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Review Article

Research Progress and Perspectives of Nitrogen Fixing Bacterium, *Gluconacetobacter diazotrophicus*, in Monocot Plants

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Gluconacetobacter diazotrophicus is a nitrogen fixing bacterium originally found in monocotyledon sugarcane plants in which the bacterium actively fixes atmosphere nitrogen and provides significant amounts of nitrogen to plants. This bacterium mainly colonizes intercellular spaces within the roots and stems of plants and does not require the formation of the complex root organ like nodule. The bacterium is less plant/crop specific and indeed *G. diazotrophicus* has been found in a number of unrelated plant species. Importantly, as the bacterium was of monocot plant origin, there exists a possibility that the nitrogen fixation feature of the bacterium may be used in many other monocot crops. This paper reviews and updates the research progress of *G. diazotrophicus* for the past 25 years but focuses on the recent research development.

1. Introduction

Nitrogen is a primary constituent of nucleotides, proteins, and chlorophyll in plants. Although the atmosphere is 78% nitrogen, its diatomic form makes it inaccessible to plants due to the presence of a triple bond [1]. Modern plant agriculture heavily relies on industry nitrogen fertilizers to maintain optimum yields [2]. However, nitrogen fertilizers are expensive, quadrupling in price from 1999 to 2008 [3]. The production and use of nitrogen fertilizers also contribute significantly to greenhouse gas emissions [4]. Additionally, many farmers apply supraoptimal amounts of fertilizers to their fields as a means of risk management and insurance against possible nitrogen losses to ensure maximum attainable yields [3, 5, 6]. Adding to this, the fact that nitrogen is mobile, reactive, and hard to contain makes it very vulnerable to losses due to denitrification, volatilization, and leaching [7–9]. Leached reactive forms of nitrogen are capable of causing widespread environmental effects and severe consequences to human health [3, 10–12]. Due to increased costs and detrimental effects on the environment associated with nitrogen fertilizers and negative field and yield effects resulting from continuous monoculture practices, farmers rely on crop rotation to both provide benefits to the agricultural system and add fixed-nitrogen into the soil through the process of biological nitrogen fixation (BNF) [2].

Biological nitrogen fixation can be defined as the reduction of dinitrogen to ammonia by means of a prokaryote [13]. However, not all plants have acquired a symbiosis with a nitrogen fixing prokaryote [14], primarily due to the fact that a large amount of prokaryotes capable of BNF, such as rhizobia, has a very selective host range [15]. One of the principal reasons for host selection by rhizobia is due to nodule formation, which requires a series of reciprocal molecular conversation signals between the bacterium and host plant which leads to changes in the transcriptional regulation of genes, structural changes, and eventually the formation of a root nodule, the epicentre of BNF in legumes [16]. However, not all prokaryotes capable of BNF require nodules to fix nitrogen. These prokaryotes, some of which are endophytic,

Table 1: Native host plants of *Gluconacetobacter diazotrophicus* and the tissues from which they were discovered.

Crop	Latin name	Tissue	Source
Sugarcane	*Saccharum* spp.	Root, stem, leaf, root hair	[18, 28, 101]
Cameroon grass	*Pennisetum purpureum*	Root, stem	[102]
Sweet potato	*Ipomoea batatas*	Root, stem	[63]
Coffee	*Coffea arabica*	Root, stem, rhizosphere	[62]
Finger millet (Ragi)	*Eleusine coracana*	Root, stem, leaf	[103]
Tea	*Camellia sinensis*	Root	[104]
Pineapple	*Ananas comosus*	Root, stem, leaf	[29]
Wetland rice	*Oryza sativa*	Root, stem, rhizosphere	[31]
Banana	*Musa acuminata* × *balbisiana*	Rhizosphere	[104]
Carrot	*Daucus carota*	Root	[99]
Radish	*Raphanus sativus*	Root	[99]
Beetroot	*Beta vulgaris*	Root	[99]

contain in many cases fewer requirements to establish a symbiotic relationship with a host plant. *Gluconacetobacter diazotrophicus* is a bacterium originally found in sugarcane plant. This bacterium can actively fix atmospheric nitrogen and provide significant amounts of nitrogen to sugarcane plants. Besides nitrogen fixation, this bacterium possesses several attractive features, including being of monocot origin, being less plant species specific, and having no nodule-structure requirement for living and nitrogen fixation. For the past years, many studies were conducted to reveal different aspects of this bacterium, aiming to explore this bacterium for nitrogen fixation in other crops, especially monocot plants. This paper will review and discuss the research progress of *G. diazotrophicus*.

2. Discovery, Classification, and Culture Media Requirements

Some basic features of *G. diazotrophicus* and related research were described in a minireview by Muthukumarasamy et al. 10 years ago [17]. This paper will provide a comprehensive review and discuss the new progress on *G. diazotrophicus* research.

Gluconacetobacter diazotrophicus was discovered within sugarcane plants in Alagoas, Brazil, by Cavalcante and Dobereiner [18]. Since then, *G. diazotrophicus* has been found in places such as Mexico and India and in crops ranging from coffee to pineapple (Table 1). The bacterium was initially named as *Saccharobacter nitrocaptans* and was later classified under acetic acid bacteria and named *Acetobacter diazotrophicus*, before being reclassified as *Gluconacetobacter diazotrophicus* based on 16S ribosomal RNA analysis [18–20]. This bacterium is accommodated with in the phylum Proteobacteria, the class Alphaproteobacteria, the order Rhodospirillales, the family Acetobacteraceae, and genus *Gluconacetobacter* [21]. *Gluconacetobacter diazotrophicus* is a Gram-negative, nonspore forming, nonnodule producing, endophytic nitrogen fixing bacterium. The bacterium is an obligate aerobe with cells measuring 0.7–0.9 μm by 2 μm and appears as single, paired, or chainlike structures when viewed under a microscope. The bacterium's cells have 1–3 lateral or peritrichous flagella used for motility. *G. diazotrophicus* is an acid-tolerant bacterium, being capable of growing at pH levels below 3.0; however its optimum pH for growth is 5.5 [18, 19].

Growth of *G. diazotrophicus* under laboratory conditions is primarily achieved through plating on LGIP medium due to the fact that it contains high sugar levels which are very similar to those found within sugarcane (quantities per litre: K_2HPO_4, 0.2 g; KH_2PO_4, 0.6 g; $MgSO_4{\cdot}7H_2O$, 0.2 g; $CaCl_2{\cdot}2H_2O$, 0.02 g; $Na_2MoO_4{\cdot}2H_2O$, 0.002 g; $FeCl_3{\cdot}6H_2O$, 0.01 g; bromothymol blue in 0.2 M KOH, 0.025 g; sucrose, 100 g; yeast extract, 0.025 g; agar, 15 g; 1% acetic acid, pH 5.5) [18]. Other media capable of sustaining *G. diazotrophicus* growth include but are not limited to DYGS, C2, ATGUS, modified potato, SYP, AcD, GYC, and EYC media [22–29]. The biochemical characteristics of *G. diazotrophicus* are listed in Table 2. Faster and more robust growth can be achieved through the addition of a nitrogen source to the LGIP medium, such as 10 mM $NH_4(SO_4)_2$. When grown on LGIP plates *G. diazotrophicus* can be visualized as smooth colonies with regular edges. Colonies on LGIP medium initially appear semitransparent but become dark orange in colour due to their uptake of bromothymol blue from within the medium following complete incubation [18, 19, 30–32]. As found within the bacterium's natural host sugarcane, *G. diazotrophicus* requires a large amount of sucrose for adequate growth [33]. With regard to growth under laboratory conditions, the bacterium is capable of growth in sucrose levels up to 30%, while optimum growth is achieved at sucrose levels of 10%. In addition to sucrose, *G. diazotrophicus* is capable of abundant growth on other carbon substrates including D-galactose, D-arabinose, D-fructose, and D-mannose [18].

3. Key Metabolic Enzymes of *G. diazotrophicus*

While *G. diazotrophicus* contains hundreds of enzymes, few have been examined as in depth as its nitrogenase, levansucrase, and pyrroloquinoline quinone-linked glucose dehydrogenase. Specifically regarding nitrogen fixation, these

Table 2: General characteristics of *Gluconacetobacter diazotrophicus*.

Characteristics	*G. diazotrophicus*
Gram reaction	−
Dark brown colonies on potato agar with 10% sugar	+
Dark orange colonies on LGIP medium with 10% sugar	+
Motility	+
N_2 fixation	+
NO_3—reduction	−
N_2 fixation with NO_3^-	+
Catalase	+
Oxidase	−
Growth on carbon sources	
Sucrose*	+
Glucose	+
Sorbitol	+
Galactose	+
Xylose	+
Ethanol	+
Sodium acetate	+
Glycerol	+
Arabinose	+
Raffinose	+
meso-Erythritol	+
Fructose	+
Maltose	+
Rhamnose	+
Trehalose	+
Cellobiose	+
Melibiose	+
Growth on L-amino acids in the presence of sorbitol as carbon source	
L-cysteine	+
L-glutamine	+
L-proline	+
L-tryptophan	+
L-aspartic acid	+

+: positive; −: negative.
*Only some strains were positive.
[18, 19, 30–32].

three enzymes are essential, as the removal or mutation of one of the three could result in either the loss of the mechanism of nitrogen fixation, the energy to power nitrogen fixation, or the environment to sustain nitrogen fixation.

3.1. Nitrogenase. The nitrogenase of *G. diazotrophicus* is a molybdenum-dependent system (Mo-nitrogenase) and is capable of providing its host with a substantial amount of fixed nitrogen [34]. ^{15}N-aided nitrogen balance studies have shown that certain genotypes of sugarcane are capable of having up to 200 kg N per hectare fixed for them by *G. diazotrophicus*, meeting approximately half of the crop's nitrogen needs without the application of additional fertilizers [35, 36]. The Mo-nitrogenase is made up of two component proteins, the Fe protein containing the ATP-binding sites and the MoFe protein containing the substrate binding sites [37]. A feature which may make nitrogen-fixation in *G. diazotrophicus* unique is that early reports indicated that it does not contain a nitrate reductase protein [18]. Without a nitrate reductase protein in the bacterium, it was hypothesized that there would not be feedback inhibition of nitrogenase by nitrate assimilation [18, 38]. However, more recent studies have suggested that the bacterium is inhibited to some extent by nitrate [39, 40]. Additionally, the nitrogenase of *G. diazotrophicus* is not completely inhibited by the addition of ammonium (Table 3). However, *G. diazotrophicus* growth under field conditions has been shown to be inhibited by high levels of nitrogen fertilization [41]. Therefore, the bacterium is capable of nitrogen fixation in crops that are supplemented with either nitrate-based fertilizers or with low amounts of ammonium-based fertilizers [34, 42].

3.2. Levansucrase. *G. diazotrophicus* is unable to transport or take up sucrose, as such it secretes an extracellular enzyme called levansucrase, a fructosyltransferase exoenzyme which hydrolyzes sucrose into fructooligosaccharides and levan [43, 44]. This enzyme is critical for the survival of the bacterium and can constitute over 70% of all secreted proteins by specific strains of *G. diazotrophicus* [43]. In addition to sucrose hydrolysis, levansucrase is also involved in tolerance to desiccation and NaCl and in biofilm formation [45]. Biofilm formation begins with the *gumD* gene homologue, an essential step in the production of exopolysaccharides, which along with levan, a product from the hydrolysis of sucrose by levansucrase, leads to the formation of biofilm in *G. diazotrophicus* [45, 46]. The removal of either of these two factors results in the bacterium being unable to form a biofilm. This leads to changes in colony morphology, tolerances, nitrogenase activity, and abilities to aggregate to abiotic and biotic surfaces, resulting in diminished colonization abilities [45–47].

3.3. Pyrroloquinoline Quinone-Linked Glucose Dehydrogenase. *G. diazotrophicus* also contains a pyrroloquinoline quinone-linked glucose dehydrogenase (PQQ-GDH), which oxidizes glucose into gluconic acid in the extracellular environment [48, 49]. More importantly, the PQQ-GDH, which is primarily synthesized under nitrogen fixing conditions, produces a large amount of energy for the bacterium. The increase of energy combined with the timing of the protein's synthesis, under nitrogen fixing condition, shows its importance in providing the bacterium with additional energy during nitrogen fixation, as there is a high energy demand associated with the conversion of dinitrogen by the nitrogenase [49]. While the principal pathway of glucose metabolism in *G. diazotrophicus* occurs through periplasmic oxidation via the PQQ-GDH, an alternate pathway exists, under specific

Table 3: Nitrogenase activity of *Gluconacetobacter diazotrophicus* strains Pal5 and Mad3A measured by acetylene reduction assay.

Nitrogen sources	Nitrogenase activity (nmol C_2H_4 h^{-1} mg^{-1} protein)		
	Pal5	Mad3A	Control
Nitrogen free	296 ± 16	0	0
1 mM NO_3^-	269 ± 15	0	0
10 mM NO_3^-	228 ± 21	0	0
1 mM NO_4^+	103 ± 19	0	0
10 mM NO_4^+	0	0	0

*Results are ±SD averages of 3 replicates for each treatment (Yoon and Tian, unpublished).

Table 4: Plant growth promoting properties of *G. diazotrophicus*.

Plant growth promotion	Traits	Capabilities	Sources
Biological nitrogen fixation	NH_4^+	400–417 nmoles of C_2H_4 hr^{-1} mg^{-1} cell protein	[105]; [106]
Phytohormone secretion	IAA	4–7 μg mL^{-1}	[87, 105]
	Gibberellin	GA_1—1.6 ng mL^{-1} GA_3—11.9 ng mL^{-1}	[52]
Mineral nutrient solubilization	Phosphorus	25–31 mm solubilization zone	[99]
	Zinc	30–48 mm solubilization zone	[99, 107]
Phytopathogen antagonism	Lysozyme-like bacteriocin	Inhibition of *X. albilineans*	[60]
	Inhibition zone of mycelial growth	*F. solani, F. solani phaseoli, F. sambucinum, F. culmorum, F. moniliforme, F. graminearum, H. carbonum*	[61]

All data are dependent on strain and carbon source.

environmental conditions, in an intracellular pathway via the nicotinamide adenine dinucleotide (NAD)-linked glucose dehydrogenase (GDH) [50].

4. Mechanism Aiding Plant Growth Promotion

As a plant-growth-promoting bacterium, *G. diazotrophicus* aids its host plant in several different ways aside from nitrogen fixation. While specifically under nitrogen limiting conditions *G. diazotrophicus* nitrogenase activity has been shown to make sugarcane plants grow better. *G. diazotrophicus* growth promotion also occurs in *nif*$^-$ mutant strains, supporting the fact of additional plant-growth promoting benefits (Table 4) [27, 51]. *G. diazotrophicus* has been found to provide its host plants with phytohormones. Indole-3-acetic acid (IAA) and gibberellins A1 and A3 have been found to be produced by *G. diazotrophicus*, both phytohormones are critical for normal plant growth and development [28, 52, 53]. *G. diazotrophicus* has also been found to have phosphorous and zinc solubilisation capabilities [22, 54–57]. Zinc and phosphorus, micro and macronutrients, respectively, are important to the growth, development, and yield of many plants. Saravanan and colleagues [57] with the aid of Fourier transform infrared spectroscopy analysis identified gluconic acid as one of the key agents involved in the solubilisation of zinc. Through transposon mutant library screening, the importance of gluconic acid and its pathway was reaffirmed for not only zinc solubilisation but phosphorus solubilisation as well [22]. More specifically, a 5-ketogluconic acid anion, a derivative of gluconic acid, appears to be the key factor associated with mineral solubilisation, as revealed through gas chromatography coupled mass spectrometry analysis [56]. Additionally, zinc solubilization appears to deform *G. diazotrophicus* cells, causing large (approximately 10 times larger than normal) pleomorphic cells and aggregate-like cells [57]. Similar cellular deformation and appearance of pleomorphic cells were also noticed when the cultures were subjected to high nitrogen (in NH_4 form) in the medium [17]. *G. diazotrophicus* has also been found to elicit a plant defence responses against *Xanthomonas albilineans*, a sugarcane pathogen [58]. *X. albilineans* causes leaf scald disease in sugarcane through its production of a xanthan-like polysaccharide product which can cause mature sugarcane plants to wilt and die [59]. *G. diazotrophicus* impedes the production of the leaf scald causing xanthan-like polysaccharide through its production of a lysozyme-like bacteriocin which results in the lysis of bacterial cells, effectively inhibiting the growth of *X. albilineans* [60]. In addition to antibacterial properties, *G. diazotrophicus* is also capable of antifungal activity against several *Fusarium* spp. and *Helminthosporium* spp. [61].

5. Natural Colonization of *G. diazotrophicus*

5.1. Native Host Plants. Aside from sugarcane, *G. diazotrophicus* has been discovered within a wide array of other organisms outside of its initial discovery within Brazil including

Table 5: Nonnative host plants of *Gluconacetobacter diazotrophicus* and the methods used for inoculation.

Crop	Latin name	Method	Source
Corn	*Zea mays*	Seed inoculation	Cocking et al. 2006 [24]; Eskin 2012 [73]; Riggs et al. 2001 [27]
		Aseptic inoculation	
		Root dip inoculation	Tian et al. 2009 [23]
Tomato	*Lycopersicon esculentum*	Aseptic inoculation	Cocking et al. 2006 [24]
Arabadopsis	*Arabidopsis thaliana*	Aseptic inoculation	Cocking et al. 2006 [24]
Sorghum	*Sorghum vulgare*	Soil drench inoculation	Paula et al. 1991 [63]
		Seed inoculation	Luna et al. 2010 [65]
		Root dip inoculation	Yoon 2011 [unpublished]
Wheat	*Triticum aestivum*	Aseptic inoculation	Cocking et al. 2006 [24]; Luna et al. 2010 [65]; Youssef et al. 2004 [32]
		Seed inoculation	
Oilseed rape	*Brassica napus*	Aseptic inoculation	Cocking et al. 2006 [24]
White clover	*Brassica napus*	Aseptic inoculation	Cocking et al. 2006 [24]
Common bean	*Phaseolus vulgaris*	Aseptic inoculation	Trujillo-López et al. 2006 [71]

coffee, pineapple, wetland rice, and many other crops as listed in Table 1. The majority of these hosts contain relatively higher levels of sucrose which appeared to be a prerequisite for colonization by this bacterium [27]. *G. diazotrophicus*, an obligate endophyte, is incapable of surviving in soil without a plant host for more than two days, with the exception of being capable of surviving within the spores of the vesicular-arbuscular mycorrhizal fungus *Glomus clarum* and within the root hairs of a host plant's rhizosphere [62, 63].

5.2. Avenues of Entry into Host. The bacterium is able to gain entry into its host plant through the roots, stems, or leaves [64]. With regard to the roots, *G. diazotrophicus* enters through the root tips and cells of the root cap and meristem, at areas of lateral root emergence and through root hairs [64–66]. Within the stems of host plants, specifically sugarcane, the bacterium is capable of entering at breaks caused by the separation of plantlets into individuals [64]. Lastly, within the leaves, the bacterium's most probable location of entry would be though damaged stomata [64]. An additional mode of entry used by *G. diazotrophicus* is achieved through an insect vector, the pink sugarcane mealybug (*Saccharicoccus sacchari*), a plant sap-sucking insect [67, 68]. Once within the host plant, *G. diazotrophicus* was found to primarily inhabit intercellular apoplastic spaces, the xylem, and the xylem parenchyma [33, 64]. However, recent findings, aided with β-glucuronidase (GUS)-labeled *G. diazotrophicus,* suggest that this bacterium is also capable of intracellular colonization within membrane-bound vesicles in its host plant [24]. Established *G. diazotrophicus* colonies are capable of growing up to 10^8 CFU per gram of tissue, as found within sugarcane [69]. The aforementioned modes of entry for *G. diazotrophicus* appear to be aided by hydrolytic enzymes [70]. Within both PAL5 and UAP5541 strains of *G. diazotrophicus,* production of endoglucanase, endopolymethylgalacturonase, and endoxygluconase was confirmed using only sucrose as their sole carbon source [70]. These enzymes have the potential to be responsible for both the bacterium's ability to enter its host plant and its mobility once inside [70].

6. Experimental Inoculation on Nonnative Host Plants

6.1. Nonnative Host Plants. *Gluconacetobacter diazotrophicus* has also been found to be capable of surviving after inoculation in a wide variety of crops, including corn, sorghum, wheat, and many others as listed in Table 5. The capability to introduce this bacterium into nonhost crops does not only provide an opportunity for further research within an aseptic environment in order to determine specifics regarding this bacterium, such as its localization within its host, points of entry, increased accuracy in hormone production, and most importantly an estimate of its nitrogen fixation, but it also provides the ability for the experimental introduction of this bacterium into novel hosts in which characteristics such as nitrogen fixation are very important. Several different inoculation methods have been used to successfully introduce *G. diazotrophicus* into crops.

6.2. Aseptic Inoculation Experiments. One of the most efficient methods of ensuring successful colonization of a bacterium into a host is under gnotobiotic/aseptic conditions. By doing so one would ensure that no competition or inhibition is occurring due to the presence of additional endophytic bacteria. Many studies used an aseptic environment for their host plants when inoculating them with *G. diazotrophicus* [24, 64–66, 71]. The majority of the host plants in the aforementioned studies were grown on varying types of modified MS medium within growth chambers to which the bacterial inoculum was added. The bacterial inoculum ranged in both amounts from 100 μL to 1 mL and CFU's mL^{-1} from 10^6 to 10^9 [24, 32, 64, 66, 71]. Seed inoculation, another method of introducing the bacterium into the host, was investigated by Luna and colleagues [65] and involved inoculating 100 g of seeds with 10 mL of a 10^8 CFU mL^{-1} bacterial culture in a

Table 6: List of DNA primer sets for PCR detection of *Gluconacetobacter diazotrophicus*.

Target	Primer name	Sequence	Source
23S rRNA	AD	TGCGGCAAAAGCCGGAT	Arencibia et al. 2006 (Arencibia et al. 2006a) [58]
	HerbaGd	TGCGGCAAAAGCCGGAT	Kirchhof et al. 1998 [108]
23S rRNA	AD	TGCGGCAAAAGCCGGAT	
	1440	GTTGGCTTAGAAGCAGCC	Sievers et al. 1998 [109]
16S rRNA	AC	CTGTTTCCCGCAAGGGAC	
	DI	CTGTTTCCCGCAAGGGAC	
16S rRNA	GDI39F	TGAGTAACGCGTAGGGATCTG	Franke-Whittle et al. 2005 [67]
	GDI916R	GGAAACAGCCATCTCTGACTG	
16S rRNA	GDI25F	TAGTGGCGGACGGGTGAGTAACG	Tian et al. 2009 [23]
	GDI923R	CCTTGCGGGAAACAGCCATCTC	

phosphate saline buffer at a pH of 6.0 [65]. Trujillo-López and colleagues [71] examined the interaction between the common bean and *G. diazotrophicus* associated with UV light stimulation as an abiotic stimulus for the promotion of secondary metabolite accumulation. While they were successful in observing secondary metabolite accumulation in the UV light stimulated seedlings, they also found that the seedlings inoculated 4 h after UV irradiation had 5.65 times the number of bacteria compared to control seedlings [71].

6.3. Greenhouse Inoculation Experiments. Studies carried out under greenhouse conditions are exposed to more factors than those grown under aseptic conditions and as such, provide a better understanding to the plant-bacterium interaction under less favorable conditions compared to those studied under aseptic conditions. Several different inoculation methods have been used in *G. diazotrophicus* studies. Tian and colleagues [23] found success with the root dip method of inoculation with corn. Plants were grown in the greenhouse to the 2-3 leaf stage at which point 10–15% of the roots were trimmed and submerged into a bacterial inoculum at 10^8 CFU mL^{-1} for 30 min [23]. Another method of inoculation, as described by James and colleagues [64], involved directly injecting 1 mL of the bacterial inoculum at approximately 10^8 CFU mL^{-1} into the growing sugarcanes "leaf pocket" at the base of the stem [64]. Seed inoculation, as mentioned earlier under the aseptic studies [65], can also be used under greenhouse conditions. Riggs and colleagues [27] successfully inoculated corn seeds by coating them with in a 10^8 CFU mL^{-1} bacterial suspension in peat which was followed by planting within 48 hrs of the coating inoculation. Paula and colleagues [63] demonstrated through soil drench inoculation an increase in bacterial numbers when sugarcane was inoculated with the spores of the vesicular-arbuscular mycorrhizal (VAM) fungus *Glomus clarum* containing *G. diazotrophicus* compared to the bacterium alone. An additional method of inoculation specifically directed towards endophytic bacteria is foliar spraying [72]. While the majority of these methods are successful at introducing the bacterium into the host under laboratory and greenhouse conditions, more research should be directed at field level application. Additionally, with regard to methods of inoculation, our lab found that the method used in inoculating a host plant appears to be more important in determining successful colonization than the plant genotype [73].

7. Identification, Quantification, and Localization of *G. diazotrophicus*

7.1. Identification and Quantification of G. diazotrophicus. Several methods exist to examine plants for the presence of *G. diazotrophicus*. One of the main methods of identification of *G. diazotrophicus* is through PCR. A list of primers used to target *G. diazotrophicus* is listed in Table 6. While a simple PCR is sufficient in identifying the bacterium at high colony numbers, a nested PCR in which a second round of PCR is used to amplify the product from the first round of PCR is instrumental in detecting the bacterium when found at very low colony numbers [23]. While PCR is capable of confirming the presence of the bacterium, it is not capable of determining the number of bacterium present within a sample. As such, the most probable number (MPN) method, using a McCrady table, has been used to quantify the amount of bacteria within a sample [63]. However, the MPN method is not considered to be very accurate and must be subjected to further testing to confirm isolates at a species level, most commonly accomplished via PCR. In order to combat the inaccuracies of the MPN method, species-specific polyclonal antibodies have been used with indirect enzyme-linked immunosorbent assay (ELISA) to quantify *G. diazotrophicus*. Da Silva-Froufe and colleagues [26] have shown that using the same *G. diazotrophicus* sample, quantification using the ELISA technique produced bacterial numbers many times greater than those calculated by the MPN method.

7.2. Localization of G. diazotrophicus. In addition to determining presence and number, researchers have used several different techniques in identifying the bacterium's localization within its host plants [24, 64–66]. James and colleagues [64] used immunogold labelling in which polyclonal antibodies were raised in rabbits against *G. diazotrophicus*, silver enhancement was also performed through the use of goat anti-rabbit antibodies [64]. With the aid of optical and transmission electron microscopy (TEM), confirmation of *G.*

diazotrophicus endophytic colonization was made [64]. In addition to immunogold labelling, other methods used for localization studies include *G. diazotrophicus* marked with *gusA* and *gfp* reporter genes from strains containing pHRGFPGUS (*gfp*::*gusA*) and pHRGFPTC (*gfp*) plasmids, respectively [24, 41, 65, 66]. *G. diazotrophicus* UAP5541/pRGS561 constitutively expressing GUS and UAP5541/pRGS562 with a *nif*H::*gus*A transcriptional fusion are two additional strains that have been used in several studies in which both intercellular and intracellular localization have been determined [24, 41, 65, 66]. More recently, in a study by Rouws and colleagues [66], *G. diazotrophicus* strain Pal5 carrying *gfp*::*gus*A plasmid pHRGFPGUS and *gfp* plasmid pHRGFPTC were proven to be valid tools in monitoring localization and colonization.

8. Molecular Research in *G. diazotrophicus*

The recent sequencing of the Pal5 genome has greatly expanded our knowledge of *G. diazotrophicus* and has opened many new doors in regard to future directions of research. The study by Bertalan and colleagues [74] was accomplished by RioGene in Brazil and funded by FAPRJ. The US DOE Joint Genome Institute has also sequenced the PAL5 genome; however, many differences exist between the two sequences [75]. *G. diazotrophicus* was only the 3rd diazotroph and the 9th endophyte to be sequenced [74]. The PAL5 genome yielded one circular chromosome (3,944,163 bp) with a G-C content of 66.19% and two plasmids pGD01 (38,818 bp) and pGD02 (16,610 bp). Overall the genome contains 3,864 putative coding sequences (CDS) [74].

One thousand and seventy-seven hypothetical proteins were found in the sequenced genome, 583 of which have already been identified and used to describe potential metabolic pathways within *G. diazotrophicus* [74, 76]. Proteome studies have observed different levels of expression and have identified the roles of specific proteins involved in *in vitro* cultures in the presence or absence of a sugarcane host and involved in the exponential and stationary phases of *G. diazotrophicus*, in the presence of high and low levels of nitrogen [76, 77].

Through thorough analysis of the genome it was found that *G. diazotrophicus* contains many genes homologous to those within other bacteria [74]. Some of these genes have been found to augment resistance to acetic acid, which undoubtedly play an important role in allowing the bacterium to grow and fix nitrogen at pH levels of 2.5 [74, 78–80]. Genes relating to production of gluconic acid can be important as the chemical is only produced during nitrogen fixing process. Other homolog genes which code for polysaccharides, including capsular polysaccharides, exopolysaccharides, and lipopolysaccharides, have been found to be involved in interactions between *rhizobia* and their host plants involved in invasion, nodule development, and protection and suppression against plant responses and antimicrobial compounds. These homologs could aid in further understanding specific actions with *G. diazotrophicus* [74, 81]. In order to accurately evaluate gene expression, reference genes are required as a comparison for normalization. Three genes have recently been suggested as suitable reference genes in *G. diazotrophicus* for real-time qPCR, *rho*, 23SrRNA, and *rpoD*, as their expression levels were shown to be very stable across different carbon sources [82]. The *G. diazotrophicus* genome has also uncovered genes which can code for several different signalling mechanisms, including those involved in the synthesis of second messenger cyclic di-GMP's, cytoplasmic, and membrane bound histidine kinase signalling proteins including response regulator genes containing several chemotaxis genes and 3 quorum sensing genes [74]. Quorum sensing refers to the ability of a bacterium to respond to autoinducers, hormone-like molecules which are capable of altering gene expression at a critical threshold population [83]. The quorum sensing genes in *G. diazotrophicus*, which consist of one *lux*I autoinducer synthase gene and two *luxR*-type transcriptional regulators genes, have been found to code for three *N*-acyl homoserine lactones (AHLs) [74, 84–86]. Analysis of *G. diazotrophicus* AHLs identified 8 different signalling molecules: C6-homoserine lactone (HSL), C8-HSL, C10-HSL, C12-HSL, C14-HSL, 3-oxo-C10-HSL, 3-oxo-C12-HSL, and 3-oxo-C14-HSL [85]. Quorum sensing has been shown to regulate and be involved in many important functions and traits, including nitrogen fixation within *Rhizobium etli* [84].

While many of the *G. diazotrophicus* plant growth-promoting traits have been investigated, their specific pathways have yet to be properly defined. Regarding IAA, studies have shown that, within *G. diazotrophicus,* an approximate 95% dropoff of IAA production occurs in cytochrome c mutants, meaning that *G. diazotrophicus* contains at least two independent pathways for IAA production [87]. Genome sequence analysis has shown that *G. diazotrophicus* does not possess indole 3-pyruvate carboxylase; as such, IAA could also be synthesized by either the trypamide pathways or by the indole-3-acetonitrole pathway [74]. *G. diazotrophicus* also appears to be capable of producing acetoin, a PGP volatile, based on the discovery of the gene homologs of key enzymes found in the acetoin pathway [74]. Volatile synthesis by *G. diazotrophicus* has yet to be investigated but could have a large impact in its PGP abilities [88]. As with the discovery of the previously discussed IAA mutant and of the more recently published flagellar mutant, Tn5 transposon mutagenesis appears to be the next step for the functional characterization of genes discovered in the genome sequence [87, 89]. New molecular methods, such as the Tn5 transposon mutagenesis and plasmid insertions, such as those discussed under localization experiments have led to the creation of several new strains of *G. diazotrophicus* which are listed in Table 7.

With the sequenced genome available to researchers, new strides have been made in understanding the many processes of *G. diazotrophicus* at a molecular level [90]. Focussing on nitrogen fixation, and more specifically on the bacterium's ability to protect its nitrogenase against inhibition due to oxygen, previous studies have suggested that nitrogenase activity is controlled by an on/off-switch mechanism for O_2 protection or that the bacterium utilizes colony mucilage, more specifically its position within it, to achieve optimal flux

Table 7: A subset of *G. diazotrophicus* mutant, plasmid, and wild-type strains.

Strain	Function	Source
Pal5	WT	Cavalcante and Dobereiner 1988 [18]
UAP5541	WT	Caballero-Mellado and Martinez-Romero 1994 [110]
MAd3A	*nifD*::*aph* mutant of Pal5 Nif-	Sevilla et al. 1997 [96]
MAd10	*ccm* mutant (6% IAA)	Lee et al. 2004 [99]
UAP5541/pRGS561	Constitutive GUS expression	Fuentes-Ramírez et al. 1999 [41]
UAP5541/pRGS562	*nif*H::*gus*A Transcriptional fusion	Fuentes-Ramírez et al. 1999 [41]
Pal5/pHRGFPGUS	*gfp*::*gusA*	Rouws et al. 2010 [66]
Pal5/pHRGFPTC	*gfp*	Rouws et al. 2010 [66]
GDP29H1, GDP9G4, GDP12F6 and GDP23A12	Unable to solubilize zinc and phosphorous	Intorne et al. 2009 [22]
AD5	*lsdA*::*nptII-ble* cassette (Levansucrase) LsdA-deffective	Hernandez et al. 1995 [43]
L-3	*lsdA* mutant	Velázquez-Hernández et al. 2011 [45]

in O_2 for aerobic respiration while not inhibiting nitrogenase activity [47, 91]. Recently, with the aid of the sequenced Pal5 genome, a putative FeSII coding gene was identified which opened the possibility of *G. diazotrophicus* using conformational protection mechanisms for nitrogenase against oxygen [92]. However, oxygen is not the only inhibitor of nitrogenase, reactive oxygen species (ROS), by-products of aerobic metabolism critical in the production of ATP for the high energy-demanding process of nitrogen fixation, have also proven to be inhibitors of nitrogenase [37, 93]. While ROS levels were expected to increase during nitrogen fixation and elevated aerobic respiration, they in fact decreased within *G. diazotrophicus*, as six ROS-detoxifying genes have been found to be upregulated within nitrogen fixing cells, a potentially adaptive mechanism by the bacterium for nitrogen fixation [94]. Asparagine, important to microbial growth promotion, is also a nitrogenase inhibitor and has been found in high amounts in many of *G. diazotrophicus* host plants [80, 94]. Genome sequencing has shown that *G. diazotrophicus* does not contain an asparagine synthetase ortholog, indicating that it requires an indirect pathway for asparagine biosynthesis [74]. Alquéres and colleagues [94] suggest the existence of a tRNA-dependent pathway for asparagine biosynthesis in *G. diazotrophicus* which ensures low intracellular levels of the amino acid.

Regarding biological nitrogen fixation, the recent sequenced genome corroborates with previous findings which have characterized the major cluster and associated genes of nitrogenase [95, 96]. Bertalan et al. [74] have also discovered that nitrogenase is not regulated at the posttranslational level and that, while the main route for ammonia assimilation is believed to occur through the glutamine synthetase/glutamate synthase pathway, alternative routes which would incorporate ammonia into different compounds can also exist.

9. Conclusions and Future Directions of Research

Over the past quarter of a century, since the original discovery of the bacterium, a lot of research has been conducted on *G. diazotrophicus*; named by some as the primary reason for the success of Brazil's bioethanol program and as the potential key to attaining BNF within nonlegume crops [40, 97]. While the majority of studies in the past have primarily focused on understanding the bacterium, its traits, and characteristics, recent studies have moved toward a more molecular focus. The recent discovery of the bacterium's genome will now only further efforts in the molecular field, potentially unlocking the door to successful BNF in nonlegume crops.

Past studies regarding BNF have only definitively shown evidence within sugarcane [35]. Studies in which the bacterium was found within additional natural host plants, such as coffee and pineapple, have not proven that BNF occurs within the plant; instead, they only demonstrate that the strain isolated from the plant is capable of nitrogen-fixation. Future studies should put more emphasis on determining *G. diazotrophicus* BNF capabilities within plants other than sugarcane. ^{15}N-aided experiments, as conducted by Boddey et al. [35], could determine the bacterium's capability in fixing nitrogen within hosts outside of sugarcane.

Studies should also continue to focus on the importance of quorum sensing. While it has been discovered that *G. diazotrophicus* contains 3 different AHLs, their exact roles have yet to be identified. Quorum sensing has been found to play pivotal roles in other bacteria; within *Rhizobium etli*, it has been found to control BNF [84]. Newly identified molecular methods used in studying *G. diazotrophicus* such as mutational studies via Tn5 transposon mutagenesis could

help identify which quorum sensing genes, if any, play any role in BNF [98]. Furthermore, overexpression of any quorum sensing genes found linked to BNF could result in BNF in hosts previously incapable of acquiring such a symbiosis.

Research into wide array of natural and nonnatural host plants of *G. diazotrophicus* has led to its discovery within radish roots in India and its capability to be successfully inoculated into plants such as Arabidopsis, a well-known and extensively studied model organism [24, 99]. While the Arabidopsis plant has provided many scientific breakthroughs, it is limited as a model organism when investigating monocot-specific processes. However, *Brachypodium distachyon* has recently emerged as a new model organism for monocots [100]. With future goals associated with *G. diazotrophicus* revolving around its potential to fix nitrogen in crops such as corn, wheat, and rice, its interactions within monocot plants must be thoroughly studied at molecular level and *B. distachyon* may provide such a system.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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