Pesquisa Agropecuária Brasileira

Microbiology/ Original Article

ISSN 1678-3921

Journal homepage: www.embrapa.br/pab

For manuscript submission and journal contents, access: www.scielo.br/pab

Azospirillum-based inoculation enhances early growth and establishment of ryegrass

Abstract – The objective of this work was to evaluate the effect of *Azospirillum brasilense* inoculation on the early growth and establishment of ryegrass (*Lolium x hybridum*) using conventional liquid inoculants and alginate-based polymeric microbeads to assess bacterial survival and root colonization. Bacterial viability was monitored over 90 days under refrigerated and ambient storage conditions. Root colonization was investigated in non-sterile soil using fluorescent in situ hybridization and scanning electron microscopy. Plant physiological responses were evaluated under controlled and greenhouse conditions. The microbeads led to a higher bacterial viability throughout storage and to a uniform colonization of root hairs during seedling establishment. Compared with the uninoculated control, the inoculation with *A. brasilense* increased shoot elongation, chlorophyll *a* and *b* concentrations, and root dry weight under controlled conditions. In the greenhouse, the microbeads maintained a higher chlorophyll *a* concentration, but did not differ significantly from the liquid inoculants regarding plant biomass. Inoculant formulation is a core determinant of *A. brasilense* storage stability and biological performance. Alginate-based polymeric carriers improve bacterial survival, sustain root colonization, and enhance early plant physiological responses, highlighting their potential use as an effective inoculant for improving forage establishment under non-sterile soil conditions.

Index terms: *Azospirillum brasilense*, *Lolium* x *hybridum,* microencapsulation, pasture establishment, plant growth-promoting rhizobacteria.

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Received
February 08, 2026

Accepted
August 26, 2026

How to cite
GONZALEZ-INFANTE, E.J.; POSADA-URIBE, L.F.; MENDOZA-LABRADOR, J.A.; LÓPEZ, B.E.R.; BONILLA-BUITRAGO, R.R. *Azospirillum*-based inoculation enhances early growth and establishment of ryegrass. **Pesquisa Agropecuária Brasileira**, v.61, e04378, 2026. DOI: https://doi.org/10.1590/S1678-3921.pab2026.v61.04378.

Inoculação com *Azospirillum* melhora o crescimento inicial e o estabelecimento de azevém

Resumo – O objetivo deste trabalho foi avaliar o efeito da inoculação com *Azospirillum brasilense* sobre o crescimento e o estabelecimento inicial do azevém (*Lolium* x *hybridum*), tendo-se utilizado inoculantes líquidos convencionais e microesferas poliméricas à base de alginato para aferir a sobrevivência e a colonização radicular bacteriana. A viabilidade bacteriana foi monitorada durante 90 dias sob condições de armazenamento refrigerado e ambiente. Já a colonização radicular foi investigada em solo não estéril por meio de hibridização in situ por fluorescência e microscopia eletrônica de varredura. As respostas fisiológicas vegetais foram avaliadas em condições controladas e em casa de vegetação. As microesferas resultaram em maior viabilidade bacteriana durante o armazenamento e em colonização radicular uniforme durante o estabelecimento das plântulas. Em comparação ao controle, a inoculação com *A. brasilense* aumentou o alongamento da parte aérea, as concentrações de clorofila *a* e *b*, e a massa seca das raízes em condições controladas. Em casa de vegetação, as microesferas mantiveram

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maiores concentrações de clorofila *a*, mas não diferiram significativamente do inoculante líquido quanto à biomassa vegetal. A formulação do inoculante é fator determinante a estabilidade durante o armazenamento e do desempenho biológico de *A. brasilense*. As microesferas poliméricas aumentam a sobrevivência bacteriana, favorecem a colonização radicular e promovem respostas fisiológicas vegetais iniciais, o que destaca seu potencial como inoculante para melhorar o estabelecimento de forrageiras em condições de solo não estéril.

Termos para indexação: *Azospirillum brasilense*, *Lolium x hybridum*, microencapsulamento, estabelecimento de pastagens, rizobactérias promotoras do crescimento vegetal.

Introduction

Grasslands constitute the foundation of livestock production systems worldwide, providing the principal feed resource for ruminants and sustaining meat and milk production, particularly in tropical and subtropical regions (Zhu et al., 2024). Globally, permanent grasslands occupy roughly 26% of Earth's land surface and nearly 70% of agricultural land, covering approximately 3.2 billion hectares, while supporting the livelihoods of more than 800 million people and providing essential ecosystem services, such as carbon sequestration, biodiversity conservation, and water regulation (FAO, 2025). Therefore, grassland establishment, persistence, and productivity are fundamental to the sustainability of these systems. However, early pasture development is frequently constrained by low soil fertility, particularly nitrogen deficiency, resulting in greater dependence on mineral fertilizers, increased production costs, and environmental impacts associated with nutrient losses (Quintero-Anzueta et al., 2021; Bourscheidt et al., 2023). Consequently, biological strategies capable of improving nutrient-use efficiency and promoting early pasture development have become increasingly important. Although plant growth-promoting microorganisms have demonstrated considerable potential to enhance forage yield, nutrient acquisition, and fertilizer-use efficiency, microbial inoculation remains underutilized in grassland systems compared with major grain crops (Bastidas et al., 2024).

Plant growth-promoting bacteria (PGPB) represent one of the most promising biological alternatives for improving crop yield while simultaneously reducing reliance on synthetic fertilizers (Beltrán-Medina et al., 2025). These microorganisms stimulate plant growth through complementary mechanisms, including biological nitrogen fixation, phytohormone production, nutrient solubilization, siderophore synthesis, modulation of root architecture, and improved tolerance to biotic and abiotic stresses. Collectively, these mechanisms enhance nutrient acquisition and support growth during critical early stages of development (Cunha et al., 2024). However, successful inoculation depends not only on the expression of these traits under laboratory conditions but also on the capacity of introduced bacteria to survive, colonize the rhizosphere, compete with indigenous microbial communities, and remain metabolically active after soil application (Reed & Glick, 2023; Biswas et al., 2024; Thakur et al., 2024).

Among PGPB, *Azospirillum brasilense* is one of the best-characterized bacterial species associated with grasses and a widely used commercial inoculant. Its beneficial effects result from the combined action of phytohormone production, stimulation of lateral and adventitious root development, increased root-hair formation, improved nutrient and water uptake, and enhanced photosynthetic performance, rather than biological nitrogen fixation alone (Cassán & Díaz-Zorita, 2016; Fukami et al., 2017; Cassán et al., 2020). Positive responses to *A. brasilense* inoculation have been reported in several forage species, including *Urochloa* spp., black oat (*Avena strigosa* Schreb.), Bermuda grass [*Cynodon dactylon* (L.) Pers.], and ryegrass (*Lolium multiflorum* Lam.), where it has promoted seedling growth, forage accumulation, nutrient acquisition, and nitrogen-use efficiency (Hungria et al., 2016; Cunha et al., 2024). Nevertheless, these responses remain inconsistent under field conditions (Piva et al., 2023).

One of the main reasons for this variability is that inoculant performance depends not only on the intrinsic plant growth-promoting capacity of the bacterial strain but also on formulation quality. An effective formulation must preserve bacterial viability during production, storage, transportation, and seed treatment while ensuring efficient delivery and successful bacterial colonization following soil application (Singh & Kumar, 2024). Liquid inoculants dominate the commercial market because they are inexpensive, easy to produce, and compatible with conventional agricultural practices. However, exposure to desiccation, temperature fluctuations, nutrient

depletion, and osmotic stress may substantially reduce bacterial viability during storage. After application to non-sterile soil, inoculated bacteria must also withstand competition from indigenous microorganisms, predation, and fluctuation of environmental conditions, thereby reducing the number of metabolically active cells capable of colonizing the rhizosphere (Khan et al., 2023; Fadiji et al., 2024; Singh & Kumar, 2024).

Among the available carrier technologies, alginate has become the reference natural polymer for microbial inoculant formulation because it combines biocompatibility, mild gelation conditions, low production cost, and the ability to protect bacterial cells during their gradual release into the rhizosphere (Bashan, 1986; Martínez-Cano et al., 2022). Alginate-based microbeads reduce desiccation stress, improve storage stability, and enhance bacterial delivery compared with conventional liquid formulations (Vassilev et al., 2020; Khan et al., 2023; Fadiji et al., 2024). Although encapsulation has consistently improved bacterial survival, formulation characteristics such as polymer composition, release kinetics, particle size, and manufacturing efficiency continue to be optimized for large-scale agricultural use (Schoebitz et al., 2013; Szopa et al., 2022).

Unlike previous studies that evaluated inoculant shelf life or plant responses independently (Pelagio-Flores et al., 2025), the present study integrates bacterial viability, microscopic evidence of root colonization, and plant physiological responses to determine whether improvements in inoculant formulation translate into greater biological effectiveness under non-sterile soil conditions.

The objective of this work was to evaluate the effect of *A. brasilense* inoculation on the early growth and establishment of ryegrass (*Lolium x hybridum*) using conventional liquid inoculants and alginate-based polymeric microbeads to assess bacterial survival and root colonization.

Materials and Methods

Azospirillum brasilense was prepared in two different inoculant formulations: a conventional liquid inoculant and an alginate-based polymeric inoculant. Both formulations were compared for inoculant stability during storage, bacterial delivery, root colonization, and early plant performance under non-sterile soil conditions. *Azospirillum brasilense* strain D7 (GenBank accession No. MN339530) was isolated from grasslands managed under traditional livestock production systems in the Colombian highlands and identified by 16S rRNA gene sequencing. The strain was deposited on accession no. 129 in the AGROSAVIA Banco de Germoplasma de Microorganismos, and registered in the Registro Único Nacional de Colecciones Biológicas of the Instituto de Investigación de Recursos Biológicos Alexander von Humboldt. The strain was reactivated on *A. brasilense* solid medium at 28°C for 48 hours (Moreno-Galván et al., 2012). Single colonies were transferred to *A. brasilense* liquid medium and incubated at 30°C with continuous agitation at 120 rpm for 48 hours until reaching an approximate optical density of 1.0 at 540 nm, equivalent to 1×10^9 CFU mL^{-1}. Centrifugation was used to harvest bacterial cells at 2,760 × g for 8 min in a 5418 R centrifuge (Eppendorf, Hamburg, Germany). The centrifuged medium was washed twice with 0.85% sterile saline solution, and resuspended in the same solution. The liquid inoculant consisted of the bacterial suspension stored in sterile sealed flasks.

Alginate-based polymeric microbeads were prepared according to Bashan et al. (2002), with minor modifications. The 3% (w/v) low-viscosity sodium alginate solution Grindsted FD/PH 120 (IFF, New York, USA) was mixed with the bacterial suspension at a 4:1 (v/v) ratio. Droplets were generated under sterile conditions using compressed air at 80 psi and sprayed into a sterile 3% (w/v) $CaCl_2$ solution to induce ionic gelation. The resulting beads were allowed to harden for 20 min, recovered using a sterile 70 µm sieve, and washed with sterile saline solution.

In order to promote bacterial recovery and physiological adaptation following encapsulation, the microbeads were incubated in fresh *A. brasilense* liquid medium and agitated at 120 rpm for 24 hours at 28°C. This procedure facilitated bacterial adaptation within the polymeric matrix before drying, thereby maximizing the number of viable encapsulated cells. Subsequently, the beads were recovered, washed, and oven-dried at 48°C until reaching a constant weight, ground, and sieved to obtain particles ranging from 70 to 250 µm in diameter. The final bacterial concentration of the dried polymeric formulation was approximately 1×10^{10} CFU g^{-1}.

To compare the storage stability of both inoculant formulations, liquid and polymeric inoculants were

stored under refrigerated (4°C) and ambient (22°C) conditions, reflecting typical storage conditions encountered during preservation, transportation, commercialization, and on-farm use. Both formulations were maintained in sterile, sealed flasks for 90 days.

Bacterial viability was determined at 30-day intervals by serial dilution and plate counting on *A. brasilense* medium. For polymeric formulations, 100 mg of microbeads were dissolved in 15 mL of citrate buffer (pH 8.0) composed of 55 mM sodium citrate, 30 mM EDTA, and 150 mM NaCl. The mixture was agitated at 100 rpm for 20 min, centrifuged at 8,161 × *g*, and resuspended in 1 mL of sterile saline solution prior to serial dilution and plating. CFU counts were quantified after 48 hours of incubation at 30°C. Viability was expressed as CFU mL^{-1} for liquid inoculants and CFU g^{-1} for polymeric inoculants.

Surface-disinfected seeds of the perennial ryegrass (*Lolium x hybridum* Hausskn.) cultivar Dorcas (Sáenz Fety S.A.S., Bogotá, Colombia) were germinated on sterile agar medium supplemented with inorganic nutrients. Seeds were inoculated with either 10 µL of liquid inoculant (1×10^7 CFU per seed) or 10 mg of polymeric microbeads (1×10^8 CFU per seed). Uninoculated seeds and seeds treated with sterile, uninoculated polymer served as control treatments.

Each experimental unit consisted of a Petri dish containing ten seeds, and each treatment was composed of ten Petri dishes, resulting in 100 seeds per treatment across two replicates under identical conditions. Maintaining ten seeds per Petri dish provided adequate spacing for unrestricted seedling growth and prevented overlap among root systems, thereby enabling individual evaluation of root colonization by fluorescence in situ hybridization (FISH) and scanning electron microscopy (SEM).

Seedlings were maintained for 20 days in a growth chamber at 22°C, 65% relative humidity, and a 16-hour photoperiod. At the end of the incubation period, shoot length, root dry biomass, photosynthetic pigment content, and bacterial root colonization were evaluated. Root dry biomass was determined after oven drying at 70°C until achieving constant weight. Shoot dry biomass was not determined for seedlings under growth chamber conditions because the amount of dry tissue obtained per seedling did not fall within the reliable range of the analytical balance used, resulting in unacceptable measurement uncertainty.

The greenhouse experiment was conducted using pots containing 2 kg of non-sterile agricultural soil collected from permanent livestock pastures. For the liquid inoculant treatment, seeds were immersed in a bacterial suspension at 1×10^9 CFU mL^{-1} for 30 min immediately prior to sowing. For the polymeric treatment, alginate microbeads were placed in direct contact with the seeds within the sowing furrow to ensure proximity between the inoculant and the emerging root system (Bashan et al., 2002). Uninoculated seeds and seeds treated with sterile, uninoculated polymer served as control treatments.

Plants were grown under greenhouse conditions for 24 days, after which a uniform harvest cut was performed to simulate routine pasture management. Plants were then allowed to regrow for an additional 48 days before shoot height, root dry biomass, and photosynthetic pigment content were evaluated.

The experimental soil was analyzed at Corporación Colombiana de Investigación Agropecuaria, and characterized as clay loam mineral soil with pH 6.07, low salinity (1.09 dS m^{-1}), high cation-exchange capacity, good structural stability, and high water and nutrient-retention capacity.

Photosynthetic pigments were quantified according to Hiscox & Israelstam (1979). 50 mg of fresh shoot tissue was incubated in dimethyl sulfoxide at 65°C for 3 hours, and absorbance was measured at 645 and 663 nm using a spectrophotometer. Chlorophyll *a*, chlorophyll *b*, and total carotenoid concentrations were calculated according to Wellburn (1994).

The identity and purity of *A. brasilense*, as well as bacterial root colonization throughout the storage period, were verified using conventional microbiological techniques and FISH. Hybridization was performed using the universal bacterial probes EUB338-I, EUB338-II, and EUB338-III, along with the *A. brasilense*-specific probe AZOI-655 (Stoffels et al., 2001; Bashan et al., 2011). Subsequently, twenty seedlings were randomly selected from each treatment for microscopic analysis.

Radicles were gently rinsed with a sterile 0.85% NaCl solution, fixed in 4% paraformaldehyde for 4 hours at 4°C, and mounted on microscope slides coated with 0.25% agarose. FISH was performed using the *A. brasilense*-specific probe AZOI-655 (5′-/FAM/-CACCATCCTCTCCGGAAC-3′) (Stoffels et al., 2001) together with an equimolar mixture of universal bacterial probes EUB338-I, EUB338-II,

and EUB338-III (Amann et al., 1990; Daims et al., 1999). Samples were incubated at 46°C for 2 hours in the hybridization buffer containing 35% formamide, followed by washing at 48°C for 5 min in pre-warmed washing buffer.

Hybridized samples were examined using an Eclipse E400 epifluorescence microscope (Nikon, Tokyo, Japan) equipped with a 100× oil-immersion objective lens and a DAPI/FITC/TRITC filter set. AF1 mountant (CitiFluor, Morgantown, PA, USA) was used as an antifade medium, and images were captured and processed through NIS-Elements software (Nikon, Tokyo, Japan). Representative micrographs showing consistent colonization patterns across treatments were selected from independent biological replicates.

Root samples were fixed in 2.5% glutaraldehyde prepared in 0.1 mol L^{-1} cacodylate buffer (pH 7.4) for 2 hours, washed, dehydrated through a graded ethanol series (20–80%), critical-point dried using a SAMDRI PVT 3B (Tousimis, Rockville, MD, USA), and sputter-coated with gold–palladium. Samples were examined using a Phenom SEM (Thermo Fisher Scientific, Waltham, MA, USA) at the Escuela de Administración, Finanzas e Instituto Tecnológico, located in the municipality of Medellín, Colombia.

Both the growth chamber and the greenhouse experiments followed a completely randomized design comprising four treatments: liquid inoculant; alginate-based polymeric inoculant; sterile, uninoculated polymer; and an uninoculated control. Because both experimental runs yielded consistent results, data were pooled for the final statistical analyses.

Data were evaluated for normality, homogeneity of variance, and independence of residuals using Shapiro-Wilk's, Levene's, and Durbin-Watson's tests, respectively. Treatment effects were analyzed by one-way analysis of variance (ANOVA), followed by Tukey's test for multiple comparisons at $\alpha = 0.05$. All statistical analyses were performed using R language version 4.3.2 (R Core Team, 2023) with RStudio interface.

Results and Discussion

The alginate-based polymeric inoculant maintained higher bacterial viability than the conventional liquid inoculant throughout the 90-day storage period under both refrigerated and ambient conditions (Figure 1). Although high numbers of viable cells were preserved in both inoculants during storage, bacterial survival was consistently higher in the polymeric formulation. Under ambient conditions, the viability of the liquid inoculant declined from approximately 1×10^9 to 7×10^7 CFU mL^{-1} after 90 days, whereas that of the polymeric formulation decreased from 1×10^{10} to 8×10^8 CFU g^{-1}. Refrigerated storage reduced viability losses in both inoculants, with reductions of approximately 20% and 11% for the polymeric and liquid inoculants, respectively. The advantage of encapsulation was particularly pronounced under ambient storage, where the polymeric inoculant retained approximately one order of magnitude more viable cells than the liquid inoculant after 90 days (Figure 1).

Typical colony morphology remained unchanged throughout the storage period, indicating phenotypic stability of the strain in both formulations. Furthermore, FISH analyses confirmed the taxonomic identity of *A. brasilense* in both liquid and polymeric inoculants after 90 days of storage (Figure 2).

FISH and SEM provided complementary evidence of root colonization by *A. brasilense* under non-sterile soil conditions (Figure 3). Fluorescent signals were consistently detected along radicles and root hair zones of inoculated seedlings, whereas no hybridization signals were observed in uninoculated controls or in seedlings treated with sterile, uninoculated polymer.

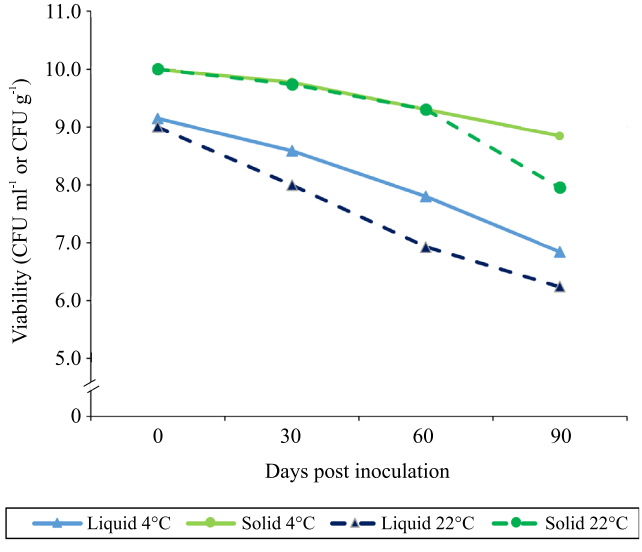

Figure 1. Viability of *Azospirillum brasilense* in solid alginate-based polymeric and liquid suspension inoculants during 90 days of storage at 4 and 22ºC.

SEM revealed distinct colonization patterns depending on the inoculant formulation (Figure 3). In seedlings treated with the polymeric inoculant, alginate microbeads remained attached to the root surface and were closely associated with the development of root hairs. Bacterial aggregates were frequently observed surrounding root hairs and elongation zones, whereas no bacterial structures were detected in either control treatment.

Inoculation with *A. brasilense* under growth chamber conditions increased early ryegrass growth and physiological performance (Table 1). Both inoculants increased shoot length relative to the uninoculated control, with gains of approximately 13 and 11% for the liquid and polymeric formulations, respectively. Similarly, chlorophyll *a* and chlorophyll *b* concentrations were significantly higher in inoculated seedlings than in the uninoculated control treatments (Table 1). Although total carotenoid concentrations in seedlings treated with the polymeric inoculant were higher than in the uninoculated controls, they did not significantly differ from other treatments. Among the evaluated variables, root dry biomass showed the greatest response to bacterial inoculation,

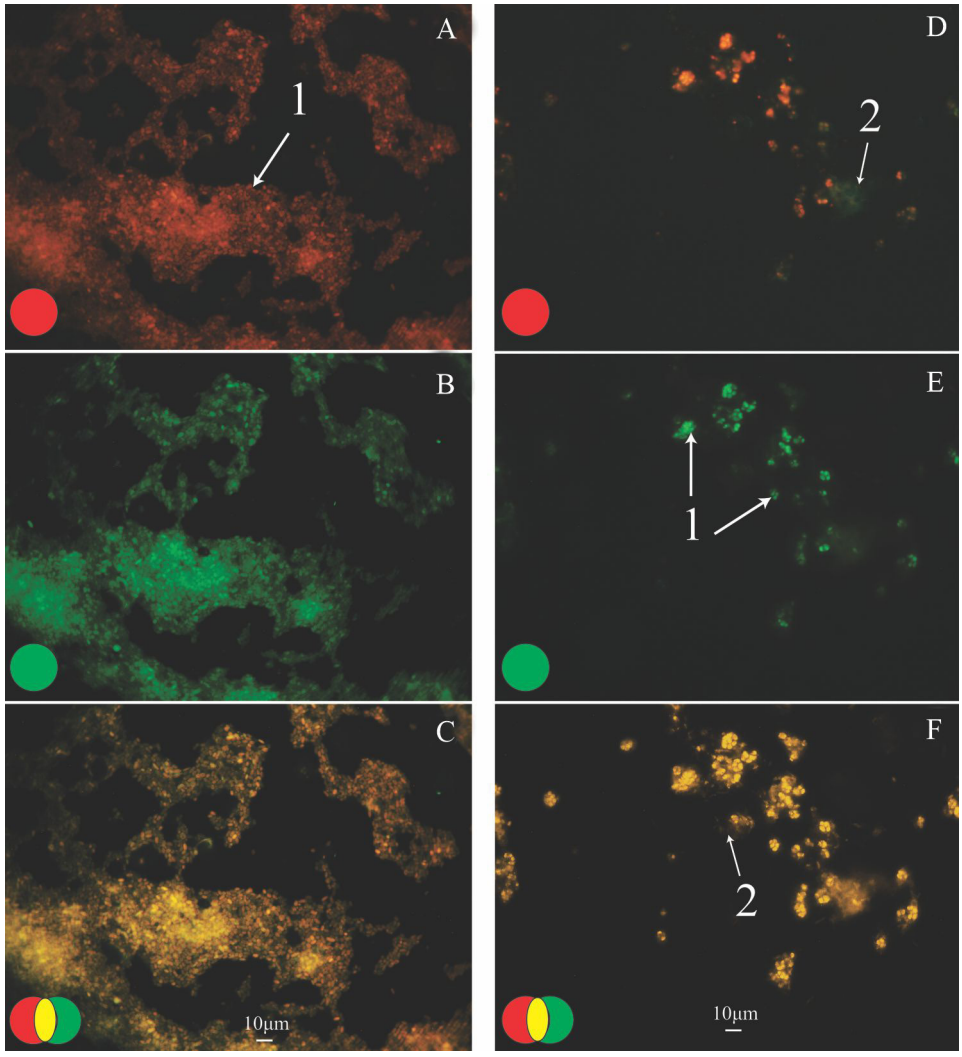

Figure 2. Fluorescence in situ hybridization micrographs showing root colonization of ryegrass (*Lolium* × *hybridum*) seedlings treated with liquid inoculant (A, B, C) or alginate-based polymeric inoculant (D, E, F). Visualizations are shown using a DAPI/FITC/TRITC filter set (A, D), a B-2A filter set (B, E), and merged filter channels (C, F). Annotations indicate bacterial cells (1) and the alginate matrix (2).

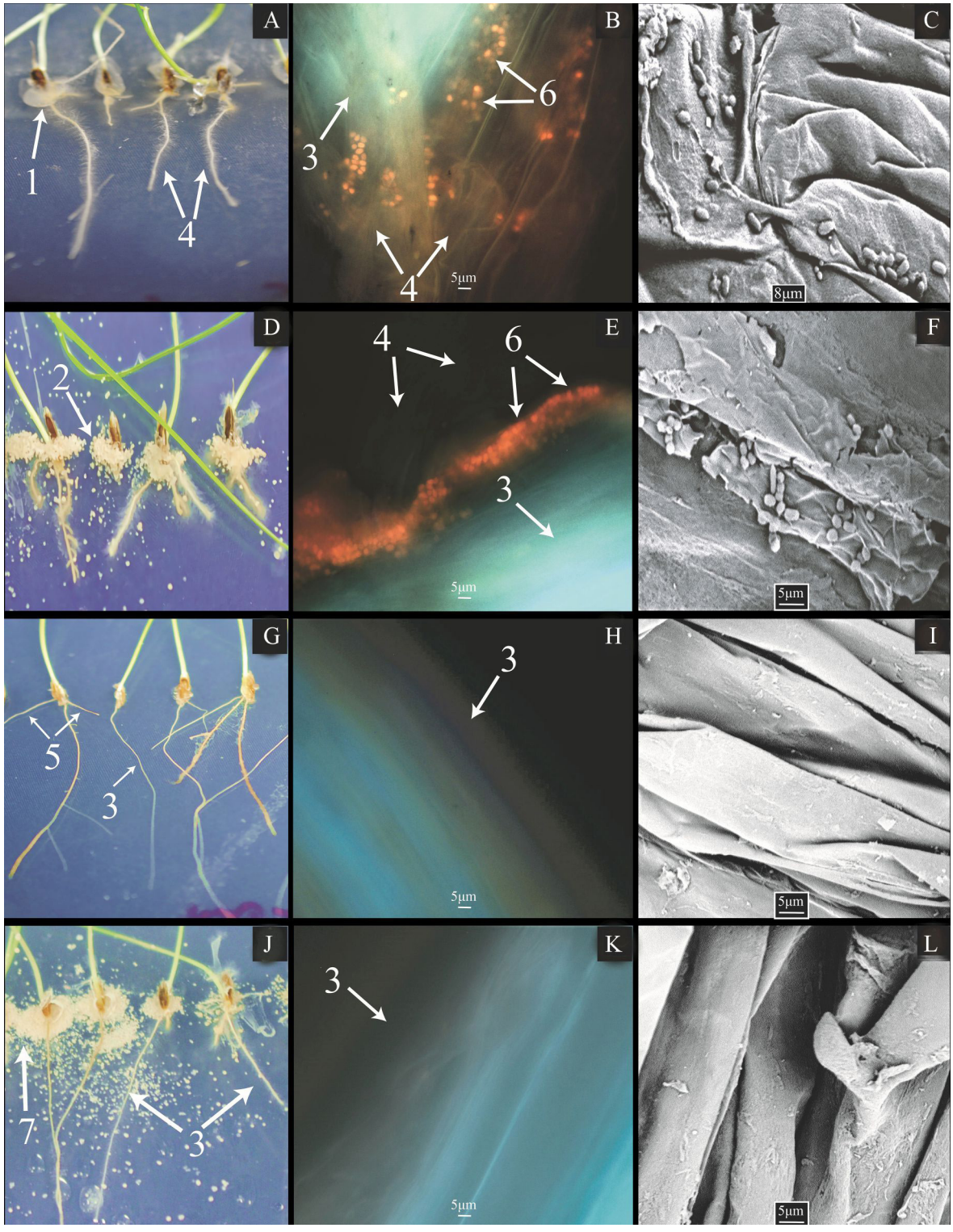

Figure 3. Macroscopic images (A, D, G, J), fluorescence in situ hybridization micrographs (B, E, H, K), and scanning electron micrographs (C, F, I, L) of ryegrass (*Lolium* × *hybridum*) seedlings subjected to *Azospirillum brasilense* inoculation treatments: liquid inoculant (A, B, C), alginate-based polymeric inoculant (D, E, F), uninoculated control (G, H, I), and sterile, uninoculated polymeric control (J, K, L). Numbered annotations indicate liquid suspension (1), microbeads containing bacteria (2), radicle (3), root hairs (4), nodal roots (5), bacterial cells (6), and sterile microbeads (7).

with seedlings exhibiting a 57.1% increase in root dry biomass compared with the uninoculated controls (Table 1).

Growth and physiological responses under greenhouse conditions were generally less pronounced than those observed under controlled growth chamber conditions (Table 1). Chlorophyll *a* concentration remained significantly higher in inoculated plants than in the uninoculated control, whereas chlorophyll *b* concentration and shoot height did not differ significantly among treatments. Total carotenoid concentration was higher in the plants receiving polymeric inoculant, although values did not differ significantly from the other treatments. Similarly, shoot dry biomass was higher in the liquid inoculant compared with the uninoculated control, even though no significant differences were detected among treatments. Root dry biomass showed no significant differences across treatments.

Results demonstrate that inoculant formulation influences the biological performance of *A. brasilense* from bacterial survival during storage to root colonization and early plant responses under non-sterile soil conditions. Although both liquid and alginate-based inoculants remained biologically effective, the polymeric inoculant consistently preserved higher bacterial viability, maintained close spatial association between bacterial cells and developing roots, and promoted enhanced physiological responses during early ryegrass establishment. The integrative approach of the present study allows inoculant performance to be evaluated as a continuous biological process, direct linking bacterial preservation during storage with rhizosphere establishment and plant responses under non-sterile soil conditions.

The principal finding of this study was the superior storage stability provided by the alginate-based polymeric formulation. Although both inoculants retained high bacterial viability throughout the 90-day storage period, viability losses were consistently lower in polymeric inoculants, particularly under ambient storage conditions. Maintaining adequate populations of viable cells prior to application is essential, as successful inoculation depends on the rapid establishment of metabolically active bacteria in the rhizosphere after seed germination. The improved preservation observed with the polymeric inoculant is consistent with the protective properties of alginate matrices, which reduce exposure to desiccation, oxidative stress, and temperature fluctuations while limiting cellular metabolic activity during storage (Vassilev et al., 2020). Similar protective effects have been reported for *Azospirillum* spp., *Bacillus* spp., *Pseudomonas* spp., and other plant growth-promoting bacteria encapsulated in natural polymers, supporting alginate as one of the most reliable carrier materials currently available for microbial inoculants (Khan et al., 2023; Fadiji et al., 2024; Singh & Kumar, 2024).

The superior stability of the polymeric inoculant under ambient conditions carries important practical implications. Although refrigerated storage remains the preferred strategy for preserving microbial inoculants, maintaining an uninterrupted cold chain throughout production, transportation, commercialization, and

Table 1. Means and standard error of physiological parameters and growth traits of ryegrass (*Lolium x hybridum*) seeds submitted to four inoculation treatments with or without *Azospirillum brasilense* under growth chamber and greenhouse conditions[1].

Condition	Treatment[2]	Physiological parameters			Plant growth traits		
		Chlorophyll *a* (mg L^{-1})	Chlorophyll *b* (mg L^{-1})	Total carotenoids (mg L^{-1})	Shoot length (cm)	Shoot dry weight (mg)	Root dry weight (mg)
Growth chamber	IL	(5.59 ± 0.45)a	(2.26 ± 0.31)b	(0.06 ± 0.001)b	(14.30 ± 0.44)a	ND	(9.67 ± 0.35)ab
	IM	(5.65 ± 0.61)a	(3.28 ± 0.25)a	(0.05 ± 0.002)b	(13.85 ± 0.43)a	ND	(11.00 ± 0.48)a
	IP	(5.06 ± 0.55)a	(2.13 ± 0.28)bc	(0.05 ± 0.001)b	(12.22 ± 0.44)b	ND	(7.66 ± 0.51)ab
	NI	(3.53 ± 0.40)b	(1.92 ± 0.19)c	(0.09 ± 0.001)a	(12.45 ± 0.45)b	ND	(7.00 ± 0.28)b
Greenhouse conditions	IL	(5.39 ± 0.40)a	(1.76 ± 0.26)a	(1.47 ± 0.10)ab	(31.42 ± 1.29)a	(3,276 ± 190)ab	(2,624 ± 190)a
	IM	(6.80 ± 0.38)a	(2.08 ± 0.18)a	(1.70 ± 0.16)a	(31.51 ± 1.18)a	(3,850 ± 240)bc	(3,257 ± 170)a
	IP	(5.58 ± 0.24)a	(1.69 ± 0.16)a	(1.37 ± 0.07)ab	(31.43 ± 1.14)a	(3,096 ± 170)ab	(2,674 ± 260)a
	NI	(3.67 ± 0.50)b	(2.57 ± 0.63)a	(1.11 ± 0.08)b	(32.86 ± 1.13)a	(2,443 ± 150)a	(2,940 ± 180)a

[1]Means followed by different letters differ significantly by Tukey's test (α = 0.05). [2]IL, inoculated with liquid formulation; IM, inoculated with alginate-based polymeric formulation; IP, inoculated with sterile polymer without bacteria; NI, uninoculated; and ND, not determined.

on-farm storage is often impractical, particularly in tropical and subtropical livestock systems (Gonzalez et al., 2018). Under these circumstances, the ability of alginate microbeads to maintain substantially higher viable bacterial populations increases the likelihood that adequate cell densities will reach the rhizosphere following sowing, thereby enhancing inoculant reliability under realistic agricultural conditions.

Conversely, storage stability alone does not ensure inoculant efficacy. Successful plant growth promotion also depends on the capacity of introduced bacteria to establish within the rhizosphere despite competition from indigenous microbial communities (Khanghahi et al., 2025). In the present study, FISH and SEM provided complementary evidence that *A. brasilense* successfully colonized ryegrass roots under non-sterile soil conditions. The close spatial association observed between alginate microbeads, bacterial aggregates, and the development of root hairs suggest that encapsulation favored localized bacterial release close to actively growing root tissues, thereby facilitating early rhizosphere establishment. Similar targeted delivery has been proposed as an important advantage of polymer-based microbial inoculants, as it enhances bacterial persistence at the root surface, where plant-microbe interactions are initiated (Rilling et al., 2019; Fadiji et al., 2024; Singh & Kumar, 2024).

The preferential association of bacterial cells with root hairs aligns with the physiological responses observed during early seedling development. Root hairs represent major sites of nutrient and water uptake, constituting key niches for bacterial colonization. Their close proximity to bacterial cells may increase the local availability of microbial metabolites involved in plant growth promotion, including auxins and other signaling compounds that regulate root development (Cortés-Patiño et al., 2021). This interpretation is supported by the increased shoot elongation, higher chlorophyll concentrations, and particularly the marked increase in root dry biomass observed in the growth chamber experiment. Furthermore, these findings corroborate the well-established capacity of *A. brasilense* to modify root architecture and stimulate plant development through mechanisms extending beyond biological nitrogen fixation (Cassán & Díaz-Zorita, 2016; Cassán et al., 2020).

As expected, plant responses were more pronounced under controlled growth chamber conditions than under greenhouse conditions in non-sterile soil. Soil environments introduce multiple ecological filters that influence inoculant performance, including microbial competition, predation, spatial heterogeneity, nutrient availability, and fluctuating physicochemical conditions. Consequently, plant responses in the field are often less pronounced than those observed under controlled conditions, representing one of the principal challenges limiting the consistency of microbial inoculants under agricultural conditions (Thakur et al., 2024). Nevertheless, chlorophyll *a* concentration remained significantly higher in inoculated plants under greenhouse conditions, indicating that bacterial activity persisted after growth in the soil. Although root biomass did not differ significantly among treatments, the observed numerical differences should be interpreted with caution, as they do not constitute statistical evidence of a treatment effect under the evaluated greenhouse conditions.

Beyond demonstrating the advantages of alginate microencapsulation, the results of this study support the concept that inoculant formulation should be regarded as a core determinant of biological performance rather than solely as a preservation strategy. Evaluating formulation effectiveness across successive biological stages — from storage stability to rhizosphere establishment and plant response — provides a more realistic framework for understanding inoculant performance and developing microbial biofertilizers with enhanced consistency under agricultural conditions. These findings underscore the critical role of formulation technology as a central component in the development of reliable microbial inoculants for sustainable forage production systems.

Several limitations of this study require consideration. First, the evaluation was restricted to a single strain of *A. brasilense* and focused on early plant establishment under controlled and greenhouse conditions. Furthermore, bacterial colonization was assessed qualitatively using FISH and SEM rather than through the direct quantification of bacterial populations in the rhizosphere. Future studies incorporating quantitative molecular approaches, such as qPCR or amplicon-based analyses, combined with long-term field experiments will provide a more comprehensive understanding of bacterial persistence, rhizosphere competence, and inoculant performance within commercial production systems.

Conclusions

1. The alginate-based polymeric inoculant maintains high viability and biological activity of *Azospirillum brasilense* throughout storage and following soil application under non-sterile conditions.

2. Inoculant formulation serves as a primary determinant of *A. brasilense* performance, facilitating localized spatial association, root hair interaction, and effective rhizospheric colonization of ryegrass (*Lolium* × *hybridum*).

3. Alginate-based encapsulation provides an effective inoculant formulation technology to support early plant performance of ryegrass in sustainable forage production systems.

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Chief editor: Edemar Corazza
Edited by: Daniel Kinpara

Data availability statement

Data available upon request: research data are only available upon reasonable request to the corresponding author.

Declaration of use of AI technologies

During the preparation of this work, the authors used OpenAI's ChatGPT to assist with English language editing, grammatical corrections, and improvements in the clarity, coherence, organization, and readability of the manuscript, including the refinement and restructuring of selected sections of the Introduction and Discussion. All AI-assisted content was critically reviewed, verified, and edited by the authors, who take full responsibility for the scientific accuracy, originality, and final content of the manuscript.

Conflict of interest statement

The authors declare no conflicts of interest.

Acknowledgments

To the Ministry of Science, Technology and Innovation of Colombia (Minciencias), for financial support within the project Strategies to Improve the Competitiveness and Sustainability of Milk and/or Meat Production Systems in the Andean Region, through Grant No. 706 – Young Researchers and Innovators Program (2015) (Contract No. 10732, Minciencias–Agrosavia); to Agencia Presidencial de Cooperación Internacional de Colombia (APC), for financial support; to Agencia Mexicana de Cooperación Internacional para el Desarrollo (AMEXCID) for financial support; to Herica Yulied Montoya, for technical assistance with scanning electron microscopy analyses; to the memory of Emeritus Professor Yoav Bashan (1951–2018), leader of the Environmental Microbiology Group at the Centro de Investigaciones Biológicas del Noroeste (CIBNOR), Mexico, and founder of the Bashan Institute of Science (BIS), for his contributions to agricultural research worldwide, and inspiration to the scientific community.

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