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Short communication
EFFECT OF DIFFERENT CARBON SOURCES ON THE GROWTH OF A RHIZOBIA STRAIN

Comunicación corta
Efecto de diferentes fuentes de carbono sobre el crecimiento de un aislado de rizobio

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ABSTRACT. Knowledge of the nutritional needs of rhizobia is important for understanding their behavior in the rhizosphere and in the biofertilizers production. The objective of this research was to determine the effect of different carbon sources on the growth of a rhizobium isolate. On five carbon sources *Rhizobium* sp. S11 was grown. Its growth was determinated by optical density (OD) measuring and the specific growth rate (μ). The highest growth of *Rhizobium* sp. S11 was obtained on the medium with mannitol. The highest μ were obtained in the media with mannitol, glucose and glycerol. These studies are the basis for a better understanding of the *Rhizobium* sp. S11 behavior in the rhizosphere. It also provides tools for the design and optimization of culture media to increase the viability and concentration of this microorganism in future inoculants.

Key words: characterization, *Rhizobium*, multiplication, carbohydrates

RESUMEN. El conocimiento de las necesidades nutricionales de los rizobios es importante para comprender su comportamiento en la rizosfera y en la producción de biofertilizantes. El objetivo de esta investigación fue determinar el efecto de diferentes fuentes de carbono sobre el crecimiento de un aislado de rizobio. *Rhizobium* sp. S11 se cultivó en cinco fuentes de carbono. Su crecimiento se determinó mediante la medición de la densidad óptica (DO) y la velocidad específica de crecimiento (μ). El mayor crecimiento de *Rhizobium* sp. S11 se obtuvo en el medio con manitol. Las mayores μ se obtuvieron en los medios con manitol, glucosa y glicerol. Estos estudios constituyen la base para una mejor comprensión del comportamiento de *Rhizobium* sp. S11 en la rizosfera. Además brinda herramientas para el diseño y la optimización de medios de cultivo que permitan incrementar la viabilidad y concentración de este microorganismo en futuros inoculantes.

Palabras clave: caracterización, *Rhizobium*, multiplicación, carbohidratos

INTRODUCTION

The rhizobia are studied mainly by the symbiosis they make with the plants belonging to the *Leguminosae* family [1,2]. These microorganisms are used as an active portion of inoculants that increase the yields of numerous crops of economic importance [3,4]. The use of biofertilizers based on rhizobia in agriculture also reduces the application of mineral fertilizers and the pollution of ecosystems (5).

The survival of the rhizobia in the soil and the rhizosphere depends, to some extent, on its ability to extract energy from the different sources of carbon available during all stages of its life cycle: as free-living microorganisms, during the process of infection of the host and as bacteroides inside the nodules where they perform the Biological Fixation of Nitrogen (6). The nutritional studies of these microorganisms provide tools of great importance for the preparation of suitable culture media, an aspect of great interest during the preparation of biofertilizers.

The design of culture media must take into account the nutritional requirements of these microorganisms by adding the nutrients in the proper form and proportion. The study of carbon sources that use heterotrophic microorganisms such as rhizobia allows a greater understanding of their ecology and behavior during the industrial production of inoculants (7).

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The choice of the optimal carbon source is one of the fundamental aspects for preparing inoculants with high bacterial viability and concentration (8). These requirements guarantee the effectiveness of the inoculant in the field. The most used carbon sources are alcohols and carbohydrates, mainly mono and disaccharides.

The standard medium for the cultivation of rhizobia includes mannitol, sucrose or glycerol as the sole carbon sources. These guarantee the multiplication of the rhizobia with a suitable nitrogen source and under certain pH and temperature parameters (9). The objective of this research was to determine the effect of different carbon sources on the growth of a rhizobium isolate.

MATERIALS AND METHODS

Microbial material

A rhizobium isolate called *Rhizobium* sp. S11 was used in this study. The microorganism came from soybean nodules (*Glyxine max* L.) and belongs to the strain collection from the Bacteriology Laboratory of the Department of Plant Physiology and Biochemistry of the National Institute of Agricultural Sciences.

Bacterial growth in different carbon sources

Pre-liquid cultures of the bacterial isolate were prepared in erlenmeyers of 100 mL volume, containing 20 mL of liquid medium Yeast Extract-Mannitol (LM) (10). For it; a roast of the microorganism, preserved in tubes with the same solid medium at 4 ºC, was placed in the erlenmeyers and these were kept under stirring conditions at 150 rpm, for 16 h at 28 + 1 ºC.

Ten milliliters of the pre-cultures were centrifuged at 10,000 rpm for 10 min. The supernatant was discarded and the pellet was re-suspended in 10 mL of sterile LM medium and lacking the carbon source traditionally used in this medium (mannitol). The optical density of the cell suspensions was adjusted to 0.25 at a wavelength of 600 nm, in a spectrophotometer (GENESYS 20).

The cell suspensions were used to inoculate erlenmeyer flasks of 250 mL volume, containing 50 mL of liquid LM medium. This medium was supplemented individually with the following carbon sources at a concentration of 10.1 g L^{-1}: mannitol; glucose; galactose; lactose; glycerol. The cultures were maintained under shaking conditions at 150 rpm, for 24 h at 28 + 1 ºC. The purity of the bacterial cultures was checked by Gram stain.

The growth of *Rhizobium* sp. S11 was evaluated every four hours until 24 hours. For this, the absorbance of the cultures was measured at a wavelength of 600 nm. In addition, the specific growth rate μ (h^{-1}) (11) was calculated in the logarithmic or exponential growth phase according to the expression:

$$\mu = \frac{\ln (DO_2 / DO_1)}{(t_2 - t_1)}$$

A completely randomized design was used, in which three repetitions were placed per treatment. Cell multiplication data were subjected to the normality and variance homogeneity test. A simple classification variance analysis was applied, using the Tukey Mean Comparison Test ($p < 0.05$) (12). All the graphics were made in the SigmaPlot 2001 program.

RESULTS

The behavior and specific growth rate of *Rhizobium* sp. S11 in the LM medium with different carbon sources is represented in the Figure 1.

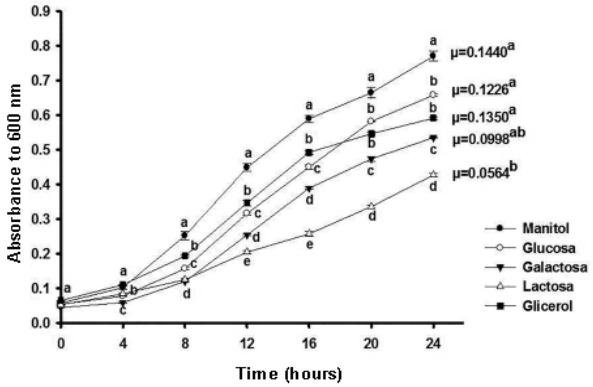

Figure 1. Dynamics of multiplication of *Rhizobium* sp. S11 in LM medium with different carbon sources

Rhizobium sp. S11 showed the highest growth in the LM medium with mannitol as a carbon source, from eight to 24 hours. In the media supplemented with mannitol and glycerol, as the sole carbon sources, no significant differences were observed in the growth of this microorganism at four hours of culture.

The growth of *Rhizobium* sp S11 showed significant differences between all carbon sources at 12 and 16 hours. However, after 20 hours of culture, no differences were observed in the growth of this microorganism between the media supplemented with glucose and with glycerol.

Rhizobium sp. S11 had the highest specific growth rate in the LM medium with mannitol. However, no significant differences in this variable were observed between media supplemented with glucose, galactose and glycerol. Lactose produced the lowest specific growth rate of the microorganism.

DISCUSSION

Rhizobium sp. S11 grew in all the sugars that were studied. The use of different organic compounds by microorganisms is an ecological advantage because it allows having alternative carbon sources to multiply and remain viable in the rhizosphere (7).

Mannitol gave the greatest growth of Rhizobium sp. S11 after eight hours of cultivation. Similar results were obtained by numerous authors (13). This compound is one of the most widely used sources of carbon for the growth of rhizobia of the *Rhizobiaceae* family, on a laboratory scale and in the production of inoculants (14). Rhizobium sp. S11 belongs to the *Rhizobiaceae* family. It groups the genera *Rhizobium*, *Ensifer* and *Shinella* (15).

The use of mannitol as a carbon source in the culture medium increases the production of polyhydroxybutyrate (PHB) by some strains of rhizobia (16). This polymer is biodegradable and therefore constitutes an alternative in the substitution of recalcitrant plastics coming from the petrochemical industry (17). The high growth of *Rhizobium* sp. S11 in the medium supplemented with mannitol could be the starting point to study the potential of this bacterial isolate in the production of PHB.

Glycerol and glucose also produced a high growth of Rhizobium sp. S11. Similar results were obtained with *Rhizobium leguminosarum* bv. *trifolii* (18). Glycerol exerts a positive effect on the competitiveness of rhizobia during the occupation of the root nodule of legumes (19) and in the production of exopolysaccharides (EPS) (18). Other authors corroborate the role of glucose (in the form of glucose 6-phosphate) as one of the main precursors of EPS in rhizobia (20). EPS are one of the determinants of the rhizobium-legume symbiosis (21).

These compounds also participate in the formation of biofilms, the collection of nutrients and protection against some abiotic stresses (22). The use of glycerol and glucose by *Rhizobium* sp. S11 not only would allow an adequate growth in the rhizosphere, but a greater adaptation to the abiotic conditions present in the soil.

Lactose produced the lowest growth of *Rhizobium* sp. S11. This sugar is a disaccharide with a single transporter in the cytoplasmic membrane of the rhizobia, so it does not degrade in its constituent monomers before penetrating the cellular cytoplasm (23). This could explain the absence of a diauxic behavior of the microorganism studied in the presence of lactose as the sole carbon source.

Rhizobium sp. S11 had a lower specific growth rate in lactose than in medium supplemented with the structural monomers of this sugar (glucose and galactose). The production by the microorganism of a β-galactosidase, an enzyme that degrades the bond between both monomers (24) could explain, to some extent, this behavior. Glucose and galactose would enter directly into the Entner-Doudoroff pathway to oxidize to pyruvate and glyceraldehyde 3-phosphate (6). However, it is necessary to carry out more in-depth molecular studies to corroborate this hypothesis and determine the rest of the factors that influence a specific growth rate of this microorganism in the presence of lactose.

CONCLUSIONS

- *Rhizobium* sp. S11 is a microorganism that has certain potentialities from the nutritional point of view because it uses different sources of carbon for its growth. Its ability to multiply considerably in the presence of mannitol, glucose and glycerol can be used to design more economical culture media.
- The use of national agricultural by-products containing these compounds could be an alternative to reduce costs in the production of inoculants for the cultivation of soybeans.

RECOMMENDATIONS

- To characterize from the chemical point of view some of the most abundant agricultural by-products in the country.
- To use those, with relatively high percentages of mannitol, glucose and glycerol, in the formulation of culture media for the growth of *Rhizobium* sp S11.
- It is also recommended to carry out inoculation tests of *Rhizobium* sp. S11 in soybean plants to determine the effect of the culture medium on the nodulation and growth of these plants.

BIBLIOGRAPHY

1. Suzaki T, Yoro E, Kawaguchi M. Leguminous Plants: Inventors of Root Nodules to Accommodate Symbiotic Bacteria. In: International Review of Cell and Molecular Biology [Internet]. Elsevier; 2015 [cited 2018 Jun 4]. p. 111–58. doi:10.1016/bs.ircmb.2015.01.004
2. Nouwen N, Arrighi JF, Cartieaux F, Chaintreuil C, Gully D, Klopp C, *et al*. The role of rhizobial (NifV) and plant (FEN1) homocitrate synthases in Aeschynomene/photosynthetic Bradyrhizobium symbiosis. Scientific Reports. 2017;7(1):439–48. doi:10.1038/s41598-017-00559-0
3. Hungria M, Araujo R S, Júnior S, Barbosa E, Zilli JÉ. Inoculum Rate Effects on the Soybean Symbiosis in New or Old Fields under Tropical Conditions. Agronomy Journal. 2017;109(3):1106-12. doi:10.2134/agronj2016.11.0641
4. Nawaz F, Khan N, Shah J A, Khan A, Liaqat A, Ullah S, *et al*. Yield and yield components of chickpea as affected by various levels of FYM and rhizobium inoculation. Pure and Applied Biology. 2017;6(1):346–51. doi:10.19045/bspab.2017.60033.
5. Adeyeye AS, Togun AO, Olaniyan AB, Akanbi WB. Effect of Fertilizer and Rhizobium Inoculation on Growth and Yield of Soyabean Variety (*Glycine max* L. Merrill). Advances in Crop Science and Technology. 2017;05(01):1–9. doi:10.4172/2329-8863.1000255
6. Geddes B A, Oresnik I J. Physiology, genetics, and biochemistry of carbon metabolism in the alphaproteobacterium Sinorhizobium meliloti. Canadian journal of microbiology. 2014;60(8):491–507. doi:10.1139/cjm-2014-0306.
7. Madigan M T, Martinko J M, Parker J. Brock biología de los microorganismos. Décima edición. United States of America: Pearson, Prentice Hall Hispanoamericana; 2011. 1011 p.
8. Singleton P, Keyser H, Sande E. Development and evaluation of liquid inoculants. In: Herridge D, editor. Inoculants and nitrogen fixation of legumes in Vietnam. Canberra,Australia: Australian Centre for International Agricultural Research Proceedings; 2002. p. 52–66.
9. Gaurav K, Singh A K, Singh G. Spent Wash as an Alternative Medium for Growth of Rhizobium. Journal of Academia and Industrial Research (JAIR). 2016;5(6):81–4.
10. Vincent JM. A Manual for the Practical Study of the Root-Nodule Bacteria. In: International Programme Handbook [Internet]. Oxford, England: Blackwele scientific publications; 1970 [cited 2018 Jun 4]. p. 440–440. Available from: https://onlinelibrary.wiley.com/doi/abs/10.1002/jobm.19720120524
11. Maier R M. Bacterial growth. In: Maier R, Pepper I, Gerba C, editors. Environmental Microbiology. 2 edition. Amsterdam ; Boston: Academic Press; 2008. p. 38–56.
12. Sigarroa A. Biometría y diseño experimental. La Habana, Cuba: Editorial Pueblo y Educación; 1985. 328 p.
13. Ouslim S, Merabet C, Boukhatem Z, Bouchentouf L, BekkiA. Phenotypic And Symbiotic Diversity, Of Nodulating Rhizobia Associated With Bean (*Vicia faba*) In West Algeria. International Journal of Technology Enhancements and Emerging Engineering Research. 2015;3(9):130–8.
14. Singh A K, Singh G, Bhat R P. Effects of salt stress on cell surface properties and symbiotic performance of root nodulating bacteria. UK J. Pharmaceut. Biosci. 2015;3(1):23–9. doi:10.20510/ukjpb/3/i1/89222
15. Berrada H, Fikri B K. Taxonomy of the Rhizobia: Current Perspectives. British Microbiology Research Journal. 2014;4(6):616–639. doi:2231-0886
16. Kıvanc M, Dombaycı N. Production of Poly-B-Hydroxybutyric Acid by Rhizobium Sp. Fresenius Environmental Bulletin. 2016;25(5):1305–11.
17. Lathwal P, Nehra K, Singh M, Jamdagni P, Rana J S. Optimization of Culture Parameters for Maximum Polyhydroxybutyrate Production by Selected Bacterial Strains Isolated from Rhizospheric Soils. Polish journal of microbiology. 2015;64(3):227–39.
18. Janczarek M, Rachwał K, Cieśla J, Ginalska G, Bieganowski A. Production of exopolysaccharide by *Rhizobium leguminosarum bv*. trifolii and its role in bacterial attachment and surface properties. Plant and Soil. 2015;388(1–2):211–27. doi:10.1007/s11104-014-2320-5
19. Vanderlinde E M, Hynes M F, Yost C K. Homoserine catabolism by *Rhizobium leguminosarum* bv. *viciae* 3841 requires a plasmid-borne gene cluster that also affects competitiveness for nodulation: *R. leguminosarum* genes for homoserine catabolism. Environmental Microbiology. 2014;16(1):205–17. doi:10.1111/1462-2920.12196
20. Gosselin I, Wattraint O, Riboul D, Barbotin J N, Portais J Ch. A deeper investigation on carbohydrate cycling in *Sinorhizobium meliloti*. FEBS Letters. 2001;499(1–2):45–9. doi:10.1016/S0014-5793(01)02518-2
21. Muszyński A, Heiss C, Hjuler CT, Sullivan J T, Kelly S J, Thygesen MB, *et al*. Structures of Exopolysaccharides Involved in Receptor-mediated Perception of *Mesorhizobium loti* by *Lotus japonicus*. Journal of Biological Chemistry. 2016;291(40):20946–61. doi:10.1074/jbc.M116.743856
22. Jaszek M, Janczarek M, Kuczynski K, Piersiak T, Grzywnowicz K. The response of the Rhizobium leguminosarum bv. trifolii wild-type and exopolysaccharide-deficient mutants to oxidative stress. Plant and Soil. 2014;376(1–2):75–94. doi:10.1007/s11104-013-1959-7
23. Glenn A R, Dilworth M J. The uptake and hydrolysis of disaccharides by fast-and slow-growing species of Rhizobium. Archives of Microbiology. 1981;129(3):238–9. doi:10.1007/BF00425257
24. Niel C, Guillaume JB, Bechet M. Mise en évidence de deux enzymes présentant une activité β-galactosidasique chez *Rhizobium meliloti*. Canadian Journal of Microbiology. 1977;23(9):1178–81. doi:10.1139/m77-177

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