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Original article

Microbiological stability of Azofert®-F and Azofert®-S biofertilizers

Estabilidad microbiológica de los biofertilizantes Azofert®-F y Azofert®-S

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ABSTRACT: Azofert® is a biofertilizer with a diazotrophic bacteria named rhizobia for the legumes inoculation. The present work aimed to evaluate the microbiological quality of the Azofert®-F and Azofert®-S biofertilizers with. Inoculants were made from two strains *Rhizobium leguminusarum* and *Brayrhizobium elkanii* and it was stored at 4 and 32 °C. The concentration of both strains was determined by the serial dilutions method. In addition, the presence of contaminants in the inoculants was determined by Gram staining. Results showed that inoculants of both strains remained pure throughout the experiment. The inoculants stored at 4 °C maintained a cell concentration of 10^8 CFU mL for longer. This concentration is suitable for the use of these products in the field. These results allow establishing a productive strategy of the Azofert®-F and Azofert®-S Cuban inoculants, according to the available temperature conditions.

Key words: conservation, Rhizobium, temperature, viability.

RESUMEN: Azofert® es un biofertilizante a base de bacterias diazotróficas denominadas rizobios para la inoculación de leguminosas de interés agrícola. El presente trabajo tuvo como objetivo evaluar la calidad microbiológica de los biofertilizantes Azofert®-F y Azofert®-S. Se elaboraron inoculantes a base de dos cepas: *Rhizobium leguminusarum* y *Brayrhizobium elkanii* y se conservaron a 4 y 32 °C. Se determinó la concentración de ambas cepas mediante el método de las diluciones decimales seriadas. Además, se determinó la presencia de contaminantes en los inoculantes, mediante tinción de Gram. Los resultados mostraron que los inoculantes de ambas cepas se mantuvieron puros durante todo el experimento. Los inoculantes que se conservaron a 4 °C mantuvieron una concentración celular en el orden de 10^8 UFC mL^{-1} durante el mayor tiempo. Esta concentración es adecuada para el empleo de estos productos en el campo. Los resultados obtenidos permitirían establecer una estrategia productiva de los inoculantes cubanos Azofert®-F y Azofert®-S, según las condiciones de temperatura disponibles.

Palabras clave: conservación, Rhizobium, temperatura, viabilidad.

INTRODUCTION

The indiscriminate use of mineral fertilizers in agriculture has caused damage to different ecosystems. Soil erosion and nitrate contamination of surface and groundwater are evidence of the poor management of fertilizers in agriculture. This constitutes a problem for the health of all living beings that inhabit these ecosystems (1). In this sense, the use of biofertilizers in agricultural practice is considered a viable alternative, as it is an economical and ecologically sound resource; and because it allows the use of mineral fertilizers to be reduced (2) and crop yields to be increased (3-6).

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Various microorganisms called Plant Growth Promoting Rhizobacteria are the active ingredient in biofertilizers. Among them are rhizobia, diazotrophic bacteria that have been studied mainly because of the symbiotic association they establish with leguminous plants. This bacterial group is able to satisfy 50-100 % of the nitrogen needs of crops of agricultural interest (6).

Inoculants based on these bacteria have been developed and applied in agricultural production systems. Such byproducts contain high concentrations of bacteria and the direct application of low doses (200 mL of inoculant per 50 kg seeds) to seeds at the time of sowing (7) saves between 50-70 % of chemical fertilizer in beans (*Phaseolus vulgaris* L.) (8,9) and up to 100 % in soybeans (*Glycine max* L.) (10).

Among the biofertilizers that are widely used in Cuba for leguminous plants is Azofert®. This byproduct is currently a liquid inoculant that differs from other national biofertilizers in that it contains high concentrations of nodulation factors, a decisive sign in the rhizobium-legume symbiosis (11,12). Azofert®, produced and marketed at the National Institute of Agricultural Sciences (INCA), has three registered products: Azofert®-S (for soybean), Azofert®-F (for bean) and Azofert®-Can (for *Canavalia ensiformis*), which have been successfully validated in different soil and climatic conditions as they increased nodulation, growth and yields of these crops (10,13,14).

However, in many cases, after the application of inoculants in the field, a positive effect on crop growth and yield is not observed. This behavior has been attributed, among other factors, to low concentrations and unfavorable physiological conditions of the bacterial cells present in the inoculants (15-17).

In view of the above, it has been established that the concentration of the micro-organisms that make up these products is one of the most important quality parameters, as it determines the colonization success and the subsequent establishment of the symbiosis between the two organisms (18). The required concentrations of rhizobia in the inoculant vary around the world. A common criterion is considered to be a minimum concentration in the order of 108 CFU mL^{-1} or g^{-1} and minimal or no contamination (19). The objective of the present work was to evaluate the viability over time of the rhizobial strains that form part of Azofert®-F and Azofert®-S biofertilizers.

MATERIALS AND METHODS

The study was carried out at the Biofertilizer Production Plant of the Plant Physiology and Biochemistry Department of INCA. The viability of *Rhizobium leguminusarum* CF1 (CF1) and *Bradyrhizobium elkanii* ICA 8001 (ICA 8001) strains from the Soil Institute's and INCA's strains, respectively, was evaluated. These strains constitute the active fraction of Azofert®-F and Azofert®-S biofertilizers, produced and marketed by INCA, for inoculation of bean and soybean crops (20,21).

Colonies isolated from strains CF1 and ICA 8001, previously grown on Petri dishes with solid yeast-mannitol medium with Congo red at 28 °C for three and seven days, respectively, were multiplied in 100 mL capacity Erlenmeyers containing 20 mL of sterile Bradyfact culture medium (18). The flasks were kept under shaking conditions at 130 rpm and 28 °C for 16 hours for strain CF1 and 72 hours for strain ICA 8001. From these pre-inocula, the multiplication of the strains by aerobic fermentation was scaled up under the same temperature and incubation conditions to a total volume of 2000 mL. 200 mL of each inoculum were packed in sterile 240 mL bottles and two treatments were established in each of them, the first one was kept at 4 °C and the second one at an average room temperature of 32 °C. The microbiological quality of the inoculants was determined, taking into account the purity and concentration of viable cells of CF1 and ICA 8001 strains in the inoculants.

Culture purity was verified by Gram staining at the beginning and at the end of the experiment. The morphological characteristics, the response to staining and the presence of endospores in the cells of both bacterial strains were taken into account. In addition, the presence of contaminating micro-organisms in the inoculum was determined (22).

To determine the concentration of viable CF1 and ICA 8001 strains, three samples were taken. From these, serial decimal dilutions were made from which 0,1 mL were grown on plates with solid yeast-mannitol medium (23). Plates were incubated at 30 °C for three days for strain CF1 and seven days for strain ICA 8001. The number of colonies per plate was quantified and the number of CFU mL^{-1} was determined according to the expression:

$$\mathrm{CFU mL^{-1}} = \mathrm{No.col x 10^{-1} xd}$$

Where:

$\mathrm{No.col}$: number of colonies

d: dilution factor

These evaluations were carried out until the concentration of the strains in the inoculants was below 10^8 CFU mL^{-1}. In the biofertilizers Azofert®-F, conserved at both temperatures, the evaluations were carried out every seven days during the first 42 days and then every 21 days, while in Azofert®-S it was sampled every 30 days.

Statistical analysis

A completely randomized experimental design was used in all experiments. The data obtained were processed by a simple rank analysis of variance. Tukey's mean comparison test for $p<0.05$ was used to discriminate differences between treatments (24). Data were processed in Statgraphics Plus version 5.1, 2001 and plotted in Microsoft Excel, 2016.

RESULTS AND DISCUSSION

Proper preservation of inoculants after processing is an important aspect to preserve the microbiological quality of inoculants and thus their marketability and effectiveness in the field. The main parameter for measuring the shelf life of

inoculants is an adequate number of viable cells capable of adapting and surviving in the medium once inoculated on the seeds (19). For this reason, in this research it was of great interest to carry out a study from the microbiological point of view of two Cuban biofertilizers, Azofert®-F and Azofert®-S.

Gram staining allowed the observation of Gram-negative bacilli without endospores, which is in agreement with the morphological characteristics of the inoculants Azofert®-F and Azofert®-S, made from rhizobial strains of the genera *Rhizobium* and *Bradyrhizobium* (20,21). In neither of the two inoculants was the presence of microorganisms with other morphology and response to staining observed.

The strain CF1 viability in Azofert®-F, preserved at the different temperatures, is shown in Figure 1.

The results showed that strain CF1 concentration was maintained at 1 x 10^8 CFU mL^{-1}, which is considered optimal for its use, for up to 126 days at 4 °C. However, this bacterial concentration was maintained in only 28 days in those inoculants that were kept at 32 °C. Both treatments differed significantly from each other after 35 days of storage in favor of those kept at refrigerated temperature.

In similar studies, two strains of *Bradyrizobium japonicum* have been reported to be stored at 5 and 28 °C, where one strain remained three times longer viable (180 days) than the other (60 days) when kept at 5 °C. In addition, this study found that evaluations of inoculants kept at room temperature showed a decrease of one logarithmic unit after 30 days for one of the *Bradyrhizobium* strains studied (25).

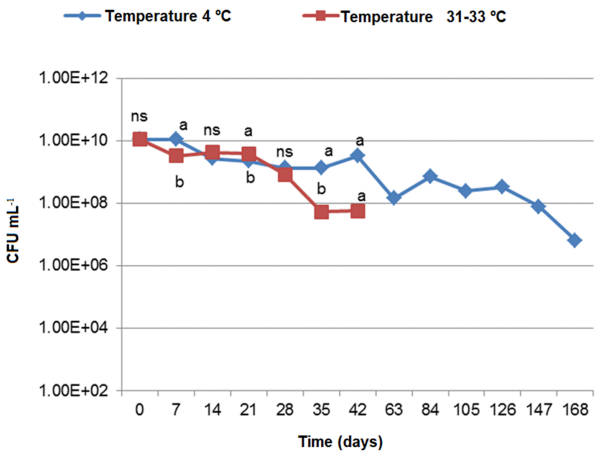
On the other hand, the viability of strain ICA 8001 in the soybean inoculant Azofert®-S, at different storage temperatures, is shown in figure 2.

In the first 30 days of evaluation, cell viability did not show significant differences between treatments. From this moment on, storage at 4 °C positively affected the number of cells in the inoculant, as it maintained, for 280 days, acceptable values for the application of the inoculant in the field. Inoculants stored at 32 °C showed viability values suitable for use only up to the first 60 days. After that time, the cell concentration decreased to values not suitable for use.

In contrast to the results obtained in this study, other authors have reported that the concentration of *Azospirillum brasiliense* in the liquid inoculant preserved at 4 °C does not exceed 60 days (26).

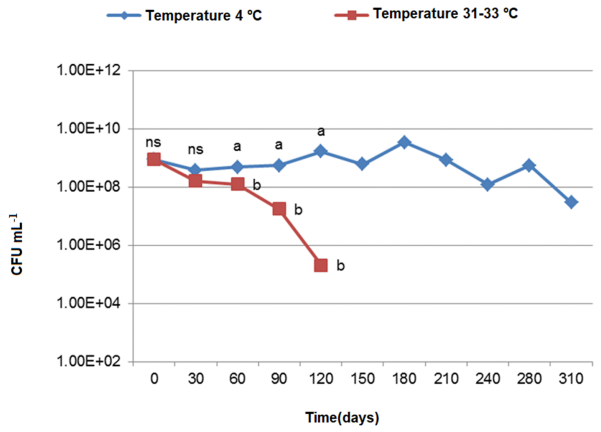
Temperature (26,27), bacterial species and formulation are some of the factors that most influence the maintenance of the cell concentration of the active agent in inoculants (28). Rhizobia have an optimum growth temperature of 30 °C (29,30). At these temperatures, the consumption of nutrients in the medium is favored (31), leading to a more accelerated depletion of nutrients, with a consequent decrease in bacterial viability (32). This may explain, to some extent, the rapid decline in viability in inoculants stored at room temperature.

At temperatures between 4 and 6 °C, the storage period of rhizobia increases, due to the reduced metabolic activity



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

Figure 1. Viability of *R. leguminosarum* CF1 strain in Azofert®-F biofertilizers, stored at 4 °C and 32 °C, for 42 days



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

Figure 2. Viability of *B. elkanii* strain ICA 8001 in Azofert®-S biofertilizers, stored at 4 °C and 32 °C, for 120 days

of the cells (33). The preservation of micro-organisms at these temperatures allows high bacterial survival, cell stability and purity of the cultures (34). This could explain the longer survival of strains CF1 and ICA 8001 when stored at 4 °C.

In the studies conducted in this investigation, two strains belonging to the rhizobial group were used, preserved in the same culture medium and at the same temperatures. However, they behaved differently under similar preservation conditions. The species (genotype) to which a micro-organism belongs is one of the factors that could explain this behavior. Previous studies have shown that the multiplication rate of *Rhizobium* sp. strains is several times higher than that of ICA strain 8001 (35), with a maximum cell multiplication at 24 and 55 hours, respectively.

Furthermore, it has been shown that rhizobia of the genus Rhizobium have a shorter half-life than slow-growing species (*Bradyrhizobium*) during storage (28).

Although this study showed a positive effect of refrigeration on cell viability, there was evidence of a decrease in the concentration of bacterial strains over time. During the life cycle of micro-organisms, they pass through various phases with different characteristics, where they may or may not find the conditions for growth and cell division. Ageing of the culture, for example, leads to the accumulation of toxic compounds in the culture medium and the depletion of existing nutrients (32), resulting in a decrease of the active agent in the inoculants. Hence the importance of developing formulations where appropriate preservatives are used to increase the shelf life of these products.

CONCLUSIONS

The storage temperature is a factor influencing the viability and concentration of the active ingredient of the Azofert®-F and Azofert®-S biofertilizers. The storage of these products at 4 °C maintains for a longer time an order of 10^8 CFU mL^{-1} of the strains *Rhizobium leguminusarum* CF1 and *Bradyrhizobium elkanii* ICA 8001, a concentration that is considered adequate for use in the field. These results allow the establishment of a productive strategy for inoculants, according to temperature conditions available.

RECOMMENDATIONS

Taking into account these results, it is essential to carry out studies on formulations with agents that allow the preservation of high concentrations of the active ingredient of these products for a longer period of time, thus increasing their shelf life.

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