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ISOLATION AND CHARACTERIZATION OF *Gluconacetobacter diazotrophicus* STRAINS

Aislamiento y caracterización de cepas de *Gluconacetobacter diazotrophicus*

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ABSTRACT. *Gluconacetobacter diazotrophicus* is an endophyte microorganism with direct and indirect mechanisms for vegetable growth promotion among its characteristics. Despite its great perspective to constitute the active principle of a bioproduct for agricultural purposes, so far there is not any product derived from this bacterial species in Cuba. The strain isolation and its characterization are important steps to obtain a biopreparation, since it enables the initial selection of strains with adequate characteristics for vegetable growth stimulation. In this research, 85 endophyte isolates were purified from different organs of 24 plant species. Four of them were identified as *Gluconacetobacter diazotrophicus,* when comparing its characteristics with those from pattern strains of the bacterial species. Microorganisms were selected from mango (*Mangifera indica* L.) and guava (*Psidium guajava* L.) fruits as well as from yucca (*Manihot esculenta* Crantz.) and beet (*Beta vulgaris* L.) stems. The presence of this microorganism was relevant in the first two crops for Cuba whereas the isolation in guava at the international level. The four strains had differences regarding their capacity to solubilize phosphorus, to produce indol acetic acid and its antagonistic activity against *Fusarium moniliforme* and *Fusarium incarnatum*. As strains were grouped according to their characteristics, mango and beet microorganisms were different, which are considered promising to study the effect of its interaction with other crops under *in vivo* conditions.

Key words: endophytes, stimulation, inoculants

RESUMEN. *Gluconacetobacter diazotrophicus* es un microorganismo endófito que presenta, dentro de sus características, mecanismos directos e indirectos de estimulación del crecimiento vegetal. A pesar de sus grandes perspectivas para constituir el principio activo de un bioproducto de uso agrícola, en Cuba no existe ninguno elaborado a partir de esta especie bacteriana. El aislamiento de cepas del microorganismo y su caracterización constituyen pasos importantes para la obtención de un biopreparado, pues permiten la selección inicial de cepas que tengan características adecuadas para la estimulación del crecimiento. En la presente investigación se purificaron 85 aislados de microorganismos endófitos, a partir de los diferentes órganos de 24 especies vegetales. Cuatro de ellos fueron identificados como *Gluconacetobacter diazotrophicus,* al comparar sus características con las de cepas patrones de la especie bacteriana. Los microorganismos seleccionados provenían de frutos de guayaba (*Psidium guajava* L.) y mango (*Mangifera indica* L.), así como de tallos de yuca (*Manihot esculenta* Crantz.) y remolacha (*Beta vulgaris* L.). Se destacó la presencia del microorganismo en los dos primeros cultivos para Cuba y el aislamiento en la guayaba a nivel internacional. Las cuatro cepas mostraron diferencias en su capacidad de solubilizar fósforo, producir ácido indol acético y en su actividad antagonista frente a *Fusarium moniliforme* y *Fusarium incarnatum*. Al agruparse las cepas por sus características, se diferenciaron los microorganismos provenientes de mango y remolacha, los que se consideran promisorios para realizar estudios en condiciones *in vivo* del efecto de su interacción con otros cultivos.

Palabras clave: endófitos, estimulación, inoculantes

INTRODUCTION

Gluconacetobacter diazotrophicus is a phylum Proteobacteria belonging to endophytic bacteria, Section Alfa, Rhodospirillales order and *Acetobacteriaceae* family. It was first isolated in 1988 (1), associated with the cultivation of sugarcane

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(*Saccharum officinarum* L.). It has great attractions for the development of stimulators bioproducts plant growth because of its ability to fix atmospheric nitrogen, producing indole acetic acid (IAA), solubilize mineral nutrients such as phosphorus and zinc and present antagonistic activity against plant pathogenic organisms (2) .

The positive results of inoculation in sugar cane (*Saccharum officinarum* L.) (3), sorghum (*Sorghum bicolor* L. Moench.) (4), corn (*Zea mays* L.) (5), taro (*Xanthosoma* spp.), sweet potato (*Ipomoea batata* L.) and cassava (*Manihot esculenta* Crantz.) (6) strengthen the prospects of use successfully this bacterium in developing inoculants. However, in Cuba there is not yet a commercial product, produced from this organism.

The isolation of strains of *G. diazotrophicus* associated with plant species grown in the country and its characterization, objectives of this research will allow for a selection of microorganisms adapted to the soil and climatic conditions of Cuba, having suitable characteristics for growth stimulation the plants. In the future, these strains could constitute the active ingredient of a byproduct, which causes positive when applied to crops of agricultural interest effects.

MATERIALS AND METHODS

Isolation of microorganisms: isolation of endophytic microorganisms was made from macerated, using a sterile mortar of a gram of different organs (root, stem, leaves, flowers and fruits) of 24 plant species (Table I).

The extracted sap was added at 0,5 mL in 10 mL vials with 5 mL of semisolid LGI medium (6). During the macerate a milliliter of distilled water was added to promote the process. Purification of microorganisms was carried out in the LGI solid medium (6), poured into Petri dishes of 90 mm, from an initial sample of film growth in semisolid variant, planted by dissemination. Both flasks with semisolid medium, such as Petri dishes were incubated for five days at a temperature of 28 ± 2 °C.

Selection of strains of *G. diazotrophicus*: the purified microorganisms were subjected to a process of selection by elimination, from the execution of four blocks of test, including within them the morphological and physiological measurements (1):
♦♦ Block I. Pigmentation in the LGI and Potato Agar media.
♦♦ Block II. Gram stain and presence of enzymes catalase (CAT) and cytochrome oxidase (x)
♦♦ Block III. Citrate was used as carbon source (cit), protein hydrolysis (starch (alm)) and gelatin (gel), indole production from tryptophan (tpr) and using mannitol as a carbon source.
♦♦ Block IV. Growth in sucrose (sac 30 %) and glucose (glu 30 %) and degradation of filter paper strips (cellulolytic activity).

The results were compared with those obtained in three strains patterns donated by the Center for Genetic Engineering and Biotechnology (CIGB), the PAL5 (ATCC 49037) of cane sugar, pineapple UAPAc 7 (*Annanas comosus* L. Merrill.) And CFNCf 13 isolated from the culture of coffee (*Coffea arabica* L.).

Table I. Cultures used in the study

Number	Culture	Scientific name	Cultivar
1	Carrot	*Daucus carota* L.	New Kuroda
2	Beet	*Beta vulgaris* L.	Detriot Dark Red
3	Okra	*Abelmoschus esculentus* L. *(*Moench)	Tropical C-17
4	Pepper	*Capsicum annuum* L.	Verano 1
5	pumpkin	*Curcubita moschata* (Duch ex Lam) Duch.	VME
6	Water melon	*Cucumis melo* L.	Gerona 1582
7	Radish	*Raphanus sativus* L.	PS-9
8	Tomato	*Solanum lycopersicum* L.	INIFAT-28
9	Corn	*Zea mayz* L.	Francisco mejorado
10	Sugar cane	*Saccharum officinarum* L.	Cristalina blanca
11	Sorghum	*Sorghum vulgare* Pres.	Criollo
12	Sweet potato	*Ipomoea batata* L.	Cemsa 78-384
13	Cassava	*Manihot esculenta* Crantz.	CMC-40
14	Taro	*Xanthosoma sagittifolium* Schott.	CMC-22
15	Banana	*Musa* sp.	Jhonson 3319
16	Mango	*Mangifera indica* L.	Keit
17	Howthorn	*Malphigia glabra* L.	Nc
18	Maracuya	*Passiflora edulis* Sims.	Nc
19	Guava	*Psidium guajaba* L.	Enana cubana
20	Papaya	*Carica papaya* L.	Maradol Roja
21	Lycheesi	*Litchi sinensis* Sonn.	Nc
22	Peanut	*Arachis hypogaea* L.	Cascajal rosado
23	Sunflower	*Helianthus annuus* L.	Cubasoy 113
24	Sesame	*Sesamun indicum* L.	Blanco 5 A

Nc: unknown

Characterization of *G. diazotrophicus strains* using the following indicators:

- Biological nitrogen fixation. The fixability of atmospheric nitrogen was determined qualitatively. Strains were inoculated semisolid LGI medium (1) in devoid of combined nitrogen. It was considered that the microorganism fixed atmospheric nitrogen to maintain growth after five successive inoculations in this culture medium. In all cases it was incubated at 30 °C temperature.
- Solubilization of phosphorus. NBRIP culture medium (7) it was used. Nutrient solubilization was determined from the measurement of yellow halo formed around the bacterial colony to 24, 48 and 72 hours incubation at 30 °C temperature.
- Indole acetic acid production (AIA). AIA production was quantified using the colorimetric method Salkowsky (8). To perform the test microorganisms were grown in Tryptone Soya Broth medium. It was used for fermentation an orbital shaker set at one 180 rpm agitation, 30 °C temperature for 24 hours. The experiment was performed with three replicates per strain as a negative control and the culture medium was used uninoculated. The absorbance of the samples was measured at a wavelength of 535 nm in a UV visible spectrophotometer.
- Antagonist activity against *Fusarium* spp. Agar Avena culture medium was inoculated with a cell suspension of 10 % of strains of *G. diazotrophicus* and allowed to stand for four hours. Then he placed in the center of the Petri dish of 90 mm, a disc of 7 mm of fungi *Fusarium moniliforme* (strain 2387) and *F. incarnatum* (strain 3188), both from the collection of fungi INIFAT (853 of WFCC).

A witness plate was remained, where only the plant pathogenic fungus was inoculated. All treatments were incubated for seven days at a temperature of 28 °C. To conduct assessments diameter mycelial growth of the fungus at three, five and seven days was measured. With these data the percent mycelial inhibition (IM) (IM = dc-dt / dc x 100 were calculated where dc: diameter or mycelial control (controls) and dt: diameter mycelium in treatments (9). Five replicates were used for each of the variants.

Statistical processing: the values obtained in each of the determinations were averaged through Microsoft Excell program run on *Windows* 2007, which was also used for calculating the standard deviation of the mean and making graphics. Statistical processing was performed with the program Statgraph version 5.1 (10). Means were compared using a "t" Student test for solubilization of nutrients, while for the rest of the trials Duncan test was used. The grouping of the strains was performed by a multivariate analysis using a cluster made with the same statistical package.

RESULTS AND DISCUSSION

From 24 plants sampled, 85 bacterial isolates were purified, of which four, as *G. diazotrophicus* were selected from the results of the morphological and physiological tests. These microorganisms from the fruits of guava (E 19) and mango (E 42) and stems beet (E 26) and cassava (E 46), presented the characteristics described for this species (1) and agreed on behavior pattern with the strain used in the investigation (Tables II and III).

Table II. Morphological characteristics of *G. diazotrophicus* isolated strains in the study and comparison with the international reference standard PAL 5

Strain	Color of the colony in culture media		Response of the Gram stain
	LGI	Potato agar	
E 19	Orange yellow	Brown	Gram negative short bacillus
E 26	Dark yellow orange	Brown	Gram negative short bacillus
E 42	Orange	Brown	Gram negative short bacillus
E 46	Orange	Brown	Gram negative short bacillus
PAL 5	Orange	Brown	Gram negative short bacillus

LGI: medium of culture

Table III. Physiological characteristics of *G. diazotrophicus* isolated strains in the study and its comparison with the international reference standard PAL 5

Strain	Cat	Ox	Ind	Cit	Mot	Alm	Gel	Sac 30 %	Glu 30 %
E 19	+	-	-	-	+	-	-	+	+
E 26	+	-	-	-	+	-	-	+	+
E 42	+	-	-	-	+	-	-	+	+
E 46	+	-	-	-	+	-	-	+	+
PAL 5	+	-	-	-	+	-	-	+	+

In other works the presence of *G. diazotrophicus* in mango (11), cassava (12) and beet (13) is recognized, so this would be the first reference to the case of guava. Optimal growth of the microorganism occurs in media with a sucrose concentration of 10 % and a pH of 5,5, so the inside of the fruits of the crop has favorable conditions for its establishment.

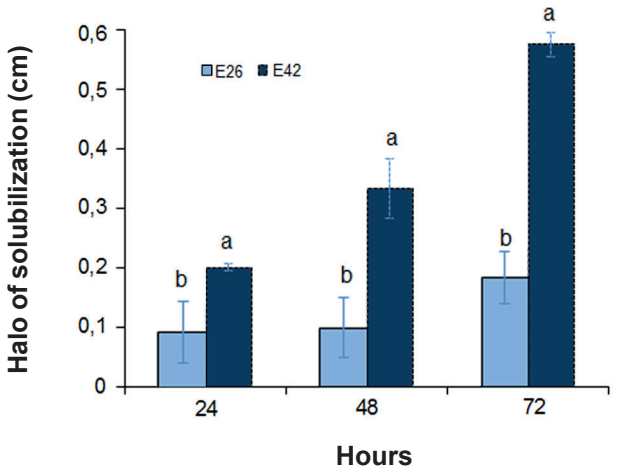
The four strains selected microorganisms as possible *G. diazotrophicus* with favorable for the stimulation of plant growth characteristics. All fix atmospheric nitrogen, considering its growth over the five inoculations on mineral medium lacking nitrogen. Biological nitrogen fixation is a feature described for the species *G. diazotrophicus* from its isolation (1). Given that this appearance was evaluated as presence/absence of bacterial growth, it is desirable to determine whether differences between the four microorganisms present in the amount of fixed nitrogen from other techniques such as acetylene reduction (ARA).

When assessing the potential solubilization of phosphorus only strains E 26 and E 42 showed positive results, highlighting the latter with significant differences from the 48 hours of incubation (Figure 1). Research *in vitro* systems demonstrate solubilization of inorganic phosphorus to the species *G. diazotrophicus*, which is directly associated to the release of gluconic acid (14).

Although generally, solubilization of nutrients is associated with rhizosphere microorganisms, the endophyte species such as *G. diazotrophicus* could increase the availability of these in the early stages of colonization and thus contribute to the stimulation of plant growth. In addition, lowering the pH in the medium by the action of the acidic compounds may constitute an adaptive advantage by lowering part of this competition in the rhizosphere environment.

Production levels of indole acetic acid (IAA) for the four microorganisms isolated in the study (Figure 2) were high (values close to 17 mg mL^{-1}), according to data reviewed by other authors (5); 35 strains belonging to the species *G. diazotrophicus*, only seven reached concentrations greater than 15 ug mL^{-1}. Given this reference, the results are still favorable for strain E 46, which released 11 ug mL^{-1} of AIA to the culture medium.

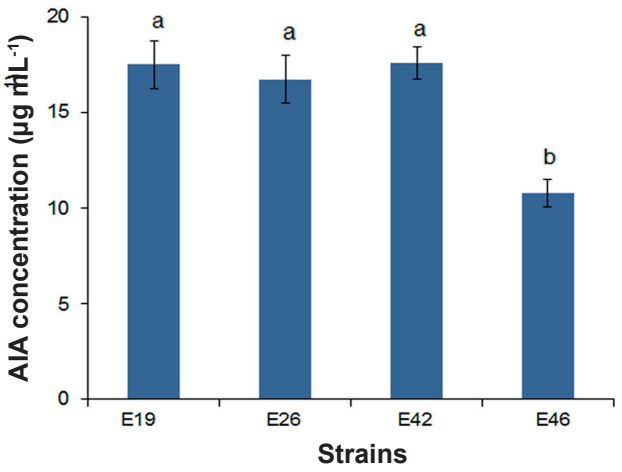
The effect of AIA in the formation of the apical domain, vascular differentiation and organ development (15) is known. In particular, *G. diazotrophicus* research shows that increases growth and root length, which results in greater access to nutrients by increasing the acreage of the crop (5). For this reason, the fact that all isolates in the study presented this feature is an important practical aspect for future use in developing inoculants for the benefit of crops of interest.



Means from the same time with different letters differ statistically for 5% significance level, according to test "t" of Student

The bars indicate the standard deviation in each case:
24 hours p: -3.1788, P: 0.01910; 48 hours p: -4.5826, P: 0.0038
72 hours: t: -14.1947, P: 0.000008

Figure 1. Solubilization of inorganic phosphorus in NBRIP supplemented by E26 and E42 isolates at 24, 48 and 72 hours

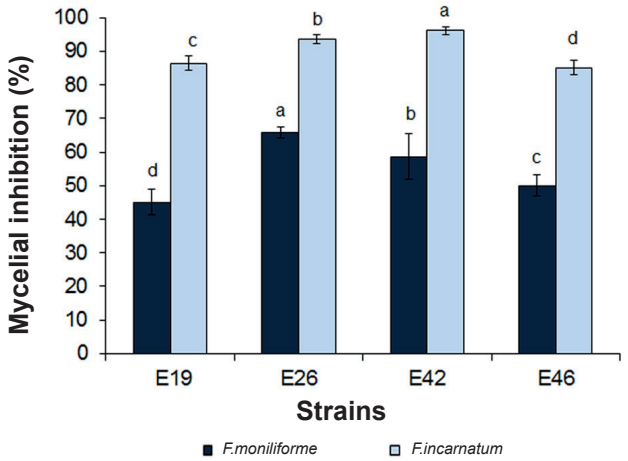


Means with different letters differ statistically for 5 % significance level, according to ANOVA by Duncan Multiple Range

Bars indicate the standard deviation in each case Esx: 0,603043, CV (%): 19,48

Figure 2. Production of indole acetic acid (AIA) in four possible isolates of *G. diazotrophicus*

Another metabolic attributes of the species *G. diazotrophicus*, useful for the production of products for agricultural use, is their antagonistic capacity. This indicator showed a positive response to all strains against *Fusarium moniliforme* and *F. incarnatum*, with 40 % more than control. Projecting the effect on *F. incarnatum* and generally stand E26 and E42 the strains caused by inhibiting the growth of pathogens (Figure 3).



F. moniliforme: Esx: 0,10673; CV (%): 16,83
F. incarnatum: Esx: 0,433785; CV (%): 5,57

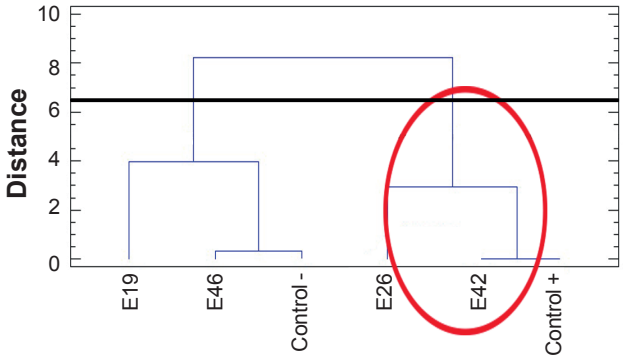
Means with different letters differ statistically 5% significance, according ANOVA by Duncan Multiple Range
Bars indicate the standard deviation in each case

Figure 3. Inhibition of mycelial growth of Fusarium species for possible strains of *G. diazotrophicus*

The effect of *G. diazotrophicus* against *Fusarium* species is an aspect little discussed at the international level, so that the results of this research are novel. Fungal species assessed are part of the complex of soil fungi and have a high impact on many crops, mainly in vegetables and fruit, the persistence of reproductive structures of the fungus in the soil and substrates (16).

The research results indicate that the purified possible isolates of *G. diazotrophicus* in the study release different metabolites; depending on the pathogen is present, taking into account the variations control rate for the same bacterial strain to the two species of fungi and similar results that other researchers have discussed the case of bacterial genera such as Bacillus (17). Most results *G. diazotrophicus* activity against *Fusarium* species is concentrated in the *F. oxysporum*, which has demonstrated the role of antibiotic metabolites character as pirrolnitrine and pioletoerine (18).

The strains were grouped according to the quantitative results of the study, two groups being formed. Stand in the branch closest to the positive control, consisting of the highest values of each of the determinations made, microorganisms from beet (E26) and mango (E42), which is recommended to evaluate *in vivo* conditions against different cultures (Figure 4).



Distance of cutting 50 %.The positive control consists of the highest values and the negative by lowest ones

Figure 4. Clustering dendrogram for possible strains of *G. diazotrophicus*, calculated from the values obtained in each of the quantitative experiments. Square Euclidean distance

From the research conducted are four possible strains of *G. diazotrophicus* isolated from plants cultivated in Cuban ecosystems, which have direct and indirect mechanisms of stimulation of plant growth. The work confirmed that the bacterial species has positive characteristics to form the basis of stimulators bioproducts of growth and demonstrated the need for research of this type, as an initial step in selecting strains, given the diversity in the expression of the metabolic potential the microorganism.

CONCLUSIONS

- The four isolated microorganisms from crops of mango, guava, cassava and sugar beet are likely to belong to the species *G. diazotrophicus*, taking into account their morphological and physiological characteristics.
- The isolated microorganisms present positive characteristics that contribute to plant growth stimulation, with a variable degree of expression.
- The isolated microorganisms from mango and beet crops stand out for their characteristics for stimulating plant growth, so they are promising to evaluate its effect on *in vivo* conditions on crops of agricultural interest.

RECOMMENDATIONS

- Identify the four isolated microorganisms.
- Determine by other methods the FBN potential of the four isolated microorganisms in the study.
- Perform tests to evaluate growth stimulation *in vivo* conditions with microorganisms from the mango and beet crops.

BIBLIOGRAPHY

1. Cavalcante, V. A. y Dobereiner, J. "A new acid-tolerant nitrogen-fixing bacterium associated with sugarcane". *Plant and Soil*, vol. 108, no. 1, mayo de 1988, pp. 23-31, ISSN 0032-079X, 1573-5036, DOI 10.1007/BF02370096.
2. Prabudoss, V. "A real multi beneficial endophytic diazotroph *Gluconacetobacter diazotrophicus* for sugarcane". *International Journal of Current Research*, vol. 3, no. 6, 2011, pp. 103-106, ISSN 0975-833X.
3. Praduboss, V. y Stella, D. "Growth enhancing association of *Gluconacetobacter diazotrophicus* and AM fungi in sugarcane". *International Journal of Current Research*, vol. 4, 2010, pp. 140-141, ISSN 0975-833X.
4. Kumarasamy, V. y Santhaguru, K. "Growth performance of *Sorghum bicolor* (L.) Moench in response to inoculation with *Gluconacetobacter diazotrophicus*". *Genetic and Plant Physiology*, vol. 1, no. 3-4, 2011, pp. 130-138, ISSN 1314-6394, 1314-5770.
5. Patil, N. B.; Gajbhiye, M.; Ahiwale, S. S.; Gunjal, A. B. y Kapadnis, B. "Optimization of Indole 3acetic acid (IAA) production by *Acetobacter diazotrophicus* L1 isolated from Sugarcane". *International Journal of Environmental Sciences*, vol. 2, no. 1, 2011, pp. 295-302, ISSN 0976–4402.
6. Dibut, B.; Martínez, R.; Ríos, Y.; Plana, L.; Rodríguez, J.; Ortega, M. y Tejada, G. "Estudio de la asociación *Gluconacetobacter diazotrophicus*-viandas tropicales establecidas sobre suelo Ferralítico Rojo. II. Determinación del método de inoculación más eficiente para la incorporación de *G. diazotrophicus* en los cultivos de boniato, yuca y malanga". *Cultivos Tropicales*, vol. 31, no. 3, septiembre de 2010, pp. 20-26, ISSN 0258-5936.
7. Nautiyal, C. S. "An efficient microbiological growth medium for screening phosphate solubilizing microorganisms". *FEMS Microbiology Letters*, vol. 170, no. 1, 1 de enero de 1999, pp. 265-270, ISSN 1574-6968, DOI 10.1111/j.1574-6968.1999.tb13383.x.
8. Glickmann, E. y Dessaux, Y. "A critical examination of the specificity of the salkowski reagent for indolic compounds produced by phytopathogenic bacteria". *Applied and Environmental Microbiology*, vol. 61, no. 2, 2 de enero de 1995, pp. 793-796, ISSN 0099-2240, 1098-5336.
9. Singh, S.; Datta, P. y Patel, R. "Survival and Growth of Diazotrophic Cyanobacterial Isolates Exposed to Rice-Field Herbicides". *Bulletin of Environmental Contamination and Toxicology*, vol. 70, no. 5, mayo de 2003, pp. 1052-1058, ISSN 0007-4861, 1432-0800, DOI 10.1007/s00128-003-0089-y.
10. 1Statistical Graphics Crop. *STATGRAPHICS® Plus* [en línea]. (ser. Profesional), versión 5.1, [Windows], 2000, Disponible en: <http://www.statgraphics.com/statgraphics/statgraphics.nsf/pd/pdpricing>.
11. Matiru, V. y Thomson, J. "Can *Acetobacter diazotrophicus* be used as a growth promoter for coffee, tea and banana plants". En: ed. Dakora F. D., *Proceedings of the 8th Congress of the African Association for Biological Nitrogen Fixation*, edit. University of Cape Town, South Africa, 1998, pp. 129–130.
12. Dibut, B.; Ortega, M.; Martínez, R.; Fey, L. y Ríos, Y. "Nuevos aislados de *Gluconacetobacter diazotrophicus* en cultivos de importancia económica para Cuba". *Cultivos tropicales*, vol. 26, no. 2, 2005, pp. 5–10, ISSN 0258-5936.
13. Crespo, J. M.; Boiardi, J. L. y Luna, M. F. "Mineral phosphate solubilization activity of *Gluconacetobacter diazotrophicus* under P-limitation and plant root environment". *Agricultural Sciences*, vol. 2, no. 1, 2011, pp. 16-22, ISSN 2156-8553, DOI http://dx.doi.org/10.4236/as.2011.21003.
14. Martínez, R. y Dibut, B. *Biofertilizantes Bacterianos*. edit. Científico Técnica, La Habana, Cuba, 2012, 279 p., ISBN 978-959-05-0659-8.
15. Velázquez, H. M. L.; Baizabal, A. V. M.; Cruz, V. F.; Trejo-, C. M. J.; Fuentes, R. L. E.; Bravo, P. A.; Cajero, J. M.; Chávez, M. M. P. y Valdez, A. J. J. "*Gluconacetobacter diazotrophicus* levansucrase is involved in tolerance to NaCl, sucrose and desiccation, and in biofilm formation". *Archives of Microbiology*, vol. 193, no. 2, 20 de noviembre de 2010, pp. 137-149, ISSN 0302-8933, 1432-072X, DOI 10.1007/s00203-010-0651-z.
16. Agrios, G. N. *Plant Pathology*. 7ª ed., edit. Elsevier Academic Press, London, 2013, 929 p., ISBN 0-12-044565-4.
17. Reyes, R. A.; Ruiz, S. E.; Yam, C. C. y Dzul, C. M. "Selección de *Bacillus* spp. con actividad antagónica *in vitro* contra *Macrophomina phaseolina* (Tassi) Goid. en diferentes medios de cultivo". *Fitosanidad*, vol. 15, no. 2, 25 de abril de 2012, pp. 117-121, ISSN 1818-1686.
18. Logeshwarn, P.; Thangarajn, M. y Rajasundari, K. "Antagonistic potential of *Gluconacetobacter diazotrophicus* in sweet potato (*Ipomoea batatus*)". *Phytopathology and Plant Protection*, vol. 44, no. 1, 2011, pp. 216-223, ISSN 1477-2906.

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