Shelf Life of *Azospirillum brasilense* in Alginate Beads Enriched With Trehalose and Humic Acid

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Abstract

Since abiotic and biotic factors can compromise the survival of bacteria and their viability, encapsulation of cells in biodegradable gel matrices, a biological macromolecule, is one alternative to have their shelf life extended. Here, it was developed a gel-based formulation of the bioinoculant *Azospirillum brasilense* strain AbV5 and determined the effect of trehalose and humic acid supplementation in viability and survival of bacteria. For each 2 ml of sodium alginate solution (3%), 1 ml of the inoculum was extruded in a solution containing sodium alginate complexed with calcium chloride, forming calcium alginate beads. Supplements were used in a ratio of 2:2:1. Treatments were peat; alginate; alginate + humic acid; alginate + trehalose 0.1 M; alginate + trehalose 1 M. Morphometric aspects, survival rate and viability were determined in 9 storage periods (3, 5, 7, 14, 21, 30, 45, 60, 90 days). As results, beads were able to sustain the growth of *A. brasilense* for 90 days. Shelf life quality decreased in all treatments and peat remained the best carrier. Encapsulation, despite promoting the greatest losses in the survival of bacteria in the first days, ensured better cell viability. Trehalose in low concentrations (0.1M) improved cell viability during storage, optimizing plant inoculation.

Keywords: additives, encapsulation, inoculants, survival and viability

1. Introduction

World food production is based on the extensive use of chemical fertilizers, which not only pollute the environment but also are expensive due to their non-renewable sources like fossil fuels, used in their exploitation, transportation and application (Schoebitz, López, & Roldán, 2013). Therefore, eco-friendly and economical alternatives have been increasingly demanded. Among some of these alternatives, plant-growth promoting bacteria (PGPB) are sustainable and low-cost biofertilizers, but need specific formulation when used in agronomical practices (Malusa & Vassilev, 2014).

Biofertilizers as inoculants must have 3 fundamental characteristics: to promote bacterial growth; to keep the cells viable for a certain period of time and to release a minimum population of bacteria, which will certainly be associated to plants (Yoav Bashan et al., 2014; Shcherbakova et al., 2018). Microbial survival after soil inoculation depends on both abiotic and biotic factors. The population of the inoculated bacteria declines progressively over time, preventing the accumulation of a bacterial pool in the rhizosphere sufficient to promote beneficial effects (Yoav Bashan, 1998; Sivakumar, Parthasarthi, & Lakshmipriya, 2014).

Nutritional conditions, humidity, temperature, and pH of soil solution are factors that compromise the survival of bacteria in the rhizosphere. In addition, the survival of the inoculated bacteria depends to a large extent on the availability of a specific niche in which competition for nutrients or substrates does not exist. It also depends on the resistance to predation and/or on the mutualistic coexistence with the native microflora, generally more adapted (Reetha, Kumaresan, & John Milton, 2014; Schoebitz et al., 2013). Peat is the most common carrier used in the inoculant industry and it is widely recommended to several plant crops (Yoav Bashan et al., 2014). It has a large water adsorption capacity, promoting a favorable microenvironment to cell growth and maintenance

(Kaljeet, Keyeo, & Amir, 2011). However, it has as disadvantages little protection to the stresses caused by storage and environmental stresses caused to the bacteria after inoculation (Yoav Bashan, 1998).

Alternatives that aim the improvement of viability of bacteria and extension of shelf life are important for the emergence of a greater number of types of inoculants that combine with different bacterial species and methods (see review Berninger, Mitter, & Preininger, 2016). One of the most successful, safe and effective methods to add bacteria to the soil is by encapsulation of cells in biodegradable gel matrices (Cassidy, Lee, & Trevors, 1996; Vassilev et al., 2015). The hydrogel is formed by the interaction between sodium alginate and Ca^{+2} ions via the ionotropic gelation mechanism (Burey, Bhandari, Howes, & Gidley, 2008). Alginate is a natural polymer composed of β-(1→4)-linked D-mannuronic acid and α-(1→4)-linked L-guluronic acid, both produced by brown algae (*Macrocystis pyrifera*), as well as by bacteria (*Pseudomonas* sp and *Azotobacter* sp) (Hay, Rehman, Ghafoor, & Rehm, 2010; Nehra & Choudhary, 2015).

The addition of supplements in the encapsulation procedure could optimize the survival and release of bacteria from the inoculant. Humic acid of high molecular weight and colloidal appearance has been efficient in improving the survival of encapsulated microorganisms (Reetha et al., 2014; Young, Rekha, Lai, & Arun, 2006). Trehalose, in turn, is a disaccharide that can be used as source of energy and as protector against dehydration. Trehalose can increase the viability of freeze-drying cells as 70% of them survived after drying (Leslie, Israeli, Lighthart, L. Crowe, & J. Crowe, 1995), compared to other adjuvants (Pereira, Oliver, Bliss, L. Crowe, & J. Crowe, 2002). Nevertheless, the effect of trehalose and humic acid on the survival and viability of alginate encapsulated cells, in a short period of storage, remains unknown.

The objective of this paper was to develop the gel-based formulation of the bioinoculant *Azospirillum brasilense* strain AbV5 and to determine if trehalose and humic acid can effectively enhance the viability and survival of bacteria along storage period.

2. Method

2.1 Encapsulation and Gel-based Bioinoculant Formulations

A. brasilense strain AbV5 was maintained by continuous cultivations in NFb Lactate solid medium at 28 °C. The pre-inoculum was prepared by transferring a bacteria colony into a 5 mL of NFb Lactate medium at 32 °C in a shaker incubator. After 24 h, 1 mL of pre-inoculum was transferred to 50 mL of NFb-Lactate medium, constituting the inoculum, which had been maintained under the same conditions as described before. The log phase of cell growth was measured by turbidimetry at 600 nm.

The encapsulation of *A. brasilense* cells in beads was performed according to the protocol proposed by Reetha et al. (2014) with modifications. The proportion was 2:1 in order to obtain beads (for each 2 mL of sodium alginate solution (3%), 1 mL of the bacterial suspension was added). The ratio was 2:2:1 when the supplement was used. To avoid fungal and other bacterial contamination, Maxim'sTM (0.01% v/v) and nalidixic acid (20 μg mL^{-1}) were added.

Before extrusion, the mixture was kept under gentle stirring for 30 min in sterile conditions for complete homogenization. The mixture was extruded through a Pasteur pipette into a beaker containing sterile 0.1 M CaCl$_2$ solution, under gently stirring at room temperature. The macrobeads were maintained in CaCl$_2$ solution for 2 h so that solid beads of homogeneous size could be formed. The CaCl$_2$ solution was drained and the beads washed twice with sterile water. After washing, the beads were incubated in NFb-Lactate liquid medium for 24 h in a shaker at 120 rpm and 32 °C to allow bacteria to multiply inside them. Afterwards, the beads were washed again twice with autoclaved distilled water, collected and left under air stream for 30 min. Aliquots of approximately 7 g of beads each were packed into 3 mm-thick plastic bags. In total, 30 packets of beads were made from each treatment.

Innocuous peat (Nitro1000^{TM}) served as control, where 30 packets of 7 g were inoculated with 1 mL of peptone solution containing *A. brasilense* (10^9 CFU mL^{-1}). All packages or aliquots containing the different formulations were stored in a dry place in the dark and at a temperature of 21±2 °C.

The experimental design was entirely randomized with 5 different formulations tested (peat; alginate; alginate + humic acid (0.8%); alginate + trehalose 0.1M; alginate + trehalose 1M). For each treatment the survival and viability (CFU mL^{-1}) of *A. brasilense* cells were evaluated at 9 different storage periods (3, 5, 7, 14, 21, 30, 45, 60, 90 days of storage) in triplicate, totalizing 27 packets for these evaluations until the last day. The 90 days were chosen because is the maximum period that usually farmers maintain inoculants stocked before sowing.

Beads were diametrically measured (mm) with a graduated ruler and weighted (mg) in an analytical balance in triplicate after the encapsulation of the bacterial cells.

2.2 Electronic Scan Microscopy (SEM)

External surfaces of the spheres were scanned using the Scanning Electron Microscope (SEM) technique (FEI Quanta 440). The bead-shaped samples were subjected to fixation with 0.2M sodium cacodylate buffer (pH = 7.2), followed by dehydration with increasing concentrations of ethanol (50% for 15 min, 60% for 15 min, 70% for 15 min, 80% for 15 min, 90% for 15 min and 100% for 15 min). After dehydration, the supernatant was discarded and the beads received 1.5 mL of cool acetone. Samples were placed on the sample set, which contained a double-sided carbon tape, and were subsequently dried and metallized with a thin layer of gold on the surface so that a photo could capture.

2.3 Evaluation of the Survival of Viable Cells or Microbial Counting

Three packages containing aliquots of beads from each treatment in different periods of storage were analyzed. From each aliquot, 10 beads were taken and dissolved in a falcon tube containing 10 mL of potassium phosphate buffer (0.25 M, pH 6.8±0.1). The tubes were kept in a BOD incubator for 16-24 h at 30±2 °C. After this period, and for complete solubilization of beads, tubes were shaken for 1 min in a vortex. Serial dilutions (10×) and plating were performed following the protocol of Romeiro (2001) that is by counting bacterial colonies that were visible on nutrient agar plates after 24 h of inoculation.

2.4 Efficiency of Encapsulation

It was proposed to measure the encapsulation efficiency by the ratio between the log-UFC mL^{-1} obtained at the inoculation day (day 0) and the log after encapsulation (1st day).

2.5 Viability and Release of A. brasilense After Inoculation of Wheat Seeds

In vitro assay was performed with 40 wheat seeds (cv CD 104) in each treatment. Seeds were washed according to a protocol suggested by Neiverth et al. (2014). Subsequently, they were placed in agar-water medium at 30±2 ℃ and kept there for 3 days for complete germination. At the 4th day, the inoculum was prepared with 20 beads diluted in 3 mL of potassium phosphate solution (0.25 M, pH 6.8±0.1). Around 20 pre-germinated wheat seeds were immersed in the inoculum for 3 h at 30±2 °C. Test tubes containing 25 mL of distilled water and 5 cm of polypropylene pellets were prepared to support the inoculated seeds. Each pre-germinated and inoculated seed was transferred to the test tubes, randomly arranged under a photoperiod of 16 h/8 h of light/dark, respectively, at temperature of 25±2 °C, where the seeds stayed for 7 days.

2.6 Counting of Epiphytic Bacterial Population

The epiphytic bacterial population was evaluated after 7 days of inoculation. Three plants had their roots washed 3 times with distilled and autoclaved water, placed in tubes containing NaCl solution (0.9%) and sonicated for 20 s. Serial dilutions (10×) and plating followed the protocol of Romeiro (2001). The plates were kept at 30±2 °C for 48 h. The evaluation was done in triplicate. The colony-forming units (CFU) obtained were counted using a stereoscopic magnifying glass (Quimis).

2.7 Molecular identification of A. brasilense

The epiphytic bacteria were detected using PCR amplification of a 648 bp region belonging to *A. brasilense* 16S rDNA region, with the primers Azo16SF (5'-GCGGTAATACGAAGGGGGGCK-3') and Azo16SR (5'TTGTCACCGGCAGTTCCACCAG-3') (Shime-Hattori et al, 2011). Bacterial samples were collected randomly at the encapsulation day (1st day) and from the epiphytic sampling, taking into consideration the period of storage (3, 5, 7, 14, 21, 30, 45, 60, 90 days of storage).

One single bacterial colony, obtained after growth in NFb-L medium, was transferred to a PCR microtube and resuspended in 20 μL of ultrapure water. Cells were lysed through heating at 96 °C for 6 min. The supernatant was separated from the cell lysate by brief centrifugation. PCR was performed in a volume of 20 μL using 2 μL of DNA, 1× PCR Buffer, 1.5 mM MgCl$_2$, 0.4 mM dNTP mix, 1 unit of Taq DNA Polymerase (4G Research and Development), and 0.2 μM of each primer. The reaction was conducted in a Bioer Life Express model MJ96 thermal cycler, with cycling conditions as follows: initial denaturation at 94 °C for 3 min, followed by 25 cycles of 94 °C for 30 s, 58 °C for 1 min, 72 °C for 1 min, with final extension of 72 ℃ for 5 min. PCR products were visualized in   agarose gels 1.5% stained by 0.5 μg mL^{-1} ethidium bromide in 1× TBE Buffer (90 mM Tris-base; 90 mM boric acid; 2 mM EDTA pH 8.0) and photo documented with the equipment Loccus Biotechnology model L.PIX. The standard molecular weight of 100 bp (NorgenTM) was used.

2.8 Statistical Analysis

The colony forming units (CFU) data were transformed into log (base 10) for comparison using the Microsoft Office Excel. Data were submitted to analysis of variance (ANOVA) and the means were compared by the Tukey test (p < 0.05), by the statistical program GENES (Cruz 2006). The data were expressed with a mean±SD (n = 3).

Individual analyses of polynomial regression were carried out in the statistical program GENES to evaluate the outcomes of the treatments, assuming time of storage and survival and viability (within epiphytic population) as variables. Statistical significance of β1 and β2 coefficients were estimated by the F-test (p ≤ 0.05).

3. Results

3.1 Morphometric Aspects of the Beads Produced

The encapsulated beads remained with the same morphological appearance throughout the evaluation period, preserving the color and shape until the 90th day (Figures 1A-1D).
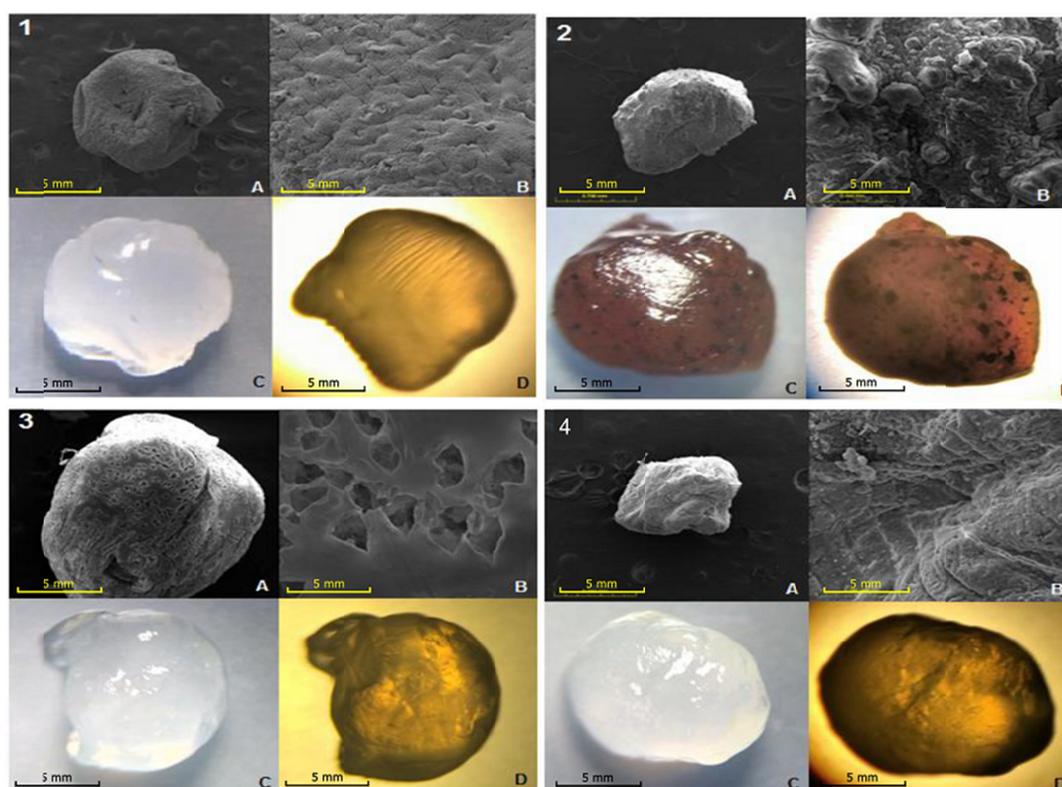

Figure 1. Morphological aspects of beads. 1. Alginate; 2. Alginate + Humic Acid 0.8%; 3. Alginate + Trehalose 0.1M; 4. Alginate + Trehalose 1M. A: External surface, increase of 120×; B. Internal surface, increase of 120×; **C.** Contre-jour picture; D. Incident light. Bar-5 mm

The morphometric data obtained at the 1st day after encapsulation are shown in Table 1. The beads had spherical shape, with their diameter measured individually. The mean size ranged from 3.3 to 4.3 mm, which confirms that the protocol used was efficient to generate beads of uniform size.

Beads weighted from 17 to 38 mg. Weight decreased when trehalose was added and increased in humic acid enriched alginate beads (Table 1).

The efficiency of encapsulation ranged from 40-61%. Trehalose (1M) decreased 15-20% the encapsulation efficiency, compared to control (alginate) (Table 1).

3.2 Survival of A. brasilense in Beads of the Different Formulations

All treatments in this experiment were planned to start with the inoculum containing a population of 10^9 CFU mL^{-1}. However, at the first day (day 0) the concentration of the bacterial population observed was approximately

10^{12} CFU mL^{-1} in all treatments (Table 1). This increase may be explained by the protocol implemented, which recommends that the solution should be gently shaken for extra 30 min after the mixture components are added. Reetha et al. (2014) observed an initial population increase of about 1000 times.

Table 1. Morphometric and encapsulation efficiency analyses

Treatments[a]	Weight (mg)	Diameter (mm)	E.E. (%)[b]	Survival (CFU mL^{-1}) (10^{12})/(Log)-Initial	Viability (CFU mL^{-1}) (10^{12})/(Log)-Initial
Peat	-	-	-	6.43/12.80	1.07/8.95
Alginate	38±3.78 a	4.30±0.57 a	59	7.86/12.90	7.86/9.85
Alginate + Humic Acid	32±0.57 ab	3.70 ±0.57a	56	7.86/12.90	7.86/9.85
Alginate + Trehalose 0.1M	17±1.52 c	3.30 ±0.59a	61	7.10/14.70	7.86/9.85
Alginate + Trehalose 1M	30±2.64 b	4.00±0.85 a	40	6.43/12.80	1.07/8.95

Note. [a]Treatments followed by different letter differ significantly by Tukey test ($p < 0.05$). [b]E.E (efficiency of encapsulation).

The treatment with peat also generated an initial population of 10^{12} CFU mL^{-1}, demonstrating a continuous bacteria growth with this carrier as well. Nevertheless, a small decrease was observed at the 1st until the last day of evaluation (Figure 2A), except for the 14th day, when the CFU mL^{-1} was around 12% higher in relation to the week before. The bacteria population at the end (90th day) was 36% lower than the initial one (2.8×10^9 CFU mL^{-1}).

In the experiment with alginate, viable bacterial cells were observed in all storage periods, which indicates that encapsulation did not prevent cell growth (Figure 2A). At the 90th day, the population of bacteria in alginate beads was 12% lower compared to the first day (5×10^7 CFU mL^{-1}).
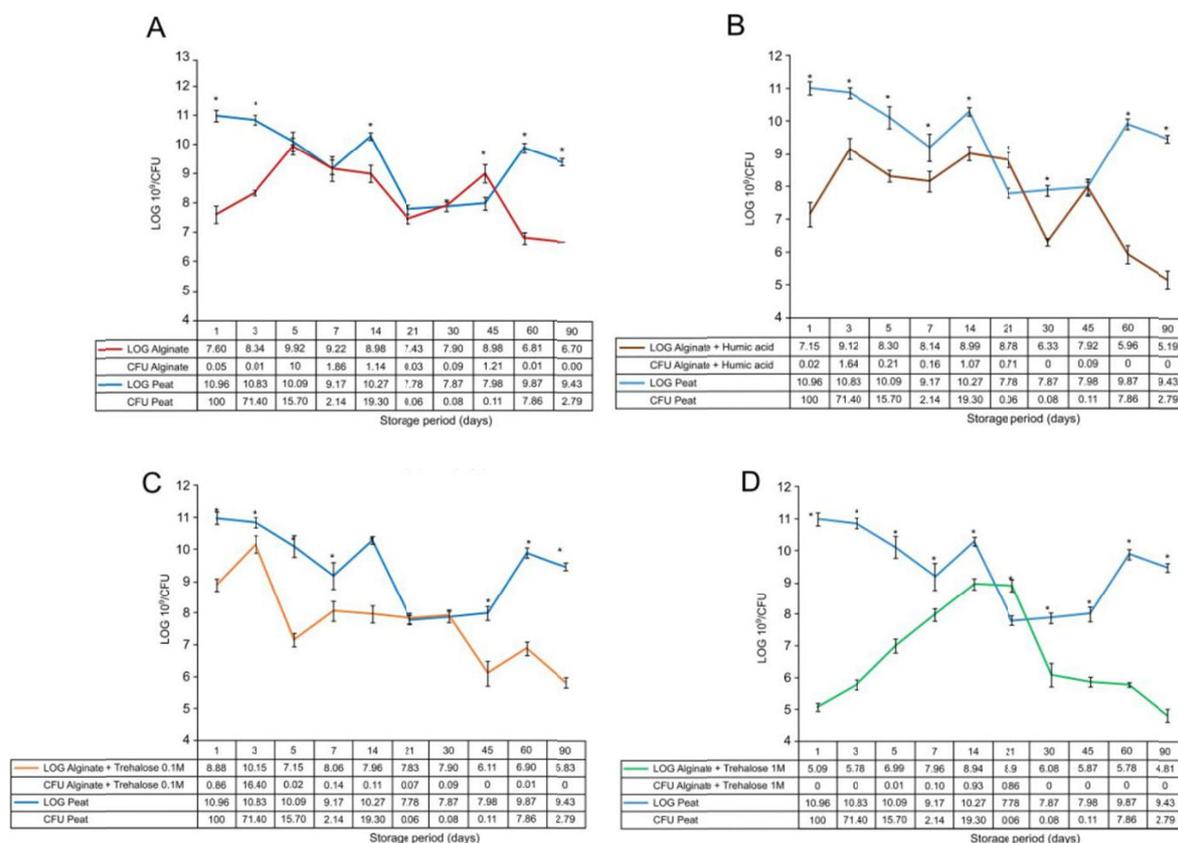

Figure 2. Survival of *A. brasilense* on different carriers matter during storage period. A. Alginate; B. Alginate + Humic Acid; C. Alginate + Trehalose 0.1M; D. Alginate + Trehalose 1M. * Treatments differ by Tukey test P > 5%. Peat was used as control. CFU is given as multiple of 10^9 mL^{-1}

Humic acid induced outcome similar to the one observed with alginate. After the first day, the microbial population found in the cells encapsulated with humic acid was 1.53 times lower than in peat (Figure 2B). After 3 days of storage, microbial population increased 27%. At the end of the observation, CFU was lower than 10^9, corresponding to 55% of the bacterial population in peat.

Trehalose was tested in 2 different concentrations (0.1 and 1M), which produced distinct effects over the encapsulated microbiota (Figures 2C-2D). At the lowest concentration (0.1M) growth acceleration was observed right after the 1st day of storage, probably because of a bigger energy source in addition to the growth phase of the encapsulated bacteria (Figure 2C). A higher concentration of carbon in the encapsulated medium when trehalose was added was confirmed by EDS (Energy-dispersive X-ray spectroscopy) (data not shown). After 72 h of storage, there was a sharp fall (41%) of the CFU compared to peat on the same evaluation day. Trehalose (0.1M) supply was over after the 3rd day of storage. After 3 months of storage, bacterial population was 38% lower than the one found in peat.

The highest concentration of trehalose (1M) (Figure 2D) promoted a significant tendency to increase survival of bacteria until the 21st day of storage (14% higher). This improvement is explained by the high concentration of C in this disaccharide, which is used by bacteria as source of energy. However, after 21 days, as this rate declined, so did the survival of bacteria. After 3 months of storage, the formulation had its CFU diminished to almost half of peat´s bacterial population.

3.3 Viability of A. brasilense During the Storage Period

The viability of *A. brasilense* after the encapsulation process and storage was evaluated through an *in vitro* test in which the bacteria that truly associated with the wheat seedlings—epiphytic bacteria—were quantified.

In all treatments, encapsulation demonstrated a beneficial effect on the epiphytic population (Figures 3A-3D). Peat presented a population ranging from 10^7 to 10^9 CFU mL^{-1} over the 90 days of storage. This population had a decrease of 22% on the 7th day after the encapsulation. At the last day of evaluation, the population adhered to the wheat roots was 10^9 CFU mL^{-1} (Figure 3A).
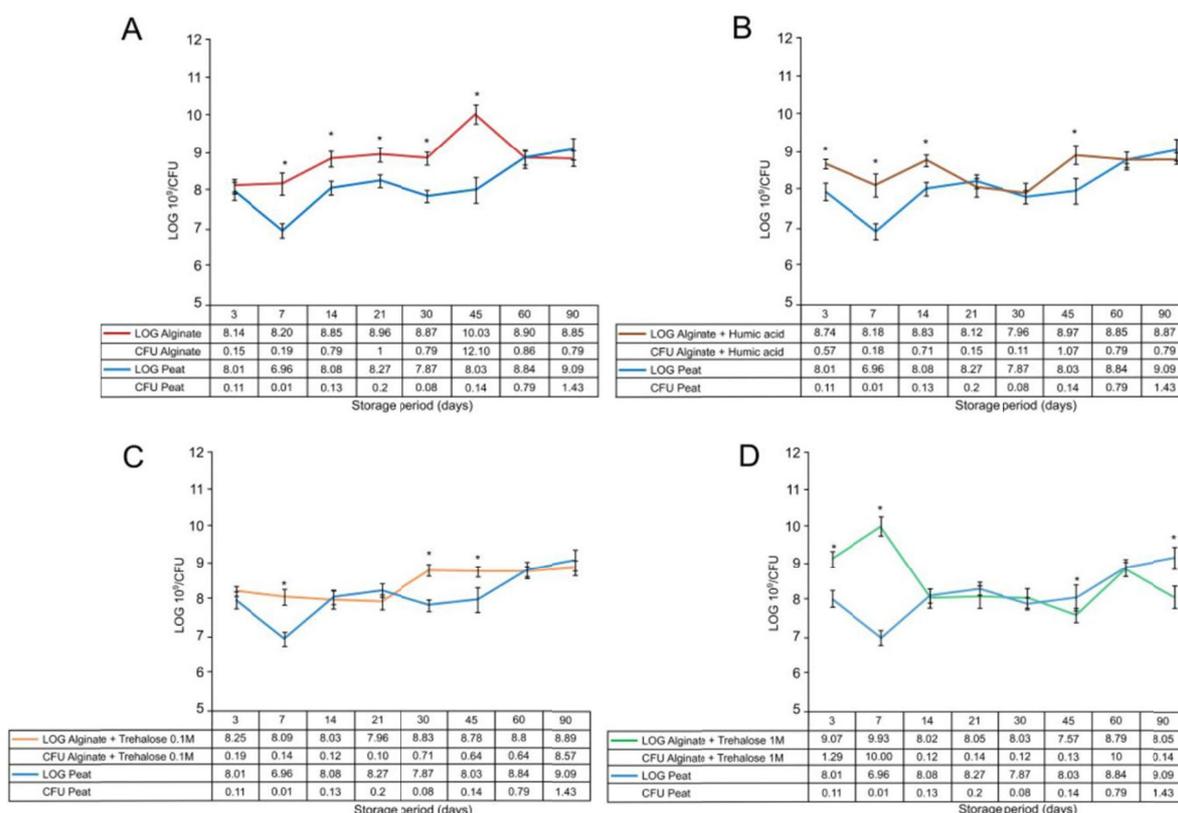

Figure 3. Viability of *A. brasilense* on different carriers matters during storage period. A. Alginate; B. Alginate + Humic Acid; C. Alginate + Trehalose 0.1M; D. Alginate + Trehalose 1M. * significative (P > 5%). Peat was used as control. CFU is given as multiple of 10^9 mL^{-1}

Encapsulation in calcium alginate matrix without supplements presented an epiphytic population superior to peat in practically all the periods of evaluation. This population was similar to peat at the 3rd day after storage. On the 45th day, the population adhered to the wheat roots was 25% higher than the one found in peat, which indicates advantages in the use of encapsulation. On the other hand, the epiphytic population was 2.7% lower than peat on the last day of evaluation.

When the carrier had humic acid added, the population of bacteria ranged from 10^8 to 10^9 CFU mL^{-1} along storage period (Figure 3B). Although the population adhered to roots increased 9% after 14 days of storage, this rate, after 3 months, was 2.5% lower than the population in peat.

The supplementation with trehalose at 0.1M during encapsulation was more effective until the 45th day (except the 21 days of storage) in relation to peat. However, epiphytic population in beads decreased at the last day (90th day) of evaluation (Figure 3C).

Trehalose at the highest concentration (1M) demonstrated to have a potent effect on bacteria viability until the 7th day of storage. After this day, the rate of CFU mL^{-1} decreased to levels similar to peat. On the 90th day of storage, the population adhered to the wheat roots was 12% lower than peat (Figure 3D).

Molecular analyses of the samples randomly collected from the epiphytic bacteria attached to seedlings roots confirmed the presence of *A. brasilense* as deriving from the inoculum used (Figure 4).
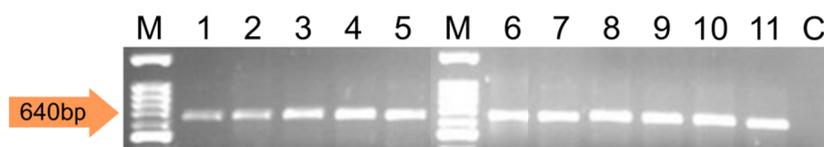

Figure 4. PCR analyses confirming *A.brasilense* presence in samples collected in different periods of storage. Bands 1 to 5 correspond to samples collected at the 1st day after encapsulation; M. Molecular Ladder 100pb; 1. Peat; 2. Alginate; 3. Alginate + Humic acid; 4. Alginate + Trehalose 0,1M; 5. Alginato + Trehalose 1M; Bands 6 to 11 were from bacteria epiphytically adhered to wheat roots: **7.** Peat (7 days); 8. Alginate (14 days); 9. Alginate + Humic acid (21 days); 10**.** Alginate+ Trehalose 0,1M (30 days); 11. Alginate + Trehalose 1M (90 days); C. Negative Control

The data were submitted to individual and joint analyses of regression, using viability and survival as variables in the functions of all evaluated treatments in order to verify if any variable is significant within treatments and to obtain a performance model (Table 2 and Figure 5). Analyses confirmed peat as the best option to support bacterial survival during 90 days of storage. Simple linear regression equations showed that treatments with alginate, alginate + humic acid and alginate + trehalose 0.1M caused significant decrease in the survival rate of *A. brasilense* (Figure 5A).
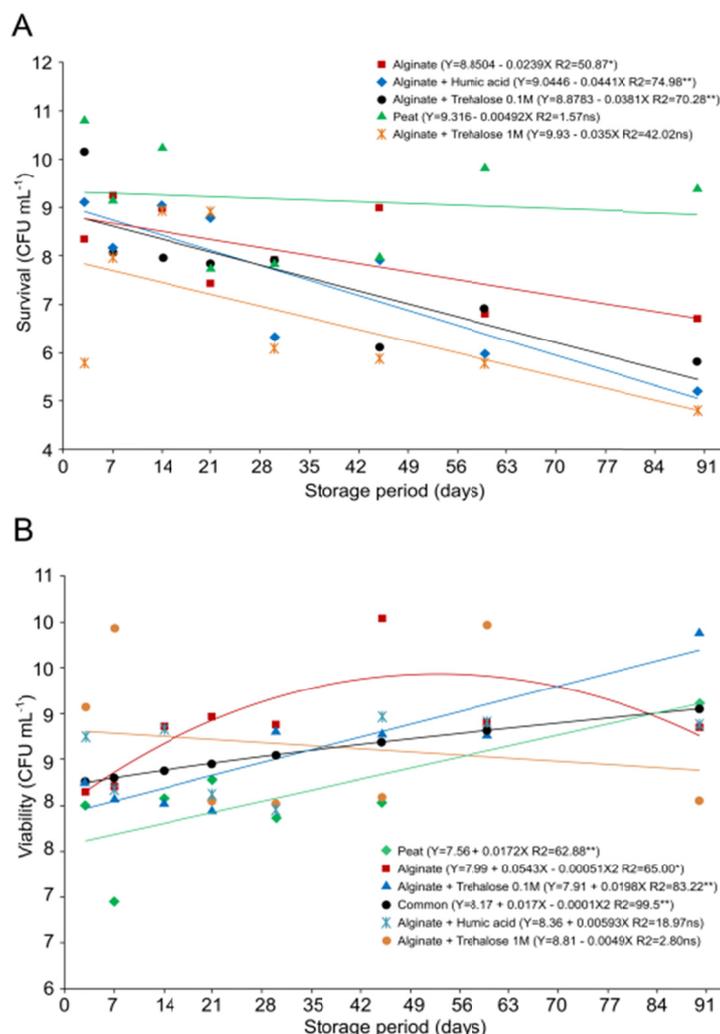
Figure 5. Projection of individual and joint regression for survival (A) and viability (B) of bacterial cells as a function of evaluated treatments. * and **indicate significant difference at $P < 0.05$ and $P < 0.01$, respectively. ns indicate no significant

On the other hand, when only alginate was used as carrier (p-value 0.0497), the results showed a tendency to decrease viability after 60 days of storage (Figure 5B). The treatments with alginate + humic acid and alginate + trehalose 1M did not present significant equations of *A. brasilense* viability.

Table 2. Joint regression analysis for viability and survival variables as a function of all evaluated treatments

Treatments	Survival (CFU mL^{-1})			Viability (CFU mL^{-1})		
	Regression Model	R^2 (%)	p-value	Regression Model	R^2 (%)	p-value
Peat	$Y = 9.31615385 - 0.00492308X$	1.57	0.7631	$Y = 7.56 + 0.0172X$	62.88	*0.0188*
	$Y = 10.3240404 - 0.0858X + 0.0009X^2$	36.55	0.1562	$Y = 7.59 + 0.0152X + 0.000022X^2$	62.94	0.9247
Alginate	$Y = 8.85 - 0.02386X^2$	50.87	0.0461*	$Y = 8.56 + 0.0084X$	18.90	0.2819
	$Y = 8.77 - 0.0178X - 0.00007X^2$	51.14	0.8688	$Y = 7.99 + 0.0543X - 0.00051X^2$	65.00	0.0497*
Alginate +Humic Acid	$Y = 9.04 - 0.0441X$	74.98	0.0057*	$Y = 8.36 + 0.00593X$	18.97	0.2810
	$Y = 9.11 - 0.0499X + 0.000064X^2$	75.08	0.8846	$Y = 8.44 - 0.00055X + 0.000072X^2$	20.83	0.7421
Alginate + Trehalose 0.1M	$Y = 8.88 - 0.0381X$	70.28	0.0095*	$Y = 7.91 + 0.0198X$	83.22	0.0018*
	$Y = 9.42 - 0.0819X + 0.00049X^2$	77.91	0.2454	$Y = 8.078 + 0.0063X + 0.00015X^2$	86.41	0.3290
Alginate + Trehalose 1M	$Y = 7.93 - 0.035X$	42.02	0.0805	$Y = 8.81 - 0.0049X$	2.80	0.6921
	$Y = 7.65 - 0.0122X - 0.00025X^2$	43.48	0.7315	$Y = 8.97 - 0.0177X + 0.00014X^2$	4.35	0.7819
Joint regression	No ajust			**$Y = 8.21 + 0.011X - 0.000025X^2$**	**99.52**	**0.0000**

Note. *Significative at 5%.

4. Discussion

To promote bacteria survival and viability is the main challenge for the inoculation technology, once shelf life is influenced by several parameters: bacterial species, culture medium, physiological condition of the microorganisms, dehydration process, storage temperature and water concentration in the inoculum (Schoebitz et al., 2013; Sivasakthivelan & Saranraj, 2013).

In order to bacteria function as promoters of plant growth, they must reach the root, survive for a certain period of time to associate with plants, and compete with other bacteria in the rhizosphere. Therefore, formulations should provide protection to bacteria longevity (Vassilev et al., 2015).

In relation to the morphological appearance, some beads exhibited striations, agreeing with other authors who observed grooves in the surface of the encapsulated beads (Berninger et al., 2016). The humic acid beads presented dark spots, probably caused by deposition of the humic acid or other impurities. The wrinkled aspect, originated from invaginations, could explain the weight gain in some treatments. This result corroborates with those obtained by other authors who observed by microphotography a wrinkled and grooved area (Bashan, Carriers, & Bashan, 1986; Sivakumar et al., 2014). The formulation of alginate enriched with humic acid presented higher porosity, similar to what Young et al. (2006) found. On the other hand, the treatment using trehalose formed beads of smoother appearance, without invaginations, which would explain their lower weights.

(Ivanova, Teunou, & Poncelet, 2005) reported another protocol in which the beads size ranged from 1 to 5 mm, and observed that by increasing the spheres size, bacteria survival enhanced 36%. Reetha et al. (2014) obtained encapsulated particles of smaller size (1.3 to 3.2 mm) and lower weight (0.5 to 10.3 mg) compared to our data, which is explained by the type of pipetting instrument used during the extrusion of the inoculum.

In the literature, different concentrations of microorganisms in inoculant formulations (CFU mL^{-1}) are reported: 10^9 (Reetha et al., 2014); 10^{10} (Young et al., 2006); 10^6 (Marcelino, Milani, Mali, Santos, & Oliveira, 2016); 10^3 (O'Callaghan, 2016). Encapsulation demands high population density during inoculation in order to ensure the minimum population desired. Shah-Smith and Burns (Shah-Smith & Burns, 1996) reported a population of 6×10^7 CFU mL^{-1} after inoculation of seeds using a cell density of 2×10^{10} of *Pseudomonas putida*.

Study by Amiet-Charpentier, Gadille, Digat, and Benoit (1998) showed that alginate matrix was not toxic or incompatible with *Pseudomonas* cells. However, in all treatments the CFU mL^{-1} decreased significantly 24 h after encapsulation. This evidence suggests that extrusion caused a harmful or stressing effect on the bacterial population encapsulated (Figures 2A-2D). After 48h of encapsulation, alginate motivated a sharp decline in the CFU of about 44% in relation to the 1st day (Table 1) and 35% compared to peat during the same period. This result corroborates the research by Ivanova et al. (2002) who observed greater decrease in the initial phase, after extrusion (7 days). Bashan et al. (2002) observed that the microencapsulation process affects the survival of bacteria due to the cross-linking of the alginate-calcium complex with the cell membrane of the bacteria, killing many of them.

Among all formulations, peat demonstrated the best capacity in maintaining bacterial survival throughout storage period. According to Kaljeet et al. (2011), peat was the only carrier that maintained 10^7 CFU mL^{-1} of rhizobia for up to 8 weeks of storage. Our results are distinct from those obtained by Reetha et al. (2014), who observed a reduction in the microbial population of around 57% in only 6 days of evaluation, but it shifted after 90 days, when the population increased to 10×10^9 CFU mL^{-1}.

Young et al., (2006) had also tested alginate supplemented with humic acid, but did not detect loss of microbiota (2×10^8 CFU g^{-1} of beads) after 24 h of encapsulation or even after 5 months of storage. The great difference between their data and ours can be partially explained by the instrument they used during the extrusion, a 26-gauge needle, smaller than one used in this work, thus forming much smaller beads (1 to 2 mm). Smaller beads would be preferable for their survival than the larger ones.

In a similar protocol, but with a concentration of 2.5% of sodium alginate, humic acid promoted a smaller reduction in the CFU mL^{-1} of *A. lipoferum* (Reetha et al., 2014). The authors reported that the beneficial effect occurred because humic acid increased porosity, enhancing oxygenation and access to nutrients, facilitating cellular metabolism within the beads.

O'Callaghan (2016) reported that the greatest benefits of supplements in the encapsulation formulations are to provide the bacteria better life conditions to withstand stresses as well as to improve cell vigor. However, data did not show improvement of CFU during periods longer than 3 weeks of storage compared to peat.

The best performance of peat regarding cell survival and viability after 90 days of storage can be explained by its high moisture holding capacity, since there may have been loss of water during encapsulation (Kaljeet et al., 2011). The calcium alginate matrix, on the other hand, is rich in water (97-98%), meaning that it would fail to provide cell protection, stated Bashan et al. (2002). Schoebitz et al. (2013) demonstrated that the addition of starch to alginate reduced the water concentration to 65% and improved significantly bacterial survival. Another factor that could explain the success of peat as carrier is that it provides better oxygenation. The inconvenience, however, is the great rates of contamination: without nalidixic acid and fungicide, it was not possible to carry out the evaluations using peat as control.

Results of viability (CFU mL^{-1}) polynomial regression analyses showed that all treatments can be represented by the common regression equation (Y = 8.21 + 0.011X − 0.000025X^2, Figure 5B). The treatments with peat (p-value 0.0188) and alginate + trehalose 0.1M (p-value 0.0018) showed linear increases in viability, and the combination of alginate + trehalose 0.1M presented the highest mean value of viability, suggesting a positive effect of this disaccharide in helping encapsulated cells become vigorous until reaching the rhizosphere and associate with plants. The results obtained with trehalose at low concentrations (Figures 2C and 5B) can open up the potential for new formulations using some PGPB strains that strive to survive, even without encapsulation.

Medium and higher concentrations of trehalose in inoculants formulations should be tested to check possible improvements in bacterial survival. The positive results of increase in cell survival and viability, found in the formulations with trehalose mainly right after encapsulation, confirm the protective effect of trehalose on proteins and components of the cell membrane at the initial phase of bacteria growing inside the beads (Leslie et al., 1995).

5. Conclusions

The gel-based formulation of *Azospirillum brasilense* developed with sodium alginate (3%) was competent to produce beads of uniform size that sustained bacteria growth and viability along 90 days of storage. Peat was the best carrier to support bacteria survival. Encapsulation in a gel matrix provided higher cell viability, mainly when low concentration (0.1M) of trehalose was added. These findings can optimize plant inoculation.

Acknowledgements

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