Dhananjaya Pratap Singh
Harikesh Bahadur Singh
Ratna Prabha *Editors*

Plant-Microbe Interactions in Agro-Ecological Perspectives

Volume 2: Microbial Interactions and Agro-Ecological Impacts

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Award by the UP Council of Science and Technology. Dr. Singh has been a fellow of the National Academy of Agricultural Sciences. Currently, he is also serving as an associate/academic/board editor for journals of international repute. Dr. Singh has more than 300 publications to his credit, including several training modules/manuals, 17 edited books, and 20 patents (USA, Canada, PCT).

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Interaction Between Beneficial Bacteria and Sugarcane

1

Guilherme Grodzki Oliveira Figueiredo, Valeria Rosa Lopes, Ricardo Cancio Fendrich, and Vivian Jaskiw Szilagyi-Zecchin

Abstract

Eco-friendly sugarcane production is constantly faced with growing demands for increased productivity. Current biotechnology, based on growth promotion through bacterial inoculants, presents us with the opportunity to increase production without an adverse environmental impact. To this end, plant growth-promoting bacteria (PGPB) with their diverse agricultural characteristics, like nitrogen fixation and production of plant regulators, are a good choice in achieving this goal. Characterization of the abilities of different strains will define their potential use, which for the most part is not limited to a single desirable feature. Therefore, our aim was to contribute to the present understanding of the principal activities of PGPB in sugarcane, to provide some simple and common methods for selecting them, and to draw attention to sugarcane breeding for selection of responsive clones for PGPB inoculation.

Keywords

PGPB • Sugarcane • Inoculation • Biological nitrogen fixation • *Saccharum* sp

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1.1 Introduction

Conventional agriculture has had a considerable negative impact on the environment in recent years, mainly on soil and water sources. Environmental degradation resulting from inappropriate agricultural practices and the indiscriminate use of agrochemicals has changed the way of modern agriculture. New cultural practices that are less aggressive to the environment are necessary and have more sustainable agriculture appeal. Agricultural techniques need to be changed, aiming at "cleaner" practices for the environment.

Many studies have led to the use of "natural products," such as beneficial bacteria for the control of pests and diseases, as well as the promotion of development of plants for greater productivity. One of these new strategies is the use of bacteria to induce plant growth, control plant disease, and produce biodegradation of xenobiotic compounds (Perry et al. 2007). This science is growing rapidly, and in turn, the new biomolecular technologists have contributed significantly to this new agriculture (Moreira and Siqueira 2006).

To date, several microorganisms have been studied and have demonstrated efficiency in controlling diseases, increasing productivity, and improving other desirable traits in various plant species, and sugarcane has been one of the most important crops in this research (Silveira 2008; Moreira and Siqueira 2006).

The interaction of sugarcane with plant growth-promoting bacteria (PGPB) has been extensively studied and various technologies have been developed in the last 50 years. Sugarcane is considered to be one of the best options among the renewable energy sources, with a promising future in a global scenario (Maule et al. 2001). In addition, sugarcane is propagated in a vegetative way, by clones (Matsuoka et al. 2005), which facilitates the selection of bacteria with greater interaction among the cultivars, ensuring greater success in obtaining inoculants.

This chapter thus covers the new knowledge about this mechanism of interaction and its implications for sugarcane agriculture.

1.2 Sugarcane

Sugarcane (*Saccharum* spp.) is one of the most important species cultivated commercially in the tropics and subtropics for renewable energy sources (Bonnett et al. 2004; Manners et al. 2004). It is propagated vegetatively by stems and produces a large amount of biomass, which requires a high application of nutrients, mainly nitrogen. Commercial sugarcane is also propagated by allowing the growth of the stems of the stools that remain in the soil after harvesting the previous crop (ratooning).

The production chain of sugarcane, its products and byproducts, is an important source of distribution of wealth (Matsuoka et al. 2005). In addition to alcohol and sugar, it has other byproducts, such as bagasse, various types of paper, pharmaceutical products, yeast, and various products resulting from the alcohol chemistry such as polyethylene, ether, acetone, and others (Vian 2009).

Brazil is the world's largest producer of sugarcane, followed by India, China, and Thailand (FAO 2016). In fact, this crop occupies an area of 8,654 hectares and has a production of 665,586 thousand tons (data from the last harvest, 2015/2016 (CONAB 2016)). Brazil is also a world leader in sugar production and is responsible for more than half of the world's sugar market (MAPA 2012), exporting to countries such as China, Russia, and Egypt (USDA 2012).

The genus *Saccharum* is characterized by high levels of polyploidy (polyploids have more than two sets of chromosomes) and frequently by unbalanced numbers of chromosomes (aneuploidy) (Blackburn 1984; Jannoo et al. 1999). These characteristics increase the genetic complexity of the cultivars (Jannoo et al. 1999), and confer to this culture a certain adaptability that allows its cultivation in different environments, soil types, and relief (Santos 2008), and adaptability is favorable for interaction with different beneficial bacteria.

1.3 The Activities of Plant Growth-Promoting Bacteria (PGPB) in Sugarcane

The binding between sugarcane culture and PGPB is of great importance for sustainable cultivation once the bacteria can promote the growth of the plant, reducing the use of chemical fertilizer by different mechanisms.

The PGPB are able to promote plant development by means of different mechanisms (Silveira 2008). These bacteria are able to fix nitrogen from the atmosphere, induce plant defense mechanisms responsible for diseases protection, solubilize phosphorus, produce siderophores that sequester and provide ferric ions, oxidize sulfur, and produce hydrocyanic acid (HCN) and other substances (Luz 1996; Rodríguez and Fraga 1999; Arencibia et al. 2006; Tortora et al. 2011).

Beyond those properties, these bacteria have the capability of producing precursor substances of plant growth regulators such as adenine derivatives (precursors in cytokinin biosynthesis) and growth-promoting compounds that have a similar activity to plant regulators (Silveira 2008). The main classes are auxins, cytokinins, gibberilines, ethylene, and abscisic acid (ABA) (Moreira and Siqueira 2006).

The first report of these beneficial mechanisms came from the fact that some commercial cultivars of sugarcane did not present symptoms of nutritional deficiency, mainly nitrogen, after many years of cultivation without fertilization (Boddey et al. 1995).

This and similar reports brought about the discovery of the nitrogen-fixing bacteria in sugarcane that have been studied since then. Recently, Magnani et al. (2010) and Moreira (2013) described the existence of a large bacterial community associated with sugarcane. This association explained the lesser requirement for soil fertility by some cultivars in the last 50 years, like the most planted sugarcane type in Brazil, the RB867515 cultivar. According to Beneduzi et al. (2013), there is a wide spectrum of bacterial populations associated with sugarcane, increasing the cultural potential in restrictive soils. The presence of these bacteria in the cane plantation is

confirmed by its survival capacity in cultivated soils and by propagation of infected stalks (Olivares et al. 1996, 1997).

There are in fact more than 40 bacterial genera that are known to be involved in growth promotion and disease occurrence in sugarcane. Among the beneficial bacteria with biological nitrogen fixation (BNF) or other properties are the genera *Azospirillum*, *Azotobacter*, *Beijerinckia*, *Burkholderia*, *Herbaspirillum,* and *Gluconacetobacter*. The most well-known genera are *Azospirillum* and *Gluconacetobacter*, both of which are endophytic (Oliveira et al. 2004; Moreira and Siqueira 2006; Hungria 2011; Mehnaz 2013). Rodrigues et al. (2016a) showed the wide spectrum of PGPB involved in sugarcane plant development. These authors isolated 136 bacteria, of which 83 bacteria presented with some plant growth mechanism.

G. diazotrophicus is the most common species associated with sugarcane and is found in leaves, stalks, and, especially, intercellular spaces, even sub-stomatal cavities. Usually in the roots this genus is presented between apoplast cells (Dong et al. 1994; James et al. 2001).

The association between PGPB and sugarcane is complex and is closely dependent on an environment-genotype-bacterium interaction. For example, the bacterium-environment interaction was studied by Pereira et al. (2012), where the genus *Burkholderia* spp. associated with sugarcane presented low bacterial growth, low BNF, and auxin production in a high salinity environment. On the other hand, *Burkholderia tropica* described by Reis et al. (2004), was demonstrated to be a good alternative for sugarcane in other environments. Oliveira et al. (2006) affirmed the necessity to combine bacteria and environmental conditions to achieve the maximum potential.

Studying the association between bacterium and genotype, Fuentes-Ramírez and Caballero-Mellado (2006) demonstrated in their review that bacteria also have an "embracing" interaction with many cultivars or specific cultivars. Schmatz et al. (2012) confirmed the response dependent on the different genotypes. For sugarcane, most distinct groups of bacteria are linked more with the rhizospheric region of sugarcane than other areas. The rhizospheric bacteria intensify root development, influencing the whole plant (Costa et al. 2014; Fuentes-Ramírez and Caballero-Mellado 2006; Oliveira et al. 2006).

For Tejera et al. (2005) working with sugarcane cultivars cultivated in Spain, the *Azospirillum* genus is more closely linked to sugarcane colonization and better associated with it than the *Azotobacter* genus, which did not show an affinity to the sugarcane rhizosphere.

Moutia et al. (2010) observed the interaction between cultivar-irrigation-inoculation of *Azospirillum* strains. These authors obtained different results for cultivars when inoculated under drought stress conditions. For cultivar R570 the inoculation did not differ from the non-inoculated treatment in both environmental conditions, while the cultivar M1167/77 presented a positive response to inoculation in drought stress. Drought tolerance in sugarcane was described by Vargas et al. (2014) using *G. diazotrophicus* species inoculated with SP70–1143 cultivar. The plants under watered conditions had less *G. diazotrophicus* concentration than

unwatered plants, 3 days after water deficit. The mechanisms that play an important role in stress tolerance were evaluated by molecular analysis, mainly in the roots. Ethylene and ABA biosynthesis were greater in non-inoculated roots, indicating tolerance to drought stress on inoculated *G. diazotrophicus* treatments.

For these reasons, the beneficial mechanisms may change depending on the cultivar, environment, and bacteria. However, the two principal mechanisms of action are described below—nitrogen fixing and the production of phytohormones.

1.3.1 Nitrogen Fixing

The measure of nitrogenase activity in sugarcane roots provided some of the earliest evidence of bacterial contribution to the rhizosphere, as related by Döbereiner et al. (1972). Some descriptions attribute about 70% of obtained nitrogen by cultivar for BNF to be *G. diazotrophicus,* one of the main bacteria that contribute to this mechanism (Boddey et al. 1991; Moreira and Siqueira 2006). Urquiaga et al. (1992) suggested through a ^{15}N enrichment method that most genotypes assimilated a large amount of nitrogen through BNF. However, Polidoro et al. (2001) observed that factors like soil fertility and plant nutrition influenced the bacterial contribution to available nitrogen to the plants. With regard to sugarcane development, Pedula et al. (2016) demonstrated recently that dry matter and nutrition increase on application of PGPB in sugarcane with or without nitrogen fertilizer.

According to Garcia et al. (2013), an inoculation of a mixture of five diazotrophic bacteria[1] strains provided an increase in chlorophyll content and plant development similar to nitrogen fertilizers. The chlorophyll content may be related to the effect of nitrogen content increasing when the BNF mechanism is activated. BNF provides organic nitrogen, which has a strong influence on the plant's photosynthesis. This may explain the higher chlorophyll content in plants inoculated with *Azospirillum*, relating to a high photosynthesis tax (Zaied et al. 2003; Donato et al. 2004; Bashan et al. 2006; Wolff and Floss 2008). For Marcos et al. (2016), the modifications in sugarcane physiology caused by PGPB inoculation do not change dry matter.

The BNF in sugarcane occurs by association, and to reach similar results to those obtained in leguminous plants may be impossible. Nevertheless, sugarcane has been even more widely studied in this area, due to promising culture associated with PGPB, either through BNF or some other mechanism (Moreira and Siqueira 2006). On the other hand, according Magnani et al. (2010), it is important to consider that not all bacteria associated with sugarcane can be considered nitrogen fixers. The authors found that only 10% of all isolates in stalks and leaves are linked to nitrogenase activity.

[1]The mix of bacteria: BR11335 (*Herbaspirillum seropedicae*), BR11504 (*Herbaspirillum rubrisubalbicans*), BR11281T (*Gluconacetobacter diazotrophicus*), BR11366T (*Burkholderia tropica*) e BR11145 (*Azospirillum amazonense*) (Garcia et al. 2013).

1.3.2 Growth-Promoting Regulators (Phytohormones)

Since the 1990s, the investigation of PGPB has been intensified by researchers, and this has revealed secondary products produced by bacteria, such as growth regulators, that bestow advantages on plant growth development and productivity. One of the growth regulators is from the auxin group, the major group linked to growth development in bacteria-sugarcane associations (Fuentes-Ramirez et al. 1993; Mirza et al. 2001; Reinhold-Hurek and Hurek 2011).

Auxin mainly alters root growth, and this aspect has been recognized as a marker of beneficial bacterial effects. The rapid establishment of roots, either by elongation of primary root or by increments in lateral roots, is "gainful" to the plants, thereby enabling the plants to absorb more nutrients and water due to the increased contact surface (Silveira 2008).

Fuentes-Ramirez et al. (1993) demonstrated a wide spectrum of the presence of *G. diazotrophicus* in sugarcane cultivars inside tissues and producing auxins (IAA), and investigated the metabolic effects on promoting growth. Mirza et al. (2001) observed auxin production by PGPB, which promoted micropropagation in sugarcane.

Beyond auxins, gibberellin production (GA1 e GA3) was found by Bastián et al. (1998) in controlled assays with *G. diazotrophicus* and *Herbaspirillum seropedicae*. Leite et al. (2014) detected PGPB salinity tolerance in soils, producing auxins, fixing nitrogen, and solubilizing phosphate, and investigated sugarcane development in soils restricted by high salinity.

The growth regulators produce secondary effects on plants, affecting sugarcane production positively or negatively. There are many related effects that have been noted in the scientific community, among which are: effect on sprouting, stalk and saccharose accumulation, height of plants, and leaf area index (de La Cruz et al. 2012; Schultz et al. 2012; Beneduzi et al. 2013; Oliver 2014; Gírio et al. 2015).

Because of the discovery of these effects, research has been intensified in sugarcane, as the application of microorganisms may be less costly and easier to handle than chemical fertilizers. Pérez and Casas (2005) isolated *Azospirillum* strains from sugarcane roots and introduced those bacteria in micropropagated sugarcane, noting greater development in the inoculated plants.

In controlled conditions, Ferrel-Caballero and Soriano (2014) applied *Rhizobium* on *Saccharum officinarum* obtaining superior results to those obtained with chemical fertilizers (33% N) applied to roots and aerial parts. Similarly, Toledo (2014) observed that micropropagated plants that had been inoculated with *G. diazotrophicus* show earlier maturity than non-inoculated plants.

The mixed strain inoculation helped the initial development in the RB867515 cultivar and, according to Gírio et al. (2015), increased sprout index and dry matter of all plants. Similarly, Chaves (2014) studied the effect of those bacteria alone and together in different cultivars, and found a positive response for some treatments according to the sprout index and macronutrient content; however, in some cases there was a reduction in biomass accumulation. Therefore, it can be speculated that the cultivar environment and cultivar genotype may influence microbiological activity.

In agreement with other obtained results, Pérez et al. (2015) studying other authors, submitted that inoculation with *G. diazotrophicus* and *Kleibisiella* sp. GR9 contributed to sugarcane biomass in an order of 50%, demonstrating the ability of the bacteria to develop beneficial conditions for plants without having to use synthetic products. These results reinforce the importance of developing standardized methods of using commercial inoculants (biofertilizers) in non-leguminous plants (Vessey 2003; Fuentes-Ramírez and Caballero-Mellado 2006).

1.4 Strain Selection of Agricultural Interest, *in vitro* Methods

Desirable characteristics of agronomic and agricultural interest are always the driving force in the selection of bacterial strains for agricultural use. Their technical features are often associated with the desire to increase yield.

It was hypothesized early on that sugarcane could benefit from nitrogen-fixing bacteria (Döbereiner et al. 1972). Ever since, many selection programs for isolation and testing have been established, as reviewed by Baldani et al. (2002). A remarkable milestone for sugarcane cultivation was the isolation of *G. diazotrophicus* (formerly known as *Acetobacter diazotrophicus*) from sugarcane, a potential plant promoter (Boddey et al. 2003). Furthermore, it has been recognized that some endophytic bacteria substantially affect sugarcane physiology but without changing plant growth (Marcos et al. 2016).

Many factors are randomized in the *in vivo* situation, whether they are beneficial or not. Nevertheless, selection always occurs under conditions that are quite different from those found in the field. These attempts are put into practice because they are a part of a process of choosing the most promising microorganisms. The more advantages it has, the better its adaptability for performance and success in the plant. To date, we have seen that these experiments under controlled conditions, e.g., *in vitro* selection, make approximate admeasurements of strain abilities, making it possible to indicate which are the most appropriate strains for undergoing *in vivo* tests.

However, this is not the only way to proceed. In conjunction with *in vitro* tests, some experiments may also indicate bacterial abilities that will certainly promote plant growth. Many are based on experiments that determine the production of some key compounds. In general, this is a stage performed *a posteriori* of the isolation and the *in vitro* tests. Nonetheless, depending on the goals and availability of resources, nothing prevents the order from being changed.

The use of both PGPB and transgenic plants will be the support basis for sustainable agriculture in the present and the future (Lucy et al. 2004; Glick 2012). The potential of the PGPB isolates can be evaluated, as widely reported, in terms of nitrogen fixation, production of plant growth-regulating substances (phytohormones), phosphorus-solubilizing activity, and siderophore production, amongst many other assays. Some strategies and their respective applied protocols are summarized in Tables 1.1 and 1.2.

Table 1.1 Evaluation according to secretion of plant growth-regulating substances

Protocol	Results of microorganisms and cultures	References
Quantitative estimation of indole-3-acetic acid (IAA) production	Detection of PGPR from roots and rhizosphere of sugarcane (Pakistan)	Ashraf et al. (2011)
(IAA) determined by Salkowski colorimetric method	*Identification of genes involved in IAA biosynthesis of Gluconacetobacter diazotrophicus*	Rodrigues et al. (2016b)
IAA colorimetrically by standard procedure (Gordon and Weber 1951)	All Endophytic bacteria isolates from sugarcane (India) were able to produce IAA (4.8–9 μg ml^{-1})	Chauhan et al. (2013)
Estimation of indolic compounds (Glickmann and Dessaux 1995)	Rhizospheric and root endophytic bacteria isolated from sugarcane (Brazil) showed high indolic compound production (N = 39) 51–100 μg ml^{-1} (N = 16) >100 μg ml^{-1}	Beneduzi et al. (2013)
Effects of exogenous abscisic acid (ABA)	Different hormone ratios influenced growth in diverse sugarcane varieties	Huang et al. (2015)
ACC deaminase (Glick 2005)	Deaminase production was mainly detected in species belonging to *Streptomyces* and *Bacillus* (from endophytes associated with sugarcane)	Kruasuwan and Thamchaipenet (2016)

One of the main targets in bacteria selection programs is to take advantage of the inoculation of sugarcane-associated nitrogen-fixing bacteria due to their capacity to reduce nitrogen fertilization and improve sugarcane production (Lin et al. 2012). Some species of bacteria are able to perform the biological fixation of nitrogen because these microorganisms have the enzyme nitrogenase, which is an enzymatic complex that breaks the triple bond of the atmospheric nitrogen (N2) allowing the formation of ammonia (NH^{4+}). To determine in the microorganism the ability to fix atmospheric nitrogen requires more than one method, since none of them can cover all the variables that this process involves, given the enormous richness of bacteria that have this characteristic.

There are tests that detect the activity of the enzyme nitrogenase by the relative reduction of acetylene (ARA) in ethylene (C_2H_4); this technique has great advantages for high sensitivity (nmoles of C_2H_4 per hour by gas chromatography) and speed. This allows the detection of nitrogenase activity in 2–3 *Azotobacter* cells (Hardy et al. 1968). In general, bacteria that reduce acetylene to ethylene also reduce nitrogen to ammonia, but the reverse is not true because it has been known for a long time that some microorganisms like *Methylosinus* oxidize ammonia into nitrate (de Bont and Mulder 1976). Therefore, it is important to conjugate more than one method to estimate the BNF. In this sense, the amplification of the *nif* genes (encoding proteins of the enzyme nitrogenase-1) has been useful, especially *nif*D, *nif*K, and *nif*H, which function as the structural genes of the nitrogenase enzyme (Dean and Jacobson 1992). In studies with sugarcane bacteria, ARA and *nif*H were used together with success to estimate nitrogen-fixing bacteria (Ashraf et al. 2011; Kruasuwan and Thamchaipenet 2016). Investigators usually find more than one

Table 1.2 Methods and protocols used for study of microorganisms with agricultural interest

Parameter evaluated	Results	References
Biological nitrogen fixation		
Nitrogenase activity by acetylene reduction assay (ARA)	Endophytic nitrogen-fixing bacteria were isolated from the leaves, stems, and roots of industrial variety (cv. U-Thong 3; UT3), wild and chewing sugarcane plants grown for 6 weeks in nitrogen (N)-free sand	Muangthong et al. (2015)
nifH gene amplification by PCR (Rösch et al. 2002)	Detection of nitrogenase producers	Kruasuwan and Thamchaipenet (2016)
Partial amplification *nifH* gene	Detection of nitrogenase producers	Ashraf et al. (2011)
Phosphorus solubilization		
Phosphorus-solubilizing activity on agar (Pikovskaya 1948)	Bacteria isolates from the rhizosphere of crop plants	Chung et al. (2005)
Siderophore production		
Chrome-azurol sulphonate assay (CAS) (Schwyn and Neilands 1987)	Field experiment on sugarcane was conducted with five plant growth-promoting bacterial endophytes Pseudomonas spp. and *Bacillus* spp.	Chauhan et al. (2013)
Chrome-azurol sulphonate including additional control (Schwyn and Neilands 1987; Beneduzi et al. 2010)	Identification of 390 siderophore producers	Beneduzi et al. (2013)

function in the same bacteria, as described by Rodrigues et al. (2016a, b), who identified genes of the beneficial nitrogen-fixing endophyte *Gluconacetobacter diazotrophicus* PAL5 in association with indole acetic acid (IAA) production and its effects on sugarcane and other important crops.

PGPB have often been selected because of their potential to secrete plant growth-regulating substances. Auxins, for instance, have effects on sugarcane dedifferentiation and embryogenic-cell initiation (Nadar et al. 1978); they promote *in vitro* sugarcane regeneration (Franklin et al. 2006) and establish lateral and adventitious root systems in grasses (McSteen 2010). Several developmental effects over sugarcane have been reviewed by Moore and Botha (2013).

There are simple tests that can be performed for qualitative and quantitative estimation of IAA production, an auxin that plays a crucial role in plant growth and development. They are easily accomplished colorimetrically with increased specificity to IAA by means of ferric chlorideperchloric acid procedure (Gordon and Weber 1951), or by adaptation of Salkowski's reagent use (Pilet and Chollet 1970; Glickmann and Dessaux 1995),or by confirming best colorimetric results by gas chromatography tandem mass spectrometry (GCMS) analysis (Ullah et al. 2013), which better indicates how to prepare and prepurify samples before colorimetric measurement. Otherwise, microplate experiments can be similarly performed with some scale adaptations (Sarwar and Kremer 1995).

In an investigation over beneficial sugarcane endophytes, the production of IAA was colorimetrically measured, according to Gordon and Weber (1951), by growing the cultures with or without tryptophan (100 μg ml^{-1}) for 48 h at 30 °C, in triplicates. All the isolates of interest were able to produce IAA ranging from 4.8 to 9 μg ml^{-1} (Chauhan et al. 2013). Several endophytes associated with sugarcane roots have also been evaluated by Kruasuwan and Thamchaipenet (2016) for IAA production, but were rather inoculated into glucose-beef extract broth supplemented with 10 mM L-tryptophan and incubated at 28 °C for 7 days in the dark using Salkowski's reagent colorimetric method (Pilet and Chollet 1970). Isolates from rhizospheric soil, roots, and stems of sugarcane from southern Brazil showed indolic production ranging from 0.16 to 160.4 μg ml^{-1} (Beneduzi et al. 2013). All these protocols used the supernatant of broth after growth and centrifugation of isolates.

In a more laborious study, quantification of IAA was performed by chromatography, comparing the retention time of samples to the IAA standard peak, using specific computer software (Ashraf et al. 2011). For this investigation, an high-performance liquid chromatography (HPLC) system with a UV detector and C-18 column was used, using as the mobile phase methanol:acetic acid:water (30:1:70 v/v/v), pumped at a rate of 0.6 ml min^{-1}. Injected samples have been obtained from the culture of bacteria isolated from the rhizosphere of sugarcane, which were extracted from ethyl acetate and re-suspended in ethanol, according to Tien et al. (1979). A more refined experiment for quantification of IAA makes use of ultra-high performance chromatography with tandem mass spectrometry (UPLC/MS/MS) (Khan et al. 2016), where details of the analysis are appropriately described. Briefly, tandem MS uses the mode of multiple reaction monitoring (MRM) to trace the transition of an IAA precursor ion from 175.65 to 129.8 m/z.

It has been proved that bacteria are able to synthesize many other plant growth regulators such as other auxins (besides IAA), gibberellins, cytokinins, and abscisic acid (Karadeniz et al. 2006). By measuring the enzymatic activity of 1-aminocyclo propane-1-carboxylate (ACC), for example, one can indirectly make an estimate of the potential of soil microorganisms to promote plant growth (Glick 2005). According to this reference, the enzyme promotes plant growth by sequestering and cleaving plant-produced ACC, and thereby lowers the level of ethylene in the plant. This allows the plant to be more resistant to a wide variety of environmental stresses.

Gene promoters are those that allow the binding of transcription factors that modulate the expression of a particular gene. There are several types of promoters, but some are directly involved with ABA. ABA is a cis-element involved in abiotic stress response. It is a phytohormone that induces leaf stomata closure and triggers the activation of many stress-related genes under abiotic stress (Lata and Prasad 2011). ABA is ubiquitous in plants, but it is also produced by some bacteria and fungi (Nambara and Marion-Poll 2005). There are many bacteria that synthesize ABA through the mevalonic acid pathway, in inter-relation with plants (Wasilewska et al. 2008). This hormone plays a pivotal role in a variety of developmental processes and adaptive stress responses to environmental stimuli in plants (Fujita et al. 2011).

Some promoters are particularly useful because they only function after being induced by certain stimuli (microorganisms, temperature, chemical compounds,

wounds) (Canhoto 2010). Thus, their manipulation can be controlled. Any foreign gene transferred to a plant can be expressed only when it has been provided with a suitable promoter sequence, many of which are already included in the commercially available vectors (Heldt and Piechulla 2004).

Sugarcane (*S. officinarum*) is worth mentioning as in it the precise sequences of plant promoters must be determined by plant genomics. However, some performances can in part be paralleled with those of maize, rice, sorghum, or wheat. According to Canhoto (2010), promoters that showed good expectation of use in monocotyledons are Ubiquitin-1 for maize and Actin-1 for rice. These promoters are activated by heat shock proteins (HSPs), which are derived from various stress factors to which the plant is subjected, including thermal stress, a factor that has been reported for its important contributions of HSPs in various abiotic stresses (Scharf et al. 2012). According to Fujita et al. (2011), the ABA-responsive element (ABRE; PyACGTG/TC) is a well-studied cis-element involved in ABA-induced gene expressions. Moreover, phytohormone ABA is involved in dehydration responsive element binding (DREBs) (Lata and Prasad 2011). Thus, ABA should be considered for bacterial selection interaction between beneficious bacteria and sugarcane, since many already culturable areas and future culturable areas of sugarcane suffer from drought. The practical and application value of ABA and DREBs in crop improvement, such as stress tolerance engineering, has been reviewed by Lata and Prasad (2011). Some physiological roles of ABA have been reviewed by Finkelstein (2013), who stated that "Although ABA has historically been thought of as a growth inhibitor, young tissues have high ABA levels."

Another role of bacteria is their phosphate-solubilizing activity. Phosphorus is among the essential nutrients applied to sugarcane (Muwamba et al. 2016). To maintain the sustainability of agriculture, it is imperative that the reliance of crops on inorganic phosphorus (P) fertilizers is reduced (George et al. 2009). This kind of bacteria, which most commonly belongs to the genera *Pseudomonas, Bacillus*, and *Rhizobium,* used as inoculants can increase P uptake by the plant and crop yield at the same time. The mechanism for mineral phosphate solubilization, in general, is the production of organic acids and acid phosphatases for the mineralization of organic phosphorous in soil (Rodríguez and Fraga 1999). In a selection program of strains for this characteristic, is very common to use the assay of solubilized insoluble phosphorus on Pikovaskaya's agar (PVK) (Pikovskaya 1948), a fast and simple method that is adequate for large samples with many isolates. Another medium, NBRIP (National Botanical Research Institute's phosphate), was used with good results for bacteria isolation; it is more efficient than PVK in a broth assay (Nautiyal 1999). It is very common to find IAA production and phosphate solubilization capability in the same bacteria, and these have been used as parameters of potential plant growth promotion (Ullah et al. 2013).

Siderophore production by the isolates is commonly qualitatively estimated by the Chrome-azurol S assay in solid medium (Schwyn and Neilands 1987). It is useful to determine its bacterial production because siderophores are a class of organic compounds with low molecular masses and with iron-chelating properties. Taken from Greek, it means "iron carrier," and this is the actual way that it increases iron

bioavailability to plants. It is produced by aerobic and facultative anaerobic bacteria (frequently by PGPB) and some fungi. Although iron is common in soils, it has a low solubility for plants and microorganism utilization.

1.5 Inoculants for Sugarcane

The PGPB inoculants available are of great importance to sustainable agriculture, as they aim to reduce the environmental impact through the use of fewer chemical fertilizers and also through a reduction in production costs. The bacteria may replace fertilizers, maintaining productivity and improving conditions for soil microbiota. In this way, the input costs can be reduced, because there is a certain fragility and dependence of the political external market on fertilizer prices (Hungria 2011).

According to Vessey (2003), inoculants can be called by biofertilizers, because their composition consists of live microorganisms that are beneficial to plant development like the chemical fertilizers but in a different way. The studies in this area are mainly from India and South America (Vessey 2003; Stamford et al. 2006; Okon et al. 2015). Biofertilizer use may reduce chemical fertilizer application, as demonstrated by Kumar and Yadav (2015) in Indian soils. The biofertilizer from that study contained BNF bacteria, phosphate-solubilizing bacteria, and bio-control agents. In South America, the commercial use of biofertilizers in sugarcane is common, therefore most of them are applied to other *Poaceae* cultures, mainly focusing on rhizospheric bacteria. Mostly of those biofertilizers are *Azospirillum* genus-based (Okon et al. 2015).

The inoculants are the vehicle in which beneficial bacteria survive, and when applied to the roots or leaves the bacteria act in symbiosis or association with plant. The inoculant must have the capacity to keep live bacteria at low metabolic activity, otherwise they will still multiply and probably compromise the stability of the inoculant when applied to plants. Thus, most bacteria provide phytohormones from bacterial "mechanisms" to plants, and on the other hand bacteria survive on plant exudates (Vessey 2003; Fuentes-Ramírez and Caballero-Mellado 2006; Moreira and Siqueira 2006).

Plants with the greatest potential to produce photoassimilates, allied carbon sources on the rhizosphere or bacteria action site, probably gain more success in the plant-bacteria association (Fuentes-Ramírez and Caballero-Mellado 2006). Accordingly Reis (2007) defined an inoculant as: "*...utilization of alive microorganisms, capable of promoting the vegetal growth in a direct or indirect way, through different mechanisms, and worldwide being named as biofertilizers...*" A good inoculant maintains the appropriate quality and quantity of bacteria to be available in the appropriate place for symbiosis or association with the plants; however, some factors may influence the viability for maintenance of the inoculant, including temperature. Inoculants based on *Azospirillum* genus present a slight decrease in the live cell quantity over time, and consequently maintain viability when inoculated in the plant. The maintenance of live cells occurs through poly-β-hydroxybutyrate production, which is produced by bacteria generally in a high C/N relation condition to maintain cell reserve. This polysaccharide also provides protection from the

deleterious effects of oxygen over nitrogenase in *Beijerinckia* genus (Barbosa and Alterthum 1992; Reis 2007).

Although viability is of great importance, the inoculant should go through many trials before being released, which involves laboratory and field tests. For laboratory tests, including *in vitro* tests and greenhouse trials, the identification of bacterial strains, microorganism benefit to the plants, and how to multiply the microorganisms are important issues. After that, agronomic efficiency must be tested, accompanied by field tests. Thus, it could result in the recommendation of an inoculant for a determined plant, place, or region (Polidoro et al. 2001; Reis Junior et al. 2000; Silva et al. 2009; Torriente 2010; Xavier 2006).

In soybean cultivation, the first Brazilian experience with inoculation with beneficial bacteria, many studies were carried out in the 1930s and 1950s. This cultivation is marked by the relationship with *Bradyrhizobium,* which participates routinely in soybean breeding. In the 1950s the commercial soybean inoculant utilization began on a large scale, initially developed as a peat inoculant and later in response to the market demand as a liquid way, oil, or polymer (Freire and Vernetti 1999).

With the inoculant success in soybean and other leguminous plants, other bacteria were discovered in association with the *Poaceae* family, *e.g.*, sugarcane, maize, and wheat. The most studied genus was *Azospirillum*, with the ability to develop inoculant with a very good response in cultures like maize and wheat, increasing the radicular system of these cultures. However, the application of the technology may modify plant response, as well as vehicle concentration (Araujo 2008; Reis 2007).

Commercially, inoculants started to make their presence known on the global market in recent years, and are aimed at *Azospirillum* utilization, and usually in association wtih wheat, maize, and rice cultures. In the Brazilian market, one of the largest, public and private partners have arisen to release and produce access to the benefits of the inoculants. Income in the order of one to two billion dollars more a year for maize and wheat may be involved (Okon et al. 2015; Hungria 2011; Parnell et al. 2016). Fuentes-Ramírez and Caballero-Mellado (2006) demonstrated that *Azospirillum* may result in profit in the order of 4–60% from increased productivity in cereals.

In recent years, research into *Azospirillum* has produced new perspectives and discoveries of species that primarily colonize the plant interior (endophytic), resulting in increases in productivity experimental trials. *G. diazotrophicus*, *Burkholderia* spp., *H. seropedicae,* and *H. rubrisubalbicans* have been well researched in sugarcane cultivation. The ecological advantage of these bacteria over naturally occurring conditions is that the inside tissues of sugarcane are protected from high concentrations of oxygen, which inhibit nitrogenase activity and reduce the ability to fix nitrogen (Perin et al. 2007). Other avenues are to mix different species and strains, reflecting more expressive effects when applied to plants (Reis 2007).

The commercial product of PGPB is commonly used for *Poaceae* cultures like wheat and maize, while for sugarcane no commercial product has been registered. Many inoculants have been developed, mainly from University research. Sugarcane presents a strong genotype-PGBG interaction, interfering directly for a better strain-plant relationship. Therefore, many field results are controversial, where in some cases PGPB works with a sugarcane genotype and in other cases it does not work.