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Original Article

Effect of temperature and storage time on the Azofert®-F´s quality

Efecto de la temperatura y el tiempo de conservación en la calidad de Azofert®-F

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ABSTRACT: The objective of this work was to determine the effect of temperature and storage time on the concentration of the active principle and the biological activity of the Azofert®-F biofertilizer. Inoculants with *Rhizobium leguminosarum* CF1 strain were stored at 4 and 29±2 °C, to determine purity and strain concentration every 30 during 180 days. Data were processed using a simple classification analysis of variance. At 40 and 120 days of conservation of the inoculants, Tazumal cultivar were applied to bean seeds and the number of nodules and their effectiveness, the dry mass of nodules, roots and aerial part and the relative content of total chlorophylls were evaluated, in controlled conditions. A completely randomized experimental design was used and the data were processed using a bifactorial arrangement. *Rhizobium leguminosarum* CF1 strain maintained a concentration of 10^8 CFU ml^{-1} or higher for 150 and 90 days, when Azofert®-F was stored at 4 and 29±2 °C, respectively. Product storage for 120 days at both temperatures did not affect the number of nodules in bean plants. Nevertheless, temperature and inoculant storage time, influenced the effectiveness of formed nodules and the relative content of total chlorophylls, respectively. This research is the firstone about the conservation influence on the efficacy of a Cuban commercial inoculant based on *Rhizobium* for beans.

Key words: *Rhizobium*, inoculant, microbiological stability, bean.

RESUMEN: El objetivo del presente trabajo fue determinar el efecto de la temperatura y el tiempo de conservación en la concentración del principio activo y la actividad biológica del biofertilizante Azofert®-F. Inoculantes a base de la cepa *Rhizobium leguminosarum* CF1 se mantuvieron a 4 y 29±2 °C, para evaluar la pureza y concentración de la cepa cada 30 días durante 180 días. Los datos se procesaron mediante un análisis de varianza de clasificación simple. A los 40 y 120 días de conservación de los inoculantes, se aplicaron en semillas de frijol cultivar Tazumal y se evalúo el número de nódulos y su efectividad, la masa seca de nódulos, raíces y parte aérea y el contenido relativo de clorofilas totales, en condiciones controladas. Se empleó un diseño experimental completamente aleatorizado y los datos se procesaron mediante un arreglo bifactorial. La cepa *Rhizobium leguminosarum* CF1 mantuvo una concentración de 10^8 UFC mL^{-1}, o superior, durante 150 y 90 días, cuando el producto Azofert®-F se conservó a 4 y 29±2 °C, respectivamente. El almacenamiento del producto hasta 120 días, en ambas temperaturas, no afectó el número de nódulos en las plantas de frijol. Sin embargo, la temperatura y el tiempo de almacenamiento del inoculante influyeron en la efectividad de los nódulos formados y en el contenido relativo de clorofilas totales, respectivamente. Esta investigación es la primera que aborda la influencia de la conservación en la eficacia de un inoculante comercial cubano a base de *Rhizobium*, para el cultivo del frijol.

Palabras clave: *Rhizobium*, biofertilizantes, estabilidad en almacenamiento, frijol.

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INTRODUCTION

The common bean (*Phaseolus vulgaris* L.) is a crop of great importance in human nutrition. Its grain is rich in protein, provides micronutrients, fiber and starch (1, 2). This legume demands high amounts of nitrogen, an element that it obtains to a great extent through Biological Nitrogen Fixation (BNF) (3, 4).

Among the microorganisms that carry out BNF are rhizobia, bacteria that are traditionally used as an active principle in the biofertilization of legumes. The use of biofertilizers in agricultural practice is considered a viable alternative for its ecological, economic and productive benefits; it allows reducing the use of mineral fertilizers (5, 6) and increasing the yield of economically important crops (7, 8).

Several biofertilizers are produced and marketed in Cuba, including Azofert® from the National Institute of Agricultural Sciences (INCA). In particular, Azofert®-F is used in the cultivation of beans; it is a liquid inoculant that contains high concentrations of the bacterial strain *Rhizobium leguminosarum* CF1 (CF1) and nodulation inducers, an attribute that distinguishes it from other commercial inoculants based on rhizobia in the country (9). Its direct application to bean seeds at the moment of sowing allows saving between 50-70 % of nitrogen fertilizer (10, 11).

The absence of contaminating microorganisms, the concentration of viable cells and their physiological state are some of the factors that influence the successful performance of biofertilizers (12). Quality standards governing the use of these biopreparations vary depending on the regulations in force in each country. However, a minimum concentration of the active ingredient of 1 x 10^9 CFU ml^{-1} or g^{-1} at the time of preparation and 1 x 10^8 CFU ml^{-1} or g^{-1} at maturity, with minimal or no presence of contaminating microorganisms, is recommended by consensus (13).

The shelf life of biofertilizers is essential for their commercialization, due to the long time that can elapse between their production and application (14). Therefore, determining the period of effectiveness of these bioproducts, according to the conservation capacities available, is essential to delimit their validity time and to be able to establish a production and commercialization strategy that satisfies the demand at the opportune moment of sowing.

There are few studies in Cuba that address the microbiological stability over time of liquid commercial inoculants. The active principles most used in the country are Plant Growth Promoting Bacteria such as *Azospirillum*, *Azotobacter* and *Mesorhizobium* (15-17). In most of these studies it is not possible to access the details of the research from the search engines available on the Internet and in many cases access to the information is restricted. Only one previous study determined the effect of temperature on the conservation of a commercial rhizobia-based inoculant for soybean (18). Considering the above, the objective of this study was to determine the effect of temperature and storage time on the concentration of the active ingredient and the biological activity of the biofertilizer Azofert®-F.

MATERIALS AND METHODS

Pre-inocula were prepared in 100 mL capacity Erlenmeyers containing 10 mL of sterile Bradyfact culture medium (9). For this purpose, a hoe of strain CF1 preserved in tubes containing solid Yeast-mannitol (LM) medium (19) with Congo red was used. Flasks were kept in agitation at 130 rpm and 28 °C, for 16 h. Subsequently, the strain multiplication by aerobic fermentation was continued under the same incubation conditions. In each case, 10 % (inoculum volume / medium volume) was inoculated until a total volume of 1000 mL of inoculum was obtained. The fermentant was formulated with 1000 mL of sterile Bradyfact medium in a 1:1 ratio.

The formulation (Azofert®-F) was packaged in ten sterile bottles of 240 mL total volume, each containing 200 mL of the product. Two treatments were established with five vials each. One treatment was stored at a refrigerated temperature of 4 °C and the other at an average room temperature of 29±2 °C.

Effect of temperature and storage time on the strain CF1 concentration in Azofert®-F

The trial ran from October 2017 to February 2018. Three flasks stored at each temperature condition were randomly selected and the microbiological purity of the inoculum was determined by Gram staining. The morpho-staining characteristics described for bacteria of the rhizobia group (20) were used as distinguishing criteria.

The number of colony forming units (CFU mL^{-1}) of strain CF1 was evaluated at the time of inoculant preparation and every 30 days under both temperature conditions. For this purpose, serial decimal dilutions of the inoculant were made and grown by spreading on plates with solid LM medium with Congo red. Cultures were incubated at 28 °C for 72 h. The calculation of the CFU number was performed using the formula:

$$\text{UFC mL}^{-1} = \text{No. col} \times 10^1 \times \text{d}$$

where:
col. no. - number of colonies
d - dilution factor

Azofert®-F application Effect stored at different temperatures and storage times on nodulation, growth and chlorophyll content of bean plants

Inoculation trials were carried out in pots of 973.90 cm^3 total volume, containing 0.2 kg of typical eutrophic Ferrallitic Red Leached soil (21), from the central area of INCA. The substrate was extracted at a depth between 0-20 cm and its chemical analysis was carried out according to the manual for soil analysis, foliar, organic fertilizers and chemical fertilizers (22). The soil had a slightly acid pH, medium

organic matter content, low Na^+ and Mg^{2+} and high K^+ and P. The Ca^{2+} content corresponded to what is normally reported for this type of soil (23) (Table 1).

The concentration of possible resident rhizobia in the substrate was also determined. For this, one gram of soil was added to 9 mL of sterile distilled water and serial decimal dilutions were performed. An aliquot of 0.1 mL was grown on plates with solid LM medium with Congo red and the culture was incubated for 10 days at 28 °C. The substrate had a concentration of 3 x 10^3 CFU g $soil^{-1}$of potential rhizobia.

To evaluate the effect of the formulation on nodulation and growth of bean plants, two pot trials were established, with five plants per treatment each. In the first, inoculants were used with 40 days of storage at 4 and 29±2 °C, with a bacterial concentration of 10^9 and 10^8 CFU ml^{-1}, respectively. In the second trial, inoculants with 120 days of conservation at 4 and 29±2 °C and with a concentration of 10^8 and 10^7 CFU ml^{-1}, respectively, were used.

Two bean seeds per pot were sown and each pot was inoculated with 200 µL of the inoculants described above. Pots were placed in trays with Hoagland's nutrient solution (24), lacking solution A (rich in nitrogen salts) in order to enhance BNF. The plants were maintained under controlled conditions, at 25±2 °C, 70 % relative humidity and photoperiod of 16 h light/8 h dark. Ten days after emergence, one seedling was removed from each pot.

Thirty days after planting, the number of total nodules (u) and the number of effective total nodules (u) were determined. The effectiveness of the nodules was assessed by cutting the nodules crosswise with a scalpel and observing the internal coloration. A red, pink or brown coloration inside the nodules was interpreted as effective in BNF (25). The dry mass of the total nodules (g), the dry mass of the aerial part of the plants (g) and the dry mass of the root system (g) were evaluated with a digital balance, model TE214S (Sartorius brand). The relative total chlorophyll content was determined in the third leaflet from bottom to top (SPAD units), with a portable chlorophyll meter (MINOLTA SPAD 502 Plus).

Design and statistical analysis

CF1 strain concentration data were analyzed by simple rank analysis of variance. Tukey's mean comparison test for $p<0.05$ was used to discriminate differences between treatments. Data were processed in Statgraphics Plus Program version 5.1 (2001) and plotted in Microsoft Excel Program, 2016.

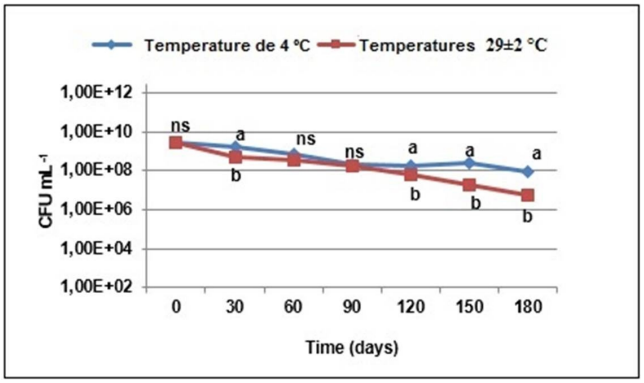
In the plant inoculation trial, a completely randomized experimental design was used and the results were subjected to a bifactorial arrangement and two factors were taken into account: temperature, with the levels: 4 and 29±2 °C and time with the levels: 40 and 120 days. The data were subjected to Between's test and processed in the statistical program SPSS version 21.

RESULTS AND DISCUSSION

Storage of Azofert®-F at 4 °C maintains higher concentrations of strain CF1 over time.

The culture of the inoculants in solid LM medium showed the formation of large and mucous colonies that do not absorb the Congo red dye. Gram staining allowed observing Gram-negative bacilli, without endospores and the absence of contaminating microorganisms. These characteristics coincide with those described for strain CF1, the active ingredient of Azofert®-F (20).

A common concern in the production of inoculants is the survival of the active ingredient, which is why the concentration of its cells is one of the most important quality parameters (26). This study addressed this problem, according to the existing storage possibilities for Azofert®-F preservation. The results showed that the storage of Azofert®-F at 4 °C maintained higher concentrations of the CF1 strain over time (Figure 1).



Statistical analysis was performed at each evaluation time. Means with equal letters do not differ statistically (Tukey p≤0.05, n=3)

Figure 1. Concentration of *Rhizobium leguminosarum* CF1 strain in Azofert®-F biofertilizer, stored at 4 °C and 29±2 °C, for 180 days

Table 1. Some chemical characteristics of the substrate Ferrallitic Red Leached Soil typical eutrophic leachate used in the inoculation trials

pH	Organic matter (g kg^{-1})	Na^+	K^+	Ca^{2+}	Mg^{2+}	P_2O_5 (mg kg^{-1})
		$cmol_c$ (kg^{-1})				
6.7	34.9	0.09	0.56	14.5	1.0	218.0

pH by potentiometry: soil/solution ratio 1:2.5; organic matter by colorimetry (Walkley Black); exchangeable cations Ca2+ and Mg2+ (complexometry by extraction with NH_4Ac at 1 mol L^{-1} at pH 7); K^+ and Na^+ by flame photometry; P by extraction with sulfuric acid at 0.1 N, Oniani's method

The concentration of strain CF1 remained above 1 x 10^8 CFU ml^{-1} when stored at 4 °C for 150 days, while at 29±2 °C it remained at those levels for only 90 days. Storage of Azofert®-F at 4 °C showed higher viability values than those obtained for the bioproduct stored at room temperature at 30, 120, 150 and 180 days after processing. In similar studies, with inoculants of *Sinorhizobium meliloti* in LM culture medium and stored at 4 °C, it is reported that the number of viable cells decreased considerably after 90 days (27). This difference in behavior could have been due to differences between the strains and the culture medium used in their multiplication for both investigations. The culture medium used in the production of Azofert® (9) is a nutrient-rich medium that differs from the chemical composition of LM medium (28).

Strain CF1 in the inoculant decreased in concentration by one logarithmic unit at 30, 120 and 180 days of storage at room temperature. Other investigations with liquid inoculants of *Bradyrhizobium japonicum* showed the same behavior at 30 and 180 days of storage at 27-29 °C (29). At the end of the trial, at 180 days at room temperature, strain CF1 in Azofert®-F showed cell concentration values of 106 CFU mL^{-1}. In contrast to other authors who reported that Pseudomonas strains at 28±2 °C did not survive in the inoculant after 150 days of storage (30).

The results show that temperature and time affect the microbiological stability of Azofert®-F. Temperature, type of formulation and storage time are known to have a significant effect on the cell viability of the active ingredient of inoculants (31). Low temperatures slow down physiological processes of the bacterial cell and its aging (32), while temperatures close to 30 °C increase the consumption of the nutrients of the medium by the bacteria, which causes a more accelerated depletion of these nutrients and thus a decrease in cell viability (33).

Knowing the shelf life of the bioproduct Azofert®-F, under the storage conditions available in the country, makes it possible to establish a production strategy that covers the greatest possible demand for the product, with sufficient availability at the time of bean planting in Cuba.

Temperature and storage time influence the biological activity of Azofert®-F in Tazumal cultivar bean plants.

Few studies have evaluated the effect of temperature and storage time on the biological activity of inoculants based on Plant Growth Promoting Bacteria. This is the first research in Cuba that addresses this issue in a commercial inoculant for beans, one of the most important crops for Cubans.

The analysis of the data from the inoculation trial under controlled conditions showed that there was interaction between the factors temperature and time in the variables dry mass of nodules, roots and aerial part of bean plants. For the rest of the variables there was no interaction between the two factors (Table 2).

Plants treated with bacterial inoculants that had been stored for 40 days at 29±2 °C showed the most favorable results in the dry mass of nodules, indicating a higher content of bacteroids established in them. The dry mass of root and aerial part was favored with the application of inoculants preserved for 40 days at 4 °C (Table 3). It is logical that the highest activity is found in the shortest time evaluated, which corresponds to the highest cell concentration values found in the inoculants: 10^8 and 10^9 CFU ml^{-1}, respectively.

Other investigations report that the radical and aerial dry mass of pea (*Pisum sativum* L.) and soybean (*Glycine max* (L.) plants is not affected, when treated with inoculants based on rhizobia that were conserved for 15, 60, 120 and 180 days at 4 and 28±2 °C, and that have a cell concentration of 10^8 CFU ml^{-1} at the moment of inoculation of the seeds (33).

On the other hand, results showed that there was an influence of the temperature factor on the number of effective nodules and of the time factor on the relative content of total chlorophylls (Table 4).

Inoculation of Azofert®-F when kept at 4 °C resulted in a higher number of effective nodules in plants. In addition, the use of inoculants that were kept for 40 days enhanced the relative content of total chlorophylls. Previous research showed a similar effect of Azofert®-F on the Cubacueto 25-9 cultivar, seven days after processing (34). Therefore,

Table 2. P value of factors and interaction terms in the ANOVA analysis for the variables of nodulation and plant growth of bean cultivar Tazumal, under controlled condition

Origin	Number of total nodules (u)	Number of total effective nodules (u)	Dry mass of total nodules (g)	Dry mass of roots (g)	Dry mass of aerial part (g)	Relative content of total chlorophylls (SPAD)
Corrected model	0.152	0.079	0.000	0.000	0.000	0.002
Intersection	0.000	0.000	0.000	0.000	0.000	0.000
[a] Temperature	0.106	0.051	0.002	0.007	0.027	0.236
[b] Time	0.511	0.282	0.000	0.003	0.000	0.000
[c] Temperature-Time	0.123	0.136	0.001*	0.007*	0.025*	0.634

[a]Temperature factor with two levels: 4 °C and 29±2 °C

[b]Time factor with two levels: 40 and 120 days.

[c]Combined effect of Temperature and Time factors

(*) Interaction between factors

Table 3. Combination effect of Temperature and Time of inoculant Azofert®-F conservation factors on the variables of nodulation and growth of bean plants that showed interaction

Treatments		Nodular dry mass (g)	Dry mass of roots (g)	Dry mass of aerial part (g)
Temperature (°C)	Time (days)			
4	40	0.033 ± 0.006 b	1.23 ± 0.32 a	1.08 ± 0.05 a
	120	0.022 ± 0.002 b	0.18 ± 0.02 b	0.40 ± 0.05 c
29±2	40	0.074 ± 0.006 a	0.24 ± 0.02 b	0.82 ± 0.05 b
	120	0.019 ± 0.005 b	0.17 ± 0.02 b	0.40 ± 0.04 c
Esx		0.008	0.22	0.07

Data show means + standard error of the mean. Equal letters in the same column show significant differences (Tukey HSD p<0.05, n=5)

Table 4. Independent effect of Temperature and Storage time of the Azofert®-F inoculant factors on the variables that did not show interaction

Treatments	Number of total nodules (u)	Number of effective nodules (u)	Relative total chlorophyll content (SPAD)
		Temperature (°C)	
4	31.1 ± 3.6 a	29.1 ± 3.8 a	27.6 ± 1.4 a
29±2	23.2 ± 3.1 a	19.8 ± 2.6 b	29.1 ± 1.2 a
		Time (days)	
40	25.6 ± 2.1 a	22.0 ± 1.7 a	31.3 ± 0.9 a
120	28.7 ± 4.6 a	26.9 ± 4.7 a	25.4 ± 0.8 b

Data show means + standard error of the mean. Equal letters in the same column show significant differences (Tukey HSD p<0.05, n=5)

this research suggests that the product is able to maintain its biological activity in plants 40 days after processing (Table 4).

Neither the storage temperature nor the storage time of the inoculants influenced the formation of nodules on the bean plants, which could be due to the fact that the ability of strain CF1 to establish symbiosis with the bean plants was maintained for a period of at least 120 days under both conditions. Previous studies, with rhizobia-based inoculants stored at 4 and 28±2 °C for 15, 60, 120 and 180 days, show similar behavior in pea and soybean crops (33).

Other research indicates that the application of *Bradyrhizobium* inoculants that were stored for 180 days at 4 °C caused an increase in the number of effective nodules in soybean plants with respect to those where inoculants stored at 29±2 °C were used (30). In addition, inoculants of *Pseudomonas fluorescens* maintained for a period of 180 days at 28±2 °C promoted the growth of tomato plants (*Lycopersicon esculentum*, Mill) and decreased the wilting of leaves by *Fusarium* (35).

The positive effect of Azofert®-F application preserved for 40 days on chlorophyll content corresponded with the best results on the nodular dry mass and the dry mass of the aerial part of bean plants (Table 3). At that time, the inoculant presented a high concentration of strain CF1 (10^8-10^9 CFU ml^{-1}). A higher concentration of the strain in the inoculant leads to a greater number of cells in contact with roots, and therefore, would increase the sites of infection, the formation of nodules and the establishment of bacteroids, which increases the nodular mass. An increase in effective nodulation in the BNF allows a greater nitrogen supply (36), which would lead to an increase in photosynthetic pigments and aerial biomass. This evidence was corroborated in previous research with cowpea (*Vigna unguiculata* L.), common bean, soybean, and canavalia (*Canavalia ensiformis*) (8, 10, 37, 38).

CONCLUSIONS

- It was demonstrated that the inoculant Azofert®-F could be conserved at 4 and 29±2 °C, for 150 and 90 days, respectively, without the need for preservatives in its formation and that up to 120 days of conservation it does not affect the formation of nodules in bean plants. These evidences would allow to establish a strategy of production and commercialization of the inoculant with adequate quality during the whole year that assures to cover the demands of the product, fundamentally in the sowing time.

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