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SPORE VIABILITY AND LIQUID INOCULANT PERFORMANCE BASED ON *Glomus cubense* IN *Sorghum bicolor* L. cv. Moench

Viabilidad de esporas y funcionamiento de un inoculante líquido a base de *Glomus cubense* en *Sorghum bicolor* L. cv. Moench

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ABSTRACT. This study aimed to evaluate arbuscular mycorrhizal fungi (*Glomus cubense*) functional viability and colonization ability in liquid medium. Two experiments were conducted, one studied spores fungal viability in liquid medium and sterile water was used in control around six months. Average 100 spores were used. In second text, we studied spores ability colonizing stored by six months in sorghum (*Sorghum vulgare* Perz.) plants. Mycorrhizal performance indicators were determined (colonization frequency and intensity and protein total) and plants growth and development indicators (dry mass root of air). Data were analyzed using Statgraphics Centurion for Windows and used the Duncan test with a significance of 5% in the cases where the ANOVA was significant. Results showed *Glomus cubense* spores viability after six months, with marked loss of viability in the variant preserved in sterile distilled water. Colonization studies demonstrated spores functional stability on sorghum plants, due to were achieved superior colonization levels in plants inoculated with respect to non-inoculated. Results demonstrated viability and functional stability of liquid inoculant retained up to six months.

Key words: spores, conservation, stability, grasses

RESUMEN. El trabajo tuvo como objetivo evaluar la viabilidad funcional y capacidad de colonización del hongo micorrízico arbuscular *Glomus cubense*, en medio líquido. Se realizaron dos experimentos, en uno se estudió la viabilidad fúngica de las esporas en un medio líquido y como control agua destilada estéril durante seis meses. Se utilizaron 100 esporas como promedio. En el segundo se estudió la capacidad de colonización de las esporas almacenadas durante seis meses inoculadas en plantas de sorgo (*Sorghum vulgare* Perz.). Se determinaron indicadores de funcionamiento micorrízico (frecuencia e intensidad de la colonización, contenido de proteínas fácilmente extraibles, producidas por los HMA) e indicadores de crecimiento y desarrollo de las plantas (masa seca de raíz y aérea). Los datos fueron analizados mediante el programa STATGRAPHICS Centurion para *Windows* y se utilizó la prueba de Duncan con una significación de un 5 % en los casos en que el ANOVA resultó significativo. Los resultados demostraron la viabilidad de las esporas de *Glomus cubense* durante seis meses, con marcadas pérdidas de viabilidad en la variante conservada en agua destilada estéril. Los estudios de colonización en plantas de sorgo demostraron la estabilidad funcional de las esporas, debido a que se alcanzaron niveles de colonización superiores en las plantas inoculadas en relación con las no inoculadas. Estos resultados demuestran la viabilidad y estabilidad funcional del inoculante líquido hasta seis meses de conservado.

Palabras clave: esporas, conservación, estabilidad, gramínea

INTRODUCTION

In the world agricultural context, there are several and extraordinary benefits of mycorrhizal symbiosis transferred to the plants. Arbuscular mycorrhizae are the most common and widely distributed in ground ecosystems (1). These evidences, in addition to the seniority of arbuscular mycorrhizal fungi (AMF), suggest their important contribution to the successful colonization of the earth by plants and the crucial role played in its evolution and diversification (2).

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AMF are considered forced biotrophs because they depend on the host plant to complete its life cycle and one of the main factors explaining this condition is the metabolism or carbon uptake at the pre-symbiotic state, due to the fact that extra-root hyphae are not able to absorb carbohydrates (3). Among the main functions made by these fungi at the soil-plant interphase, its role in the nutrients uptake stands out, specifically phosphorus and other macro and micronutrients (4). It happens because of the increased soil volume to explore (5); its role in restoring the diversity of biological communities thus contributing to the sustainability of agro-ecosystems (1 y 6); its positive effect to biotic stresses (pathogens) and abiotic (salinity and drought) since they act on various physiological plant processes (7 and 8) and plant tolerance to heavy metals (9).

Based on the above-mentioned criteria, in Cuba, since the 90s, the National Institute of Agricultural Sciences (INCA) embarked upon a wide program of basic research with these symbionts and as a result, a solid formulation biofertilizer registered as EcoMic®, was produced. It has a high purity degree and biological stability, several studies with successful results were conducted with EcoMic® in soybean (10), also in developing rootstocks for avocado nurseries (11) and in cassava (12).

Taking into account the effectiveness shown by this solid inoculant, 2000 marked the beginning of new studies, but this time with the purpose of formulating a new product from AMF fungi in liquid support to diversify the inoculation ways of these symbionts thus assuring future applications through fertigation. Recently, promising results have been reached with the use of AMF in liquid formulation in cereals as rice under salt stress (13).

A crucial element that limits the use of microbial inoculants is the functional stability of the product once formulated, therefore, the main objective if this study has been to prove the functional viability of AMF fungi of the *Glomus genus* in liquid medium as well as their capability of colonizing sorghum plants *Sorghum bicolor* L. Moench.

MATERIALS AND METHODS

Experiment 1

Production of fungi material for the liquid inoculant

For the execution of this research the isolate INCAM-4 of *Glomus cubense* as used (14), it came from the isolate collection of INCA, Cuba. The certified inoculum of *Glomus cubense* (100 g), that contains a mixture of kaolinistic clay and fungal propagules (spores, hyphae, micelium), was used in planting soghum plants in culture pots with an sterile mineral substrate. Ninety days after planting, the aereal part of the plants was removed and the substrate with mycorrhizae roots was used as spore source. The material was manually homogenized, dried at room temperature and stored for 15 weeks at 4°C.

For this spore isolation, 50 g of the homogenized material was taken and a humid screening was done (15), from a paste prepared by mixing soil and water, between two screens (40 and 400 µm of light) with the addition of water to facilitate the process. The residue was collected in the 40 µm screen, it was transferred to a centrifuge pipe with a spatula and in there, was mixed with a sucrose solution (720 g of sucrose and 20 g of Tween 80 L^{-1}), it was then centrifuged at 2000 rpm for five minutes. Immediately afterward, the liquid fraction was decanted with the fungal propapules which were deposited in eppendorf pipes of 1,5 mL, with 300 µL of Ringer solution to preserve them till disinfection. One liter of Ringer solution contained NaCl 7,5 g, KCl 0,75 g, $CaCl_2$ 0,1 g and $NaHCO_3$ 0,1 g.

Stability of the liquid inoculant

In order to evaluate the viability of fungal germplasm contained in the osmoprotective solution, a storing trial was conducted by placing 400 viable spores in 300 mL of solution in amber flasks under refrigeration. This trial extended for six months, five observations per treatment were done. Treatments followed a complete random design and as control, the same quantity of propagules was used, but preserved in sterile distilled water.

Determinations

On a monthly basis, morphological indicators of the AMF spores were evaluated, namely color and shape with the help of an optical microscope. The production of new spores was also quantified using a stereomicroscope (MEIJI TECHNO) and the percentage increase of total protein content produced in a liquid medium was determined (16).

Experiment 2

In order to prove the effectiveness of this inoculant after preserving it for six months, an inoculation trial was conducted in areas of the greenhouse adscript to the Department of Biofertilizers and Plan Nutrition at INCA. Sorghum was used (*Sorghum bicolor* L) Moench as the model crop, seeds were disinfected with a

sodium hypochlorite solution at 10 % for 10 minutes[A] . After this time, the solution was decanted, seeds were washed three times with distilled water and two of them were placed in each pot.

Plastic pots of 1 kg of capacity were used and as substrate, an Hydromorphic Gley Vertico Carbonated soil (17), whose features are shown in Table I. Sterilization was done in autoclave at 121 ºC for two hours, in three consecutive cycles.

Description of the mycorrhizal inoculant, experimental design and treatment description

Plants grew up at an average temperature of 25±3 ºC, relative humidity of 75-80 % and natural photoperiod. The trial extended for 60 days and was repeated twice during 2013 (April and June).

The liquid inoculant was formulated taking into account the above-mentioned procedures and 1 mL (40 spores average) were inoculated in each pot at planting time. A variant including certified solid inoculant at the rate of two grams per pot was used and with equal content as average and control, a non-inoculated treatment. Manual irrigation was applied according to plant needs. A totally random design was used with eight treatments described in Table II. Ten pots per treatments were used.

Determinations and Statistical analysis

Samplings were performed 30 and 60 days after seed germination (ddg), five pots per treatment were taken and the following evaluations were done:

- Mycorrhizal functioning indicators: For the estimation of fungal indicators, rootlets were dried at 70 ºC and dyed 18). The frequency and intensity of the mycorrhizal colonization was determined by the intercept method[C].
- Plant growth indicators: Root dry mass and aereal dry mass were determined by placing samples on the heater at 70 ºC till reaching constant weight.

Table II. Description of the treatments used in the experiment

Treatments	Time preservation (months)
T1	Liquid inoculum (1 month in storage)
T2	Liquid inoculum (2 month in storage)
T3	Liquid inoculum (3 month in storage)
T4	Liquid inoculum (4 month in storage)
T5	Liquid inoculum (5 month in storage)
T6	Liquid inoculum (6 month in storage)
T7	Certified solid inoculum
T8	Control without inoculation

All characters met the assumptions of normality and variance homogeneity so an analysis of variance was done with a bifactorial arrangement in Experiment 1, and ANOVA of simple classification in Experiment 2. Data were analyzed with the software STATGRAPHICS Centurion for Windows. Mean discrimination used the Duncan test with a 5 % significance in cases where ANOVA was significative.

RESULTS AND DISCUSION

Data shown meet the means of a repetition since the behavior was similar in the second one.

Experiment 1

The observation of *Glomus cubense* propagules preserved cold for six months through the optical microscope revealed that spores maintained a hyaline yellow color with a very pale tendency. As to spores shape, there was diversity: ovoid, ellipsoid, pyriform, irregular, rarely globose. Spore walls were made up of two layers and not hatching was seen.

Table III shows the number of spores in liquid medium and sterile distilled water during the time of the trial. The bifactorial analysis showed that the number of viable spores of *Glomus cubense* depended on the interaction between the type of solution used and the preservation time.

[A] Ortega, E. y Rodés, R. *Manual de prácticas de laboratorio de fisiología vegetal*, Pueblo y Educación, Ciudad de la Habana, 1986, p. 196.

[B] Herrera, R. A.; Ferrer, R. L.; Furrazola, E. y Orozco, M. O. Estrategia de funcionamiento de las micorrizas VA en un bosque tropical. Biodiversidad en Iberoamérica. Ecosistemas, Evolución y Procesos sociales, (ed. Monasterio, M.), edit. Programa Iberoamericano de Ciencia y Tecnología para el desarrollo, 1995.

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Table I. Main chemical features of the soil used for the experiment

pH	MO (%)	P (mg kg^{-1})	Ca	Mg	K (cmol kg^{-1})	Na	Espore AMF/50 g soil
7,5	1,04	17	12,5	3,7	0,48	0,10	21

Chemical determinations: pH H_2O, power meter; organic matter (MO), Walkley Black; phosphorus (P), Oniani; cations, Ca, Mg, Na and K, Maslova method; AMF spores (15) with modifications[B]

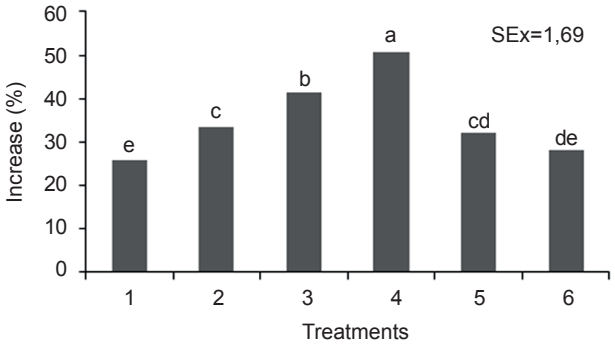
Table III. Number of viable spores of Glomus cubense in liquid medium (ML) and in water for six months

Treatments	Spore preservation time (months)					
	1	2	3	4	5	6
Glomus cubense (ML)	398,8 a	398,8 a	398,8 a	398,8 a	398,2 a	398,4 a
Glomus cubense (water)	245,8 b	230,8 b	225,2 b	220,2 b	212,4 b	203,6 b
SEx			1,11			

For the liquid medium it was found that the spore content remained stable during the study, while the value of preserved spores in sterile distilled water reduced practically half since the first month of evaluation, a tendency that decreased as the study time went by.

This behavior found in the spores preserved in sterile distilled water could be related to the osmotic pressure difference between the interior of the spore and the external medium surrounded by water.

Figure 1 describes the percentage increase of easily extracted protein content produced in liquid medium. There is a tendency to increase with higher percentages after four months of spores preservation (50 %) and a sharp decrease from the fifth month on.



1: liquid inoculum (one month); 2: liquid inoculum (two months); 3: liquid inoculum (three months); 4: liquid inoculum (four months); 5: liquid inoculum (five months) and 6: liquid inoculum (six months)

Figure 1. Percentage increase of the easily extracted protetin content in liquid medium

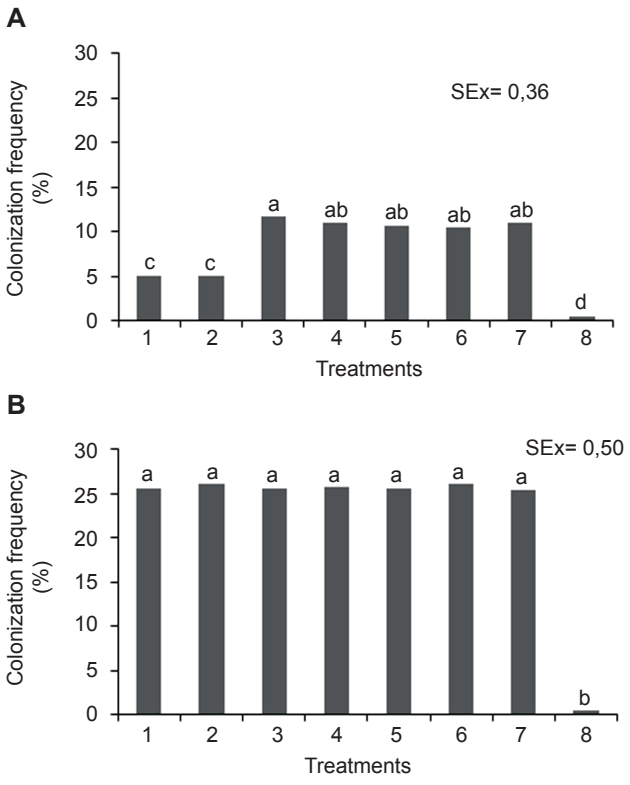
It should not be bypassed that disinfection done to spores used in this experiment removed part of the microbial flora associated to the external wall and some other studies confirm that the presence of microorganism on the spore walls stimulated the germination and development of the germinating tube and hyphal ramification, processes inherent in the fungus metabolism that as a whole, encourage the release of substances to the surrounding medium (19).

This study showed the ability of *Glomus cubense* spores to maintain viability after 6 months in liquid medium, a situation that could be related to the stimulation of germination mechanisms from the creation of stressing conditions during preservation.

Experiment 2

The influence of *Glomus cubense* inoculation on indicators of mycorrhizal functioning

Figure 2 describes the behavior of the mycorrhizal colonization frequency reached for sorghum plants in the two sampling done. In the first one, there were differences among treatments and the non-inoculated control.



A: 30 ddg B: 60 ddg
1: liquid inoculum (one month); 2: liquid inoculum (two months); 3: liquid inoculum (three months); 4: liquid inoculum (four months); 5: liquid inoculum (five months); 6: liquid inoculum (six months)
7: liquid inoculum and 8: control without inoculation

Figure 2. Inoculation influence on the mycorrhizal colonization frequency

The treatments inoculated with spores preserved for three, four, five and six months reached higher values (11 %) and in turn, did not differ from the treatment with solid inoculum. On the other hand, it was found that treatments inoculated with spores preserved for one and two months, showed the lowest values.

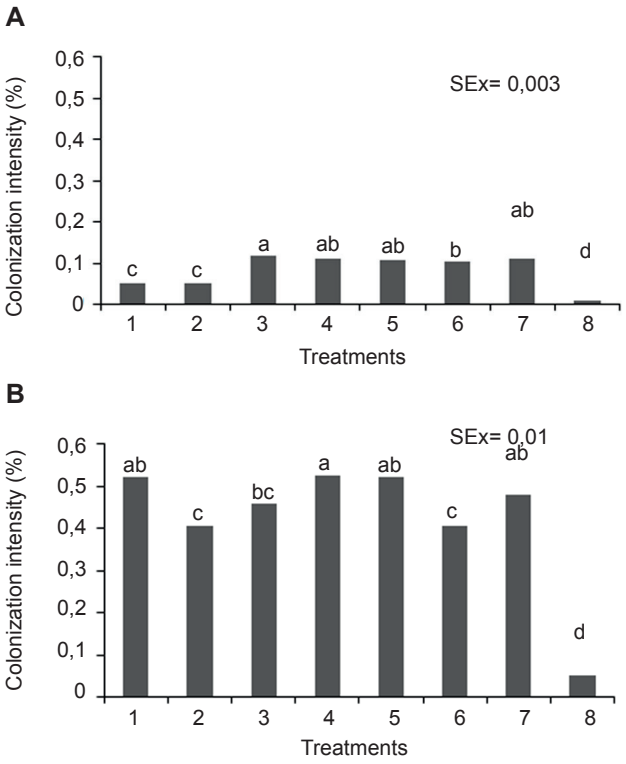
In the second sampling, there were differences compared to the non-inoculated control. Maximum values of inoculated treatments (25 %) were lower if compared to those reported for rice plants under salinity stress conditions, where figures close to 38% were reported 60 days after germination in those mycorrhized treatments with liquid inoculum (14).

The response found for this indicator in the non-inoculated treatment could be related to the presence of some fungal structures that persisted after the soil sterilization process, being such propagules less infective than the inoculated specie in this trial.

The intensity behavior of mycorrhizal colonization during the trial is shown in Figure 3. The first sampling found that inoculated treatments with spores stored for three, four, five and six months, expressed a similar behavior to the treatment with solid inoculum (T7), reaching values close to 0,13 %. On the other hand, treatments inoculated with spores stored for one and two months (T1 and T2) expressed a similar performance with values ranging around 0,05 % and all inoculated treatments significantly differed as compared to the non-inoculated control.

The second sampling showed that treatments T1, T4, T5 and T7 (inoculated with spores stored for one, four, five and seven months, respectively) reached higher values (0,53 %) significantly differing from treatments T2, T3 and T6 (inoculated with spores stored for two, three and six months, respectively) with values from 0,40 - 0,45 %. The non-inoculated treatment had the same behavior than the previous sampling. Studies made with wheat plants resulted in colonization intensity values of 0,49 % when they were inoculated with AMF in liquid formulation, which was lower than the one found in this study (20).

A comprehensive analysis of the mycorrhizal functioning indicators shows that both forms of inoculation (liquid or solid) resulted promising for sorghum plants.



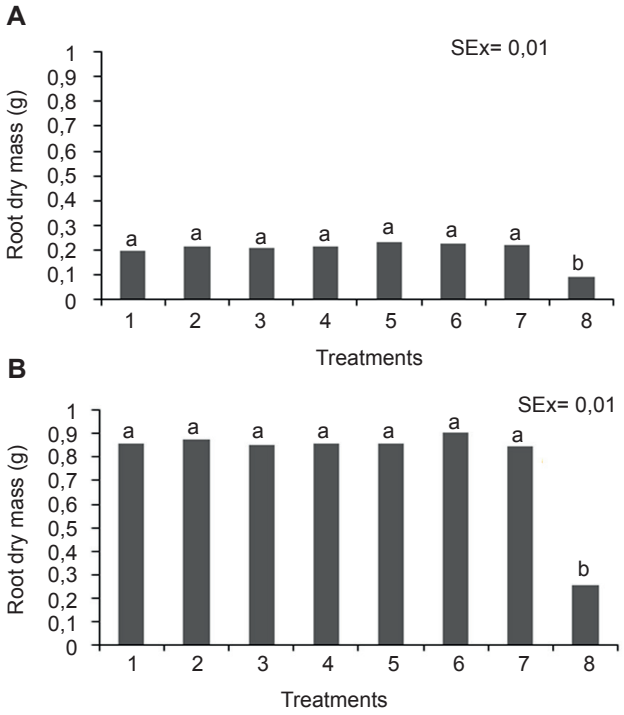
A: 30 ddg B: 60 ddg
1: liquid inoculum (one month); 2: liquid inoculum (two months);
3: liquid inoculum (three months); 4: liquid inoculum (four months);
5: liquid inoculum (five months); 6: liquid inoculum (six months)
7: liquid inoculum and 8: control without inoculation

Figure 3. Inoculation influence on the intensity of mycorrhizal colonization

Inoculation influence of *Glomus cubense* in plants growth indicators

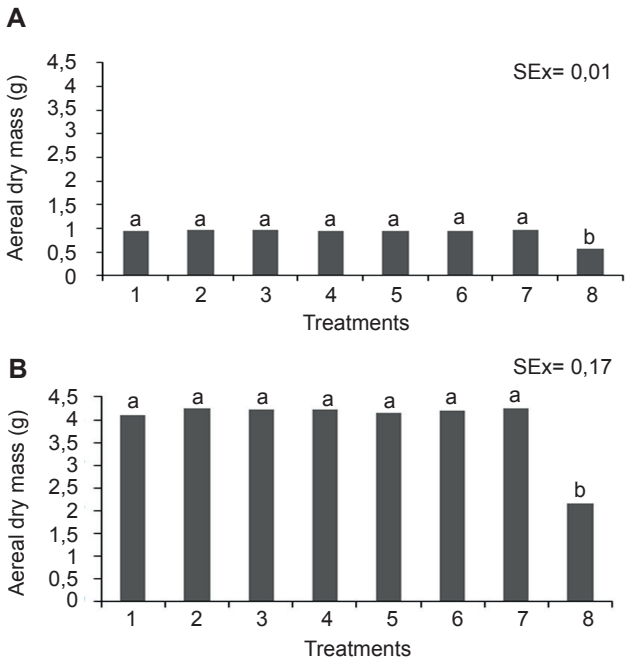
One of the benefits attributed to AMF fungi is their capacity to promote plant growth and development (21), Figures 4 and 5 show the influence of inoculation on the root dry mass and foliar dry mass, respectively. A similar behavior was found in both sampling as to the behavior of inoculated treatments in relation to the control which allows to confirm the effectiveness of symbiosis occurrence in both inoculation forms (solid and liquid) for the two evaluated indicators.

The inoculation effect of AMF fungi on the plant growth and development indicators has been widely supported by several studies, not only for the ability of such symbionts to colonize a root, but for taking nutrients from the soil and transfer them to the plant, namely, phosphorus and nitrogen. This is one of the key factors determining the effectiveness of symbiosis (4, 5).



30 ddg B: 60 ddg
1: liquid inoculum (one month); 2: liquid inoculum (two months);
3: liquid inoculum (three months); 4: liquid inoculum (four months);
5: liquid inoculum (five months); 6: liquid inoculum (six months)
7: liquid inoculum and 8: control without inoculation

Figure 4. Treatment influence on the root dry mass (g)



30 ddg B: 60 ddg
1: liquid inoculum (one month); 2: liquid inoculum (two months);
3: liquid inoculum (three months); 4: liquid inoculum (four months);
5: liquid inoculum (five months); 6: liquid inoculum (six months)
7: liquid inoculum and 8: control without inoculation

Figure 5. Treatment influence on the aereal dry mass (g)

The results of this experiment showed that spores from *Glomus cubense* do not lose their ability to colonize plants after six months of preservation; a crucial element to provide guarantee terms for the inoculant effectiveness once it has been formulated.

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