Environmental Technology & Innovation 34 (2024) 103627

Contents lists available at ScienceDirect

Environmental Technology & Innovation

journal homepage: www.elsevier.com/locate/eti

Polyglycerol citrate: A novel coating and inoculation material for soybean seeds

Amanda S. Giroto [a,b,*], Stella F. Valle [a], Gelton G.F. Guimarães [d], Benedict Ohrem [b], Joana Bresolin [a], Andreas Lücke [c], Holger Wissel [c], Mariangela Hungria [e], Caue Ribeiro [a,**], Luiz H.C. Mattoso [a], Nicolai D. Jablonowski [b,**]

[a] Embrapa Instrumentation, XV de Novembro Street, 1452, São Carlos, SP 13560-970, Brazil
[b] Forschungszentrum Jülich GmbH, Institute of Bio- and Geosciences, IBG-2: Plant Sciences, Jülich 52425, Germany
[c] Forschungszentrum Jülich GmbH, Institute of Bio- und Geosciences, IBG-3: Agrosphere, Jülich 52425, Germany
[d] EPAGRI - Agricultural Research and Rural Extension Company of Santa Catarina, 6800 Rd. Antônio Heil, Itajaí, Santa Catarina 88318112, Brazil
[e] Embrapa Soybeans, Soil Biotechnology Laboratory, Embrapa Soja, C.P. 4006, Londrina, PR 86085-981, Brazil

ARTICLE INFO

Keywords:
Polymer
Seed-coating
Inoculant
Rhizobia
Soybean

ABSTRACT

The microbial inoculation of legumes such as soybeans is crucial for thriving plant growth due to symbiotic nitrogen (N) fixation and biological plant N fertilization. Soybean requires microbial pre-inoculation before sowing using the rhizobia strain *Bradyrhizobium japonicum*. Peat is typical for this purpose, although not sustainable since it is a finite resource. Here, we propose a straightforwarding route to prepare and apply polyglycerol-citrate polymer (PGC), a biodegradable and fully renewable polymer, as a carrier for *Bradyrhizobium japonicum* inoculants for soybeans. This novel eco-friendly polymer combines the advantages of a polymeric, water-soluble structure based on biopolymers, which can protect the inoculant cells during the seed inoculation process, with protective properties of glycerol for bacterial cells and the contribution of citric acid for metabolic processes. A greenhouse study was conducted using soybean seeds coated with three different proportions of PGC with *B. japonicum* planted in a sand substrate free of external interference. Comparative results of N content and $\delta^{15}N$ signature in soybean plant parts calculated from the natural abundance method associated with viability tests showed equal or superior symbiotic performance and nitrogen fixation rates to peat-based inoculants, considered the gold-standard carrier for inoculants. It ensured the shelf life of the inoculant formulations, offering convenience for farmers and environmental benefits through reduced fertilization.

1. Introduction

The rise of microbiological products marks a crucial shift towards eco-friendly, sustainable agriculture, benefiting both productivity and the environment (Saleem et al., 2023; Sarpong et al., 2021; Shameem M et al., 2023). Developing commercial rhizobia strains, which act in symbiosis with legumes, was vital for replacing chemical fertilizers. They are referred to as inoculants or biofertilizers, and they provide the nitrogen plants' needs through biological atmospheric N_2 fixation (Taghizadeh et al., 2013, 2023).

* Corresponding author at: Embrapa Instrumentation, XV de Novembro Street, 1452, São Carlos, SP 13560-970, Brazil.
** Corresponding authors.
E-mail addresses: asgiroto@gmail.com (A.S. Giroto), caue.ribeiro@embrapa.br (C. Ribeiro), n.d.jablonowski@fz-juelich.de (N.D. Jablonowski).

https://doi.org/10.1016/j.eti.2024.103627
Received 4 January 2024; Received in revised form 15 March 2024; Accepted 2 April 2024
Available online 4 April 2024

Still, their survival in the field depends on adequate formulations to keep them active, protecting against external stresses (e.g., temperature, low moisture, or chemical agents) (Garcia et al., 2021; Hungria et al., 2020; Zilli et al., 2021). Several protective materials are proposed (Allouzi et al., 2022; Sivaram et al., 2023), but peat is still considered the most efficient (Hungria et al., 2020; Santos et al., 2019). Since 1920, peat has been used for manufacturing legume inoculants (Albareda et al., 2008; Deaker et al., 2007; Singleton, 2002). It consists of solid biogenic organic matter, serving as both a source of nutrients and physical protection for the microorganisms (Hungria et al., 2007; Hungria et al., 2006). However, peat is a non-renewable resource, scarce in many regions like the tropics (Zhang et al., 2022) since it occurs in specific environments and is formed after a long geological period (Głodowska et al., 2016). In addition to its exploitation impacts, peat requires significant processing such as milling, pH correction, and sterilization, making it unfeasible for smaller production.

Thus, readily available alternative carriers for inoculant production and application, predominantly liquid inoculant formulations, have been recently investigated. These included broth cultures, yeast-mannitol, and micronutrients, amended with agents (i.e., biopolymers) that promote cell survival and facilitate their application to the seed and soil (Albareda et al., 2008; Allouzi et al., 2022; Fernandes Júnior et al., 2009; Hungria et al., 2020) though few promote cell viability and field performance comparable to peat inoculants (Gopi et al., 2019; Hungria et al., 2007; Santos et al., 2019). The primary impediment to enhancing the utilization of inoculants stems from the limited availability of formulation knowledge, attributed to the scarcity of public research on this subject. Additionally, these inoculants exhibit an extended shelf life and reduced susceptibility to contamination, rendering them a preferred choice for a variety of applications (Bashan et al., 2016; Egamberdieva et al., 2020; Palhares Farias et al., 2022). In this scenario, polyglycerol citrate emerges as an eco-friendly candidate. It could protect the microorganisms with a suitable medium for survival, staying effective over a prolonged storage period at neutral or near-neutral pH (Giroto et al., 2023). Glycerol is a cell cryoprotectant for bacteria in culture collections, protecting cells against abiotic stress by promoting osmotic pressure balance and regulation of transmembrane traffic (Whaley et al., 2021). Moreover, it is a carbon source with high water-binding capacity and fluidity that promotes fast seed covering (Singleton, 2002; Tittabutr et al., 2007). Additionally, citric acid is a carboxylic acid involved in the Krebs cycle, a central metabolic pathway for plants and bacteria.

Thus, here we propose a straightforward route to produce polyglycerol-citrate (PGC) protective carriers for *Bradyrhizobium japonicum* inoculants on soybean seeds, showing the PGC's role in protecting the microorganisms in liquid and dried seed films. A greenhouse experiment assessed the symbiotic nitrogen-fixing ability of *B. japonicum* via PGC-coated and inoculated seeds compared to peat and untreated controls. Overall, the experiment aims to demonstrate the potential of PGC as a novel, eco-friendly seed coating and inoculation material for enhancing sustainable soybean production while minimizing the environmental impact associated with peat exploitation.

2. Materials and methods

2.1. Inoculant preparation and inoculation procedure

Bradyrhizobium japonicum DSM 30131^{T} (=USDA 6 ^{T}, =ATCC10324^{T}), (Leibniz Institute DSMZ GmbH, Germany) were stored in a modified yeast-mannitol (YM) liquid medium containing 20% glycerol at −80 °C. For the evaluations, an aliquot was grown in a modified YM-broth (per liter: mannitol, 5.0 g; yeast extract, 0.4 g; K_2HPO_4, 0.5 g; $MgSO_4{\cdot}7\ H_2O$, 0.2 g; NaCl, 0.1 g, pH 6.8, Hungria et al., 2006) with shaking at 120 rpm, at 28 °C, for 5 days. The cultures were centrifuged for 15 min at 7000 rpm (Centrifuge 5425/5425 R, Eppendorf, Hamburg, Germany). The supernatant was discarded, and the cells were resuspended in 0.85% (m/v) NaCl saline solution. Cell concentration was estimated by plating in Petri plates containing modified YM-medium. Colony forming units (CFU) were counted after five days of incubation at 28 °C.

2.2. Preparation of the polymer solution carriers

Polyglycerol citrate (PGC) polymers were synthesized as described by Giroto et al. (2023). The polymeric solutions were prepared in three different concentrations, namely 25, 50, and 66% (m/v) of polymer in water, to study the effect of polymer concentration on cell viability, seed coating ability, and resulting plant performance. The pH was adjusted to neutral with $CaCO_3$ (Merck, Germany) and some dropwise of NaOH (6 M). Modified-YM medium was subsequently added into the solutions lacking mannitol. The decision to omit mannitol was deliberate, aiming to compel the microorganism to utilize glycerol as the primary carbon source from the inoculation matrix. The polymeric solutions were sterilized at 121 °C for 20 min, during which the original clear solution changed into a white viscous gel. After cooling, each of the three polymer gels with the different PGC concentrations were inoculated with *B. japonicum* at approximately 10^9 cells mL^{-1}. The added cells were thoroughly homogenized with the PGC gels and stored at 5 °C for further use.

2.3. Coating soybean seeds with PGC gels

Soybean seeds (*Glycine max* L., Eiko cultivar; Asgrow, United States) were treated with PGC gel at a dose of 200 mL_{PGC} per 100 kg $_{seed}$ (Hungria et al., 2020) by adding an appropriate amount of each PGC to seeds in a glass flask, followed by 15 min homogenization on a rotating table (MIXER 10, Greiner Labortechnik, Germany) until the seed's surface was dry. A control without coating treatment and a PGC polymer at the three used concentrations without rhizobia cells were also prepared. Seeds (S) coated with PGC polymers were named SPGC25, SPGC50, and SPGC66 according to the concentration of PGC used and the inclusion of *B. japonicum*.

Seeds were also inoculated with peat inoculant (Einheitserde Typ 0, Germany) as a positive control. The peat substrate used for inoculation was prepared by drying overnight in an oven at 50 °C, pulverized to > 2 μm using a ball mill (MM 400, Retsch, Germany) before being used. The used peat initially possessed a neutral pH, and therefore no additional pH-adjustment was necessary. For seed treatments, 220 g_{Peat}/50 kg_{seeds} were used after seed pre-moistening with 300 mL of 10% sucrose solution to create a sufficient adhesive surface for the peat powder over the soy surface (Campo et al., 2009). The rhizobia cells were introduced into the saccharose solution, which was subsequently applied to the seeds. Following this step, the peat powder was incorporated into the system. The rhizobia survival rate after seed coating with PGC polymers or peat was assessed after 0, 1, 3, 7, and 14 days of seed storage at 5 °C.

2.4. Recovery of viable cells from inoculated polymer

A sufficient number of viable rhizobia cells is crucial for successful nodulation at the plant roots and symbiotic N_2 fixation by the microorganisms. Therefore, the survival of rhizobia in the PGC gels was assessed after each day by using the dilution method starting with 1 mL added to a sterile flask along with 9 mL of physiological solution 0.85% NaCl (m/v) + 0.01% (m/v) Tween 80 solution (Vetec, Brazil) and counting with the drop plate method (Howieson and Dilworth, 2016). The plates were incubated at 28 °C for five days, and the number of CFUs was counted. The recovery and counting of viable *B. japonicum* cells from inoculated seeds followed the procedure described by Santos et al. (2019). For the evaluation, 100 coated seeds were transferred to a 250-mL Erlenmeyer flask containing 100 mL of sterile 0.85% (w/v) NaCl saline solution + 0.01% (m/v) Tween 80 solution, kept stirring for 2 hours, and following as described by Moretti et al. (2020).

2.5. Seed surface morphology analysis

Scanning electron microscopy (SEM) was employed to analyze the seeds' PGC-coated and uncoated surface morphology using a Zeiss model SUPRA 50VP. The samples were dispersed over carbon tape on the surface of a metallic disc (stub), carbon-coated in a Leica EM SCD050 chamber, and imaged using the secondary electron mode.

2.6. Greenhouse experiments

The greenhouse experiment was performed under controlled conditions at the Institute of Bio- and Geosciences, IBG-2: Plant Sciences, Forschungszentrum Jülich GmbH, Germany (location: 50°54'36" N, 6°24'49" E), from January to March 2023. To evaluate the effect of PGC polymer as seed inoculant (named SPGC), the following treatments were applied: (*i*) non-inoculated receiving 100 mg of N as urea kg^{-1} substrate; (*ii*) non-inoculated and non-N-fertilized control (control); (*iii*) peat inoculant, traditionally considered as the best inoculation medium (Hungria et al., 2020), (peat); (*iv*) pure PG polymer without rhizobia (PGC); (*v* to *vii*) seeds coated with inoculants SPGC25, SPGC50, and SPGC66. All inoculant-based treatments were prepared on the day of sowing to deliver at least 1.2 million vital *B. japonicum* cells $seed^{-1}$, as recommended by Hungria et al. (2020). After that, seeds were directly sown into 1.5 L pots containing 1 kg of a sandy substrate (RBS GmbH, Inden, Germany; particle size: ≤1 mm; pH_{H2O} 6.6; WHC: 24%; no detectable amounts of N, P, K, and C), which was used also earlier in numerous fertilizer studies as a model substrate for a marginal soil (Nabel et al., 2016; Valle et al., 2022; Giroto et al., 2022). This substrate was chosen to avoid unpredictable influences of other soil organisms and nutrients as potentially present in natural soils or organic growing media. Plants were kept in the greenhouse, ensuring a daily light period of 16 h, a constant day/night temperature of 23°C, and air humidity of 48%. Five replicates for each treatment were employed in a completely randomized block design, and pot positions were randomly changed every week to circumvent microclimatic effects on plant growth. Plants were watered twice a week using an N-free Hoagland solution (50 mL). Harvest was conducted after 60 days of cultivation.

Before harvest, SPAD values were measured using Chlorophyll Meter SPAD-502Plus (Konica Minolta), allowing for an estimation of leaves' greenness as an indicator for plant N availability.

2.7. Post-harvest analysis

Following harvest, total shoot length and leaf area were measured for all plants using a leaf area meter (LI-3100, LI-COR). Subsequently, shoots were dried at 60°C until complete dryness. Before drying, roots were washed carefully, and all nodules were removed from the roots, counted, dried, and weighed to obtain total nodule dry weight per plant. Assessment of nodulation (number and mass), shoot and root dry mass, N concentration, and N contents in each part of the plants was conducted, and N content of the dry biomass (shoots, roots, and nodules) was determined via CHN elemental analysis (Leco TCH 600).

2.8. Quantitative estimation of nitrogen fixation: ^{15}N natural abundance analysis

Nitrogen fixation was evaluated by the ^{15}N natural abundance method, with sampling as described by Shearer and Kohl, 1988. Plant materials were divided into shoots, roots, and nodules, and each biomass fraction was milled to a fine powder after drying using a ball mill (MM 400, Retsch, Germany). For total-N and $\delta^{15}N$ determinations, samples of 1.5–2 mg per fraction were analyzed using an elemental analyzer coupled with an isotope ratio mass spectrometer (EAIRMS; IRMS IsoPrime, Micromass UK Limited, USA). The term $\delta^{15}N$ refers to a sample's ratio of the heavier to the lighter stable isotope of nitrogen (^{15}N over ^{14}N) in comparison to a reference value (atmospheric N_2) (Nabel et al., 2016). Generally, the ^{15}N abundance of the N derived from the air in the whole plant, including roots

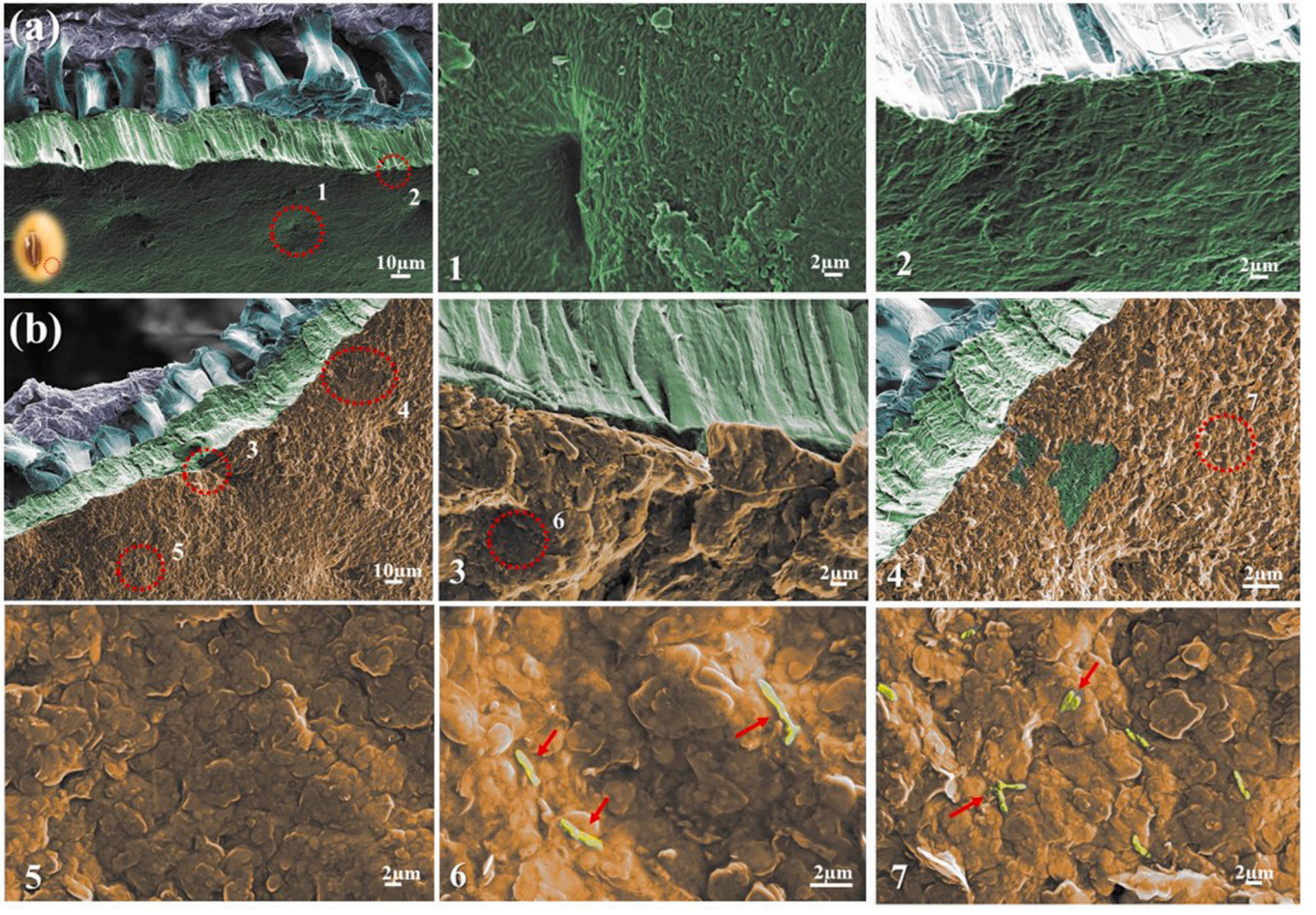

Fig. 1. (a) Soybean seed untreated, illustrating the region of the surface analyzed and allowing for a visual comparison between untreated and the PGC-treated seeds, showing the seed coat (green), followed by the hypodermis (green-turquoise) and parenchyma layer (grey); (b) seed coated with PGC 50 including inoculated *Bradyrhizobium japonicum*. (1) a region showing the natural porosity of the seed, (2) cross-sections of the seed coat, (3) a region of the seed surface coated with the PGC polymer, (4) coating defect on soybean surface, (5) "cornflakes-shape" seed surface as a result of the SPGC50 polymer coating, (6) and (7) presence of *B. japonicum* on the PGC polymer-coated seed surface.

and nodules, is close to that of the atmosphere, i.e., 0.0‰ using standard notation (Unkovich, 2013).

$$\delta^{15}N = \left(\frac{R_{sample}}{R_{standard}} - 1\right) \times 1000$$

R_{sample} or $R_{standard}$ is the ratio of ^{15}N over ^{14}N for sample or standard, respectively. $\delta^{15}N$ values were calibrated against IAEA-N-2, IAEA-N-1, and USGS25 (ammonium sulfate standard) and are reported scale-normalized to air. The relative share of N derived from the atmosphere ($\%N_{dfa}$) can be calculated using the method of Shearer and Kohl, 1988, with the equation:

$$\%Ndfa = \left(1 - \frac{\delta^{15}N_{fixplant}}{\delta^{15}N_{refplant}}\right) \times 100$$

N_{dfa} was calculated for soy growing in the control setups, i.e., i) the negative control (no N fertilization and no inoculation), and ii) treatments with rhizobia inoculations, i.e., peat, and the SPGC treatments (Chalk, 2016; Unkovich, 2013).

2.9. Statistical analysis

All results were submitted to one-way statistical analysis (ANOVA) with Tukey's test at the significance level $p < 0.05$ (Statistic R Software, R Core Team (2019) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria).

3. Results and discussion

3.1. PGC seed coating analysis using scanning electron microscopy

The PGC film coating over the seeds was confirmed by scanning electron microscopy (SEM). The polymeric coating protected the seed by an almost evenly PGC layer on the seed surface. Fig. 1, (b) shows in detail a "cornflake-shape" appearance at the seed surface (Fig. 1, (b) 5–7). Only a few regions were not covered by the polymer, as shown in Fig. 1(b), region 4, due to local inhomogeneities. This coating thickness is expected to guarantee moisture preservation and gas exchange with the external medium. The presence of

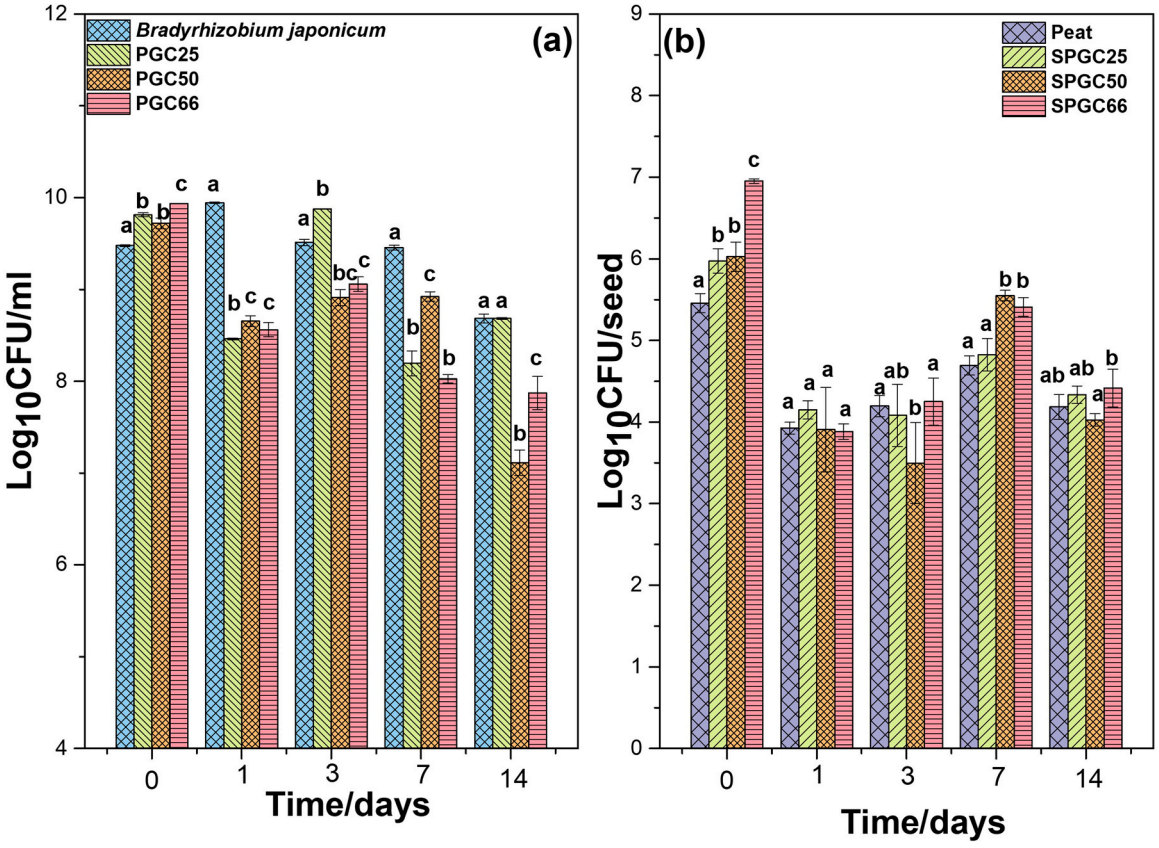

Fig. 2. Survival of rhizobia in (a) liquid inoculants PGC25, PGC50, and PGC66, (b) seeds coated peat, SPGC25, SPGC50, and SPGC66 over 14 days.

bacteria was seen in high magnification SEM images of seeds coated with PGC50, showing rod-shaped bacteria with uniform cellular surface texture (Fig. 1(b), regions 6 and 7), consistent with the inoculated *B. japonicum* strain (Thapa et al., 2023).

3.2. Viability Tests of Bradyrhizobium japonicum in PGC formulations

The survival of *Bradyrhizobium japonicum* was evaluated in suspensions of PGC25, PGC50, and PGC66 after seed coating (Fig. 2). The net losses of viable cells were determined by calculating the mean difference in log10 CFU/mL between 0 and 14 days across three replicate samples. *B. japonicum* strain DSM 30131 demonstrated robust survival across all PGC formulations (Fig. 2a), consistently maintaining over 10^9 CFU/mL, affirming the safety of the polymer. Although a slight decline in CFU/mL was observed after 7 days, the count remained above 10^8 CFU/mL. By day 14, the PGC25 treatment exhibited sustained levels of viable cells, while the counts in the other treatments decreased. The effect on strain survival of pre-inoculated soybean seeds was also followed over 14 days of storage (Fig. 2b), with peat as the positive control. After coating, SPGC66 attained the highest CFU/seed level of 10^7, as opposed to 10^5 for peat-treated seeds. After 24 hours, CFU drastically decreased but remained stable over the subsequent 7 days. SPGC50 and SPGC66 slightly increased (10^6) after this period, returning to 10^4 as for the other treatments. Similar results were reported for other biodegradable polymers using *B. japonicum* (Allouzi et al., 2022; Fernandes Júnior et al., 2009; Mortvedt and Giordano, 1967; Tittabutr et al., 2007), but, distinct from them, PGC has a dual function as a cell protector and a direct nutrient source, facilitating the survival of the bacterial strain (De Gregorio et al., 2017; Garcia et al., 2021; Vassilev et al., 2017). After cells enter the stationary phase, nutrient depletion may be severe and cause a higher reduction of viable cells in liquid inoculants than in peat (Garcia et al., 2021; Hungria et al., 2020; Santos et al., 2019). In our approach, this can be avoided by the polymer as a carbon source. Although polymers kept the cell viability at around 10^4 cells/seed after 14 days, it is reported that concentrations of even 100–1000 cells per seed produce satisfactory nodulation (Brockwell et al., 1980; Tittabutr et al., 2007).

3.3. Greenhouse experiment

A greenhouse experiment was conducted to test the inoculated and SPGC-coated soybean seeds. Table 1 shows the number of nodules, leaf area, plant height, SPAD (indication for chlorophyll content), plant dry weight, and N content of each plant part compared to peat-based inoculant. Results show that SPGC25 and SPGC66 had statistically similar CFU per seed, and SPGC50 was superior compared to the peat control. However, SPGC66 and SPGC25 presented a similar number to peat, considered as gold control. Seeds coated with a pure polymer (SPGC, no inoculant, and no N fertilization) were also evaluated to understand if the material would affect plant development.

Plants grown from SPGC-treated seeds had vigorous growth, presenting the highest leaf area superior to the control, confirming the polymer non-toxicity and non-adverse effects. However, the SPAD value of the pure SPGC was statistically similar to the control treatment, indicating a lower nitrogen supply for them. Most importantly, all seed treatments with SPGC inoculated with the *B. japonicum* had superior leaf area production (> 114.61 cm^2) compared to the negative control (70.43 cm^2) and similar to the urea treatment (147.31 cm^2), despite the similar plant heights and chlorophyll content values (Table 1).

Concerning the dry weight, no difference was observed between the peat and the SPGC treatments in the biomass from the nodules for all individual plant parts. Additionally, the total dry biomass of the pure SPGC treatment without rhizobia addition was similar to urea and peat treatments and superior to the control. This result suggests that the simple PGC polymer coating of the soybean seeds provided an increment in plant growth, similar to the N fertilization and rhizobia inoculation with peat.

Nitrogen content results of the various plant parts revealed that inoculated PGC seed treatments, namely SPGC25, SPGC50, and SPGC66, resulted in values comparable to those of plants with peat inoculant (Table 2), confirming the SPGC treatment's effectiveness for nodulation resulted in adequate supply of N to plants by the microbial-plant symbiosis. In the peat and SPGC treatments, the determined N content was mainly obtained via symbiotic N_2 fixation, accounting for 21–23 mg N plant^{-1}, less than half of the fertilized amount. However, the N uptake from N_2 fixation was enough for the plants' N supply and promoted soybean growth equivalently to plants fertilized with urea. It suggests that the fertilized plant with urea absorbed an N excess in the vegetative period. On the other hand, biological fixation provided N in a more synchronized manner with plant demand. Thus, the PGC polymer matrix

Table 1
Viable cells in colony forming units (CFU) per seed, nodule number, leaf area, plant height, SPAD (indication for chlorophyll content), and dry biomass content of different soybean plants grown in the sand culture at different treatments. SPGC seeds inoculated (seeds coated with PGC polymer solution with rhizobia cells).

Treatments	$\log_{10}$ CFU (seed^{-1})	Nodule number (plant^{-1})	Leaf area (cm^2)	Height (cm)	SPAD	Dry biomass (g)			
						Nodule	Root	Shoot	Total
Control*	-	-	70.4c	29.3 a	23.1 b	-	0.19 b	0.73c	0.83 b
Urea*	-	-	147.3 a	33.4 a	39.5 a	-	0.32 a	1.32 a	1.58 a
Pure PGC*	-	-	115.6 ab	33.6 a	27.1 b	-	0.37 a	1.10 ab	1.45 a
Peat	6.87 b	15.6 a	103.7 bc	34.2 a	38.2 a	0.031 a	0.29 ab	0.98 bc	1.30 ab
SPGC25	6.83 b	18.4 a	117.1 ab	30.4 a	36.7 a	0.038 a	0.28 ab	1.02 bc	0.87 b
SPGC50	7.19 a	15.2 a	117.9 ab	38.3 a	36.7 a	0.038 a	0.29 ab	1.06 ab	1.38 a
SPGC66	6.93 ab	19.0 a	114.6 ab	34.8 a	39.1 a	0.034 a	0.28 ab	0.98 bc	1.30 ab

*without rhizobia addition, Different letters indicate significant mean differences at $p < 0.05$.

Table 2
Comparative results of N content and $\delta^{15}N$ signature in soybean plant parts calculated from the natural abundance method. SPGC25/50/66: seeds coated with PGC polymer solution containing 25, 50, and 66% PGC, inoculated with rhizobia cells. %Ndfa: relative share of N derived from the atmosphere. n = 4.

Treatments	N content (mg N plant^{-1})				$\delta^{15}N$ value (‰ AIR)				%Ndfa
	Nodule	Root	Shoot	Total	Nodule	Root	Shoot	Total	
Control*	-	2.31c	6.89 d	9.72c	-	1.18 b	1.14 b	1.16 b	-
Urea*	-	14.35 a	34.41 a	46.51 a	-	3.36 a	2.78 a	2.94 a	-
Pure PGC*	-	4.48 b	8.72 cd	13.07c	-	0.25c	0.54c	0.44c	-
Peat	1.51 a	4.58 b	15.22 bc	21.07 b	3.16 a	0.64 bc	-0.38 de	0.11 cd	90.26a
SPGC25	1.83 a	3.87 bc	16.06 b	23.25 b	3.12 a	0.23c	-0.08 d	0.27 cd	77.01a
SPGC50	1.91 a	3.53 bc	17.40 b	21.58 b	3.89 a	0.40c	-0.49 de	0.18 cd	84.73a
SPGC66	1.54 a	3.98 bc	15.66 bc	21.18 b	3.82 a	0.15c	-0.59 e	-0.10 d	100.0a

*without rhizobia addition; Different letters indicate significant differences at $p < 0.05$.

provided physical protection to the inoculum, water holding capacity, and nutrient provision, not requiring the use of adhesives (as required by peat-based), reducing the time and workforce demands (Santos et al., 2019).

Concerning the N content in the plant biomass, shoots had the highest N concentration, accounting for more than 15 mg/plant, which is likely associated with the higher N content in the chloroplasts. This is reflected by the measured SPAD values (Table 1), showing the greenness of the leaves as an indirect measure for N content (Table 2). This result confirms the efficiency of this analysis for indirect measurement of the plant N content, being, therefore, an essential tool for continuously measuring the N nutritional status in commercial crops such as soybean.

The pattern of ^{15}N distribution within soybean plants markedly changed among the treatments. It is assumed that shoots are ^{15}N depleted, while nodules and, to a lesser extent, roots depict a significantly higher share of ^{15}N (Wanek and Arndt, 2002). A ^{15}N enrichment in the nodules was earlier described for numerous legume species, including soybeans, lupins, and *Vigna* species (Shearer and Kohl, 1988; Wanek and Arndt, 2002). ^{15}N increase is linked to nodule metabolism and was demonstrated to vary with rhizobial strains and to increase with nodule age (Unkovich, 2013). Therefore, the ^{15}N content was higher in roots than in shoots (Kohl et al., 1989). In contrast, under favorable conditions for nodulation, as in the case of peat and SPGC treatments, shoots tend to be enriched in ^{14}N, and the $\delta^{15}N$ showed lower values. Shoots' ^{15}N content is higher when plants rely on mineral N, as in the case of urea treatment, with $\delta^{15}N$ higher than 2.7‰. These modifications in internal ^{15}N distribution patterns across nodules, roots, and shoots follow the changes in the ^{15}N constitution among plant components that rely or not on exclusive N_2 fixation. This behavior was observed for all plants from the peat and the three SPGC seed treatments that rely on the symbiotic, bacterially-induced biological nitrogen fixation (BNF) process. We observed that the ^{15}N accumulation followed the order nodule > roots > shoots for these treatments. In plant parts based on BNF, as in the case of peat and SPGC treatments, the $\delta^{15}N$ values in shoots were negative. On the contrary, in the control, PGC, and urea treatments, the shoot and root $\delta^{15}N$ values were always positive.

SPGC seed treatments significantly improved the soybean nodulation and plants' BNF capacity, resulting in increased N accumulation in the shoots and roots in the same range as peat (Table 2). SPGC treatments resulted in increased nitrogen accumulation in the shoots and roots and increased the biological nitrogen fixation capacity. The $\delta^{15}N$ and %Ndfa values suggest the SPGC66 treatment was superior. In all regions studied in Brazil, the %Ndfa for soybeans has been found to range from 60% to 90%, and the $\delta^{15}N$ composition of N derived from the soil is often less than 4.0‰ (Pauferro et al., 2010).

Taking into account the very harsh environment used to test the formulations (i.e., sand with no organic matter), our results demonstrate a promising approach to sustainable and improved soybean seed treatment and plant growth. The BNF in two of the three proposed seed treatments had similar plant growth and fertilization responses compared to the traditional peat inoculation technique. The novelty of our study relies on being the first to use polyglycerol as a carrier for microbial inoculants for seed coating purposes, with both a simultaneous protective and energy source for *B. japonium.* More studies will be needed to improve the nodulation and symbiotic atmospheric N-fixing efficiency using the proposed technology. Soluble polymers containing the necessary inoculant formulations are convenient for both large-scale industrial applications and manageability at the farm level. The developed PGC polymer (Giroto et al., 2023) used in this study was selected based on its advantageous properties, such as easy and economic accessibility, solubility in water, non-toxicity, biological degradability and simultaneously complex chemical nature, which prevents soil microorganisms from rapidly degrading the polymeric coating.

4. Conclusions

Polyglycerol-citrate (PGC) has been demonstrated to be an effective carrier and protective agent for the rhizobia strain *B. japonicum* inoculation for soybean seeds. SEM imaging confirmed the presence of a uniform and adherent polymeric coating on the seed surface, effectively safeguarding both the seeds and the enclosed microorganisms. The survival of *B. japonicum* cells in PGC formulations exceeded agricultural application requirements, being vital even after 14 days. Greenhouse experiments revealed that coated seeds positively impacted soybean plant development, with sufficient nodule formation on par with peat-based inoculants. Notably, seeds coated solely with the pure polymer (SPGC) had no adverse effects on plant growth, confirming its non-toxic nature.

Most importantly, the enhanced biological nitrogen fixation capacity of soybean plants, particularly with SPGC66 and SPGC50 treatments, was evident. This was supported by the detected N content and distribution in the plant tissues, with negative $\delta^{15}N$ values

in shoot parts confirming the effectiveness of the symbiotic nitrogen fixation process. The findings and the innovative approach presented in this study propose PGC as a promising alternative to traditional inoculation carriers like peat, offering protection for microorganisms and serving as a direct nutrient source for bacteria. The environmentally friendly and sustainable formulation and production of PGC could help to protect finite peat resources. These results underscore the potential of PGC as an inoculant and seed coating material for improved nitrogen fixation and enhanced plant growth, positioning PGC as an environmentally friendly and practical choice for soybean cultivation. While further research is necessary to optimize this technology, this study paves the way for more sustainable and efficient soybean production.

Author statement

All authors have read and agreed to the submitted version of the manuscript.

CRediT authorship contribution statement

Gelton G.F. Guimarães: Writing – original draft, Validation, Methodology, Formal analysis, Data curation. **Stella F. Valle:** Data curation, Formal analysis, Investigation, Methodology, Writing – review & editing. **Amanda S. Giroto:** Funding acquisition, Formal analysis, Data curation, Conceptualization, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. **Nicolai Jablonowski:** Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Writing – original draft, Writing – review & editing. **Luiz H. C. Mattoso:** Funding acquisition, Project administration, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing. **Caue Ribeiro:** Conceptualization, Data curation, Funding acquisition, Investigation, Project administration, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing. **Mariangela Hungria:** Methodology, Validation, Writing – review & editing. **Holger Wissel:** Data curation, Formal analysis, Methodology. **Andreas Lücke:** Data curation, Formal analysis, Investigation, Methodology, Resources, Supervision, Validation, Writing – review & editing. **Joana Bresolin:** Data curation, Formal analysis, Methodology, Validation. **Benedict Ohrem:** Methodology, Investigation, Data curation, Writing – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Amanda Soares Giroto reports financial support was provided by State of Sao Paulo Research Foundation. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

Acknowledgment

This work was supported by FAPESP (São Paulo State Research Foundation, grants #2016/10636-8, #2018/10104-1, and #2021/13645-6), CNPq (Brazilian National Council for Scientific and Technological Development, grant #2014/142348-7), and CAPES (Coordination for the Improvement of Higher Education Personnel, Finance Code 001). The authors thank the Agronano Network (Embrapa Research Network), SISNANO/MCTI, and the National Nanotechnology Laboratory for Agribusiness (LNNA) for providing institutional support and facilities. The institutional funding and support by IBG-2: Plant Sciences, Forschungszentrum Jülich GmbH, a member of the Helmholtz Association, is highly appreciated. We kindly acknowledge the help and assistance in experimentation by Michael Gitzen and Klara Grünewald, and the greenhouse assistance by Beate Uhlig, Katharina Wolter-Heinen, and Marina Heinen. Many thanks to Sabine Willbold, Nadine Wettengl, Jennifer Pelikan, Daniela Gesekus, and colleagues from ZEA-3 for the chemical analysis of the plant materials, and soil samples. We thank Dr. Daniel Grüner (IEK-2) for the SEM images.

References

Albareda, M., Rodríguez-Navarro, D.N., Camacho, M., Temprano, F.J., 2008. Alternatives to peat as a carrier for rhizobia inoculants: Solid and liquid formulations. Soil Biol. Biochem 40, 2771–2779. https://doi.org/10.1016/j.soilbio.2008.07.021.

Allouzi, M.M.A., Allouzi, S.M.A., Keng, Z.X., Supramaniam, C.V., Singh, A., Chong, S., 2022. Liquid biofertilizers as a sustainable solution for agriculture. Heliyon. https://doi.org/10.1016/j.heliyon.2022.e12609.

Bashan, Y., de-Bashan, L.E., Prabhu, S.R., 2016. Superior polymeric formulations and emerging innovative products of bacterial inoculants for sustainable agriculture and the environment. in: Agriculturally Important Microorganisms: Commercialization and Regulatory Requirements in Asia. Springer Singapore, pp. 15–46. https://doi.org/10.1007/978-981-10-2576-1_2.

Brockwell, J., Gault, R., Chase, D., Hely, F., Zorin, M., Corbin, E., 1980. An appraisal of practical alternatives to legume seed inoculation: field experiments on seed bed inoculation with solid and liquid inoculants. Aust. J. Agric. Res 31, 47. https://doi.org/10.1071/AR9800047.

Campo, R.J., Araujo, R.S., Hungria, M., 2009. Nitrogen fixation with the soybean crop in Brazil: Compatibility between seed treatment with fungicides and bradyrhizobial inoculants, in: Symbiosis. Balaban Publishers, pp. 154–163. https://doi.org/10.1007/BF03179994.

Chalk, P.M., 2016. The strategic role of 15N in quantifying the contribution of endophytic N2 fixation to the N nutrition of non-legumes. Symbiosis. https://doi.org/10.1007/s13199-016-0397-8.

De Gregorio, P.R., Michavila, G., Muller, L.R., De Souza Borges, C., Pomares, M.F., De Sá, E.L.S., Pereira, C., Vincent, P.A., 2017. Bene-cial rhizobacteria immobilized in nano-bers for potential application as soybean seed bioinoculants. PLoS One 12. https://doi.org/10.1371/journal.pone.0176930.

Deaker, R., Roughley, R.J., Kennedy, I.R., 2007. Desiccation tolerance of rhizobia when protected by synthetic polymers. Soil Biol. Biochem 39, 573–580. https://doi.org/10.1016/j.soilbio.2006.09.005.

Egamberdieva, D., Ma, H., Alimov, J., Reckling, M., Wirth, S., Bellingrath-Kimura, S.D., 2020. Response of soybean to hydrochar-based rhizobium inoculation in loamy sandy soil. Microorganisms 8, 1–10. https://doi.org/10.3390/microorganisms8111674.

Fernandes Júnior, P.I., Rohr, T.G., Oliveira, P.J. de, Xavier, G.R., Rumjanek, N.G., 2009. Polymers as carriers for rhizobial inoculant formulations. Pesqui. Agropecu. Bras. 44, 1184–1190. https://doi.org/10.1590/S0100-204×2009000900017.

Garcia, M.V.C., Nogueira, M.A., Hungria, M., 2021. Combining microorganisms in inoculants is agronomically important but industrially challenging: case study of a composite inoculant containing Bradyrhizobium and Azospirillum for the soybean crop. AMB Express 11. https://doi.org/10.1186/s13568-021-01230-8.

Giroto, A.S., Do Valle, S.F., Guimarães, G.F.G., Wuyts, N., Ohrem, B., Jablonowski, N.D., Ribeiro, C., Mattoso, L.H.C., 2022. Zinc loading in urea-formaldehyde nanocomposites increases nitrogen and zinc micronutrient fertilization efficiencies in poor sand substrate. Sci. Total Environ. 841, 156688 https://doi.org/10.1016/j.scitotenv.2022.156688.

Giroto, A.S., Valle, S.F., Borges, R., Colnago, L.A., Ribeiro, T.S., Jablonowski, N.D., Ribeiro, C., Mattoso, L.H.C., 2023. Revealing the Structure Formation on Polyglycerol Citrate Polymers—An Environmentally Friendly Polyester as a Seed-Coating Material. Polym. (Basel) 15. https://doi.org/10.3390/polym15214303.

Głodowska, M., Husk, B., Schwinghamer, T., Smith, D., 2016. Biochar is a growth-promoting alternative to peat moss for the inoculation of corn with a pseudomonad. Agron. Sustain Dev. 36, 1–10. https://doi.org/10.1007/s13593-016-0356-z.

Gopi, G.K., Meenakumari, K.S., Nysanth, N.S., Subha, P., 2019. An optimized standard liquid carrier formulation for extended shelf-life of plant growth promoting bacteria. Rhizosphere 11, 100160. https://doi.org/10.1016/j.rhisph.2019.100160.

Howieson, J.G., Dilworth, M.J., 2016. Working with rhizobia. Australian Centre for International Agricultural Research, Canberra.

Hungria, M., Campo, R., Mendes, I., 2007. A importância do processo de fixaçâo biológica do nitrogênio para a cultura da soja componente essencial para a competitividade de produto brasileiro, 80. Documentos - EMBRAPA Soja, Brazil.

Hungria, M., Nogueira, M.A., Campos, L.J.M., Menna, P., Brandi, F., Ramos, Y.G., 2020. Seed pre-inoculation with Bradyrhizobium as time-optimizing option for large-scale soybean cropping systems. Agron. J. 112, 5222–5236. https://doi.org/10.1002/agj2.20392.

Hungria, Mariangela, De, I., Mendes, C., Hungria, M., Franchini, J.C., Campo, R.J., Graham, P.H., 2006. Chapter 3 THE IMPORTANCE OF NITROGEN FIXATION TO SOYBEAN CROPPING IN SOUTH AMERICA.

Kohl, D.H., Reynolds, P.H.S., Shearer, G., 1989. Distribution of 15N within Pea, Lupin, and Soybean Nodules. Plant Physiol. 1.

Moretti, L.G., Crusciol, C.A.C., Kuramae, E.E., Bossolani, J.W., Moreira, A., Costa, N.R., Alves, C.J., Pascoaloto, I.M., Rondina, A.B.L., Hungria, M., 2020. Effects of growth-promoting bacteria on soybean root activity, plant development, and yield. Agron. J. 112, 418–428. https://doi.org/10.1002/agj2.20010.

Mortvedt, J.J., Giordano, P.M., 1967. Crop Response to Zinc Oxide Applied in Liquid and Granular Fertilizers. J. Agric. Food Chem. 15, 118–122. https://doi.org/10.1021/jf60149a031.

Nabel, M., Temperton, V.M., Poorter, H., Lücke, A., Jablonowski, N.D., 2016. Energizing marginal soils – The establishment of the energy crop *Sida hermaphrodita* as dependent on digestate fertilization, NPK, and legume intercropping. Biomass and Bioenergy 87, 9–16. https://doi.org/10.1016/j.biombioe.2016.02.010.

Palhares Farias, T., Lima Soares, B., Barbosa D'Eça, C.S., de Souza Moreira, F.M., 2022. Polymeric formulations of liquid inoculants with rhizobia exopolysaccharides increase the survival and symbiotic efficiency of elite Bradyrhizobium strains. Arch. Microbiol 204. https://doi.org/10.1007/s00203-022-02779-z.

Pauferro, N., Guimarães, A.P., Jantalia, C.P., Urquiaga, S., Alves, B.J.R., Boddey, R.M., 2010. 15N natural abundance of biologically fixed N2 in soybean is controlled more by the Bradyrhizobium strain than by the variety of the host plant. Soil Biol. Biochem 42, 1694–1700. https://doi.org/10.1016/j.soilbio.2010.05.032.

Saleem, S., Malik, A., Khan, S.T., 2023. ZnO nanoparticles in combination with Zn biofertilizer improve wheat plant growth and grain Zn content without significantly changing the rhizospheric microbiome. Environ. Exp. Bot. 213, 105446 https://doi.org/10.1016/j.envexpbot.2023.105446.

Santos, M.S., Nogueira, M.A., Hungria, M., 2019. Microbial inoculants: reviewing the past, discussing the present and previewing an outstanding future for the use of beneficial bacteria in agriculture. AMB Express 9, 205. https://doi.org/10.1186/s13568-019-0932-0.

Sarpong, C.K., Zhang, X., Wang, Q., Wang, W., Jamali, Z.H., Yong, T., Chang, X., Yang, W., Wang, Y., Song, C., 2021. Improvement of plant microbiome using inoculants for agricultural production: a sustainable approach for reducing fertilizer application. Can. J. Soil Sci. 101, 1–11. https://doi.org/10.1139/cjss-2019-0146.

Shameem M, R., Sonali J, M.I., Kumar, P.S., Rangasamy, G., Gayathri, K.V., Parthasarathy, V., 2023. Rhizobium mayense sp. Nov., an efficient plant growth-promoting nitrogen-fixing bacteria isolated from rhizosphere soil. Environ. Res 220, 115200. https://doi.org/10.1016/j.envres.2022.115200.

Shearer, G., Kohl, D.H., 1988. Natural15N abundance as a method of estimating the contribution of biologically fixed nitrogen to N2-fixing systems: Potential for non-legumes. Plant Soil 110, 317–327. https://doi.org/10.1007/BF02226812.

Singleton, P.W., 2002. Development and Evaluation of Liquid Inoculants.

Sivaram, A.K., Abinandan, S., Chen, C., Venkateswartlu, K., Megharaj, M., 2023. Microbial inoculant carriers: Soil health improvement and moisture retention in sustainable agriculture. pp. 35–91. https://doi.org/10.1016/bs.agron.2023.03.001.

Taghizadeh, A., Sarazin, P., Favis, B.D., 2013. High molecular weight plasticizers in thermoplastic starch/polyethylene blends. J. Mater. Sci. 48, 1799–1811. https://doi.org/10.1007/s10853-012-6943-8.

Taghizadeh, Y., Amirnia, R., Rezaei-Chiyaneh, E., Ghiyasi, M., Razavi, B.S., Siddique, K.H.M., 2023. Co-Inoculation of Mycorrhizal Fungi with Bacterial Fertilizer Along with Intercropping Scenarios Improves Seed Yield and Oil Constituents of Sesame. J. Soil Sci. Plant Nutr. 23, 2258–2272. https://doi.org/10.1007/s42729-023-01177-8.

Thapa, M., Sadhukhan, R., Mukherjee, A., Biswas, P.K., 2023. Effects of nZnS vs. nZnO and ZnCl2 on mungbean [Vigna radiata (L.) R. Wilczek] plant and Bradyrhizobium symbiosis: A life cycle study. NanoImpact 29, 100440. https://doi.org/10.1016/j.impact.2022.100440.

Tittabutr, P., Payakapong, W., Teaumroong, N., Singleton, P.W., Boonkerd, N., 2007. Growth, survival and field performance of bradyrhizobial liquid inoculant formulations with polymeric additives. ScienceAsia 33, 69–77. https://doi.org/10.2306/scienceasia1513-1874.2007.33.069.

Unkovich, M., 2013. Isotope discrimination provides new insight into biological nitrogen fixation. N. Phytol. https://doi.org/10.1111/nph.12227.

Valle, S.F., Giroto, A.S., Dombinov, V., et al., 2022. Struvite-based composites for slow-release fertilization: a case study in sand. Sci Rep 12, 14176. https://doi.org/10.1038/s41598-022-18214-8.

Vassilev, N., Malusa, E., Requena, A.R., Martos, V., López, A., Maksimovic, I., Vassileva, M., 2017. Potential application of glycerol in the production of plant beneficial microorganisms. J Ind Microbiol Biotechnol 44, 735743. https://doi.org/10.1007/s10295-016-1810-2.

Wanek, W., Arndt, S.K., 2002. Difference in δ15N signatures between nodulated roots and shoots of soybean is indicative of the contribution of symbiotic N2 fixation to plant N. J. Exp. Bot. 53, 1109–1118.

Whaley, D., Damyar, K., Witek, R.P., Mendoza, A., Alexander, M., Lakey, J.R., 2021. Cryopreservation: An Overview of Principles and Cell-Specific Considerations, 096368972199961 Cell Transpl. 30. https://doi.org/10.1177/0963689721999617.

Zhang, K., Khan, Z., Yu, Q., Qu, Z., Liu, J., Luo, T., Zhu, K., Bi, J., Hu, L., Luo, L., 2022. Biochar Coating Is a Sustainable and Economical Approach to Promote Seed Coating Technology, Seed Germination, Plant Performance, and Soil Health. Plants. https://doi.org/10.3390/plants11212864.

Zilli, J.É., Pacheco, R.S., Gianluppi, V., Smiderle, O.J., Urquiaga, S., Hungria, M., 2021. Biological N2 fixation and yield performance of soybean inoculated with Bradyrhizobium. Nutr. Cycl. Agroecosyst 119, 323–336. https://doi.org/10.1007/s10705-021-10128-7.