Influencia de diferentes factores en el crecimiento de bacterias endófitas de caña de azúcar

Influence of different factors in the growth of endophytic bacteria of sugarcane

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Resumen

En Cuba, entre los esfuerzos por lograr la sostenibilidad en la agricultura se han empleado biopreparados a gran escala, los cuales han tenido un gran impacto económico, ecológico y social. La caña de azúcar constituye uno de los principales cultivos agrícolas para nuestro país y tiene gran importancia desde los puntos de vista económico y ecológico a nivel mundial. En el presente trabajo se demostró el efecto de diferentes fuentes de carbono y nitrógeno en el crecimiento de 5 cepas endófitas de caña de azúcar, 3 de *Gluconacetobacter diazotrophicus*, una de *Bacillus licheniformis* y una de *Enterobacter agglomerans*. De igual forma, se estudió la influencia de jugos provenientes de cinco variedades, así como diferentes concentraciones de las fitohormonas ácido 3 indolacético (AIA) y ácido giberélico (GA) en el crecimiento. Se demostró que la asparagina y el sulfato de amonio como fuentes de nitrógeno adicionadas al medio LGI posibilitan un mayor crecimiento de las bacterias endófitas estudiadas. El medio LGI suplementado con jugo de caña de azúcar favorece significativamente ($p \leq 0,05$) el crecimiento de los microorganismos endófitos y no existe relación directa entre el origen varietal del jugo y de las cepas. Por otra parte las fitohormonas en bajas concentraciones favorecieron el crecimiento, no ocurriendo así cuando se encuentran a elevadas concentraciones en el medio de cultivo. Es necesario estudiar todos los factores que pueden influir en la interacción entre la planta y los endófitos para poder utilizar sus potencialidades como promotores del crecimiento vegetal.

Palabras clave: PGPB, *Saccharum* sp. híbrido, jugo de caña.

Abstract

Among the efforts done in Cuba to the sustainability in the agricultural system, one of them is the use of bioproducts, which have a relevant economic, ecological and social impact. The sugarcane is one of main crops in our country and it has a great importance at world level. In the present work is demonstrated the effect of different carbon and nitrogen sources in the growth of 5 entophytic bacteria (three of *Gluconacetobacter diazotrophicus*, one of *Bacillus licheniformis* and one of *Enterobacter agglomerans*) were demonstrated. As the same form are studied the influence of juices from five varieties, as well as, different concentrations of fitohormones indole3acetic acid and giberelic acid on the growth. Was demonstrated that asparagine and ammonium sulfate as nitrogen sources added to LGI medium enhance the growth a major growth of the studied endophytic bacteria. The LGI medium supplied with juices of sugarcane enhance the growth of microorganisms ($p \leq 0,05$) and don't exist any relationships among the origin of the juice and the strains. On the other hand, the fitohormones at low concentrations don´t affect the growth but at high levels of these hormones inhibit the growth. It's necessary to study the factors that have influence on the interaction between the plant and endophytes to use their potentialities as plant growth promoters.

Key words: PGPB, *Saccharum* sp. hybrid, juice of cane.

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Introduction

Sugarcane (*Saccharum* sp. hybrid) is a plant with a high sucrose content that currently represents one of the world's most important sources of sugar, which is why it is farmed in more than 50 countries. The interest in maintaining and increasing the productivity of this crop is not only sustained by its use in human food, but also by its use in animal feed (Correa *et al*. 2003; Martín *et al.* 2005). Specifically in Cuba, work is being carried out on the diversification of the sugar industry, which gives this crop high added value.

Agricultural biotechnology and the use of natural processes play an important role in the implementation of sustainable agriculture. With this objective, plant growth-promoting bacteria (PGPB) are studied, including the bacteria that live in the rhizosphere, as well as rhizobia and endophytic bacteria, which can all benefit the crops (Acemad and Kribet, 2014).

Velázquez *et al*. (2008) described the genetic diversity of the endophytic bacterial community of apoplastic sap from the medullary parenchyma of sugarcane stems cultivated in Cuban soil. The *Gluconacetobacter*, *Bacillus* and *Enterobacter* genera were isolated from this crop, and the first two were found to be the most abundant in the bacterial community.

Studying the effect of different sources of carbon and nitrogen related to the natural environment, as well as other components of it, will allow to explain the endophytic microorganisms' behavior in their natural habitat. This will allow more in-depth knowledge of the plant-bacterium interaction and support for the bacterium's use in agricultural biotechnology. Additionally, this study lays the foundations for the formulation of efficient culture media based on natural sources for the endophytes' growth.

This work aims to demonstrate the effect of different sources of carbon and nitrogen on the growth of five endophytic strains found in sugarcane. Additionally, the influence of juices from the different varieties on the growth of these bacteria was studied, as well as different concentrations of the plant hormones indole-3-acetic acid (IAA) and gibberellic acid (GA).

Materials and Methods

Strains Used

The following bacterial strains were used: *Gluconacetobacter diazotrophicus* PAl5 (ATCC 49037), a strain isolated from roots of sugarcane in Brazil (Cavalcante and Dobereiner, 1988); *G. diazotrophicus* 1-05 and 4-02 strains isolated from the stems of the Jaronú 60-5 and Media Luna 318 sugarcane cultivars, respectively (Rojas, 2005); *Bacillus licheniformis* isolated from the stems of the Cuba 323-68 sugarcane variety (Rojas *et al*. 2001) and *Enterobacter agglomerans* isolated from the stems of the Media Luna 318 sugarcane variety (Rojas *et al*. 2001).

Influence of the Source of Carbon and Nitrogen on Growth

The strains were cultivated in liquid LGI medium (in g/l, 0.2 K_2HPO_4; 0.6 KH_2PO_4; 0.2 $MgSO_4$ $7H_2O$; 0.02 $CACl_2$ $2H_2O$; 0.02 $NaMoO_4$ $2H_2O$; 0.01 $FeCl_3$ $6H_2O$; 5 ml of bromothymol blue (0.05% solution in 0.2 N KOH)) with 10 g/l of sucrose as a source of carbon (Cavalcante and Dobereiner, 1988) for 48 hours at 30 °C and 150 rpm. The inoculums were adjusted to 10^8 UFC/ml, according to the McFarland standard (CLSI, 2005) with 0.8% saline solution (0.8 g NaCl in 100 ml of H_2O). A semisolid LGI medium was prepared (2 g/l of agar) with the following sources of carbon in a final concentration of 1%: xylose, sucrose, inositol, glucose, fructose and sugarcane juice.

Culture media were prepared with 1% sucrose as a source of carbon and the following sources of nitrogen in a final concentration of 5 mM: ammonium sulfate, potassium nitrate, asparagine, tryptophan, threonine, arginine and a control without the addition of nitrogen.

In every 5 ml of medium, 100 µl of inoculum were planted and incubated for 72 hours at 30 °C. The absorbency was measured at 600 nm. Everything was determined three times.

Influence of Juices of Different Sugarcane Varieties on Growth

An inoculum similar to the one described in the summary above was prepared, and it was also adjusted to 10^8 UFC/ml. From this preparation, 100 µl were taken to inoculate tubes with cultures that

contained 5 ml of liquid LGI medium and sugarcane juice from different sugarcane cultivars for a final concentration of 20 and 30%, as well as a control with commercial sugar as a source of carbon. The juice of five different sugarcane cultivars was used. The samples were incubated at 150 rpm, and 30 °C, and the absorbency was measured at 600 nm after 24, 48 and 72 hours. Everything was determined three times.

Influence of Indole-3-acetic Acid and Gibberellic Acid on Growth

A similar procedure was used to the one described in the summary above, using the same SYP (in g/l, 10 sucrose, 3 yeast extract, 1 K_2HPO_4, 3 KH_2PO_4, pH = 6.2) (Caballero-Mellado and Martínez-Romero, 1994) liquid medium with different final concentrations of indole-3-acetic acid (IAA) and gibberellic acid (GA) of 0.025, 0.25 and 2.5 mg/l, and the medium without hormones as a control. The samples were incubated at 150 rpm and 30 °C, and the absorbency was measured at 600 nm after 24, 48 and 72 hours. Everything was determined three times.

Biometric Analysis

The results of the described physiological studies were analyzed using the Tonystat (9) statistical package. Parametric and non-parametric tests were conducted: analysis of variance, Kruskal-Wallis test, Duncan's new multiple range test and non-parametric multiple comparison test.

Results and Discussion

Influence of the Source of Carbon and Nitrogen on Growth

In the studied strains, significant differences can be observed in growth between the media with different sources of nitrogen (Table 1). The media with ammonium sulfate and asparagine are highlighted as sources of nitrogen for the strains of *G. diazotrophicus*. For *B. licheniformis*, the media with ammonium sulfate, potassium nitrate, asparagine and arginine show the highest growth values. The last two sources of nitrogen are repeated for *E. agglomerans*.

Stephan *et al*. (1991) proved the action of several amino acids on the nitrogenase activity of *G. diazotrophicus*. Rodríguez *et al*. (2005) demonstrated that with threonine, tryptophan, arginine and asparagine as sources of nitrogen, there is evidence of partial inhibition of this activity. This is perhaps a result of similar deactivation mechanisms of the biological fixing of nitrogen, which are possibly not so efficient, which is consistent with the results obtained in growth inhibition in this study.

This bacterium has shown its promoting effect on the growth of different crops of economic importance such as *Colocasia esculenta*, cassava and papaya. Field studies have demonstrated its effect on increasing the yields and quality of the fruits or tubers (Dibut *et al*. 2009).

After 24 hours of incubation in media with different sources of carbon, there are no significant differences in the growth of the strains of *G. diazotrophicus* (Table 2). However, for *B. licheniformis* and *E. agglomerans*, differences in growth are observed from 24 hours. This was to be expected, because these bacteria had a higher growth rate than *G. diazotrophicus*.

The results obtained with the strains of *G. diazotrophicus* are consistent with those presented by Ureta *et al*. (1995). These authors observed that there are differences between strains of the same species in terms of the use of sources of carbon. In this research, differences in growth were observed in the media with xylose, fructose, inositol, glucose and pectin. When identifying 46 isolated of this species from several cultivars of sugarcane, Rojas *et al*. (2012) demonstrated that there are strains that do not grow in the media with glycerol, mannitol, xylose and inositol as sources of carbon, which is consistent with our results. This work demonstrates that there are different behaviors in the growth for the studied strains after being analyzed quantitatively, even when the strains grow in the analyzed media. This is important for the future use of these bacteria in agricultural biotechnology. As demonstrated by Dibut *et al*. (2011), the best results are obtained by sprinkling the leaves and the soil of the *G. diazotrophicus* crop. Therefore, the use of a wide range of sources of carbon could sustain the bacterium's survival.

Through poteomic studies, Lery *et al*. (2008) identified numerous proteins involved in the metabolism of amino acids, carbohydrates and others in the Pal5 strain, which supports the use of the compounds

Table 1. Growth of strains of sugarcane endophytes in LGI medium with different sources of nitrogen measured in cultures of 5 ml through absorbency at 600 nm.

Sources of Nitrogen	Strains									
	Gluconacetobacter diazotrophicus						*Bacillus licheniformis*		*Enterobacter agglomerans*	
	Strain Pal-5		Strain 1-05		Strain 4-02					
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
$(NH_4)_2SO_4$	0.093a	0.623b	0.038a	0.325a	0.048ab	0.308ab	0.080cd	0.393b	0.223c	0.355d
KNO_3	0.000b	0.000e	0.000a	0.000d	0.000b	0.000c	0.027e	0.536a	0.079d	0.439c
Asparagine	0.084a	0.730a	0.009a	0.288ab	0.053ab	0.347a	0.320a	0.399b	0.530a	0.741b
Tryptophan	0.074a	0.469c	0.013a	0.216bc	0.071a	0.257b	0.005c	0.89c	0.084d	0.107e
Threonine	0.000b	0.061e	0.032a	0.000d	0.003b	0.018c	0.020e	0.068c	0.030e	0.060ef
Arginine	0.103a	0.370d	0.027a	0.173c	0.032a	0.244b	0.265b	0.419b	0.346b	0.910a
LGI	0.000b	0.000e	0.000a	0.000d	0.000b	0.000c	0.038de	0.029c	0.001e	0.000f

Different letters indicate significant differences ($p < 0.05$) using Duncan's new multiple range test. Mean of three repetitions.

Table 2. Growth of sugarcane endophytes in LGI medium with different sources of carbon measured in 5 ml cultures through absorbency at 600 nm.

Sources of Carbon	Strains									
	Gluconacetobacter diazotrophicus						*Bacillus licheniformis*		*Enterobacter agglomerans*	
	Strain PAl5		Strain 1-05		Strain 4-02					
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
Xylose	0.008a	0.019c	0.010a	0.004b	0.005a	0.002b	0.018b	0.200bc	0.144cd	0.268bc
Sucrose	0.015a	0.140bc	0.021a	0.032b	0.016a	0.053ab	0.052b	0.177cb	0.272a	0.376ab
Sugarcane juice	0.010a	0.392a	0.006a	0.241a	0.008a	0.244a	0.175a	0.322ab	0.306a	0.516a
Fructose	0.027a	0.210b	0.018a	0.100b	0.029a	0.074a	0.014b	0.104d	0.133d	0.155c
Inositol	0.000a	0.061c	0.000a	0.005b	0.005a	0.031b	0.015b	0.267ab	0.285a	0.417a
Glucose	0.000a	0.198b	0.000a	0.000b	0.002a	0.000b	0.006b	0.165b	0.199bc	0.276bc
Pectin	0.006a	0.096bc	0.006a	0.079b	0.038a	0.151a	0.043b	0.170b	0.100de	0.208bc
Cellulose	0.016a	0.021c	0.015a	0.027b	0.057a	0.071ab	0.031b	0.034c	0.020e	0.026d
LGI	0.014a	0.055c	0.019a	0.045b	0.001a	0.016b	0.009b	0.348a	0.250ab	0.438a

Mean of three repetitions. Different letters indicate significant differences ($p < 0.05$) using Duncan's new multiple range test.

analyzed in this study. Even Luna and Boiardi (2007) demonstrated that the use of glucose depends on the medium's pH.

Using RT-qPCR, Galisa *et al*. (2012) demonstrated the differentiated expression of genes of maintenance and others related to biosynthesis and catabolism in LGI medium supplemented with different sources of carbon. This corroborates the differences in growth found in this research.

The difference between the medium that has sugarcane juice as a source of carbon and the other sources of carbon used is observed after 48 hours of growth for *G. diazotrophicus* and from 24 hours for *B. licheniformis* and *E. agglomerans*. The elevated growth in LGI medium with sugarcane juice could be due to the presence of vitamins, proteins and other compounds that facilitate the growth of the microorganisms. Taking into account the results obtained in the LGI medium with sugarcane juice, and that there are differences in the composition of the juice of the sugarcane cultivars, it was

decided to test whether the juice's variety of origin had an influence on the growth of this species. This is due to the importance this could have when formulating a culture medium for the biotechnological application of these bacteria in agriculture.

Influence of Juices of Different Sugarcane Cultivars on Growth

After 24 and 48 hours of culture in the LGI medium supplemented with juice of different varieties of sugarcane at concentrations of 20 and 30%, there are no differences in the growth of the analyzed strains.

After 72 hours, in the case of the 4-02 strain of *G. diazotrophicus,* growth is still significantly higher in the medium without juice using commercial sugar at 20 and 30% (Figure 1). This could suggest that the compounds present in sugarcane juice at certain concentrations stimulate microbial growth, but that from a determined concentration, toxic substances may be produced that inhibit the growth of some strains.

After 72 hours of growth in all the media with juice, *Enterobacter agglomerans* and *B. subtilis* exceeded the LGI medium with commercial sugar in growth. In this case, it could be considered that sugarcane juice has a positive influence on the growth of the bacteria, because the juice contains glucose and fructose (0.2-0.6%), organic nitrogen (0.02-0.04%) and vitamins (Reis *et al*. 1994). Furthermore, the poor growth of these microorganisms in the medium with commercial sugar compared to the growth in sugarcane juice could indicate the presence of industrial waste from the sugarcane industry that harms the bacteria's growth.

The difference in growth is notable in the media with juice from the cultivars C1051-73 and Ja605 at 30% for the strain of *E. agglomerans*, which have the indicator of total soluble solids (Brix) and the pol percentage (pol: mainly formed of sucrose) in common (Naranjo, S.).

The presence of large amounts of ions such as K^+ and organic acids (Wealbun *et al*. 1990) among the soluble solids could stimulate the growth of *E. agglomerans*, but it is more reasonable to believe that the pol percentage has a bigger influence. This essentially takes into account the percentage of sucrose, but carbohydrates of medium molecular weight could also be present, which are optically active substances, and it has been demonstrated that they divert the plane of polarized light and could influence the measurements of sucrose by polarimetry (De Armas *et al*. 1999). In turn, these carbohydrates could be used by the microorganism, because they are polymers of fructose and galactitol (Legaz *et al*. 1992). It is worth clarifying that no literature has been found on researches that compare the growth of bacteria in different sugarcane juices, and that these studies could lay the foundations for the formulation of culture media with cheaper sources for the agricultural biotechnological application of plant growth-promoting bacteria.

Influence of Indole-3-acetic Acid and Gibberellic Acid on Growth

There are very few studies that analyze endophyte-plant interrelationship with respect to the plant hormones' influence on the bacteria's physiology.

For E. agglomerans, it is observed that concentrations of 0.025 and 0.25 mg/l of both plant hormones stimulate growth compared to the SYP medium without hormones after 72 hours (Figure 2). Therefore, it can be inferred that induction of growth occurs at these concentrations of IAA and GA, as it occurs with Azospirillum brasilense in the presence of IAA. This is explained by stimulated regulation of the genes that codify for the indole-3-pyruvate decarboxylase enzyme, which is involved in the pathway of IAA synthesis by the microorganism (Vande Broek et al. 1999).

In turn, the IAA in the plant stimulates protein synthesis because it stabilizes the messenger RNA, which increases the rate of transcription, and the GA_3 empowers the presence of polyribosomes and increases the number of ribosomes per cell (De Armas et al. 1988), which may exercise a similar action over the microbial cell.

Conclusions

It may be posed that the use of asparagine and ammonium sulfate as sources of nitrogen added to the LGI medium facilitates greater growth of the endophytic bacteria in sugarcane.

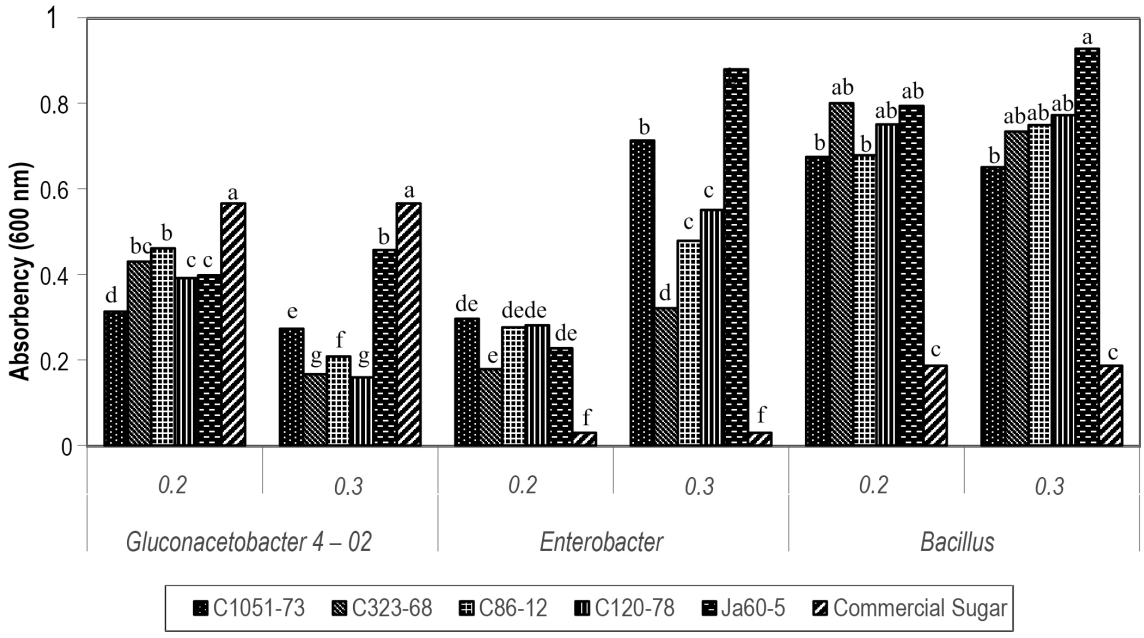

Figure 1. Growth of endophytic strains of sugarcane in juices of different cultivars (C1051-73, C323-68, C86-12 and Ja605) added at 20 and 30% to the LGI medium after 72 hours of culture, shaken at 150 rpm and 30 °C. Different letters indicate significant differences within the same bacterial strain using Duncan's new multiple range test ($p < 0.05$).

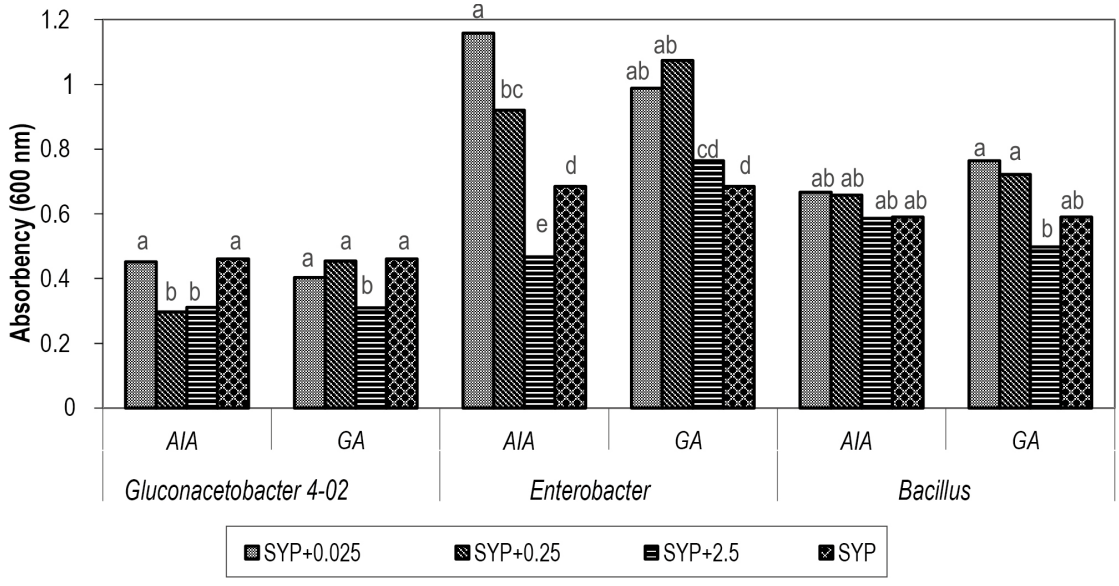

Figure 2. Influence of indole-3-acetic acid (IAA) and gibberellic acid (GA) on the growth of sugarcane endophytes after 72 hours of culture in SYP medium supplemented with different concentrations of hormones (mg/l). Different letters indicate significant differences within the same bacterial strain using Duncan's new multiple range test ($p < 0.05$).

The LGI medium supplemented with sugarcane juice significantly facilitates the growth of endophytic microorganisms and there is no direct relationship between the juice's variety of origin and the bacteria's growth. Therefore, it could be used in a culture medium for the biotechnological production of the endophytes.

The study of the ecology on the interaction between the endophytes and the plants is of great importance. More specifically, the study of the relations of the plant's components, whether the juice or the

plant hormones, could be extremely interesting and open doors to analyze the *in vivo* plant-endophyte interaction in future work.

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