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ANOTHER NITROGEN-FIXING MICROORGANISM IN SUGARCANE STALKS: *Bacillus brevis*?

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ABSTRACT. In order to identify a white colony-growing microorganism in an LGI medium, coming from sugarcane stalk apoplastic sap, some experiments were carried out in which ML-318 variety was used. Then, apoplastic sap was extracted and the microorganism of interest was isolated as well as some morphological, cultural and biochemical tests were carried out, which revealed 65 % probability that the microorganism of interest belongs to *Bacillus brevis*. Nitrogenase activity was measured by acetylene to ethylene reduction. Results also indicate interaction between *Acetobacter diazotrophicus* and the white colony-growing microorganism identified as *Bacillus brevis.*

Key words: sugarcane, *Bacillus brevis*, microorganisms, biological N fixation

RESUMEN. Con el objetivo de identificar un microorganismo formador de colonias blancas en medio de cultivo LGI, proveniente de savia apoplástica del tallo de la caña de azúcar, se realizaron diferentes experimentos en los que se utilizó la variedad ML-318. De la misma se extrajo savia apoplástica, de la cual se aisló el microorganismo de interés, al que se le realizaron pruebas morfológicas, culturales y bioquímicas, las que revelaron un 65 % de probabilidad de que el microorganismo de interés se corresponde con *Bacillus brevis.* Se detectó actividad nitrogenasa por la reducción de acetileno a etileno. Además, los resultados indican interacción entre *Acetobacter diazotrophicus* y el microorganismo formador de colonias blancas identificado como *Bacillus brevis.*

Palabras clave: caña de azúcar, *Bacillus brevis*, microorganismos, fijación biológica del nitrógeno

INTRODUCTION

Sugarcane crop has developed in Cuba for many centuries and as any other crop, it requires abundant nitrogen fertilizer which is very expensive (1). Nitrogen is the macroelement used in greater proportions by plants (2, 3).

In the tropics many graminae, especially sugarcane, have good yields, even in soils with low amounts of ammonium or nitrate which gives us an idea of the possibility of biological fixation of nitrogen (4).

A great number of endophytic microorganisms have been isolated from sugarcane, also diazotrophic endophytes (5), providing nitrogen to the crop. Among the genera are *Pseudomonas, Bacillus, Klebsiella, Vibrio, Acetobacter, Rhizobium, Azospirillum, Herbaspirillum*, and others (6, 7). *Acetobacter diazotrophicus* is an important endophyte in sugarcane (7, 8) and coffee (9) crops.

In studies previously carried out at the Laboratory of Plant Physiology of the University of Havana (10), white colonies were found in apoplastic sap culture on an LGI medium. The colonies were the same shape as *Acetobacter diazotrophicus* colonies. It was reported that white colonies accompany *Acetobacter diazotrophicus* colonies in successive subcultures, making the purification of both difficult, even though when LGI medium, specific for *Acetobacter diazotrophicus* (11) was used.

The aim of this work was to study the relationship between sugarcane and endophytic white colony-growing microorganisms on an LGI medium, and to determine if it is able to carry out nitrogen fixation as well as its relation with *Acetobacter diazotrophicus*.

MATERIALS AND METHODS

Sugarcane variety ML-318 was used for the experiments. Adult plants with millable and variable-aged stalks were sampled. Then they were harvested, and the internodes were separated from the top of the stalk.

Apoplastic sap was extracted following a methodology (12) and LGI medium free from nitrogen (13) with 10 mL sugarcane juice/L and pH 5,5 adjusted with acetic acid was used.

The microorganisms of interest were purified and characterized by morphologic and cultural methods (14). Different biochemical tests were done; catalase and oxidase activities, starch (15), casein and gelatin were hydrolyzed.

Nitrogenase activity was detected as hydrogen production in the culture and also for the reduction of acetylene to ethylene, by gas chromatography, using a Taguchi detector, with air as gas-like carrier at 55 kPa, with columns filled by Porapak.

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Counts of viable colonies from *Acetobacter diazotrophicus* and from white colony-growing microorganisms were made every nine days, then four subcultures were carried out. The culture medium used was LGI.

RESULTS AND DISCUSSION

The method for apoplastic sap extraction has already been described in detail (12). The colonies of *Acetobacter diazotrophicus* and the other microorganism, which grew in white colonies, were isolated.

The white colonies appeared with the yellowish center in LGI approximately at the seventh day after inoculation, and 15 days after they were completely yellow. In an LGI medium they reached a relatively small size (X =1.2 mm ± 0.2); each colony was also surrounded by fine filaments. An important characteristic for its morphologic identification in dishes with LGI was its penetrative power into the agar.

From the outline (10) and the authors' observations, the white colony-growing microorganism have conditions to be located in the Bacterium Kingdom.

The unknown white colony-growing microorganism of our interest has bacillus-shape with 6.5 ± 4.5 µm long and 0.8 ± 0.3 µm wide, most of which is Gram-positive. Although isolated bacilli can be found, they grow making long chains (Figure 1).

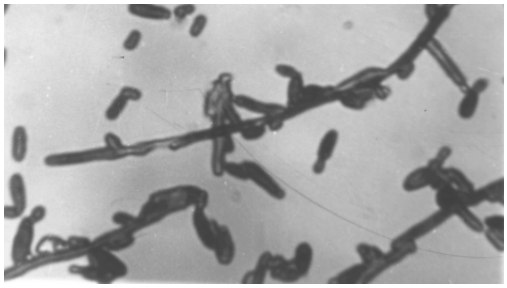


Figure 1. The bacillar form of the microorganism and long chain formation

With the hanging drop method, it can be seen that the bacterium is motile; therefore, it flagellates. In relation to the spores, using (1000X) light microscope the sporangium has been observed. It shows elliptic spores with central position or terminal in some cases.

The colony shape and border are rhizoid, with a mate color and the diameter is about 1.2 mm. It is difficult to emulsify in water.

At the time our research was being accomplished, comparing it with literature results, emerged a series of evidence that probably the white colony-growing microorganism obtained from sugarcane apoplastic sap would be *Bacillus brevis*. Distinguishing features from *Bacillus brevis* on Bergey's book and the microorganism studied are shown in Table I. It can be seen that there are many coincident features in both and a high probability that the microorganism studied will precisely be *Bacillus brevis*.

Table I. Comparison of a group of characteristics from *Bacillus brevis* (16) and the microorganism object of study

Tests	Microorganism studied	*Bacillus brevis*
Catalase	+	+
Oxidase	-	
Hydrolysis of starch	+	+
Hydrolysis of gelatin	+	+
Hydrolysis of casein	+	+
Formation of indole	-	-
Utilization of citrate	-	d
Voges-Proskauer test	-	-
Hydrolysis of lecithinase	-	-
Oxidation and/or fermentation	+	
Nitrate reduced to nitrite	-	d
Ammonia formation	-	
Growth at 45°C	-	+
Growth at 2% in NaCl	+	+
Growth at 5, 7, 10 % in NaCl	-	-
Production of acid:		
Glucose	+	d
Arabinosa	-	-
Xilosa	-	-
Manitol	+	d
Oxygen requirement	Aerobe	Aerobe

d: 11-89 % of the culture are positive. The blank spaces must be to the fact that results of those tests have not been specifically reported for this *Bacillus*

So far, identification was based on Bergey's Manual of Determinative Bacteriology. Furthermore, we worked with GIDEON Program, Gold Crop Software. Israel, which reports only two possible kinds of microorganisms, both located within *Bacillus* genus: *Bacillus stearothermophilus* and *Bacillus brevis*. However, the program reports a greater probability that this microorganism will be *Bacillus brevis*, with a probability value about 65 % and 37 %, respectively.

So there is great probability that this work will be the first to report *Bacillus brevis* as an endophytic organism of sugarcane. The presence of microorganisms from *Bacillus* genus in sugarcane had been previously reported (7), but reports on the species *B . brevis* in this gramineous had not yet been found.

Upon accomplishing the determination of nitrogenase activity through testing hydrogen released by the crop, positive values of the slopes were not obtained in three successive subcultures of *Bacillus brevis* (Table II), as it was obtained by *Acetobacter diazotrophicus.* It indicates the nonexistence of hydrogen or sufficient quantities to be detected in the time evaluated. Another cause could be that even the hydrogen produced would be reused for the action of a hydrogenase enzyme. N-fixing organisms can have at least three enzymes that participate in hydrogen metabolism (17).

That means:

- There can be a nitrogenase producing hydrogen along with the fixing of N_2 at NH_3, at a speed of at least 25 % of the electron flow through nitrogenase.
- A hydrogenase at membrane level oxidizes the hydrogen produced by nitrogenase.
- A soluble hydrogenase of reversible physiological function is able to reabsorbe the hydrogen produced.

Table II. Slope values (nL of H_2/tube per minute) in three successive subcultures of *Bacillus brevis*. The columns are different crop replies from the purified microorganism

Subcultures		
First	Second	Third
-96.00	-1.2	3.6
-15.60	-1.2	-1.5
-7.83	-2.4	-0.6
-8.70	-16.2	-3.3
-5.70	-9.6	-12.6
-7.50	-9.0	-3.0
-3.00	-4.8	-7.2
9.90	-56.1	13.2
14.10	-4.2	-0.6
-11.10	-9.3	1.5

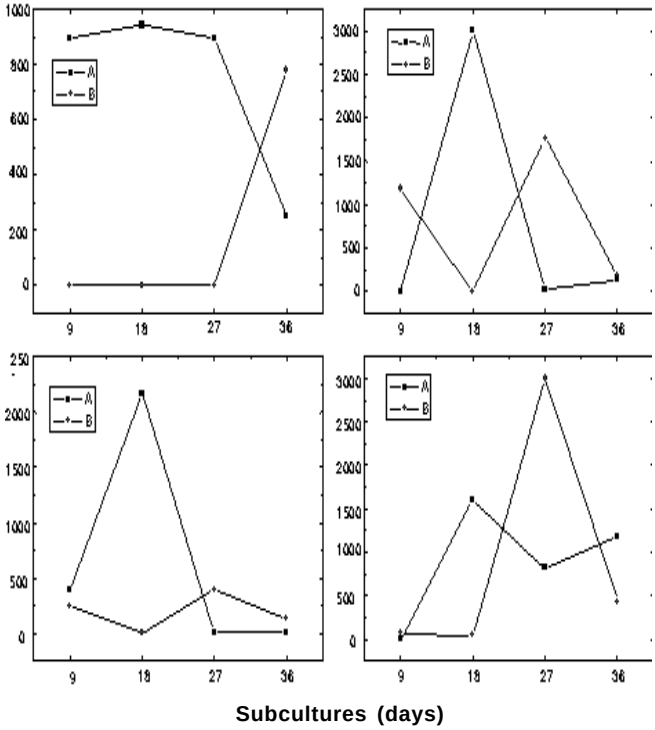
To detect if our strains, that is to say, the strains object of study, actually fix nitrogen, nitrogenase enzyme activity was analyzed according to acetylene to ethylene reduction, since this enzyme is also capable of reducing this substrate up to ethylene production. Results are shown in Table III.

Table III. Reducing activity of the acetylene (nmol ethylene produced/hour per mL culture medium). The columns refer to different inoculated tubes (0.1 mL) with the microorganism object of study (six cultivation days)

Sample	ARA
1	20
2	91
3	16
4	12
5	43
6	113
7	49
8	96
9	25
10	12
11	22
Mean ± SD	45±35

To compare the nitrogen-fixing results of this microorganism, the reported results (7) of reducing activity of the acetylene (ARA) by *Acetobacter diazotrophicus* were used. In the LGI medium with 10 % sucrose, the PAL 3 line had an ARA of 88.7 nmol ethylene/strain per hour, while the PAL 5 line had an ARA of 114.3 nmol ethylene/strain per hour. Even though we have tried to express our results in a very similar unit to the one reported (7), units are not exactly equal. To determine ARA, vials with 5 mL of inoculated media were used (11). If the results of those researches are divided by 5 in order to equalize units the values would be 17.7 and 22.9 nmol ethylene/mL medium per hour respectively. Even though the comparison is difficult, crop results of the endophytic microorganism from sugarcane stalk have the same values of nitrogen fixation to the one reported for *Acetobacter diazotrophicus* in the article above mentioned.

The viable cell counts of both microorganisms in the LGI medium were achieved, and it could be appreciated that there exists an inverse relationship (Figure 2) between them. Figure 2 shows the number of viable cells that grew of each dish, independently of the sample or the subculture. The relationship is clearly negative though it does not continue a linear function.



A: *Acetobacter diazotrophicus*, B: *Bacillus brevis*

Figure 2. Viable cell count of *Acetobacter diazotrophicus* and *Bacillus brevis* in the LGI medium

This could be due to the fact that there are yet unknown aspects of the physiology of the white colony growing microorganism; for example, its optimum pH of growth. It is known that *Acetobacter diazotrophicus* acidifies the medium during vital activity. A lack of coincidence in the values of optimum pH of both growths could have an effect in the sense found.

CONCLUSIONS

- In the apoplastic sap of sugarcane stalk, variety ML-318, white colony-growing microorganism is found in the LGI cultural medium, with a high probability of being *Bacillus brevis.*
- The microorganism identified as *Bacillus brevis* fixes nitrogen from air, but hydrogen production is not detected during its growth, which could be indicative of the presence of an absorbing hydrogenase.
- In sugarcane apoplastic sap culture, an inverse relationship between colony-growing units of *Acetobacter diazotrophicus* and *Bacillus brevis* is observed, which seems to indicate a microorganism interaction.

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