# Chapter 4
# Acetic Acid Bacteria as Plant Growth Promoters

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**Abstract** Different genera and species of the family *Acetobacteraceae* (e.g., *Gluconacetobacter diazotrophicus*, *Gluconacetobacter johannae*, *Gluconacetobacter azotocaptans*, *Swaminathania salitolerans*, *Acetobacter peroxydans*, and *Acetobacter nitrogenifigens*) were found associated with diverse plant species, colonizing the inner tissues and roots. Several of these species are capable of promoting plant growth through special mechanisms such as the biological nitrogen-fixing process, phytohormone production, mineral solubilization, and antagonistic effect against pathogens, among others. Plant growth-promoting bacteria (PGPB) are valuable for agriculture as a tool for improving crop performance and environmental conditions, as they may reduce or avoid the use of chemical fertilizers and pesticides.

From an agricultural point of view, to achieve a sustainable crop production to feed the growing human population, strategic biotechnological approaches should be considered in crop management, including nutritional and phyto-sanitary aspects. Therefore, the use of PGPB is one of the possible approaches. Research and field trials of PGPB over decades in the present and past century have opened up new horizons for their biotechnological application. In this chapter, information on $N_2$-fixing acetic acid bacteria (AAB), their ecology, their physiological and genetic characteristics, the mode of action of AAB as plant growth promoters, and their biotechnological application is presented.

**Keywords** *Gluconacetobacter diazotrophicus* • *Gluconacetobacter johannae* • *Gluconacetobacter azotocaptans* • *Gluconacetobacter kombuchae (Komagataeibacter kombuchae)* • *Swaminathania salitolerans* • *Acetobacter peroxydans* • *Acetobacter nitrogenifigens*

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## 4.1 Introduction

Plant growth-promoting bacteria (PGPB) are free-living soil, rhizosphere, rhizoplane, and phylosphere bacteria that, under some conditions, are beneficial for plants (Bashan and Holguin 1998). Most of the activities of PGPB have been studied in the rhizosphere (the soil area influenced by roots), and to a lesser extent, on leaf surfaces. Presently, endophytic PGPB (those colonizing plant inner tissues without producing damage) constitute an important area of PGPB study also.

PGPB can promote plant growth in two ways: by directly affecting the metabolism of the plants by providing substances that are usually in scant supply, or indirectly by preventing the deleterious effects of phytopathogenic bacteria, fungi, nematodes, and viruses (Bashan et al. 2008). In the first situation, bacteria are capable of fixing atmospheric dinitrogen, solubilizing phosphorus, zinc, or other metals, and enhancing production of plant hormones (e.g., auxins). Furthermore, they can improve plant tolerance to different environmental stresses, such as drought, high salinity of soils, metal toxicity, and pesticide load. One or more of these features may contribute to increase plant growth and development more than that of plants grown under standard cultivation conditions. In addition to the agricultural usefulness of PGPB, there are potential benefits in environmental applications: for example, increased bioremediation of wastewater, prevention of soil erosion in arid zones by improving the growth of desert plants, or extraction of hazardous materials from the soil (Bashan et al. 2008).

Bacteria capable of increasing plant growth and productivity in agriculture have been known for more than a century. Prominent among these organisms are members of the rhizobia whose potential and biotechnological use in agriculture is at present beyond doubt. However, although being not all well known, diverse genera belong to the PGPB, such as *Azospirillum*, *Azotobacter*, *Azoarcus*, *Bacillus*, *Burkholderia*, *Frankia*, *Herbaspirillum*, *Pseudomonas*, *Rhizobium*, *Serratia*, and *Sinorhizobium*. Additionally, in the past three decades some members of the family *Acetobacteraceae* have drawn attention to the scientific community as PGPB: *Gluconacetobacter*, *Swaminathania*, and *Acetobacter*. In this chapter, $N_2$-fixing acetic acid bacteria (AAB), their ecology, physiological and genetic characteristics, mode of action of AAB as plant-growth promoters, and biotechnological application, especially their contribution in agriculture, are described.

## 4.2 Contribution of Acetic Acid Bacteria as Plant Growth Promoters

### *4.2.1 Background: Nitrogen-Fixing AAB*

In the family *Acetobacteraceae*, only three genera (*Gluconacetobacter*, *Acetobacter*, and *Swaminathania*) include nitrogen-fixing species. Presently,

some of these species are considered as PGPB for harboring different mechanisms to promote plant growth, in addition to the capacity to fix atmospheric dinitrogen.

The first $N_2$-fixing AAB was described in Brazil by Dr. Johanna Döbereiner and her research team (Cavalcante and Döbereiner 1988); since then, it has been investigated thoroughly with regard to its nitrogen-fixing ability. It was found inside sugarcane plant tissues and was first named *Acetobacter diazotrophicus* (Gillis et al. 1989), then renamed as *Gluconacetobacter diazotrophicus* (Yamada et al. 1997). Later, three new species within the genus *Gluconacetobacter* were described: *Ga. johannae* and *Ga. azotocaptans* associated with coffee plants in Mexico (Fuentes-Ramírez et al. 2001), and *Gluconacetobacter kombuchae*, isolated from Kombucha tea in India (Dutta and Gachhui 2007). However, it was reported that *Ga. kombuchae* (LMG 23726$^T$) should be reclassified as *Gluconacetobacter hansenii* (now further changed to *Komagataeibacter kombuchae*), according to analyses of amplified fragment length polymorphism (AFLP) DNA fingerprinting (Cleenwerck et al. 2009).

In 2004, *Swaminathania salitolerans* was described (Loganathan and Nair 2004). Then, *Acetobacter peroxydans* and *A. nitrogenifigens*, associated with rice plants and Kombucha tea, respectively, were described as $N_2$-fixing AAB in India (Dutta and Gachhui 2006; Muthukumarasamy et al. 2005). The identification of *A. peroxydans* as a nitrogen-fixing species was surprising as this species had been already described in 1925 and had thus far never been reported as diazotrophic. Although the type strain and novel isolates of *A. peroxydans* showed low and inconsistent acetylene reduction activity, compared to *Ga. diazotrophicus*, their diazotrophic ability was confirmed by the presence of *nifH* genes (Muthukumarasamy et al. 2005). Some important characteristics of dinitrogen-fixing AAB are shown in Table 4.1.

### *4.2.2 Ecology*

Many reports on $N_2$-fixing AAB mention that they are mostly associated with sugar- or ethanol-rich environments; however, recent studies show that their distribution is wider.

*Gluconacetobacter diazotrophicus* was first isolated from sugarcane, colonizing the inner tissues of roots, stems, and leaves (Cavalcante and Döbereiner 1988). It was originally described as an endophytic species, although its natural occurrence in the rhizosphere of different plants has been documented (Jiménez-Salgado et al. 1997; Loganathan et al. 1999; Muthukumarasamy et al. 2005; Santos et al. 2006). Since its first report (Cavalcante and Döbereiner 1988), *G. diazotrophicus* has been isolated from sweet potato (Paula et al. 1991), *Pennisetum purpureum* (Reis et al. 1994), coffee plants in Mexico (Jiménez-Salgado et al. 1997), field-grown pineapples in Mexico (Tapia-Hernández et al. 2000), a grass called "finger millet" (*Eleusine coracana* L. Gaertn) (Loganathan et al. 1999), tea plants (roots), mango (fruits), the rhizosphere of banana plants (Muthukumarasamy et al. 2002), and from wetland rice

**Table 4.1** Some important characteristics of dinitrogen-fixing acetic acid bacteria (AAB)*

| Characteristics | *Gluconacetobacter diazotrophicus* | *Ga. johannae* | *Ga. azotocaptans* | *K. kombuchae* | *Swaminathania salitolerans* | *Acetobacter peroxydans* | *A. nitrogenifigens* |
|---|---|---|---|---|---|---|---|
| Cells | Straight rods | Straight rods | Straight rods | Straight rods | Straight rods | Straight rods | Straight rods |
| | 2.0 μm long | 1.5–1.9 μm long | 1.6–2.0 μm long | 2.0–3.0 μm long | 1.9–3.1 μm long | 2.0–3.0 μm long | 1.5–2.0 μm long |
| | 0.7–0.6 μm wide | 0.5–0.6 μm wide | 0.5–0.6 μm wide | 0.5–0.6 μm wide | 0.7–0.9 μm wide | 0.5–0.6 μm wide | 0.1–0.2 μm wide |
| Motile | + | + | + | + | + | + | + |
| Gram | – | – | – | – | – | – | – |
| Colonies on potato agar medium | Dark brown | Brownish | Beige-light-brownish | Light-brown | n.d. | Light-brown | Light-brown |
| $N_2$-fixation | + | + | + | + | + | + | + |
| Optimum growth temperature | 30 °C | 29 °C | 29 °C | 30 °C | 30 °C | 32 °C | 30 °C |
| Optimum pH | 5.5 | 5.5 | 5.5 | 4.5 | 5.5 | 6.0 | 4.5 |
| G+C content | 61–63 mol% | 57.96 mol% | 64.01 mol% | 55.8 mol% | 57.6–59.9 mol% | 60.5 mol% | 64.1 mol% |
| First source of isolation | Sugarcane roots and stems | Rhizosphere of coffee plants | Rhizosphere of coffee plants | Kombucha tea | Mangrove-associated wild rice (rhizosphere) | Roots and stems of wet-land rice | Kombucha tea |

*Data obtained from Gillis et al. (1989), Fuentes-Ramírez et al. (2001), Dutta and Gachhui (2007), Loganathan and Nair (2004); Muthukumarasamy et al. (2005), and Dutta and Gachhui (2006)
*n.d.* not determined

(Muthukumarasamy et al. 2005). *Ga. diazotrophicus* was found also in trash of sugarcane (e.g., senescent leaves left on the ground from the last cut) (Reis et al. 1994), and it was also isolated from a mealy bug (*Saccharococcus sacchari*) associated with sugarcane plants (Ashbolt and Inkerman 1990). Recently, the endophytic existence of *Ga. diazotrophicus* in different xerophytic plants of the Sinai desert (Egypt) has been reported (Hanna et al. 2013). This finding speaks well about the possible role of endophytic bacterial populations in the survival, nutrition, and health of existing plants in semiarid environments.

Different plants from locations where *Ga. diazotrophicus* was isolated (e.g., sugarcane, rice, elephant grass, sweet potato, coffee, and pineapple) have a high level of asparagine, which promotes microbial growth and inhibits nitrogenase activity. The regulation of intracellular concentrations of this amino acid is essential for growth and biological nitrogen fixation (BNF) in this diazotroph. Analysis carried out in the asparagine metabolic pathway by Alquéres et al. (2012) showed that intracellular levels of asparagine regulate the expression of nitrogenase *nifD* gene, suggesting that the presence of an alternative route to produce asparagine might give *Ga. diazotrophicus* a tighter control over cell growth and BNF and may be of importance in the regulation of the endophytic plant–microbe interaction.

In contrast to *Ga. diazotrophicus*, which can inhabits inner plant tissues as an endophyte, *Ga. johannae* and *Ga. azotocaptans* were only found colonizing the rhizosphere of coffee plants (Fuentes-Ramírez et al. 2001). Also, *Ga. azotocaptans* was isolated from the rhizosphere of corn (Mehnaz et al. 2006). The last species described within this genus, the $N_2$-fixing and cellulose-producing *K. kombuchae*, was isolated from Kombucha tea in India (Dutta and Gachhui 2007).

Regarding the other genera and species of $N_2$-fixing AAB, *Swaminathania salitolerans* was isolated from the rhizosphere, roots, and stems of salt-tolerant, mangrove-associated wild rice (*Porteresia coarctata* Tateoka); as a particular feature, it can solubilize phosphate in the presence of NaCl (Loganathan and Nair 2004). *Acetobacter peroxydans* was found associated with cultivated wetland rice varieties in India (Muthukumarasamy et al. 2005). Then, within this genus, *A. nitrogenifigens*, also isolated from Kombucha tea in India, was described as a novel $N_2$-fixing acetic acid bacterium (Dutta and Gachhui 2006).

Since the first description of *Ga. diazotrophicus* in 1988 (Cavalcante and Döbereiner 1988), it has been investigated thoroughly, first with regard to its nitrogen-fixing capacity and lately because it has additional abilities to enhance plant growth. Therefore, literature is abundant on this species, compared with the other AAB mentioned herein, and consequently the information in this chapter refers mainly to *Ga. diazotrophicus*.

### *4.2.3 Physiological Characteristics*

The most common physiological characteristics of *Ga. diazotrophicus* are its high sucrose tolerance (10 %), growth and nitrogen fixation at low pH (5.0 or less),

chocolate colonies on potato agar medium with 10 % sucrose, absence of nitrate reductase, nitrogen fixation not affected by high concentrations of $NO_3^-$ (25 mM), and partial inhibition by $NH_4^+$, especially at high sucrose concentrations (Boddey et al. 1991; Stephan et al. 1991). These characteristics enable *Ga. diazotrophicus* to fix $N_2$ in the presence of soil nitrogen, thus making it an even more interesting plant-associated diazotroph. For instance, within sugarcane stems, where *Ga. diazotrophicus* occurs in numbers up to $10^7$ $g^{-1}$ of plant tissue (Cavalcante and Döbereiner 1988), considerable amounts of nitrogen could be made available to the plant, hence providing a basis for the high estimates of $N_2$ fixation reported in certain sugarcane genotypes under field conditions in Brazil (Boddey et al. 1991; Urquiaga et al. 1992).

Acid production by *Ga. diazotrophicus* has an additional value as it can solubilize insoluble P and Zn compounds (Muthukumarasamy et al. 2002; Madhaiyan et al. 2004), although this feature is reduced when the organism is in contact with pesticides, as reported by Madhaiyan et al. (2006).

The respiratory system and diazotrophic activity of *Ga. diazotrophicus* PAL5 (the most studied strain) were investigated by Flores-Encarnación et al. (1999). Spectral and high-pressure liquid chromatography analysis of membranes revealed the presence of cytochrome *ba* as a putative oxidase in cells obtained from diazotrophically active cultures, concluding that glucose dehydrogenase and cytochrome *ba* are key components of the respiratory system of *Ga. diazotrophicus* during aerobic diazotrophy.

Levansucrase (LsdA, EC 2.4.1.10) is a constitutive exoenzyme in *Ga. diazotrophicus* that hydrolyzes sucrose to synthesize oligofructans and levan (Hernández et al. 2000). By targeted disruption of the *lsdA* gene it was observed that *Ga. diazotrophicus* utilizes plant sucrose via levansucrase. Arrieta et al. (2004) reported that LsdA, differing from other extracellular levansucrases from gram-negative bacteria, is transported to the periplasm by a signal peptide-dependent pathway: this was the first description of a type II pathway for protein secretion in the *Acetobacteraceae*. Also, the purification and characterization of a levansucrase enzyme produced by *A. nitrogenifigens* have been reported (Paul et al. 2011).

Extracellular glucose oxidation is considered the main route for glucose catabolism in *Ga. diazotrophicus* (Alvarez and Martínez-Drets 1995; Attwood et al. 1991). However, low hexokinase activities have been also reported in this organism; moreover, a nicotinamide adenine dinucleotide-linked glucose dehydrogenase (NADGDH) was found to be actively synthesized in glucose-containing cultures (Alvarez and Martínez-Drets 1995; Attwood et al. 1991). Consequently, two oxidative routes seem to be simultaneously expressed in *Ga. 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 reported also that a PQQ-GDH was primarily responsible for the high rates of gluconic acid formation exhibited by *Ga. diazotrophicus* (Attwood et al. 1991). About the regulation of both enzymes, Luna et al. (2006) observed that *Ga. diazotrophicus* metabolizes glucose mainly by way of a PQQ-GDH, particularly under BNF or limited conditions. Studies conducted in batch and continuous

cultures (Luna et al. 2006) showed that glutamate is the central molecule of carbon metabolism in *Ga. diazotrophicus*, and that metabolic flux proceeds mainly by the pentose–phosphate pathway, as has already been reported for other AAB (Matsushita et al. 1994).

It has been demonstrated that exopolysaccharides (EPS) produced by *Ga. diazotrophicus* are important in plant infection. Further studies revealed that, when grown in liquid medium containing mannitol as the sole carbon source, EPS were composed of Glc, Gal, and Man in a molar ratio of 6:3:1, respectively (Serrato et al. 2013). Nuclear magnetic resonance spectroscopy and chemical derivatization showed that the EPS structure has 4-*O*-substituted units of β-glucose, 3-*O*-substituted units of β-galactose, and 2-*O*-substituted units of α-mannose. Glucose and galactose units linked at C6 were also found. The structure proposed by Serrato et al. (2013) is different from EPS produced by other species of *Gluconacetobacter* published so far.

It was determined that reactive oxygen species (ROS) scavenging enzymes of *Ga. diazotrophicus* strain PAL5 are important in the endophytic colonization of rice plants (Alquéres et al. 2013). In that work, they observed that ROS were produced at early stages of rice root colonization, a typical plant defense response against pathogens. The transcription of the pathogen-related-10 gene of the jasmonic acid pathway, but not of the PR-1 gene of the salicylic acid pathway, was activated by the endophytic colonization of rice roots by *Ga. diazotrophicus* strain PAL5. Quantitative polymerase chain reaction analyses showed that, at early stages of colonization, the bacteria upregulated the transcript levels of ROS-detoxifying genes such as superoxide dismutase and glutathione reductase.

The mechanisms of cadmium, cobalt, and zinc resistance were characterized in *Gluconacetobacter diazotrophicus* PAL5 by Intorne et al. (2012). They found that this bacterium was resistant to high concentrations of Cd, Co, and Zn, with minimum inhibitory concentrations of 1.2, 20, and 20 mM, respectively. Therefore, they provided evidence for the high tolerance of *Ga. diazotrophicus* PAL5 to heavy metals and that the *czc* gene (which encodes a protein involved in metal efflux) is a determinant for metal resistance in this bacterium.

### *4.2.4 Genetic Characteristics*

It has been reported that some *Ga. diazotrophicus* strains carry plasmids, and that the *nif* genes (involved in the $N_2$-fixing process) are not located in plasmids but on the chromosome (Teixeira et al. 1994). Also, Caballero-Mellado and Martínez-Romero (1994) observed that not all *Ga. diazotrophicus* strains harbor plasmids, and that two plasmids were highly conserved among the isolates examined. Although their functions are yet to be identified, the fact that some strains do not harbor plasmids may indicate that fundamental phenotypic characteristics of *Ga. diazotrophicus* such as nitrogen fixation, indole-3-acetic acid (IAA) production, and the use of different carbon substrates are not plasmid encoded. The same

authors have reported that, regardless of the presence of plasmids, all the *Ga. diazotrophicus* isolates analyzed shared a common pattern of *nif* structural gene organization on the chromosome. Different genes involved in the $N_2$-fixing process and its regulation, such as *nifHDK*, *nifA*, *nifB*, *nifV*, *nifE*, and *ntrBC*, have been identified by Sevilla et al. (1997).

More recently, the complete genome of *Ga. diazotrophicus* PAL5 was reported (Bertalan et al. 2009). It is composed of one circular chromosome of 3,944,163 base pairs (bp) with an average G+C content of 66.19 %, and two plasmids of 38,818 and 16,610 bp, respectively. The circular chromosome has a total of 3864 putative coding sequences, with an overall coding capacity of 90.67 %. Among the predicted genes, 2861 were assigned a putative function, and 1077 encode hypothetical proteins. Regarding noncoding RNA genes, 12 rRNAs (4 rRNA operons) and 55 tRNAs were identified. The larger plasmid (pGD01) has 53 coding sequences; approximately 70 % encode hypothetical or conserved hypothetical proteins, and 5 encode proteins involved in plasmid-related functions. The remaining 11 coding sequences encode putative components of the type IV secretion system. It was found also that the small plasmid (pGD02) has 21 coding sequences, and about 50 % are hypothetical proteins.

The complete genomic sequence of *Ga. diazotrophicus* PAL5 (Bertalan et al. 2009) also revealed the presence of a quorum sensing system, among other features: these are regulatory mechanisms that, through the production of signal molecules or auto-inducers, allow the regulation of the physiology of a microbial population in a coordinated way. In gram-negative bacteria, the *N*-acyl homoserine lactones (AHL) are the most studied auto-inducers, and the presence of AHL-like molecules in cultures of *Ga. diazotrophicus* PAL5 grown in complex and synthetic media has been reported (Nieto-Peñalver et al. 2012). At present, new information on this interesting bacterium is expected after the publication of its genome sequence.

## 4.3 Mode of Action of AAB as Plant Growth Promoters

It has been reported by different authors that *Ga. diazotrophicus* can promote plant growth through more than a few independent direct or indirect mechanisms besides $N_2$ fixation, including synthesis of phytohormones, solubilization of nutrients, and antagonistic effects against phytopathogens.

### *4.3.1 Biological Nitrogen Fixation*

The biological reaction that counterbalances the loss of nitrogen from soils or agro-ecosystems is biological nitrogen fixation (BNF), which is the enzymatic reduction of the atmospheric dinitrogen ($N_2$) to ammonia, catalyzed by the nitrogenase

complex. This process is exclusive to Bacteria and Archaea, and the microorganisms that fix nitrogen are named diazotrophs.

The genetics and biochemistry of BNF and nitrogen utilization by *G. diazotrophicus* have been previously investigated to some extent (revised by Pedraza 2008; Saravanan et al. 2008). Corroborating previous studies through the genome sequence analysis of *G. diazotrophicus* PAL5, Bertalan et al. (2009) have found that the structural genes for nitrogenase *nifHDK* are arranged in a cluster, which also contains other $N_2$ fixation-related genes, such as *fixABCX*, *modABC*, and *nifAB*. They found also that other related genes (*ntrX*, *ntrY*, and *ntrC*, implicated in regulatory systems) are localized elsewhere in the chromosome in a 5.2-kb cluster. There are three copies of *nifU* homologous genes: one localized in the *nif* cluster and the other two scattered on the bacterial chromosome. No *draT* or *draG* homologues were found in this bacterium, confirming that nitrogenase activity is not regulated at the posttranslational level. It has been suggested that posttranslational modulation in *G. diazotrophicus* might be mediated by a FeSII Shethna protein (Ureta and Nordlund 2002), but no such coding sequence was identified. However, many other FeSII protein genes are present, and they are possible candidates for this function. The apparent absence of *nifL* as a *nifA* activity modulator in response to the cell $O_2$ status in *Ga. diazotrophicus* (Perlova et al. 2002) is in agreement with the lack of a *nifL* homologue on the genome. The *nifA* protein appears to be inherently sensitive to $O_2$. In *Ga. diazotrophicus*, the main route for assimilation of ammonia is believed to occur through the glutamine synthetase/glutamate synthase pathway (GS/GOGAT encoded by *glnA* and *gltDB*, respectively) (Dow et al. 2006). However, the genome analysis suggests the existence of alternative routes, wherein the putative enzymes NAD-synthase, aminomethyltransferase, histidine ammonia-lyase, and D-amino acid dehydrogenase would incorporate ammonia into different compounds (Bertalan et al. 2009). The enzymatic activity of GS is known to be regulated by an adenylyltransferase enzyme, which is probably encoded by *glnE*. The glutamate dehydrogenase gene was not found in *Ga. diazotrophicus* PAL5, although its activity was demonstrated for the strain PAL3 (Perlova et al. 2002).

### *4.3.2 Phytohormones*

It is well known that phytohormones are important as signals and regulators of growth and development in plants. The capacity to produce these hormones is frequently considered to be a peculiarity of the plant kingdom. Nevertheless, that characteristic is also extensive among soil- and plant-associated prokaryotes (Costacurta and Vanderleyden 1995). Furthermore, the production of hormonal substances such as auxins and gibberellins by different PGPB has been proposed as one of the mechanisms to explain plant growth promotion.

Indole-3-acetic acid (IAA) is a naturally occurring auxin with broad physiological effects. The production of IAA by *Ga. diazotrophicus* was first reported by

Fuentes-Ramírez et al. (1993). Later, the detection of aromatic amino acid amino-transferase (AAT) activity in *Ga. diazotrophicus*, *Ga. johannae*, and *Ga. azotocaptans*, as well as their IAA production, was reported by Pedraza et al. (2004). The AATs are ubiquitous enzymes that reversibly catalyze the conversion of amino acids to the corresponding α-keto acids; they can participate in multiple metabolic pathways, such as IAA synthesis. The presence of genes encoding enzymes such as aromatic-L-amino-acid decarboxylase, amine oxidase, and aldehyde dehydrogenases in the genome sequence of *Ga. diazotrophicus* suggests that this bacterium might synthesize IAA via the trypamide pathway (Bertalan et al. 2009). Also, the presence of two genes coding for putative nitrilases suggests that IAA might also be produced by the indole-3-acetonitrile pathway.

In addition to IAA, the second hormonal substances detected in *Ga. diazotrophicus* are the gibberellins A1 and A3. They were characterized by capillary gas chromatography–mass spectrometry from chemically defined culture media containing 10 % sucrose (Bastián et al. 1998).

The presence of genes coding for enzymes for the synthesis and secretion of spermidine in the *Ga. diazotrophicus* PAL5 genome sequence suggests that this polyamine may also contribute to promote plant growth (Bertalan et al. 2009).

### *4.3.3 Mineral Nutrients Solubilization*

Mineral phosphate solubilization is generally considered to be a plant growth-promoting characteristic for PGPB. This activity has been observed in different strains of *Ga. diazotrophicus*, including PAL5 recovered from sugarcane and from other crops, in the presence of sucrose or glucose as carbon sources (Fig. 4.1). Also, *Ga. azotocaptans* and *S. salitolerans* are able to solubilize insoluble mineral phosphate (reviewed by Saravanan et al. 2008).

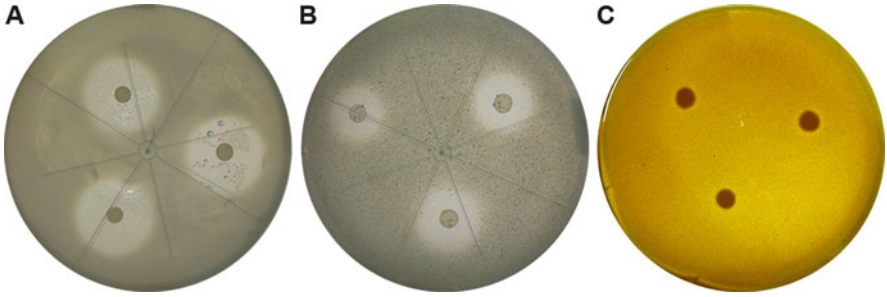


**Fig. 4.1** Phosphate solubilization by *Gluconacetobacter diazotrophicus* PAL5 is observed by the formation of a clear halo around the bacterial colony (shown in triplicate in each Petri dish). The growth culture medium was supplemented with different P-sources (5 g $l^{-1}$): (**a**) tricalcium phosphate [$Ca_3(PO4)_2$]; (**b**) hydroxyapatite [$Ca_5(PO_4)_3OH$]; (**c**) iron-phosphate ($FePO_4$). The solubility of the P-compounds decreases from (**a**) to (**c**)

Zinc is an essential micronutrient in crop production; its deficiency is widespread in arable soils and is also frequent in the host crops of *Ga. diazotrophicus*, such as sugarcane, rice, and coffee. Therefore, solubilization of insoluble Zn compounds by *Ga. diazotrophicus* may enhance Zn nutrition of host crops (reviewed by Saravanan et al. 2008). It was reported that different isolates of *Ga. diazotrophicus* effectively solubilized ZnO over $ZnCO_3$ or $ZnSO_4$ in plate and broth assays, showing variations in the solubilization capacity. After inoculation, those isolates were also able to improve sugarcane growth (Natheer and Muthukkaruppan 2012).

Another essential micronutrient is iron. To maintain growth, bacteria have developed different strategies to obtain iron from the iron-limited environment, but siderophore-mediated iron uptake is probably the most common form of iron acquisition (Braun and Winkelmann 1987). It has been reported that *Ga. diazotrophicus* produces these, being hydroxamate-type siderophores (Logeshwaran et al. 2009). However, their function in the *Ga. diazotrophicus*–plant association was not yet elucidated. Soto Urzúa et al. (2013) have reported the identification and characterization of an ATP-binding cassette (ABC) transport system in *Ga. diazotrophicus* PAL5, comprising three genes (*feuABC*) that encode a periplasmic-binding protein, a permease, and a traffic ATPase, and the involvement of this system in iron acquisition by this bacterium.

### *4.3.4 Antagonistic Effects Against Pathogens*

It has been documented that the ability of *Ga. diazotrophicus* to antagonize diverse plant pathogens, such as fungi and bacteria, contributes to increasing its ability to survive under environmental stress and leads to an improvement in plant fitness, which may have significant consequences for agricultural productivity (Pedraza 2008; Saravanan et al. 2008). On these features, the genome sequence of *Ga. diazotrophicus* encodes a large stock of genes whose products oppose attack from competing microbes, such as drug efflux systems, and acriflavin and fusaric acid resistance proteins (Bertalan et al. 2009). On the other hand, this bacterium may also produce a wide variety of proteins such as lytic enzymes and phospholipases and antibiotic biosynthetic pathways that could be toxic to other organisms. Also, it was observed in the genome sequence that *Ga. diazotrophicus* encodes a putative lysozyme-like bacteriocin, coinciding with data on its secretion of a lysozyme-like bacteriocin that inhibits the growth of *Xanthomonas albilineans* growth, the causal agent of leaf scald disease in sugarcane.

## 4.4 Biotechnological Applications

From an agricultural point of view, to achieve sustainable crop production to feed the growing human population, strategic biotechnological approaches should be considered in crop management, including nutritional and phyto-sanitary aspects. The use of PGPB is one of the possible approaches. Research and field trials of PGPB over decades in the present and past century have opened up new horizons to their biotechnological application. Even more, in many countries this is a very attractive activity for the agricultural industry of biostimulants. Also, the development of superior or novel PGPB strains with improved plant growth-promoting characteristics, and the development of transgenic crop plants expressing some special PGPB features, is possible by genetic manipulations. Therefore, these PGPB biotechnologies can be now exploited as a low-input, sustainable, and environmental friendly strategy for crop management.

### *4.4.1 Biostimulants*

Recently, the emerging definitions of plant biostimulants (Calvo et al. 2014) were revised. As a general definition, they are diverse substances and microorganisms used to enhance plant growth, including five categories: microbial inoculants, humic acids, fulvic acids, protein hydrolysates and amino acids, and seaweed extracts. Presently, there is growing scientific evidence supporting the use of biostimulants as agricultural inputs on diverse plant species. According to Calvo et al. (2014), the global market for biostimulants is projected to increase 12 % per year and surpass US$ 2200 million by 2018. Also, they reported increased root growth, enhanced nutrient uptake, and stress tolerance as examples of common features in plant responses to different biostimulants.

### *4.4.2 Inoculants and Inoculation*

Bacterial inoculation of plants to enhance yield of crops is a century-old proven technology for rhizobia and a newer venue for PGPB. The two main aspects dominating the success of inoculation are the effectiveness of the bacterial isolate and the proper application technology (Bashan et al. 2014). In general, “bacterial isolates” refer to specific bacterial strains (PGPB) that can promote plant growth after inoculation. “Carrier” refers to the abiotic substrate (e.g., solid, liquid, or gel) that is used in the formulation process. “Formulation” refers to the laboratory or industrial process of unifying the carrier with the bacterial strain. Last, “inoculant” refers to the final product of formulation containing a carrier and bacterial agent or consortium of microorganisms. Presently, there exist different carriers and

formulations of inoculants including liquid, organic, inorganic, polymeric, and encapsulated formulations. Technical aspects of this issue include inoculation techniques (soil and seed application), mass culture production, bulk sterilization, seed coating, shelf life, and effects of moisture (Bashan et al. 2014).

The use of AAB as inoculants in agriculture is something new. In Brazil, studies have been conducted to evaluate maintenance of cell viability and stability, as well as to select cheap carriers to extend the shelf life of plant beneficial bacterial inoculants for agricultural crops (da Silva et al. 2012). Thus, the shelf life and the colonization efficiency of novel liquid- and gel-based inoculant formulations for sugarcane were evaluated. The different inoculant formulations were all composed of a mixture of five strains of diazotrophic bacteria (*Gluconacetobacter diazotrophicus*, *Herbaspirillum seropedicae*, *H. rubrisubalbicans*, *Azospirillum amazonense*, and *Burkholderia tropica*), which are recognized as sugarcane growth promoters. Different inoculant formulations containing as carrier the polymers carboxymethylcellulose (CMC) and cornstarch (60/40 ratio) at five different concentrations (named PIC, for polymeric inoculant carrier) were supplemented, or not, with 2 % MgO, an interfacial stabilizing agent. Laboratory tests showed that in the formulation composed of 0.8 g of the polymeric mixture per 100 g of the final product (PIC 0.8), survival of *Ga. diazotrophicus* and *A. amazonense* was around $10^9$ CFU $ml^{-1}$ after 120 days of storage, regardless of supplementation with MgO. The other formulation (2.2 g of polymeric mixture, PIC 2.2) presented survival levels of $10^8$ CFU $ml^{-1}$ for up to 60 days of storage for all the individual strains. In the greenhouse, sugarcane seedlings showed a positive growth response 50 days after inoculation when inoculated with the mixture of five bacteria, with and without PIC 2.2. Hence, the polymer carriers used allowed for the long-term survival of the five different bacterial strains tested. Additionally, short-term experiments in the greenhouse showed that their application as part of an inoculant on sugarcane cuttings was at least as effective, in terms of bacterial colonization and the promotion of plant growth, as that of the bacterial mixture without carriers.

Also, the sprouting, survival and growth of young sugarcane var. VMC-86-550 treated with *Ga. diazotrophicus* was investigated in the Philippines (De La Cruz et al. 2012). Three bacterial concentrations ($10^8$, $10^{10}$, and $10^{12}$ cells $ml^{-1}$) and three methods of inoculation (spraying, soaking, and dipping) were used. The inoculated plants showed a significant increase in percent survival, plant height, and shoot/root biomass compared with the uninoculated control at 45 days after planting (DAP). However, no significant differences were observed in percent sprouting between inoculated plants and the uninoculated controls at 30 DAP. It was observed that introduction of *Ga. diazotrophicus* at $10^{12}$ cells $ml^{-1}$ by means of the dipping method had constantly yielded taller plants with greater shoot and root biomass, relative to the other treatments and to the uninoculated controls. This experiment showed the potential use of *Ga. diazotrophicus* in the development of a cost-effective technology in sugarcane production.

## 4.5 Application in Agriculture

The use of AAB as PGPB was reviewed by Pedraza (2008) and Saravanan et al. (2008), including data on $N_2$-fixing AAB interacting with agronomically important crops, such as sugarcane, sorghum, rice, maize, and wheat. In most cases, positive plant response after bacterial inoculation was observed; therefore, only recent information on this topic is reported in this chapter.

A field experiment to evaluate the performance of in vitro micropropagated plantlets inoculated with *Ga. diazotrophicus* on the population of diazotrophs, plant growth, yield, quality, and planting material of sugarcane cultivar CoS 96268 was carried out in India (Singh et al. 2012). Maximum cell counts ($5.8 \times 10^5$ cells $g^{-1}$ fresh weight) were obtained in the inoculated micropropagated plantlets, followed by untreated micropropagated plantlets ($4.7 \times 10^4$ cells $g^{-1}$ fresh weight), but only $3.7 \times 10^4$ cells $g^{-1}$ fresh weight in conventionally grown plants. Bacterial inoculation improved dry matter accumulation, especially in the roots, accompanied by higher uptake of nitrogen and potassium in micropropagated plantlets. There was significant increase in number of millable canes, cane length, number of nodes, cane diameter, and cane yield. Further, treated plantlets produced 5.45 and 1.52 times higher planting material (three budded setts) over conventionally grown plants and untreated micropropagated plantlets, respectively. This result signifies that inoculation with diazotrophic bacteria in micropropagated plantlets for faster multiplication of disease-free healthy seed cane of sugarcane varieties constitutes an interesting biotechnology to be adopted.

Also, a field experiment conducted on sugarcane in India analyzed the effect of pre-planting sett treatment and/or soil application with *Ga. diazotrophicus* or composite culture consisting of *Azotobacter*, *Azospirillum*, *Ga. diazotrophicus*, and phosphate-solubilizing bacteria with varying levels of N fertilizer (Nalawade et al. 2013). They observed that sett treatment with *Ga. diazotrophicus* at the time of planting could save 50 % of chemical N fertilizer with equivalent cane yield and higher monetary returns as compared to only the recommended dose of fertilizers (100 % NPK). In line with this, another field experiment on sugarcane was conducted in India with five PGPB (*Pseudomonas* spp., *Bacillus* spp., *Azospirillum* spp., and *Ga. diazotrophicus*) (Chauhan et al. 2013). As the conclusion, the values of plant height, chlorophyll content, total nitrogen, and cane length were significantly higher in almost all inoculated plants compared with the uninoculated control. Particularly, an increase of 42 % in cane yield over the control was obtained after inoculation only with *Ga. diazotrophicus*.

In another trial, three PGPB of diverse habitats (endophytic *Ga. diazotrophicus*, free-living *Azotobacter chrococcum*, and associative *Azospirillum brasilense*) were tested at field conditions during a complete sugarcane crop cycle (plant and a ratoon) (Suman et al. 2013). Different levels of N input for enhancing crop productivity and soil N balance were also considered. In general, the inoculation with PGPB was highly beneficial and, in particular, *Ga. diazotrophicus* was the

most efficient in terms of obtaining a higher cane yield, commercial cane sugar production, efficient N utilization, and maintaining positive soil N balance.

Continuing with experiences in sugarcane, the effects of subsequent sugarcane ratooning on soil quality and the crop yields under four treatments [an absolute control, application of recommended dose of NPK, application of sulphitation press mud (SPM), a sugar factory by-product, and SPM along with *Ga. diazotrophicus*] were evaluated for 7 years (Singh et al. 2013). In the control and NPK-fertilized plots, an increase in soil compaction (5.4 %), decrease in infiltration rate (6.04 %), lower microbial activities, and increased soil phenolic contents (72.4 %) rendered the nutrients unavailable, leading to significant declines in the crop yields at the rate of 5.47 Mg $ha^{-1}$ $year^{-1}$ and 4.67 Mg $ha^{-1}$ $year^{-1}$, respectively. Also, a crop yield decline from 53 kg $ha^{-1}$ in plant crop to 18 kg $ha^{-1}$ in the sixth ratoon crop under the absolute control was observed. However, the rates of yield decline were minimized in plots including SPM and SPM plus *Ga. diazotrophicus* to 3.54 and 3.51 Mg $ha^{-1}$ $year^{-1}$, respectively. It was also reported that *Ga. diazotrophicus* together with the fungus *Trichoderma*, in combination with trash and farmyard manure, were used as recycling amendments to sustain soil quality and sugarcane ratoon yield in an Udic Ustochrep soil of India (Shukla et al. 2012). Recently, the tolerance of *Ga. diazotrophicus* to different herbicides used in the sugarcane crop was also reported (Procópio et al. 2013).

Considering crops other than sugarcane, $N_2$-fixing AAB, for example, *Ga. diazotrophicus* and *A. peroxydans*, were also found in natural association with rice plants, and they may be considered important for agriculture because both species may supply a part of the nitrogen that is required by rice (Muthukumarasamy et al. 2005).

Concerning the application of *Ga. diazotrophicus* in non-gramineous plants, it was reported by Luna et al. (2012) that inoculation of tomato (*Lycopersicum esculentum*) with this bacterium could confer beneficial effects to this crop after efficient plant colonization. These authors observed that both numbers and weight of fruit production significantly increased in inoculated plants as compared to non-inoculated controls. Although the growth promotion mechanisms involved were not evaluated, *Ga. diazotrophicus* enhanced tomato fruit yield under greenhouse conditions. Also, effective colonization of *Ga. diazotrophicus* in inner tissues of tomato plantlets was observed by Botta et al. (2013).

## 4.6 Concluding Remarks

This chapter has shown that AAB as plant growth promoters are of beneficial importance to agriculture. Some of these bacteria have more than one mechanism of accomplishing increased plant growth, such as the biological $N_2$-fixing process, production of phytohormones, and solubilization of mineral nutrients. Although variability of yield in field performance is common, as they are influenced by the environment and plant and bacterial genotypes, PGPB are environmentally

friendly, in contrast to the overuse of the chemical fertilizers and pesticides applied in modern agriculture.

It is well known that chemical fertilizers increase yield in agriculture, but these are expensive, and their inappropriate use may harm the environment. They can deplete nonrenewable energy via side effects, such as leaching out, and polluting water sources, destroying microorganisms and beneficial insects, making the crops more susceptible to diseases, reducing soil fertility and biodiversity, and, consequently, causing irreversible damage to the overall agroecosystem. Therefore, the use of PGPB could be a better alternative to agrochemicals, considering that they are economical, not harmful to the environment, and can easily be found in different habitats.

Regarding the biotechnological application of AAB as plant growth promoters, the formulation and field application of inoculants need further studies. The isolation, characterization, and identification of new bacterial isolates having plant growth-promoting capacities is often not difficult and such are frequently reported. However, most new PGPB strains remain there, without ever reaching the formulation stage. Therefore, new insights for this issue should include not only *Ga. diazotrophicus*, but the other members of the *Acetobacteraceae* family with PGPB characteristics. According to Bashan et al. (2014), the development of PGPB inoculants involves a technological platform with knowledge that is based on fundamental principles of microbiology and material sciences. The unification of these fields, however, presently creates useful products that have important input for sustainable agriculture.

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