation, for carbon metabolism, for plant growth, as well as genes for a limited plant defense were induced (de Matos Nogueira *et al.*, 2001). Santos *et al.* (2010) used a proteomic approach to analyze proteins differentially expressed in the presence and absence of sugarcane plantlets. Two-dimensional gel electrophoresis (2-DE) showed 42 spots with altered levels of expression. Analysis of these spots by matrix-assisted laser desorption ionization time-of-flight in tandem (MALDI-TOF-TOF) identified 38 proteins. Differentially expressed proteins were associated with carbohydrate and energy metabolism, folding, sorting and degradation processes, and transcription and translation. Among proteins expressed in co-cultivated bacteria, four belong to membrane systems; others, like a transcription elongation factor (GreA), a 60 kDa chaperonin (GroEL), and an outer membrane lipoprotein (Omp16) have also been described in other plant-bacteria associations, indicating a common protein expression pattern as a result of symbiosis. A high protein content of 60kDa chaperonin isoforms was detected as non-differentially expressed proteins of the bacteria proteome. These results allow the assessment of the physiological significance of specific proteins to *G. diazotrophicus* metabolism and to the pathways involved in bacteria-host endophytic interaction. Recently, Lery *et al.* (2011) investigated molecular aspects of the *G. diazotrophicus*-sugarcane interaction. They performed a quantitative mass spectrometry-based proteomic analysis by (15 N) metabolic labeling of bacteria and root samples of sugarcane. Overall, more than 400 proteins were analyzed and 78 were differentially expressed between the plant-bacterium interaction model. In addition, they identified 30 bacterial proteins in the roots of the plant samples; from those, nine were specifically induced by plant signals.

Association of *G. diazotrophicus* with Other Hosts: Rhizosphere Occurrence and Survival

G. diazotrophicus, originally isolated as an endosymbiont of sugarcane. Previous studies have considered *G. diazotrophicus* to be an obligate endophyte since it has not been isolated from soil or rhizosphere regions in significant numbers, although it is cultured and manipulated easily in the laboratory (Sevilla *et al.*, 2001). However, various studies have observed the rhizospheric occurrence and survival of *G. diazotrophicus* in agricultural crops apart from sugarcane. The

occurrence of *G. diazotrophicus* in various hosts is listed in Table 1. Besides sugarcane, Do**ber**einer *et al.* (1988) identified its association with other sugar and starch-rich plants like cameroon grass (*Pennisetum purpureum*) and sweet potato (*Ipomoea batatas*). The occurrence of *G. diazotrophicus* as endophyte and rhizosphere dwellers in coffee revealed it to be a preferential host other than sugar crops (Jimenez-salgado *et al.*, 1997). In a subsequent study conducted in India, a species-specific oligonucleotide primer approach permitted the identification of *G. diazotrophicus* in rhizosphere soil apart from roots, stems and leaves of finger millet (*Eleusine coracana* L.) (Loganathan *et al.*, 1999). Endophytic colonization was also elucidated in pineapple roots, stems and leaves with a high frequency of isolation from nitrogen unfertilized propagative buds (Tapia-Hernandez *et al.*, 2000). In a review by Fuentes *et al.* (2003), *G. diazotrophicus* was stated to occur in plants belonging to family *Poaceae*, *Rubiaceae*, *Bromeliaceae*, *Rosaceae* and *Malphigiaceae*. The ability to colonize a number of families suggests that *G. diazotrophicus* possibly has the potential to benefit different plant hosts (Dobereiner *et al.*, 1988; Reis *et al.*, 1994; Estrada-de los Santos *et al.*, 2001; Munoz-Rojas *et al.*, 2005). An extensive study on 50 different tropical and subtropical plants of Western Ghats, one of the biodiversity hot spots in India, revealed the endophytic association of *G. diazotrophicus* with carrot, radish, beet root and coffee (Madhaiyan *et al.*, 2004). Studies have also observed the occurrence of *G. diazotrophicus* as endophyte and rhizosphere dwellers in wetland rice (Muthukumarasamy *et al.*, 2005, 2007). As already mentioned, the rhizospheric occurrence of *G. diazotrophicus* is less emphasized for the reasons that efforts of isolation from soil were unsuccessful or may be due to its low survival in soil (Baldani *et al.*, 1997; Cavalcante and Do**ber**einer, 1988). However, when inoculated to micropropagated sterile sugarcane plantlets, under suitable conditions this putative endophyte was able to survive in rhizosphere in higher numbers than the population and persist up to the end of cropping period (Munoz-Rojas *et al.*, 2003). In the rice ecosystems (Muthukumarasamy *et al.*, 2007), the presence of *G. diazotrophicus* was attributed to the left-out trashes of previously cropped sugarcane in the same field. However, this could not be true for the fact that in the same study its occurrence was also observed in rice crop grown in field where sugarcane was not previously cultivated and thus they could persist in soil in a non-vegetatively propagated crop like rice (Muthukumarasamy *et al.*, 2005). Another factor that may govern its survival in soil is the increased soil moisture (93.5%) level (Oliveira *et al.*, 2004), and further, its ability to utilize certain organic acids (Carrizo de Bellone, 2006) may be advantageous, since both simple sugar and organic acids are available in soil as products of organic matter degradation. Presence of certain cell wall degrading enzymes such as endoglucanase, endoxyloglucanase and endopolymethylgalacturonase in *G. diazotrophicus* (Adriano-Anaya *et al.*, 2005, 2006) raises the speculation that these enzymes may help the bacteria to successfully outcompete other rhizosphere microorganisms and colonize the internal tissues of plant systems, but the common mode of transmission is spread through setts to new canes (Do**ber**einer, 1990). Finally, the possibility that cannot be ruled out for its low detection in rhizosphere soil is the viable but non-culturable state of this bacterium. The possible modes of dissemination of *G. diazotrophicus* have been clearly reviewed by Baldani *et al.* (2002), in which the presence of this bacterium in soil was also suggested. The bacterial cells in soil could be

stimulated by plant root exudates and are able to colonize the plants, or alternatively, their population might be very low to achieve an efficient colonization (Baldani *et al.*, 2002, 2005). However, these types of studies are preliminary and additional experiments are needed to reveal the fact. Nevertheless, the above said investigations have clearly proved the rhizosphere occurrence of *G. diazotrophicus* and rhizosphere soil should also be considered as the possible source for their spread at least in non vegetatively propagated plants like coffee and rice. *G. diazotrophicus* was reported in the rhizo-and-phylo spheres of a number of desert plants in Sinai environment (Hanna *et al.*, 2012). Apart from its occurrence as endophyte, it was also been detected in pink sugarcane mealybug *Saccharococcus sacchari*, the sucking pest commonly. *Gluconacetobacter* sp as a natural colonizer of the wild rice (*Porteresia cocarctata* Tateoka, formerly *Oryza coarctata* Roxbi) and a salt tolerant rice variety (Loganathan *et al.*, 2003). Tian *et al.*, (2009) studied colonization of this bacterium in corn (*Zea mays*). The overall colonization in corn was 74.1%. The bacterium was detected in stems and leaves from the primary site of inoculation, indicating the bacterium could move to other organs and the bacterium showed a positive adaptation to this host plant. Several different studies have observed the ability of *G. diazotrophicus* to colonize corn plants under field, greenhouse, and aseptic conditions. The bacterium is capable of inhabiting several corn genotypes through several different means of inoculation: seed coating, applications to the base of stems, and root dipping (Riggs *et al.*, 2001; Cocking *et al.*, 2006; Tian *et al.*, 2009). Some of these studies have shown that under both field and greenhouse conditions *G. diazotrophicus* is capable of enhancing corn productivity, resulting in an increased yield (Riggs *et al.*, 2001). Other studies have proven through β -glucuronidase (GUS)-labeling that in addition to being capable of intracellular colonization of the roots, the bacterium is capable of expressing nitrogenase genes within corn plants. Unfortunately, research has yet to show any nitrogen fixation by *G. diazotrophicus* within corn plants. No nitrogenase activity was detected in this study in plants and tissues colonized by *G. diazotrophicus*. If the cause of the enzyme's inactivity was a result of insufficient quorum sensing signals resulting from low bacterial numbers, potential future experiments should investigate this pathway and pursue means of overriding it (Bertalan *et al.*, 2009). The potential benefits from the successful introduction of *G. diazotrophicus* into corn are too great to not continue research into this field. With the recent sequencing of the *G. diazotrophicus* genome, many new directions for future research exist in attaining successful colonization and nitrogen fixation within corn (Bertalan *et al.*, 2009).

Agronomic Importance

The potentially beneficial effects promoted by this bacterium on plants are nitrogen-fixation, production of phytohormones, action against pathogens and mineral nutrient solubilization. In 2009 the genome of *G. diazotrophicus* strain PAL5 was completely sequenced by Bertalan *et al.* (2009) and genes involved in nitrogen fixation, sugar metabolism, transport systems, biosynthesis of polysaccharides, quorum sensing and auxin biosynthesis were identified again confirming its importance.

Biological Nitrogen Fixation

Biological nitrogen fixation (BNF) is the potential biological process that maintains the soil nitrogen status under normal conditions. The process of BNF can be defined as the reduction of dinitrogen to ammonia by means of a prokaryote (Mylona *et al.*, 1995). This process can be symbiotic and is considered to be a monospecific association

Table 1. Occurrence of *G. diazotrophicus* in various hosts.

Host	References
Sugarcane	Cavalcante and Dobereiner, 1988; Li and MacRae, 1991; Fuentes-Ramírez <i>et al.</i> , 1993; Caballero-Mellado <i>et al.</i> , 1995
Cameroon grass	Reis <i>et al.</i> , 1994
Sweet potato	Paula <i>et al.</i> , 1991
Coffee	Jimenez-Salgado <i>et al.</i> , 1997
Pineapple	Tapia-Hernández <i>et al.</i> , 2000
Eleusine coracana	Loganathan <i>et al.</i> , 1999
Tea	Muthukumarasamy <i>et al.</i> , 2002a
Mango	Muthukumarasamy <i>et al.</i> , 2002a
Banana	Muthukumarasamy <i>et al.</i> , 2002a
Mealy bugs	Ashbolt and Inkerman, 1990; Caballero-Mellado <i>et al.</i> , 1995
Rice	Muthukumarasamy <i>et al.</i> , 2005
Sorgham	Isopi <i>et al.</i> , 2005
Corn	Tian <i>et al.</i> , 2009
AM Spores	Dobereiner, 1988
Carrot, raddish, Beet root	Madhaiyan <i>et al.</i> , 2004

which evolved over 60 million years ago (Hirsch, 2004; Geetanjali, 2006). BNF is accomplished by a wide variety of prokaryotes; some can accomplish this as free living organisms, while others require a symbiotic association with plants (Mylona *et al.*, 1995; Gothwal *et al.*, 2008). The two main types that require associations with host plants are endophytes and rhizobacteria, which can be classified as plant growth promoting bacteria because they are beneficial to their host plants (Saharan and Nehra, 2011). In recent years nitrogen fixation by *G. diazotrophicus* in sugar rich crops has been well established. BNF effectively supplemented the need of nitrogen and minimize the cost of production by reducing doses of nitrogenous fertilizers. The contribution of endophytic nitrogen fixation in sugarcane by *G. diazotrophicus* is as high as 150 Kg N ha⁻¹yr⁻¹ (Cavalcante and Dobereiner, 1988; Boddey *et al.*, 1991; Muthukumarasamy *et al.*, 2005). However, contribution of BNF to host may vary with the genotype of host. Proteomic analyses of sugarcane variety SP70-1143 grown with *G. diazotrophicus* revealed up-regulated expression of ammonia lyase which indicates increased metabolism resulted from increased uptake of nitrogen contributed by bacteria (Lery *et al.*, 2011).

In *G. diazotrophicus* component proteins are synthesized from a set of highly conserved nitrogen fixation (*nif*) structural genes, very similar to other members of the class Alpha Proteobacteria (Fisher and Newton, 2005; Bertalan *et al.*, 2009). What makes *G. diazotrophicus* unique is that it does not contain a nitrate reductase protein (Cavalcante and Dobereiner, 1988). Without the nitrate reductase protein in the bacterium, the nitrogenase does not become inhibited by rising levels of nitrites (Trinchant and Rigaud, 1982; Cavalcante and Dobereiner, 1988). Additionally, the nitrogenase of *G. diazotrophicus* is not completely inhibited by the addition of ammonium, meaning that the bacterium is capable of undergoing nitrogen fixation in crops that are supplemented with low amounts of ammonium-based nitrogen fertilizers (Stephan *et al.*, 1991; Fisher and Newton, 2005).

G. diazotrophicus has the capacity to fix N₂ at environmental atmospheric partial O₂ pressures (pO₂) (e. g. approximately 20 kPa of O₂) when grown in semisolid medium (Cavalcante and Dobereiner, 1988) and as colonies on solid medium (Dong *et al.*, 1995). The influence

of high sugar content on nitrogenase activity of *G. diazotrophicus* in the presence of O₂ and of N added in the form of ammonium and amino acids was studied by Reis and Dobereiner (1998). They observed that 10% sucrose protected nitrogenase against inhibition by oxygen, ammonium, some amino acids, and also to some extent by salt stress, indicating the presence of different osmotolerance mechanisms for sucrose and salt (Reis and Dobereiner, 1998). When evaluating the effect of the addition of increasing amounts of two sources of mineral nitrogen (ammonium sulphate and calcium nitrate) on the nitrogenase (acetylene reduction) activity of *G. diazotrophicus* in two sugarcane hybrids, SP70-1143 and SP79-2312, Medeiros *et al.* (2006) determined that it was inhibited by both sources, especially in variety SP79-2312. Likewise, Madhaiyan *et al.* (2006) noticed that addition of pesticides to the growth media substantially reduced the nitrogenase activity of pure cultures of *G. diazotrophicus*. Burries *et al.* (1991) reported that ammonia affects the growth of *G. diazotrophicus* and a level of about 100 μM NH₄⁺ was required to switch off the system. The enzymes dinitrogenase reductase, ADP-ribosyl transferase (DRAT) and dinitrogenase reductase activating glycohydrazidase (DRAG) systems did not appear to function in the presence of ammonia. Chapman *et al.* (1992) conducted field assays for two years employing acetylene reduction assay (ARA) technique which indicated presence of 10⁶ diazotrophs (*G. diazotrophicus* and *Klebsilla spp.*) per gram stem and roots. They demonstrated significant levels of nitrogen-fixation by the bacteria. Twenty four isolates of *G. diazotrophicus* isolated from sugarcane (Root, stem and leaves) showed appreciable amount of nitrogenase activity and the strain isolated from sugarcane stem showed ARA of maximum 410.92 nmoles of C₂H₄/hr/mg cell protein followed by the strain from sugarcane leaf to the amount of 400.17 n moles of C₂H₄/hr/mg cell protein and other strains were intermediate in their nitrogen fixation and hence the strain from sugarcane stem was recommended as a effective biofertilizers (Meenakshisundaram *et al.* 2010).

Phytohormones Production

Indole-3-acetic acid (IAA) is an important auxin involved in plant growth promotion and its production by microbes is considered to be an important PGP activity. Production of IAA by *G. diazotrophicus* in defined culture medium was observed and it was suggested that it could promote rooting and improve sugarcane growth by direct effect on metabolic processes apart from their role of N₂ fixation (Fuentes-Ramirez *et al.*, 1993). IAA production was present in almost all the *G. diazotrophicus* strains isolated from the diverse sugarcane genotypes maintained in the Indian Institute of Sugarcane Research, India (Suman *et al.*, 2001). Fuentes-Ramirez *et al.* (1993) isolated 18 strains of *G. diazotrophicus* from roots of sugarcane varieties and found that all 18 strains produced indole acetic acid when grown in a defined culture medium. HPLC analysis of the culture media revealed that IAA production varied from 0.1 to 1.42 μg ml⁻¹. As to the role of IAA, the authors suggested that due to this property the bacteria might be promoting growth of root system as well as shoot system besides helping in nitrogen-fixation. Madhaiyan *et al.* (2004) also confirmed the IAA production trait among the *G. diazotrophicus* isolates recovered from various crop plants. Molecular analysis of the interrelationship between cytochrome synthesis genes (*ccm*) and IAA production proved that *ccm* genes, despite their role in cytochrome c biosynthesis, are also involved in IAA synthesis (Lee *et al.*, 2004). The involvement of *G. diazotrophicus* in the production of the other plant hormone gibberellins

(GA1 and GA3) was also studied in a chemically defined medium (Bastián *et al.*, 1998). But under laboratory conditions, it was also observed that the addition of pesticides to the growth media of pure cultures of *G. diazotrophicus* reduced the production of IAA and gibberellin A3 (Madhaiyan *et al.*, 2006).

Solubilization of Mineral Nutrients

Several reports show the ability of different bacteria to solubilize inorganic phosphate compounds such as tricalcium phosphate, dicalcium phosphate, hydroxyapatite and rock phosphate (Sashidhar and Podile, 2010). Mineral phosphate solubilization is generally considered to be a plant growth promoting trait for rhizosphere bacteria. However, mineral phosphate solubilization by an endophytic bacteria (Dias *et al.*, 2009; Puente *et al.*, 2009) could aid in availability of phosphorus to the host crop during initial colonization and subsequently promote plant growth (Kuklinsky-Sobral *et al.*, 2004). This activity has been observed in various strains of *G. diazotrophicus* recovered from sugarcane and from other crops in the presence of sucrose or glucose as carbon sources (Maheshkumar *et al.*, 1999; Suman *et al.*, 2001; Madhaiyan *et al.*, 2004; Intorne *et al.*, 2009).

The ability to solubilize insoluble inorganic phosphate compounds by *Gluconacetobacter diazotrophicus* was studied by Crespo *et al.* (2011), using different culture approaches. Qualitative plate assays using tricalcium phosphate as the sole P-source showed that *G. diazotrophicus* produced solubilization only when aldoses were used as the C-source. Extracellular aldose oxidation via a pyrroloquinoline quinone-linked glucose dehydrogenase (PQQ-GDH) is the main pathway for glucose metabolism in *G. diazotrophicus*. In batch cultures with 5 g l⁻¹ of hydroxyapatite as the P-source and glucose as the C-source, more than 98% of insoluble P was solubilized. No solubilization was observed neither using glycerol nor culturing a PQQ-GDH mutant of *G. diazotrophicus*. Solubilization was not affected by adding 100 mmol l⁻¹ of MES buffer. Continuous cultures of *G. diazotrophicus* showed significant activities of PQQ-GDH either under C or P limitation. An intense acidification in the root environment of tomato and wheat seedlings inoculated with a *G. diazotrophicus* PAL5 was observed. Seedlings inoculated with a PQQ-GDH mutant strain of *G. diazotrophicus* showed no acidification.

Solubilization of Heavy Metals

Zinc is an essential micronutrient in crop production; its deficiency symptoms in plants include premature yellowing, drying of leaf tips and leaf margin. Zn deficiency is widespread in arable soils and is also common in the host crops of *G. diazotrophicus*, such as sugarcane, rice and coffee. Solubilization of insoluble Zn compounds by *G. diazotrophicus* may augment Zn nutrition of host crops. Variation in Zn solubilization potential was commonly noticed among the *G. diazotrophicus* isolates in various studies. In particular, Madhaiyan *et al.* (2004) proposed that all the isolates were unable to solubilize insoluble Zn₃(PO₄)₂ except that obtained from coffee plants. Saravanan *et al.* (2007) showed that *G. diazotrophicus* strain PAL5 was able to solubilize most of the insoluble Zn compounds, including ZnO, ZnCO₃ and Zn₃(PO₄)₂, using 5-ketogluconic acid. In addition, it solubilized Fe, Mn and Si compounds like FeO, FePO₄, MnO and MgO. SiO₂, and analysis of the culture supernatants unequivocally suggested the presence of ketogluconates, in huge quantities (Saravanan *et al.*, 2004). They also clearly indicated that it has the ability to solubilize Zn metal and during this process the cells

were transformed into pleomorphic, aggregate-like forms, especially in the later stages of solubilization when the available Zn concentration increased rapidly. Similar results were previously observed when the cells were subjected to high concentration of ammonia or pesticides in the growth medium (Madhaiyan *et al.*, 2006; Muthukumarasamy *et al.*, 2002).

Introne *et al.*, (2009) studied the molecular mechanisms associated with phosphorus and zinc solubilization. A transposon mutant library was constructed and screened to select for mutants defective for phosphorous [Ca₅(PO₄)₃(OH)] and zinc (Zn) solubilization. A total of five mutants were identified in each screen. Both screenings, performed independently, allowed to select the same mutants. The interrupted gene in each mutant was identified by sequencing and the results demonstrated that the production of gluconic acid is a required pathway for solubilization of such nutrients in *G. diazotrophicus*. Sarathambal *et al.*, (2010) confirmed solubilization of insoluble zinc compounds like ZnCO₃ and ZnO by *G. diazotrophicus* using radiotracers. The zinc compounds (ZnCO₃ and ZnO) were tagged with ⁶⁵Zn. ⁶⁵ZnCO₃ and ⁶⁵ZnO was effectively solubilized and the uptake of Zn by the plants also more in *G. diazotrophicus* inoculated treatments compared to the uninoculated treatments. The production of gluconic acid is a required pathway for solubilization of phosphorus and zinc nutrients in *G. diazotrophicus*.

Production of Siderophores

G. diazotrophicus produced salicylate and catecholate type siderophores (Logeshwaran *et al.*, 2009). In addition, *G. diazotrophicus* can also promote biomass gain induced by hormone and/or siderophore production [Urquiaga *et al.*, 1992; Logeshwaran *et al.*, 2009].

Antagonistic Activity Against Phytopathogens

The antagonistic potential of *G. diazotrophicus* against *Colletotrichum falcatum*, the red rot fungal pathogen in sugarcane was first demonstrated by Muthukumarasamy *et al.*, (2000). When both *G. diazotrophicus* and the pathogenic red rot fungi were cultured in the same medium, a clear zone of inhibition against rot fungi was visualized. Similar to red rot pathogen, antagonistic potential was also demonstrated against the phytopathogenic bacterium *Xanthomonas albilineans*, the causative organism of leaf scald disease in sugarcane leaves (Pinon *et al.*, 2006). *G. diazotrophicus* secretes certain proteins (bacteriocins) that impede the growth of *X. albilineans* and impart a lysozyme-like activity on the innermost thick cell wall layer of the pathogen (Blanco *et al.*, 2005). Recently, Blanco *et al.* (2010) studied the mechanism of inhibition of *X. albilineans* by *G. diazotrophicus* and concluded that xanthan production is achieved by the pathogen *X. albilineans* infecting sugarcane stalks but it was nullified by *G. diazotrophicus* when the endosymbiont is growing in plant tissues together the pathogen.

Mehnaz and Lazarovits (2006) also demonstrated the presence of anti-fungal activity by *G. diazotrophicus* (wild-type and nifD strains) and *G. azotocaptans* (strain DS1) against various *Fusarium* spp. (except *Fusarium culmorum* and *Helminthosporium carbonum*) when they were cultivated in PDA medium. In contrast, both *G. diazotrophicus* and *G. azotocaptans* were completely ineffective against *Pythium aphanidermatum* and *P. ultimum*. *G. diazotrophicus* was also found to be able to impart antagonistic activity on the nematode *Meloidogyne incognita* by the production volatile fatty acids. In addition to the paralyzing effect (Bansal *et al.*, 1998) and disruption in the movement of juveniles (Djian *et al.*, 1991), volatile fatty acids are known to reduce

egg hatching by impairing embryogenesis (Bansal and Bajaj, 2003) of *M. incognita*. *G. diazotrophicus* inoculation was useful in reducing root galling due to *M. javanica* in bottle guard. Castro et al. (1990) found that ammonia excreted by *G. diazotrophicus* had diminished attractiveness of root-tips and delayed root invasion and egg deposition resulting in reduced egg content per egg mass thus affected total reproduction in comparison to uninoculated control.

Field evaluation of *G. diazotrophicus* against *M. incognita* in cotton also revealed significant increase in yield of cotton and reduction in root-knot disease severity (Bansal et al., 2005). The cell free culture filtrate reduced egg hatching by more than 95% and was capable of effectively paralyzing the infective juveniles of *M. incognita*. On the same lines, an in vitro study from our laboratory indicated that Zn²⁺ ions and Zn chelates could be formed due to solubilization of insoluble ZnO, ZnCO₃ and Zn₃(PO₄)₂ by *G. diazotrophicus* (Saravanan et al., 2007). The deleterious effect could be incited by the available Zn on the nematode *M. incognita*. Thus, *G. diazotrophicus* could mediate a dual role by solubilizing insoluble Zn compounds and enhancing the mortality and reduced root penetration by *M. incognita* due to significant release of Zn ions. Bansal et al. (1999) have investigated antagonistic efficacy of *G. diazotrophicus* and reported its mitigatory effect against root-knot nematode, *Meloidogyne javanica* and plant growth promoting ability in bottle guard.

G. diazotrophicus strains have the additional property of biocontrol potential against soil borne pathogenic fungus. Antibiotic, pyoluteorin produced by *Gluconacetobacter diazotrophicus* helped in the suppression of soil borne pathogenic fungus such as *F. oxysporum* in Sweet potato (Logeshwaran et al., 2011)

Conclusion

In conclusion, the importance of this endophytic, nitrogen fixing, plant growth promoting bacterium *G. diazotrophicus* is unquestionable. Extensive and intensive research on the understanding of associative and endophytic ecology will be major determinant to maximize benefit from these bacteria and if we understand more about this bacterium, we will have a better potential to use this bacterium.

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