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Formulations of polymeric biodegradable low-cost foam by melt extrusion to deliver plant growth-promoting bacteria in agricultural systems

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Abstract The extrusion technology of blends formed by compounds with different physicochemical properties often results in new materials that present properties distinctive from its original individual constituents. Here, we report the use of melt extrusion of blends made from low-cost materials to produce a biodegradable foam suitable for use as an inoculant carrier of plant growth-promoting bacteria (PGPB). Six formulations were prepared with variable proportions of the raw materials; the resulting physicochemical and structural properties are described, as well as formulation performance in the maintenance of bacterial viability during 120 days of storage. Differences in blend composition influenced foam density, porosity, expansion index, and water absorption. Additionally, differences in the capability of sustaining bacterial viability for long periods of time were more related to the foam composition than to the resulting physicochemical characteristics. Microscopic analyses showed that the inoculant bacteria had firmly attached to the extruded material by forming biofilms. Inoculation assays using maize plants demonstrated that the bacteria attached to the extruded foams could survive in the soil for up to 10 days before maize sowing, without diminishing its ability to promote plant growth. The results presented demonstrate the viability of the new matrix as a biotechnological material for bacterial delivery not only in agriculture but also in other biotechnological applications, according to the selected bacterial strains.

Keywords Inoculants · Biocomposites · Bacterial immobilization · *Azospirillum brasilense*

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Introduction

Modern agriculture is facing a paradigm shift. There is a search for new management strategies that allow reduced use and dependence on system inputs that demand large quantities of non-renewable materials and fossil energy; however, maintaining and even increasing current productivity levels is paramount for assuring that growing worldwide demands for food and energy are met. A feasible and factual sustainable agriculture alternative to lower the energy input of agroecosystems is the biotechnological exploitation of natural and beneficial plant-microbe relationships found in different crops. Several bacterial taxonomic groups are reported to exert a positive influence on plant development and productivity by fully or partially supporting the nutrient demands and providing protection against biotic and abiotic stresses (Glick 2015; Pii et al. 2015). The broad application of natural plant-bacteria interactions in agricultural crops can be best exemplified by the *Rhizobium* inoculants, which have been used for more than a century in leguminous crops such as soybeans. In addition to the well-known growth-promoting effects resulting from the use of rhizobial inoculants in leguminous plants, a group of free-living bacterial species named plant growth-promoting bacteria (PGPB) has shown potential to constitute and are currently being commercially formulated as new inoculants for non-leguminous crops.

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In contrast with the symbiosis observed with legume-rhizobia, where the growth promotion is largely based on BNF inputs, non-rhizobial PGPB bacteria can increase plant growth by direct and indirect mechanisms (Glick 2015). Therefore, the development of PGPB inoculants as a biotechnological input for agriculture should consider the ecophysiological requirements of establishing beneficial plant-bacteria relationships, where inoculated PGPB must have the ability to recognize the target plant as a potential partner, leading to plant colonization and bacterial gene expression to promote plant growth (Glick 2015; De-La-Peña and Loyola-Vargas 2014). The incorporation of additives to liquid formulations and the vehiculation of inoculant bacteria to a proper carrier can assure the conditions required to avoid the decline of inoculum populations and contribute to an efficient plant colonization, hence improving the field performance of PGPB inoculants (Bashan et al. 2014).

Although many diverse bacterial species with the ability to promote plant growth have continuously been identified and described, commercial applications largely remain in development; to date, only a few non-rhizobial commercial inoculants have been formulated with bacterial strains belonging to genera other than *Azospirillum* and *Bacillus* (Bashan et al. 2004; Herrmann and Lesueur 2013; Okon et al. 2015). Furthermore, the low quality of commercial inoculants is historically known and well discussed in the literature (Calvo et al. 2014; Bashan et al. 2014), impairing the establishment of this technology as a regular practice for non-leguminous crops. Formulations are the final step, and probably one of the main steps, of an inoculant life cycle, comprising both the microbial agent and its appropriate carrier. Neglecting the quality of the inoculant formulation and its feasibility for practical field application may lead to inefficient use and hence a lack of intended benefits, assuming it is used by farmers. Despite the lack of an international inoculant quality standard, high-quality inoculants must show common characteristics regarding the source and acquisition of raw materials, cost (should be low), uniformity (chemical and physical), ease of handling/transport/storage, non-toxicity to human/plants/animals/environment, and biodegradability. Concerning the formulation, it should promote the long-term viability of the added PGPB cells during storage periods and at the inoculation site; it should also be stable under diverse edaphoclimatic conditions and for different bacterial strains (Bashan 1998; Albareda et al. 2008; Herrmann and Lesueur 2013; Bashan et al. 2014).

The development of a high-quality inoculant formulation presenting all the desirable characteristics is not a trivial task and is probably the biggest challenge for the consolidation of crop inoculation technology. As stated by Bashan et al. (2014), most commercial inoculants are treated as an "industrial secret," and in-depth scientific evaluations of inoculant carrier performances are barely available, making it difficult to perform a comparative analysis of the currently available carrier materials. Inoculant formulations based on the use of polymers and polymeric materials have been introduced to substitute peat and liquid formulations, which normally suffer from poor viability and longevity of PGPB cells (Bashan and Gonzalez 1999; Denardin and Freire 2000; Silva et al. 2012). Polymers used for the production of bacterial inoculants include carboxymethylcellulose, alginate, and starch, in which the bacterial cells are entrapped in microcapsules formed by different processes. The immobilization of bacterial cells in a polymeric matrix presents considerable advantages including increased bacterial survival and viability over time and in soil near the target plant, which results in increased inoculation efficiency compared with seed inoculation or with direct soil spreading using bacterial suspensions. Although inoculant formulations based on polymers and/or polymeric blends raise product costs (Schoebitz et al. 2013), they are easy to handle and provide PGPB cell protection against unfavorable conditions such as those found in soil; in addition, they can gradually release the bacterial inoculant strains (Bashan 1986; Covarrubias et al. 2012; Schoebitz et al. 2013).

The use of melt extrusion to produce expanded starch foams has been reported as a partial substitute for the synthetic plastics derived from petroleum (Mitrus and Moscicki 2014). Extrusion technology is a high-temperature, short-duration process with the advantage of high versatility and the absence of effluents. These materials present high porosity, low density, low cost, and biodegradability; however, starch foams have a limited application due to a high hygroscopicity that results in rapid deterioration and weakening of its functional properties. The combination of starch with other raw materials and waste residues, such as plant fibers, mineral adjuvants in the clay fraction, and glycerol, improved the mechanical properties of starch-based extruded foams (Faruk et al. 2012). During the melt extrusion of starch blended with complex immiscible materials, novel homogeneous materials are generated resulting from the crosslinking of the polymeric material with the other blend compounds because of the thermomechanical and mechanical energy applied to the raw materials (Yu et al. 2006; Faruk et al. 2012). However, the use of different substances to produce a homogeneous mixture may present constraints inherent to the physicochemical properties of each particular substance, which may result in an instable mixture as a result of partial (or even absent) interactions between the individual components.

We postulate that biodegradable foams produced by melt extrusion of low-cost raw materials can be applied to the delivery of selected PGPB in agricultural systems as an inoculant carrier. This material may favor the viability of PGPB during the storage time and in the soil, as the result of the immobilization of cells on this matrix and also the putative nutritional role of the materials used to produce it. To test this, starch-based biodegradable foam referred to as the biocomposite (or BioC) throughout this

work was produced and characterized, as well as its fitness in sustaining the viability of *Azospirillum* cells and the effectiveness of its use as inoculant carrier to maize plants under a greenhouse trial.

Materials and methods

Plant growth-promoting bacterial strain and growth conditions

Azospirillum brasilense strain Ab-V5, a plant growth-promoting bacteria originally isolated from maize plants and recommended by the Brazilian Ministry of Agriculture for the production of commercial inoculants for maize, wheat, and rice crops (Hungria et al. 2010), was used in this study. This strain is deposited at the "Culture Collection of Diazotrophic and Plant Growth Promoting Bacteria" of Embrapa Soja, Londrina, Paraná, Brazil. When needed, bacteria were grown in DYGS liquid medium (g L^{-1}, glucose, 2.0; malic acid, 2.0; yeast extract, 2.0; glutamic acid, 1.5; peptone, 1.5; K_2HPO_4, 0.5; $MgSO_4$, 0.5) in test tubes (5 mL DYGS) as a pre-inoculum and in 250 mL Erlenmeyer flasks (50 mL) for the plant inoculation assay. Additionally, bacteria were grown in a defined liquid medium (patent pending, INPI protocol no. BR 1020140171746, Brazil) named MCA4, a culture medium with low C:N prepared with defined amounts of glycerol (46.6 %), sucrose (23.3 %), yeast extract (23.3 %), xanthan gum (0.47 %), polyvinylpyrrolidone (0.47 %), minerals (K_2HPO_4, KH_2PO_4, NH_4NO_3, $MgSO_4$, Fe-EDTA), and micronutrients (Mo, Mn, B, Cu, Zn), which promotes high accumulation of poly-hydroxybutyrate and high production of exopolysaccharides. All bacterial cultures were grown on Erlenmeyer flasks (250 mL volume) containing 50 mL of culture media in a rotary shaker at 150 rpm and maintained at 28 ± 2 °C for 36 h.

Production of biocomposites by melt extrusion

A total of six formulations were employed to produce the biocomposites, and they differed in the proportions of the following materials: cassava starch (19 % amylose, Hiraki, São Paulo, Brazil), sugarcane bagasse (Usina Bandeirantes, Paraná, Brazil), glycerol (Labsynth, São Paulo, Brazil), rock phosphate (Fosforita Alvorada, Socal S.A., São Paulo, Brazil), crystal sugar (Usina Alto Alegre S.A., Presidente Prudente, Brazil), powdered skim milk (Piracanjuba, Santa Catarina, Brazil), yeast extract (Sigma-Aldrich, São Paulo, Brazil), and phosphate-buffered saline (1.0 M, pH 7.0). The range selected for each component was based on previous experience and the need to produce a matrix capable of positively interacting with the inoculant PGPB. The materials used to produce the biocomposites were defined according to local availability and price with considerations for using renewable, biodegradable, and non-toxic materials capable of acting as anchoring sites for the inoculant bacterial cells; anchoring site was required to immobilize the bacteria inside the produced biocomposite matrix (Table 1). The materials were mechanically mixed using a food mixer (Arno, São Paulo, Brazil) in the following order. The dry materials were added first and mixed for 5 min at 780 rpm, followed by addition of the wet materials (glycerol and PBS) with further mixing for 10 min at 780 rpm, and an overnight incubation at 8 °C. The mixtures were extruded using a single-screw extruder (BGM EL-25, São Paulo, Brazil) with a barrel that was 700 mm long and 25 mm in diameter. Temperatures were kept at 120 °C from the feeding zone to the die zone, and a screw speed of 70 rpm and two 4-mm die nozzles were used to produce cylindrical extrudates; use of a rotary cutter operating at 200 rpm resulted in extrudates that were 0.5 cm long. A schematic representation of biocomposites production is presented Fig. S1 in the Supplementary Material.

Table 1 Composition of mixtures used for the production of biocomposites (BioC) by melt extrusion

Materials[a]		Formulations (biocomposites—BioC)					
		BioC1	BioC2	BioC3	BioC4	BioC5	BioC6
Cassava starch	(g%)	72.4	58.5	65.0	69.7	56.7	62.8
Sugarcane bagasse	(g%)	9.4	23.0	17.1	9.2	22.4	16.5
Glycerol	(g%)	9.5	7.7	8.5	9.2	7.5	8.3
Rock phosphate	(g%)	4.8	7.7	5.9	4.6	7.5	5.8
Crystal sugar	(g%)	1.9	1.5	1.7	1.8	1.5	1.7
Powdered skim milk	(g%)	1.0	0.8	0.9	0.9	0.7	0.8
Yeast extract	(g%)	1.0	0.8	0.9	4.6	3.7	4.1
PBS	(mL)	10.9	11.7	10.1	10.9	11.7	10.1

[a] Values are presented as g % (*w*/*w*) except for the phosphate-buffered saline (PBS; 1.0 M, pH 7.0), which is presented as the volume of applied solution (mL/100 g)

Preparation of the biocomposites with the PGPB *A. brasilense* Ab-V5

Extruded biocomposite samples were placed for at least 1 week in an environmentally controlled chamber at a temperature of 25 °C and a relative humidity of 60 % using a saturated sodium bromide (NaBr) solution (Rockland 1960) to establish moisture equilibrium in all samples. Prior to performing the inoculation with *A. brasilense* Ab-V5 or the physicochemical characterization (pH, EI, density and water absorption), the biocomposites were wrapped in cellophane packages (20 × 10 cm) containing 20.0 g of each formulation. The packages were used during sterilization by moist heat (autoclaving at 121 °C and 15 psi pressure for 20 min) or dry heat in a forced-draft oven (a 4-h treatment at 160 °C). After sterilization treatments, the biocomposite packages remained stored at room temperature (26 ± 4 °C) for at least 48 h before physicochemical characterization. Inoculation of extruded biocomposites was accomplished using biomass of *A. brasilense* AbV5 grown in MCA4 liquid medium, as stated above. Following the growth period, the bacterial broth was diluted in fresh MCA4 medium to reach a population density of 1×10^7 cells mL^{-1}. From this suspension, aliquots of 4.0 mL were introduced with the aid of a sterile needle into the previously sterilized cellophane packages of each formulation, resulting in a final density of 2×10^6 cells g^{-1} biocomposite. After inoculation, the biocomposite packages were manually homogenized for approximately 1 min and stored at 25 °C in the dark until use. Fresh MCA4 liquid medium in the same proportion as in the culture broth was used to prepare control extruded biocomposites for the effectiveness test described below.

Biocomposite characterization

PH The pH of extruded biocomposites was determined after crushing the materials in a blender to a fine powder, mixing 1 g with 9 mL of deionized water and incubating for 1 h at room temperature (26 ± 4 °C). Direct determination of pH of these solutions was performed in triplicate.

Expansion index (EI) The reported expansion indices (EI) were determined by the ratio of each extrudate diameter to the diameter of the die orifice, and each expansion index represents the mean of 20 determinations performed for each formulation, as described by Gujska and Khan (1991).

Density The density of the extruded biocomposites was determined as the ratio between the weight and volume of the samples and is expressed in g cm^{-3}; reported densities represent the mean of five replicates for each formulation (Shogren et al. 1998).

Water absorption capacity The amount of water absorbed by the extruded biocomposites was measured according to the standard ABNT NBR NM ISO 535 (1999). Briefly, 5.0 g of sample 0.5 cm in length previously stored in a desiccator for 7 days over anhydrous calcium chloride was weighed and immersed in distilled water for different times (1, 5, 10, 20, 30, and 60 min). After each immersion time, the biocomposites samples were collected, the water excess of biocomposites were removed using tissue paper, and samples were reweighed. The results were expressed as g water g^{-1} biocomposite at each time. Determinations were performed for extruded biocomposites before and after sterilization treatments and represented the mean of five replicates.

Scanning electron microscopy (SEM)

The interactions among the *A. brasilense* cells and the biocomposite formulations were determined by scanning electron microscopy. A total of 20 inoculated biocomposite specimens were randomly sampled from four different packs of each BioC formulation after 10 days of storage at room temperature (26 ± 4 °C); these samples were used for surface and cross section visualizations after manipulation under aseptic conditions. Samples were fixed by immersion in 3 % glutaraldehyde in 0.1 M phosphate buffer for 24 h, washed with 0.1 M phosphate buffer three times for 5 min, and impregnated with 1 % osmium tetroxide overnight. After fixation, the BioC samples were dehydrated throughout a graded ethanol series. This was followed by critical point drying with liquefied carbon dioxide. The samples were then mounted on bronze chucks using double-sided carbon tape and were coated with gold (40–50 nm). The sections were examined with a FEI Quanta 200 microscope (Hillsboro, Oregon, USA) at 20 kV.

Survival of *A. brasilense* Ab-V5 on the biocomposites

The survival of *A. brasilense* on the biocomposites was determined at 5, 10, 20, 40, 60, 90, and 120 days of storage at room temperature (26 ± 4 °C). The number of viable cells was determined by most probable number (MPN) counts by placing 5 g of inoculated biocomposites in Erlenmeyer flasks (125 mL volume) containing 45 mL of sterile saline solution (0.9 % NaCl, *w*/*v*). These suspensions were stirred at high speed under orbital shaking (200 rpm) for 30 min, followed by a serial dilution and the counting of dilutions from 10^{-3} to 10^{-6} using NFb semi-solid N-free media (Döbereiner 1988). After 7 days of incubation at 30 ± 2 °C, bacterial quantification was performed using the McCrady table. Determinations were performed in quadruplicate for each MPN count.

Effectiveness of biocomposite plant growth promotion

The biocomposite formulation that showed the best performance in sustaining bacterial viability over 120 days of storage was further investigated to determine its effectiveness in promoting plant growth when used as a bacterial carrier. To this end, a completely randomized experimental design was carried out with maize (hybrid P30F53H, Pioneer, Johnston, Iowa, USA) and the plant growth-promoting (PGPB) bacteria *A. brasilense* Ab-V5. The trial was conducted in a greenhouse using pots containing 2 kg of a 2:1 (*v*:*v*) mixture of unsterilized sand and oxisol with a high-clay content (78.2 %) as a substrate. The substrate contained the following: pH (in H_2O), 5.3; H + Al ($cmol_c\ dm^{-3}$), 8.36; K ($cmol_c\ dm^{-3}$), 0.55; Ca ($cmol_c\ dm^{-3}$), 4.2; Mg ($cmol_c\ dm^{-3}$), 1.2; Al ($cmol_c\ dm^{-3}$), 0.19; P ($mg\ dm^{-3}$), 10.0; and organic matter (%), 2.41. Maize growth was evaluated under four conditions: pure substrate (control treatment); substrate with 2 g of uninoculated BioC (BioC treatment); substrate inoculated with 2 mL of *A. brasilense* Ab-V5 cell suspension (2×10^6 cells mL^{-1}; Azo treatment); and substrates with 2 g of inoculated BioC (*A. brasilense* Ab-V5, 2×10^6 cells g^{-1} BioC; Azo+BioC treatment). The substrate treatments (BioC, Azo and Azo+BioC) were carried out by directly applying these treatments to the pots, in a single spot 2 cm below the substrate surface. After preparing the different substrate conditions, each pot was watered with 400 mL of distilled sterilized water previous seed planting. The maize seeds were planted at increasing intervals of time counted as days elapsed from the date of substrate preparation, starting from day 0 (1 h after substrate preparation) and thereafter at 2, 4, 6, and 10 days after substrate preparation while control treatment was sown only at day 0. Therefore, there were five different sowing dates for a single substrate preparation time except for the control treatment. The four substrate conditions tested remained wet regardless of the presence or absence of maize seeds. In addition to watering the substrates with sterilized distilled water at day 0, each pot received 200 mL of distilled water twice a week, and 200 mL of Hoagland solution (Hoagland and Arnon 1951) modified by depleting nitrogen on a weekly basis. In this way, inoculant bacteria applied as a suspension or as an inoculated biocomposite remained in a wet substrate without a plant for up to 10 days.

Maize plants were harvested 30 days after the sowing date, and the effects of the treatments were evaluated by determinations of the root volume, root dry weight, and shoot dry weight. All bacterial cultures were grown on Erlenmeyer flasks (250 mL volume) containing 50 mL of DYGS (Azo treatment) or MCA4 (Azo+BioC treatment) culture media in a rotary shaker at 150 rpm and maintained at 28 ± 2 °C for 36 h (late lag-growth phase). Four replicates were used for each substrate treatment and sowing date, resulting in 64 pots distributed across 16 treatments: four replicates of control treatments sown at day 0, and four replicates of each BioC, Azo, and BioC + Azo treatment sown at days 0, 2, 4, 6 and 10 days after preparing the different substrate conditions.

Statistical analysis

Experimental data were submitted to normality (Lilliefors) and homogeneity (Cochran) testing. An analysis of variance (ANOVA) and the Scott-Knott test, with significance defined by $p \leq 0.05$, were performed for mean comparisons using Statistica software version 7.0 (Statsoft, Tulsa, OK, USA). Principal component analysis was performed using the R software version 3.2.1 (R Foundation for Statistical Computing, Vienna, Austria).

Results

Due to the use of nonsterile raw organic materials to produce the biocomposites by melt extrusion, and to avoid the presence of contaminant microorganisms that could shorten the viability and performance of *A. brasilense*, we evaluated both moist and dry heat sterilization methods aiming to define a proper sterilization method for the carrier produced. Both sterilization methods used induced changes in the physicochemical properties of the biocomposites, as presented in Table 2. A principal component analysis (PCA) was performed to better understand the influence of each material on the physicochemical property variations observed after the melt extrusion and sterilization treatments (Fig. 1). The biplot of the first two PCs accounted for 84.49 % of the total variability with principal component 1 explaining 67.43 % of the total variance. It is interesting to note that the BioC formulations were different from each other, which was indicative of dissimilar properties among them.

pH of the biocomposites

The pH of inoculant carriers exerts great influence on the survival of bacterial cells over long-term storage, as previously observed in formulations containing *Herbaspirillum* and *Azospirillum* (Silva et al. 2012; Trujillo-Roldán et al. 2013). The BioCs produced by melt extrusion were designed to reach an approximately neutral final pH, and although the formulations contained similar amounts of phosphate buffer (10.1–11.7 mL 100 g^{-1}), the final pH values ranged from 6.80 to 7.29 (Table 2). Although the phosphate buffer used to moisten the materials prior to extrusion was pH-adjusted to 7.0, the melt extrusion process resulted in weak modifications to final biocomposite pH, with three formulations showing basic pH values (BioC1, BioC3, and BioC4) and three other formulations (BioC2, BioC5, and BioC6) demonstrating acidic pH values. In addition, according to the PCA, a positive

Table 2 Physicochemical parameters of biocomposite formulations produced through melt extrusion, before (unsterilized biocomposites) and after sterilization by moist heat (autoclaving) and dry heat (4 h at 160 °C in a forced-draft oven). The formulation compositions are shown in Table 1

Formulations	Unsterilized biocomposites			Autoclaved biocomposites			Dry-heated biocomposites		
	pH	Density (g/cm^3)	Expansion index (EI)	pH	Density (g/cm^3)	Expansion index (EI)	pH	Density (g/cm^3)	Expansion index (EI)
BioC1	7.29	0.265 ± 0.016a	1.450 ± 0.192a	6.54	0.319 ± 0.020a	1.375 ± 0.222a	7.33	0.263 ± 0.018a	1.713 ± 0.203a
BioC2	6.85	0.244 ± 0.012b	1.025 ± 0.138b	6.37	0.276 ± 0.011b	0.900 ± 0.170b	6.80	0.246 ± 0.009b	1.050 ± 0.154c
BioC3	7.10	0.237 ± 0.013c	1.100 ± 0.150b	6.41	0.277 ± 0.014b	0.988 ± 0.190b	6.98	0.237 ± 0.017c	1.062 ± 0.138c
BioC4	7.14	0.278 ± 0.008a	1.188 ± 0.168a	6.68	0.284 ± 0.012b	0.988 ± 0.172b	7.03	0.276 ± 0.005a	1.700 ± 0.208a
BioC5	6.80	0.244 ± 0.016b	0.938 ± 0.160b	6.54	0.278 ± 0.012b	0.888 ± 0.206b	6.71	0.242 ± 0.009b	1.388 ± 0.128b
BioC6	6.82	0.237 ± 0.012c	1.075 ± 0.143b	6.56	0.278 ± 0.012b	0.938 ± 0.111b	6.76	0.235 ± 0.013c	1.050 ± 0.154c

Data presented are means of three (pH), five (density), or 20 (EI) replicates with standard deviation. Different small letters in the columns indicate statistically significant differences ($p \leq 0.05$) according to the Scott-Knott test

correlation was found between the pH and the powdered skim milk, cassava starch, glycerol, and crystal sugar amounts. Remarkably, the biocomposites containing higher quantities of such materials reached a basic final pH, as stated in Table 1, suggesting that the melt extrusion led to a thermal hydrolysis and/or phosphorylation of at least one of these substances, and consequently increased the pH of these formulations (Moad 2011). Both sterilization processes influenced the pH of the BioCs in different manners, according to the method applied. Overall, the autoclaved biocomposites demonstrated decreased pH values, with a maximum ΔpH of 0.75 (BioC1) and a minimum ΔpH of 0.26 (BioC5 and BioC6). In contrast, the dry heat sterilization had a nearly neutral effect on biocomposite pH, with ΔpH varying from 0.12 (BioC3) to 0.04 (BioC1). These effects are better demonstrated by the principal component analysis (Fig. 1), where the pH-vector of the autoclaved BioCs (pH-MH) was found at opposite part of the graph where the pH-vectors of unsterilized and dry-heated BioCs were placed (pH-NT and pH-DH).

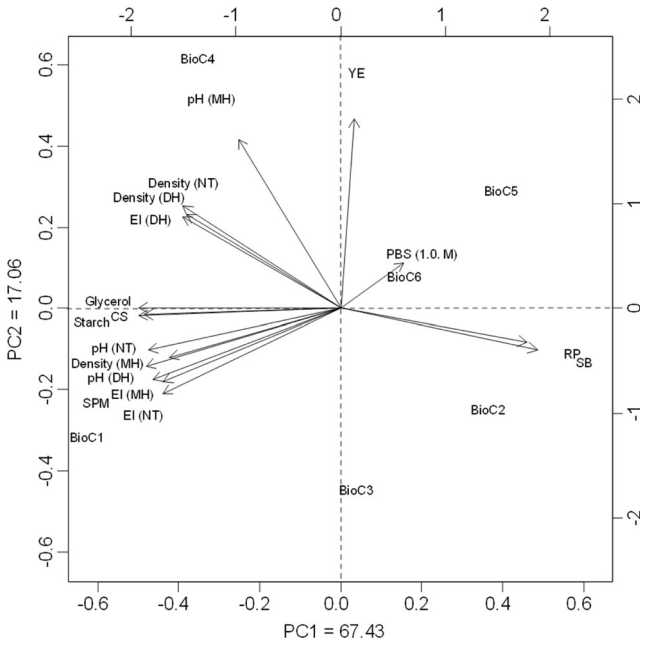

Fig. 1 Principal component analysis (PCA) of extruded biocomposite physicochemical properties as affected by the amount of materials employed in each formulation and by the sterilization treatments. *NT* unsterilized biocomposites, *MH* autoclaved biocomposites, *DH* dry-heated biocomposites, *CS* crystal sugar, *SPM* powdered skim milk, *RP* rock phosphate, *SB* sugarcane bagasse, *YE* yeast extract, *PBS* phosphate-buffered saline. The biocomposites (BioCs) compositions are shown in Table 1

Expansion index (EI)

While expansion index experimental results showed low expansion for all the extruded biocomposite blends, the values for the BioC1 (1.450 ± 0.192) and BioC4 (1.188 ± 0.168) formulations were significantly higher. The EI of the other formulations varied between 0.938 ± 0.16 and 1.100 ± 0.15 (Table 2). The PCA analysis (Fig. 1) grouped the EI together with the pH and the powdered skim milk and opposite from the sugarcane bagasse and rock phosphate. Application of heat to the extruded materials induced modification of the biocomposite EI, as was observed with the pH. While autoclaving reduced the EI of all formulations by factors ranging from 5.2 (BioC1) to 16.8 % (BioC4), sterilization under dry heat led to an increase in the EI of all formulations except BioC3 and BioC6, which showed EI decreases of 3.5 and 2.3 %, respectively. Interestingly, the BioC4 and BioC5 formulations demonstrated EI increases of approximately 43.1 and 48 %, respectively, after sterilization by dry heat (Table 2). Indeed, the effect of dry-heat sterilization on BioC expansion index is graphically indicated by the principal component analysis, where the EI of BioCs subjected to dry-heat

sterilization is shown to be distant from the EI of unsterilized or autoclaved BioCs (Fig. 1).

Density

The densities of the biocomposites studied in this work ranged from 0.237 ± 0.012 to 0.278 ± 0.008 g cm^{-3} (Table 2), which falls in the range observed in similar extruded foams (Kaewtatip et al. 2013; Polat et al. 2013). Though small density variations between different BioCs formulations were recorded, three distinct groups were observed: higher density biocomposites (BioC1 and BioC4), biocomposites with intermediate densities (BioC2 and BioC5), and biocomposites with lower densities (BioC3 and BioC6). Sterilization by autoclaving increased the density of biocomposites, as expected, due to the decrease in the EI observed by autoclaving and discussed above. The density increases varied from 2.2 (BioC4) to 20.4 % (BioC1), corresponding to the formulations with higher amounts of starch. Application of dry heat to the biocomposites had no effect on the density of the extruded foams (Table 2). In fact, according to the principal component analysis (Fig. 1), the density of autoclaved BioCs was close to the starch vector and distant from the density vectors of both the unsterilized and dry-heated BioCs, which were close to each other.

Water absorption capacity

The water absorption kinetics of the unsterilized and sterilized biocomposites through humid (autoclaving) and dry heat (hot air oven) processes are presented in Fig. 2 and Table S1 in the Supplementary Material. Both sterilization processes modified the absorption kinetics of all biocomposite formulations, even taking into account the inherent differences related to formulation interactions with water. The unsterilized formulations reached saturation (BioC3, BioC4, and BioC5) or showed slow absorption of water after the first 30 min of immersion, while formulations sterilized by autoclave or dry-heat demonstrated continuous water absorption throughout the duration of the experiment (60 min). Dry-heat biocomposite sterilization resulted in samples with the highest water absorption capacities, reaching average values of 1.47 g H_2O g^{-1} BioC after 30 min and 2.42 g H_2O g^{-1} BioC after 60 min of water immersion. Autoclaved BioCs showed lower water absorption and the unsterilized BioCs demonstrated intermediate behavior. Autoclaved biocomposites showed mean water absorption values of 0.62 g H_2O g^{-1} BioC after 30 min and 0.97 g H_2O g^{-1} BioC after 60 min of immersion. These last values from autoclaved BioCs were similar to those observed for unsterilized BioC, which absorbed 0.84 g H_2O g^{-1} BioC after 30 min and 0.94 g H_2O g^{-1} BioC after 60 min of immersion, indicating that the autoclave sterilization delayed BioC water absorption.

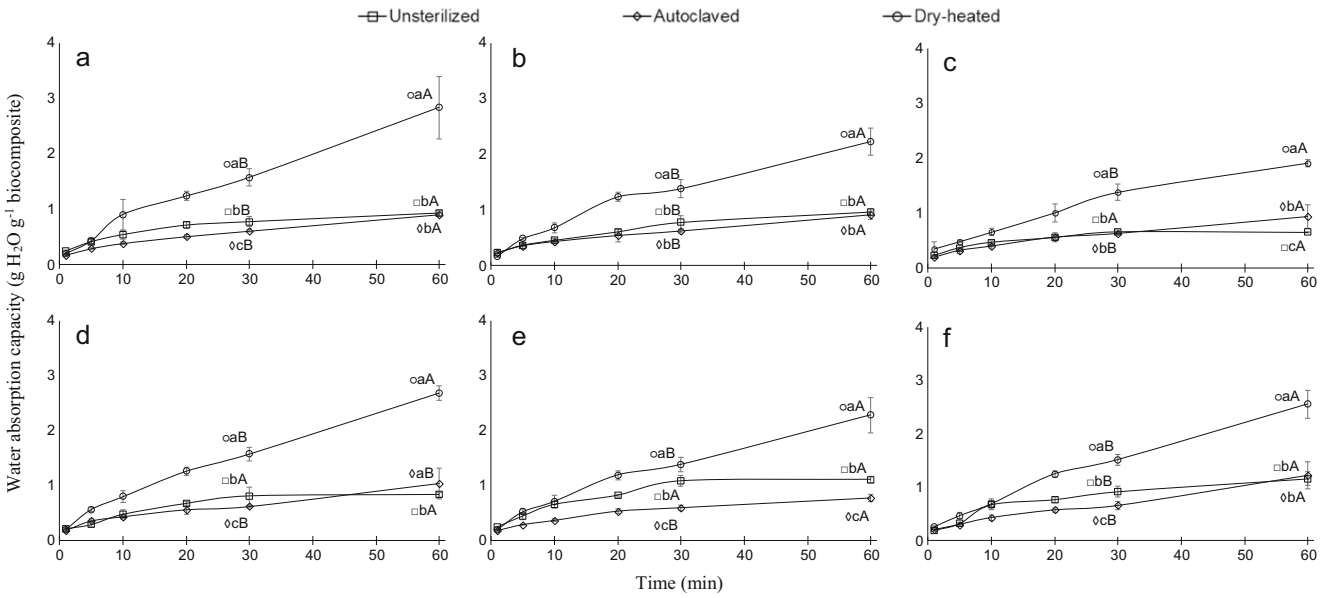

Fig. 2 Water absorption capacity of different biocomposite formulations as affected by humid heat (autoclaving at 121 °C and 15 psi pressure for 20 min) or dry heat (4 h in a forced-draft oven at 160 °C). **a** BioC1, **b** BioC2, **c** BioC3, **d** BioC4, **e** BioC5, and **f** BioC6. Data represent the means of five replicates with standard deviation. *Different small letters* indicate statistically significant differences in the amount of water absorbed between biocomposite formulations in the same time of immersion in distilled water as affected by the sterilization treatment, according to the Scott-Knott test ($p \leq 0.05$). *Different capital letters* indicate statistically significant differences in the amount of water absorbed for each biocomposite formulation as affected by the period of time of immersion in distilled water, according to the Scott-Knott test ($p \leq 0.05$). The complete statistical analysis is presented in the Table S1 in the Supplementary Material

Dry-heated samples did not reach water saturation by the end of the 60 min test.

Visualization of biocomposites by SEM

Biocomposites produced with the same batch of *A. brasilense* Ab-V5 culture were evaluated by SEM (Fig. 3). After 10 days of storage, BioC2 was the only sample among the six formulations to display a decrease in cell viability, though only minor differences among the formulations in foam structure and bacterial attachment were observed using electronic microscopy (data not shown). SEM images show the BioCs have a homogeneous but rough outer surface (Fig. 3a) and a highly porous interior with both fiber-rich areas (Fig. 3b) and large air pockets similar to those that have been observed in previous studies (Mali et al. 2010). *A. brasilense* densely

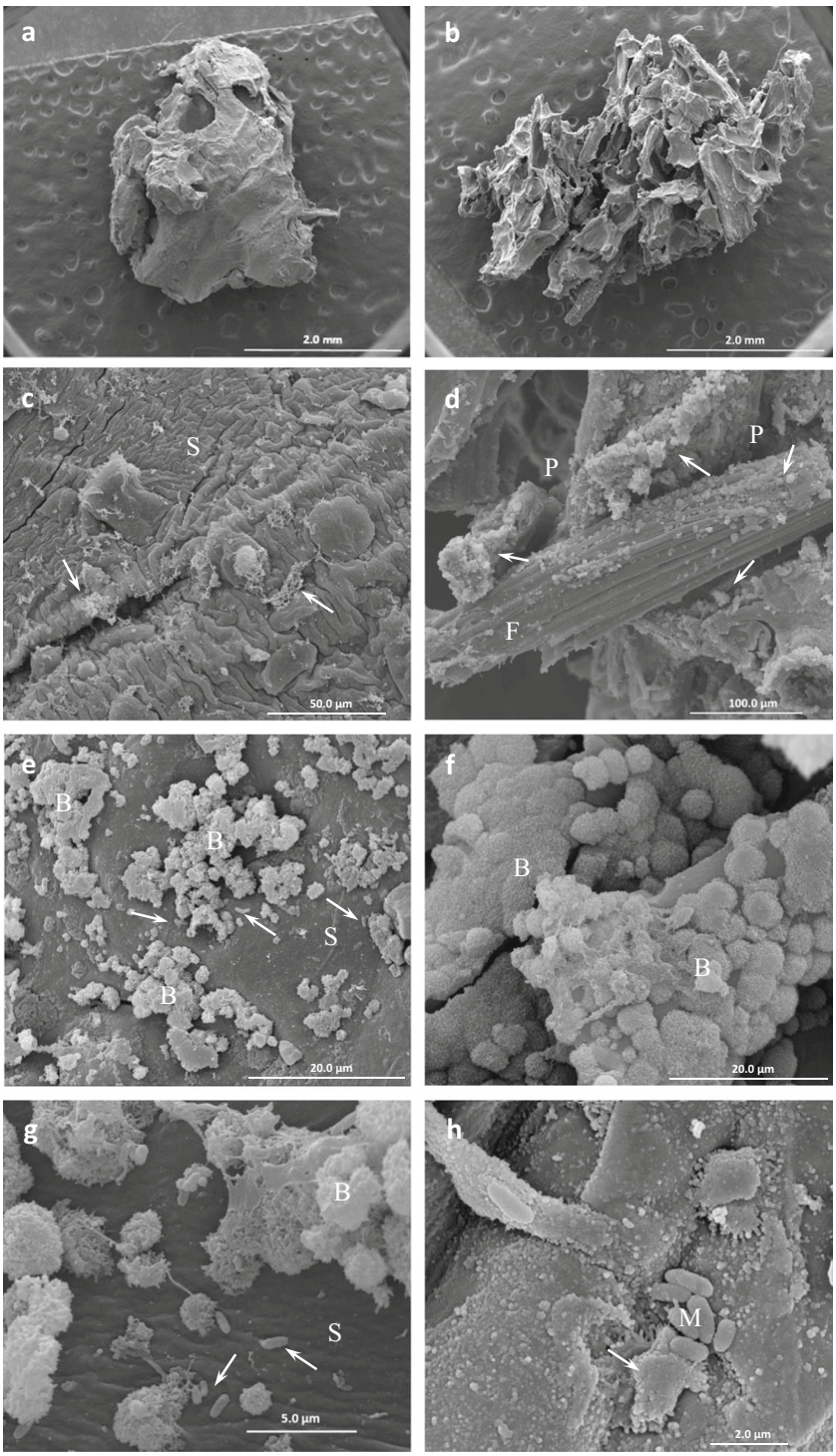

Fig. 3 Scanning electron microscopy (SEM) of extruded biocomposite foams inoculated with *Azospirillum brasilense* Ab-V5. The biocomposites were packaged in cellophane and evaluated 10 days after inoculation and dark storage at room temperature (26 ± 4 °C). All the formulations presented a similar appearance by SEM. **a** Surface appearance of a biocomposite foam (BioC) after melt extrusion (formulation BioC4). **b** Internal view of BioC5 showing high porosity. **c** Close-up of the surface of inoculated BioC6 showing a rough surface with a bacterial EPS matrix (*arrows*). **d** Internal view of BioC3 where a sugarcane fiber and pore surfaces have been colonized by *A. brasilense* biofilms (*arrows*). **e** Surface view of BioC2 densely colonized by *A. brasilense* biofilms and single bacterial cells (*arrows*). **f** Internal view of BioC1 with pore surfaces densely colonized by *A. brasilense* biofilms. **g** Surface view of BioC5 colonized by *A. brasilense* biofilms and single bacterial cells (*arrows*). **h** Internal view of BioC5 pore surface presenting *A. brasilense* microcolony covered with bacterial EPS (*arrow*). *B* biofilm, *F* sugarcane fiber, *M* Microcolony, *P* biocomposite pore, *S* biocomposite surface

colonized all the BioC formulations, both on the outer surface (Fig. 3c, e) and in the interior of the extruded foams (Fig. 3d, f–h). Most bacteria were found to be firmly anchored to the BioC surface and involved in a dense exopolysaccharide matrix; these observations indicated that enough water was available in the early stages of storage for the bacteria to form biofilm.

Survival of *A. brasilense* Ab-V5 in the biocomposites: bioproduct shelf-life

The viability of *A. brasilense* Ab-V5 cells in each biocomposite formulation is presented in Fig. 4, estimated as the most probable number (MPN) of cells grown in NFb N-free semi-solid media over 120 days of storage at room temperature (26 ± 4 °C). It is important to point out that the procedure adopted to perform the MPN completely disrupts the structure of the extruded foams but do not result in a homogeneous solution, due to the presence of insoluble materials in the biocomposite formulations. Even considering that *A. brasilense* cells could remain firmly attached to such insoluble materials, this fraction was kept in suspension throughout the serial dilutions procedure applied to the MPN counts and should not biased the counts at great extent. In addition, even though the use of culture media to determine the bacterial viability in different environments and materials are being applied until today, includding inoculant formulations, the MPN used in this work could result in underestimated counts since part of the *Azospirillum* cells could be in a viable but non-culturable state (Kushneruk et al. 2013).

In the first 10 days of storage, the population densities of *A. brasilense* remained stable in all formulations but BioC2, which showed a decrease from $2.0 \pm 0.48 \times 10^6$ cells g^{-1} (initial population density) to $2.6 \pm 2.12 \times 10^5$ cells g^{-1} biocomposite. During the storage period, total populations declined to densities below 1×10^5 cells g^{-1} of biocomposite except in the BioC4 and BioC5 formulations, which sustained population densities of $1.2 \pm 6.11 \times 10^5$ and $1.6 \pm 0.82 \times 10^6$ cells g^{-1} of biocomposite, respectively, for up to 120 days. The lowest bacterial counts were observed in the BioC1 and BioC6 formulations, which showed populations of $2.5 \pm 4.36 \times 10^3$ and $4.5 \pm 10.10 \times 10^3$ cells g^{-1} of biocomposite, respectively, at the end of the storage period. It is important to note that according to the PCA analysis (Fig. 1), the formulations that showed the higher bacterial counts after 120 days storage, BioC4 and BioC5, are closer to the yeast extract (YE) vector, while the formulations that showed the lower bacterial counts, BioC1 and BioC6, are positioned far from the YE vector in the PCA. Population decay over time also varied across the BioC formulations. The *A. brasilense* counts in BioC1, BioC2, BioC4, and BioC6 indicated lower populations after just 20 days of storage; however, while the populations of BioC1 and BioC6 remained constant until 60 days of storage (Fig. 4a, f), a further decay in *A. brasilense* population was observed after 90 days of storage in BioC2 (Fig. 4b) with no further decline in BioC4 was noted throughout the experimental period (Fig. 4d). The viability of *A. brasilense* in the BioC3

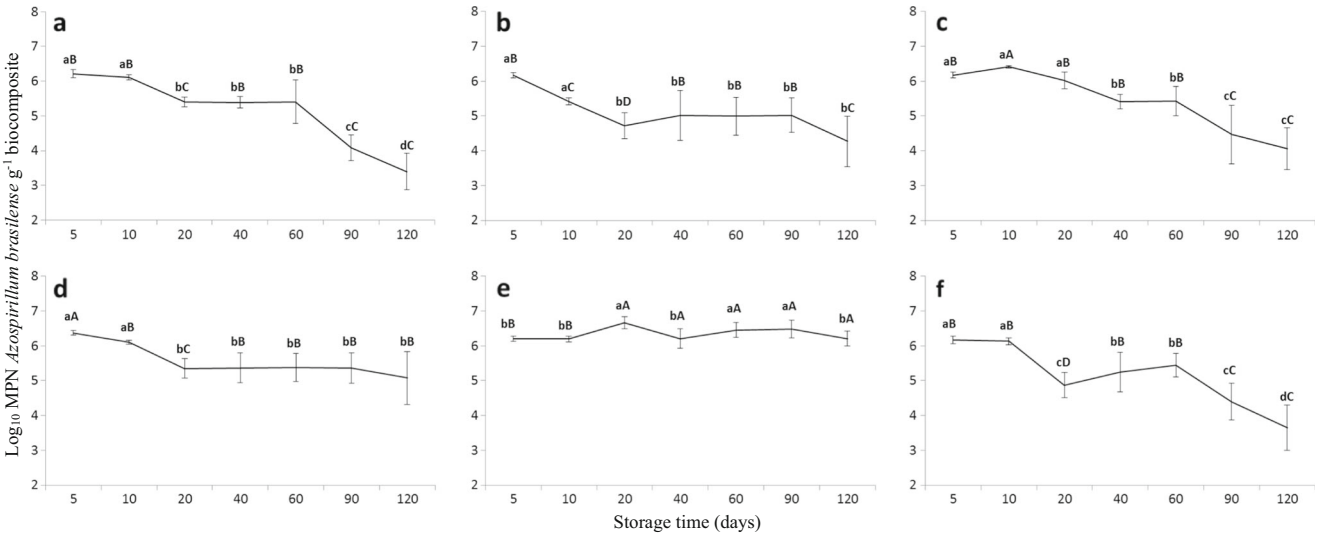

Fig. 4 Viability of *Azospirillum brasilense* Ab-V5 in different biocomposite formulations over 120 days of dark storage at room temperature (26 ± 4 °C). MPN data represent means of four replicates. Biocomposite formulations are presented in Table 1. **a** BioC1, **b** BioC2, **c** BioC3, **d** BioC4, **e** BioC5, and **f** BioC6. *Different small letters* indicate statistically significant differences between MPN counts for each biocomposite formulation at different storage times, according to the Scott-Knott test ($p \leq 0.05$). *Different capital letters* indicate statistically significant differences between MPN counts at each storage time between the biocomposite formulations, according to the Scott-Knott test ($p \leq 0.05$)

formulation did not differ from the initial population until day 60 of storage, but a decrease in population was found after this period (Fig. 4c). Only the BioC5 formulation maintained the *A. brasilense* population without decline throughout the experimental period (Fig. 4e), and for that reason, this formulation was chosen to evaluate the efficacy of the biocomposite as a PGPB inoculant carrier in a pot trial with maize. The intellectual property of the process to prepare the BioC5 as carrier for PGPB in agricultural crops has its patent pending at INPI, Brazil, under the protocol no. BR 1320120298626.

Biocomposite plant growth-promotion effectiveness

The evaluation of maize development performed after 30 days of growth in pots under different substrate conditions indicated that *A. brasilense* Ab-V5 successfully promoted root development and dry mass accumulation (Fig. 5). It is clear that maize plants grown on substrates that received the inoculated BioC5 10 days before planting have shown increases in root volume and root dry weight, which was not observed for the plants grown on substrates treated with the same PGPB

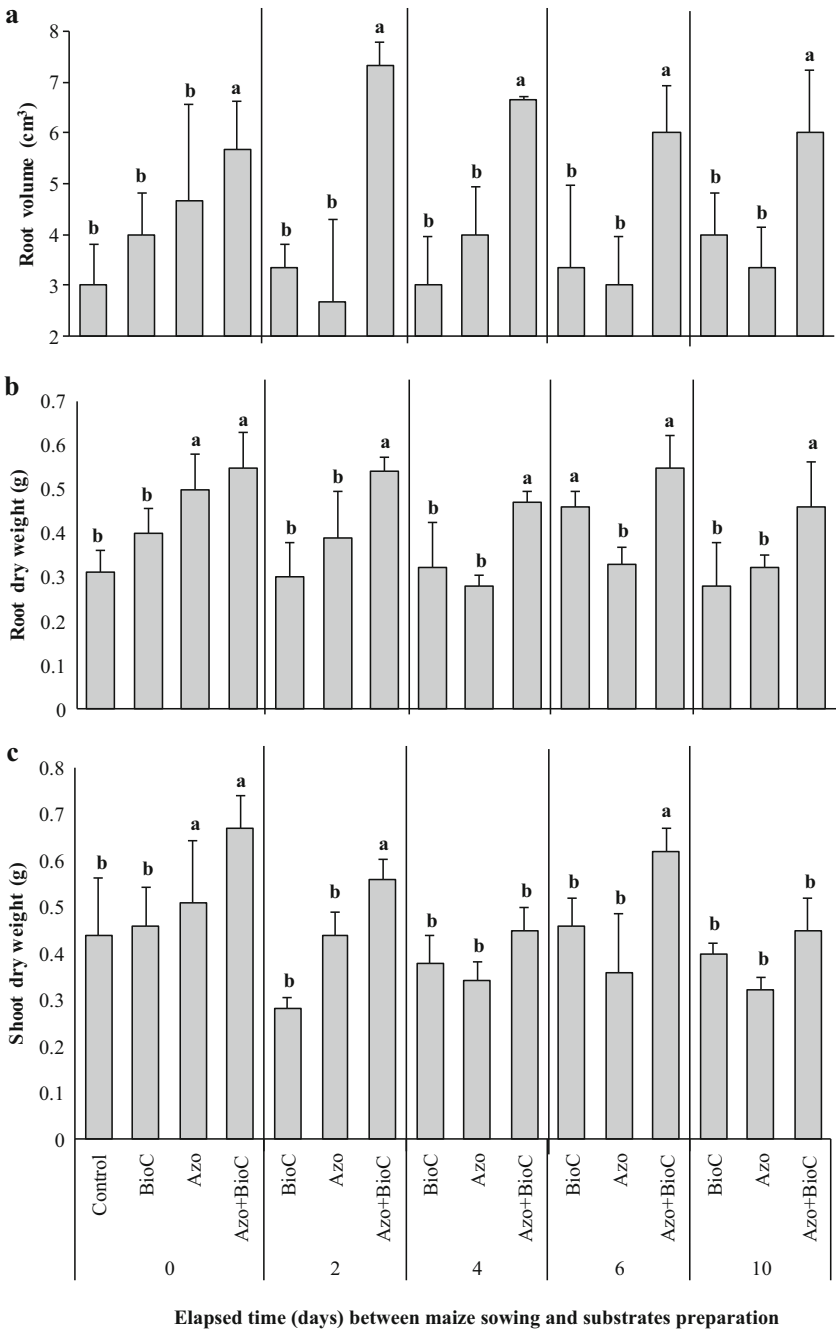

Fig. 5 Root volume (**a**), root dry weight (**b**), and dry shoot weight (**c**) of maize plants grown in pots containing substrate inoculated with *Azospirillum brasilense* Ab-V5 through different treatments and sown on different dates after substrate preparation. Significant differences ($p < 0.05$) according to the Scott-Knott test are indicated by *lowercase letters* ($n = 4$). a Treatments were as follows: control, substrate without bacterium or biocomposite; BioC, substrates added with 2 g of uninoculated BioC per pot; Azo, substrate inoculated with 2 mL of *A. brasilense* Ab-V5 cell suspension (1×10^6 cells mL^{-1}) per pot; Azo+BioC, substrates added with 2 g of inoculated BioC (*A. brasilense* Ab-V5, 1×10^6 cells g^{-1} BioC) per pot. Maize sowing started 1 h after the preparation of substrates (0) and was performed on four subsequent days (2, 4, 6, and 10 days after substrate preparation) except for the control treatment, which was sown only on day 0

applied as a bacterial suspension. This result indicates a protective and nutritional effect of the biocomposite and suggests its potential to sustain the viability of *A. brasilense* Ab-V5 up to 10 days after its introduction to the substrate in the absence of a host plant. The volume of the maize root system was increased by up to 89 % in groups where the substrate was seeded 1 h after the introduction of the inoculated BioC, and this value reached 144 % in groups where the substrate was seeded 2 days after the introduction of the inoculated BioC. Ten days after substrate treatment with the inoculated BioC5, the continued growth-promotion effect of *A. brasilense* was indicated by a 100 % increase in the root system volume of the maize plants (Fig. 5a). The root system volume of the plants grown in substrates treated with an *A. brasilense* cell suspension or plants grown in substrates treated with the uninoculated BioC5 did not differ from that of the control plants at any seeding date. Plants sown in substrates treated with uninoculated BioC5 or with an *A. brasilense* suspension at later dates (2, 4, 6, or 10 days after substrate preparation) did not shown any increase in root system volume.

In addition to the increases in root system volume, maize plants grown in the presence of the PGPB *A. brasilense* showed higher dry mass accumulation compared with those plants grown on substrates that did not receive the PGPB (Fig. 5b, c). Although plants sown in substrates that received a bacterial suspension demonstrated that root and shoot dry masses increased by up to 61.3 and 15.9 %, respectively, this was only observed when the maize was seeded 1 h after the substrate inoculation (day 0). In this condition, when maize seeding was delayed for 2 days after substrate inoculation, the growth-promotion effect caused by *A. brasilense* was eliminated. However, the maize plants grown on substrates that received the inoculated BioC5 showed significant increases in root dry mass when compared with the control plants, even when seeding was performed 10 days after substrates treatment. Such increases varied, ranging from 48.4 % for maize sown 10 days after the substrate treatment to 77.4 % for maize sown 1 h after substrate treatment with inoculated BioC5. In this same condition, significant increases in the maize shoot dry mass varied from up to 27.3 % in plants sown 2 days after substrate treatment to 52.3 % in plants sown at day 0; this effect was restricted to those plants sown up to 6 days after substrate treatment (40.9 % increase compared to control plants).

Discussion

Production of biocomposites by melt extrusion: a new carrier for PGPB

Traditionally, peat has been used as main carrier for rhizobia as well as in many PGPB inoculant formulations because of the advantages related to its high content of organic matter, buffering capacity and ability to retain water, functioning as a protective and nutritional carrier to the inoculant bacteria. By the other hand, peat is made of decomposed plant material and varies in composition from different deposits, is a non-renewable material, and its extraction may cause negative environmental impact (Herrmann and Lesueur 2013). There are important alternatives to the use of peat as a vehicle for carrying inoculant bacterial cells, such as organic materials and polymer-based carriers; these latter has the advantage to entrap the bacterial cells (bioencapsulation) allowing its gradual delivery close to the plant (Bashan and de-Bashan and LE 2015). Different materials have been used for bioencapsulation of microorganisms, particularly to immobilize plant growth-promoting bacteria (PGPB). Among the polymeric materials employed, there are some natural polymers, such as alginate, carrageenan, and agarose, and there are synthetic polymeric materials, such as polyacrylamide and polyurethane (Schoebitz et al. 2013; Bashan et al. 2014). Originally, starch-based foams produced by melt extrusion were developed to substitute polymeric materials derived from non-renewable sources, such as loose-fill packaging (Mali et al. 2010). Preliminary results obtained by our research group have demonstrated the possibility of using starch-based extruded foams to immobilize PGPB, although the viability of immobilized bacterial cells was observed to be quite short (our unpublished results). Based on these preliminary experiments, a set of mixtures was prepared using materials intended to produce biodegradable foams and provide for the long-term survival of the immobilized bacterial cells. The formulations studied contained different amounts of cassava starch (56.7–72.4 %), cellulosic sugarcane bagasse fibers (9.2–23 %), glycerol (7.5–9.5 %), and yeast extract (0.8–4.6 %); these materials constitute up to 90–92.7 % of the materials used. Although the biodegradation of the biocomposites was not addressed in this work, similar starch-based foams showed to be readily biodegradable (Vercelheze et al. 2013). The major drawbacks to the use of biocomposites as a carrier for PGPB inoculant strains are related to the availability of raw material needed to produce the extruded foams. Despite cassava starch and sugarcane bagasse are easily available and inexpensive in Brazil, the general idea to use biocomposites as inoculant carrier is suitable and viable to be broadly adopted. The production of biocomposites using starch and fibers has been studied for more than a decade, and different biocomposite formulations are described according to the availability of raw materials for distinct geographical regions (Yu et al. 2006; Faruk et al. 2012).

Biocomposite pH

Inoculant carrier pH exerts a great influence on bacterial cell survival during long-term storage, as previously observed in

formulations containing *Herbaspirillum* and *Azospirillum* (Silva et al. 2012; Trujillo-Roldán et al. 2013). The pH decrease induced by autoclaving the biocomposites suggests that an additional hydrolysis of mixture components occurred as a result of the moist heat and high-pressure combination. It is important to note that the characterization of the extruded materials presented in this study cannot be applied to any type of chemical modification or degradation of raw materials used to produce the BioCs. Survival of bacterial inoculants in soil is thought to be influenced by pH variations, although acid-tolerant and alkaline-tolerant PGPB strains have also been identified (Karagöz et al. 2012). In addition, inoculant carriers have been stated to have an approximately neutral pH; regardless, a direct relationship between inoculant formulation pH and *Azospirilla* viability has yet to be proven (Trujillo-Roldán et al. 2013).

Expansion index (EI)

Extruded composites with alkaline pH and small amounts of milk protein have been shown to exhibit increased expansion, though moisture content and barrel temperature exert greater influence over this parameter (Amaya-Llano et al. 2007). In this work, all the composites were extruded at the same barrel temperature (120 °C), and moisture was kept nearly constant (10.1 to 11.7 mL 100 g^{-1} materials), reinforcing the effects of pH and amount of powdered skim milk on the observed EI. Proteins can interact with the water in the blends by lowering the vapor pressure and diminishing the degradation of the starch melt; in addition, protein can form covalent bonds and non-bonded interactions with the blend compounds because of its macromolecular structure. Moreover, the amount of fiber was proven to be inversely related to the EI of the extruded starch blends (Guan and Hanna 2004; Mali et al. 2010), probably due to an increase in the density and viscosity of such composites. The decrease of the expansion index in the autoclaved BioC formulations could be due to the collapse of the foam cells, which led to foam structure degradation. However, BioC sterilization using dry heat led to an additional expansion of the biocomposites, which was likely caused by a further elimination of moisture content, as has been observed during microwave-induced expansion (Lopez-Gil et al. 2015).

Density

Polymer composite density has been shown to directly correlate with starch and fiber content (Salgado et al. 2008; Mali et al. 2010). While the addition of nano-size minerals lowers the density of fiber-starch foams (Mali et al. 2010; Polat et al. 2013), increases in foam density by the addition of clay minerals have also been reported (Kaewtatip et al. 2013). In this study, the higher density BioCs (BioC1 and BioC4) were formulated with the smallest amounts of rock phosphate and sugarcane bagasse, while the lower density BioCs received larger amounts of these compounds. The results suggest that the addition of nano-size minerals more strongly influences the density of extruded foams than the addition of plant fibers, at least considering the assortment of materials and the amounts used in the formulations presented in this work. This can be visualized using the PCA (Fig. 1), where the density vector of biocomposites is placed in contraposition with the sugarcane bagasse and rock phosphate vectors. Modifications in BioC density were more pronounced in the autoclaved samples, reinforcing the possible effect of moist heat on foam cell collapse. The BioCs sterilized by dry-heat showed minor density variations compared to the unsterilized samples, generally lower than 1 %. As the BioCs are intended to entrap bacterial cells inside the extruded foams, the shrinking of its pores could limit the bacteria to the outer material surface, thus leaving them unprotected and subject to soil factors that could lead to decreased viability (Rathore et al. 2013). Low-density foams have been stated to diminish the production cost of biodegradable materials, but to the best of our knowledge, there have been no studies on the effect of melt-extruded foam density on bacterial immobilization and long-term viability.

Water absorption capacity

The dry-heat sterilization led to an increase in water absorption in all formulations, which can be related to the higher EI of these samples compared with the other samples (Table 2). Foam material water absorption is related to the porosity and formulation of the foam (Sjöqvist et al. 2010); the higher EI was a consequence of the higher porosity of these samples, which were able to absorb more water through their pores. Along the same lines, the delayed water absorption of the BioCs sterilized using humid heat (autoclave) reinforces the possibility of foam cell collapse occurring as a result of the high temperature (121 °C) and pressure (15 psi) applied during autoclaving; these observations are in accordance with the observed EI and density changes. In addition to other modifications, it has been shown that acetylation (Xu et al. 2005) and hydrolysis (Niba and Hoffman 2003) cause decreased starch granule water absorption. These modifications could have contributed to the decreased water absorption exhibited by the autoclaved BioCs, as shown in Fig. 2. It is interesting to note that the amount of bacterial broth used for the *A. brasilense* inoculation of the BioC packs (0.2 mL bacteria broth g^{-1} BioC) was absorbed in approximately 1 min by all BioCs.

Biocomposite visualization by SEM

Production of bacterial biomass in culture media developed to induce *A. brasilense* EPS production and PHB

accumulation could have had a positive effect on bacterial adhesion and survival in the biocomposites because no striking differences were evident on the SEM images of the different formulations. However, the presence of high EPS and PHB content could not be considered the ultimate influencing factor of bacterial survival in each formulation during storage because differences in biocomposite composition led to highly variable bacterial survival. In this sense, the physicochemical differences found in the BioCs could have influenced bacterial metabolic behavior after the packs were prepared. Biopolymers such as polyhydroxybutyrate (PHB) and exopolysaccharides (EPS) were demonstrated to play important role in *Azospirillum*-plant interactions by protecting *A. brasilense* cells submitted to abiotic stresses, preventing its viability decline in carrier materials, and helping its establishment and proliferation in the rhizosphere (Tal and Okon 1985; Okon and Itzigsohn 1992; Konnova et al. 2001; Kadouri et al. 2003; Fibach-Paldi et al. 2012). Conditions that induce high amounts of both the secondary metabolites PHB and EPS in *A. brasilense* cells are related to nutritional factors and can be achieved by growing the bacteria under high C:N ratio (Kadouri et al. 2003), and inoculant formulations prepared with *A. brasilense* containing high concentration of EPS and/or PHB have demonstrated superior performance in field trials (Fallik and Okon 1996; Joe et al. 2012). Furthermore, the addition of extracellular lipochitooligosaccharides produced by *Rhizobium tropici* has shown to increase the performance *A. brasilense* inoculant (Marks et al. 2015). The introduction of *A. brasilense* cells into the BioCs with high amounts of EPS and PHB may provide protection to the cells and support to its long-term viability, favoring an immobilization-like mechanism by the irreversible attachment of bacteria due to the biofilm formation on the BioCs.

The immobilization of the PGPB provides the advantage of slow bacterial release during composite matrix biodegradation and physical protection against both competition and predation by native microorganisms, which favors the colonization of plant roots and assures the presence of inoculated bacteria near the plant for longer periods of time (Bashan et al. 2014). Unlike processes of bacteria immobilization in alginate and carboxymethylcellulose, the use of extruded foams prepared with nutritional materials such as starch, sucrose, and glycerol can promote the inoculant bacteria proliferation once its moisture is raised due to application in the soil and the beginning of biodegradation. In addition, the composition of the biocomposites can be easily modified to include micronutrients and other molecules that could serve as nutritional sources for the inoculant bacteria, increasing the inoculation efficacy and reducing the need for fertilizer application to agricultural crops.

Survival of *A. brasilense* Ab-V5 on the biocomposites: bioproduct shelf-life

Observing the composition of each formulation, the amount of YE appears to exert a major influence on the viability of *A. brasilense* cells over the storage period. This can be better pictured by comparing the *A. brasilense* viability of biocomposite BioC2, which presented a population density of $1.9 \pm 3.45 \times 10^4$ cells g^{-1} after 120 days of storage, with that of the BioC5 formulation, where populations remained constant throughout the storage period. The main difference between these formulations lies in the amount of YE, where BioC5 was enriched with 4.6 times more YE than BioC2, and other constituents were present in relatively equivalent amounts (Table 1, Fig. 1). In addition, the BioC4 formulation and its counterpart, BioC1, also differed mainly in the quantity of YE (as described for the BioC2 and BioC5 formulations), as well as in the capability to preserve *A. brasilense* viability throughout the storage period; these results support the importance of the YE for achieving a high quality formulation for extruded biocomposite foams. Yeast extracts can provide nutrients, such as nitrogen, vitamins, amino acids, and sugars, and have been related to increased resistance against abiotic stressors, such as desiccation (Streeter 2003) and acidic conditions (Charalampopoulus et al. 2003). Other materials that could influence the maintenance of *A. brasilense* viability are rock phosphate and sugarcane bagasse because the BioC4 and BioC5 formulations, which showed higher population densities after 120 days of storage, contained largely different amounts of these substances. Properties of starch-based extruded foams are difficult to predict due to the several different interactions that take place between the materials under the extrusion process (Moad 2011), leading the composite to behave as a completely new material. Indeed, the role of a single particular material and its relative amount plays in the maintenance of *A. brasilense* viability in each BioC formulation is not clear because the experimental design was not intended to define it. At the beginning of the storage period, the BioC packs contained enough humidity to allow the cells to reach the inner pores of the biocomposites, and at this time some nutrients could be acquired from the extruded foams. Nevertheless, it was not expected that the bacteria would remain metabolically active and able to use the BioC components as a nutrient or energy source in a significant manner during the entire storage period. Limited water activity can improve bacterial viability during storage (Gallarato et al. 2015); however, a low water activity also reduces the metabolism of microorganisms (Bossio and Scow 1995) preventing a broad utilization of the BioC constituents by the *A. brasilense* cells.

The maintenance of bacterial viability is the ultimate achievement for the success of an inoculant aimed to improve crop productivity. The presence of inoculated bacterial cells in

high population density at the time of plant development in the field leads to plant colonization and consequently to the expression of plant growth-promoting traits (Pii et al. 2015). By modifying the composition of starch-based extruded foams, a bacterial carrier was designed and showed high stability by maintaining the viability of *A. brasilense* Ab-V5 cells for up to 4 months; however, the shelf-life could be longer because a decay period was not identified for the formulation BioC5 in this study. In addition, the equipment needed for its production is largely used by the industry. The physical characteristics of BioC also allows its application with the regular implements used for fertilizer application because immobilized PGPB inside the pores of the extruded foam are expected to be protected against biotic and abiotic factors that decrease bacterial viability both while in storage and in the soil.

Biocomposite plant growth-promotion effectiveness

The inoculation practice with PGPBs in non-leguminous crops has gained increasing attention worldwide, and its use as a complementary practice for cereals such as maize is becoming popular in Brazil. Nevertheless, the inoculation of seeds with PGPB is mostly presented as an additional agricultural recommendation because the effectiveness of the BNF contribution in non-legumes is still controversial, even though the transference of biologically fixed nitrogen from *A. brasilense* to plants has robust scientific evidences (Bashan and de-Bashan 2010; Pankievicz et al. 2015). Furthermore, the variability of plant growth in response to PGPB inoculation is the primary concern of diminishing the amount of N-fertilizer used in non-leguminous crops, but the literature has shown that the inoculation effect of diazotrophic PGPB is more pronounced under low N-fertilizer inputs and can actually diminish N-fertilization without a decrease in crop productivity (Fallik and Okon 1996; Baris et al. 2014; Matsumura et al. 2015). Immobilized PGPB in different matrices has been presented as an alternative to reach more consistent inoculation responses by supporting bacterial viability during longer periods of storage and allowing the slow release of PGPB in soil, hence favoring plant colonization (Dommergues et al. 1979; Bashan 1986; Silva et al. 2012). In addition to the use of suitable carriers as PGPB vehicles, the production of high physiological quality bacterial biomass has also been considered to be of great importance for obtaining effective inoculants (Herrmann and Lesueur 2013; Bashan et al. 2014; Carrasco-Espinosa et al. 2015).

The results presented in this work introduce an innovative bacterial carrier which works as an immobilizer matrix able to sustain the viability of *A. brasilense* cells for up to 120 days of storage at room temperature. Bacteria immobilized in the BioC5 could fully contribute to maize growth even when they were applied to the soil 10 days before the maize was sown, suggesting that the BioC provides a protective and nutritional environment that supports the bacteria for periods in soil longer than those observed for liquid inoculant formulations. The biocomposites indeed allow the introduction of minor nutritional elements and/or bioactive compounds, such as micronutrients and growth factors, to improve plant performance and productivity in the field. The amount of BioC that was used in the greenhouse trial presented in this work is suggested as a base dose to be used in commercial crops, meaning approximately 120–150 kg ha^{-1} that can be distributed using the same implements that farmers commonly adopt to distribute solid fertilizers. The increase in efficiency of benefits from diazotrophic PGPB when a high quality inoculant is provided to cereals should also lead to, at least, a partial substitution of industrial fertilizers by PGPB inoculants, strengthening its value as an agricultural input. Field application of BioC is underway to confirm the results presented in this paper.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical statement This article does not contain any studies with human participants or animals performed by any of the authors.

References

ABNT–Associacão Brasileira de Normas Técnicas (1999) Papel e Cartão. Determinacão da capacidade de absorção de água. Método de Cobb. Associacão Brasileira de Normas Técnicas, Rio de Janeiro

Albareda M, Rodríguez-Navarro DN, Camacho M, Temprano FJ (2008) Alternatives to peat as a carrier for rhizobia inoculants: solid and liquid formulations. Soil Biol Biochem 40:2771–2779

Amaya-Llano SL, Hernández NM, Tostado EC, Martínez-Bustos F (2007) Functional characteristics of extruded blends of whey protein concentrate and corn starch. Cereal Chem 84:195–201

Baris O, Sahin F, Turan M, Orhan F, Gulluce M (2014) Use of plant-growth-promoting rhizobacteria (PGPR) seed inoculation as alternative fertilizer inputs in wheat and barley production. Commun Soil Sci Plan 45:2457–2467

Bashan Y (1986) Alginate beads as synthetic inoculant carriers for slow release of bacteria that affect plant growth. Appl Environ Microbiol 51:1089–1098

Bashan Y (1998) Inoculants of plant growth-promoting bacteria for use in agriculture. Biotechnol Adv 16:729–770

Bashan Y, Gonzalez LE (1999) Long-term survival of the plant growth-promoting bacteria *Azospirillum brasilense* and *Pseudomonas*

fluorescens in dry alginate beads. Appl Microbiol Biotechnol 51: 262–266

Bashan Y, LE d-B (2015) Inoculant preparation and formulations for *Azospirillum* spp. In: Cassán FD, Okon Y, Creus CM (eds) Handbook for *Azospirillum*. Springer International Publishing, Cham, pp. 469–485

Bashan Y, Holguin G, LE d-B (2004) *Azospirillum*-plant relationships: physiological, molecular, agricultural, and environmental advances (1997-2003). Can J Microbiol 50:521–577

Bashan Y, LE d-B, SR P, JP H (2014) Advances in plant growth-promoting bacterial inoculant technology: formulation and practical perspectives. Plant Soil 378:1–33

Bossio DA, Scow KM (1995) Impact of carbon and flooding on the metabolic diversity of microbial communities in soil. Appl Environ Microbiol 61:4043–4050

Calvo P, Nelson L, Kloepper JW (2014) Agricultural uses of plant biostimulants. Plant Soil 383:3–41

Carrasco-Espinosa K, García-Cabrera RI, Bedoya-López A, Trujillo-Roldán MA, Valdez-Cruz NA (2015) Positive effect of reduced aeration rate on growth and stereospecificity of DL-malic acid consumption by *Azospirillum brasilense*: improving the shelf-life of a liquid inoculant formulation. J Biotechnol 195:74–81

Charalampopoulus D, Pandiella SS, Webb C (2003) Evaluation of the effect of malt, wheat and barley extracts on the viability of potential probiotic lactic acid bacteria under acidic conditions. Int J Food Microbiol 82:133–141

Covarrubias SA, LE d-B, Moreno M, Bashan Y (2012) Alginate beads provide a beneficial physical barrier against native microorganisms in wastewater treated with immobilized bacteria and microalgae. Appl Microbiol Biotechnol 93:2669–2680

De-La-Peña C, Loyola-Vargas VM (2014) Biotic interactions in the rhizosphere: a diverse cooperative enterprise for plant productivity. Plant Physiol 16:701–719

Denardin ND, Freire JRJ (2000) Assessment of polymers for the formulation of legume inoculants. World J Microbiol Biotechnol 16:215–217

Döbereiner J (1988) Isolation and identification of root associated diazotrophs. Plant Soil 110:207–212

Dommergues YR, Diem HG, Divies C (1979) Polyacrylamide-entrapped *Rhizobium* as an inoculant for legumes. Appl Environ Microbiol 37: 779–781

Fallik E, Okon Y (1996) The response of maize (*Zea mays*) to *Azospirillum* inoculation in various types of soils in the field. World J Microbiol Biotechnol 12:511–515

Faruk O, Bledzki AK, Fink HP, Sain M (2012) Biocomposites reinforced with natural fibers: 2000-2010. Prog Polym Sci 37:1552–1596

Fibach-Paldi S, Burdman S, Okon Y (2012) Key physiological properties contributing to rhizosphere adaptation and plant growth promotion abilities of *Azospirillum brasilense*. FEMS Microbiol Lett 326:99–108

Gallarato LA, Paulucci NS, Chiappero J, Cesari AB, Reguera YB, Vicario JC, Dardanelli MS (2015) Stress physiology in *Azospirillum* and other PGPRs. In: Cassán FD, Okon Y, Creus CM (eds) Handbook for *Azospirillum*. Springer International Publishing, Cham, pp. 319–330

Glick BR (2015) Issues regarding the use of PGPB. In: Glick BR (ed) Beneficial plant-bacterial interactions. Springer International Publishing, Cham, pp. 223–243

Guan J, Hanna MA (2004) Functional properties of extruded foam composites of starch acetate and corn cob fiber. Ind Crop Prod 19:255–269

Gujska E, Khan K (1991) Functional properties of extrudates from high starch fractions of navy and pinto beans and corn meal blended with legume high protein fractions. J Food Sci 56:431–435

Herrmann L, Lesueur D (2013) Challenges of formulation and quality of biofertilizers for successful inoculation. Appl Microbiol Biotechnol 97:8859–8873

Hoagland DR, Arnon DI (1951) The water culture method for growing plants without soil. Calif Agric Exp Stat Univ Calif Berkeley Circ 347:1–32

Hungria M, Campo RJ, Souza EM, Pedrosa FO (2010) Inoculation with selected strains of *Azospirillum brasilense* and *A. Lipoferum* improves yields of maize and wheat in Brazil. Plant Soil 331:413–425

Joe MM, Karthikeyan B, Chauhan PS, Shagol C, Islam MR, Deiveekasundaram M, Sa T (2012) Survival of *Azospirillum brasilense* flocculated cells in alginate and its inoculation effect on growth and yield of maize under water deficit conditions. Eur J Soil Biol 50:198–206

Kadouri D, Jurkevitch E, Okon Y (2003) Involvement of the reserve material poly-β-hydroxybutirate in *Azospirillum brasilense* stress endurance and root colonization. Appl Environ Microbiol 69: 3244–3250

Kaewtatip K, Tanrattanakul V, Phetrat W (2013) Preparation and characterization of kaolin/starch foam. Appl Clay Sci 80-81:413–416

Karagöz K, Ates F, Karagöz H, Kotan R, Çakmakçi R (2012) Characterization of plant growth-promoting traits of bacteria isolated from the rhizosphere of grapevine grown in alkaline and acidic soils. Eur J Soil Biol 50:144–150

Konnova SA, Brykova OS, Sachkova OA, Egorenkova IV, Ignatov VV (2001) Protective role of the polysaccharide containing capsular components of *Azospirillum brasilense*. Microbiology 70:436–440

Kushneruk MA, Tugarova AV, II'chukova AV, Slavkina EA, Starichkova NI, Bogatyrev VA, Antonyuk LP (2013) Factors inducing transition from growth to dormancy in rhizobacteria *Azospirillum brasilense*. Microbiology 82:572–578

Lopez-Gil A, Silva-Bellucci F, Velasco D, Ardanuy M, Rodriguez-Perez MA (2015) Cellular structure and mechanical properties of starch-based foamed blocks reinforced with natural fibers and produced by microwave heating. Ind Crop Prod 66:194–205

Mali S, Debiagi F, Grossmann MVE, Yamashita F (2010) Starch, sugarcane bagasse fibre, and polyvinyl alcohol effects on extruded foam properties: a mixture design approach. Ind Crop Prod 32:353–359

Marks BB, Megías M, Ollero FJ, Nogueira MA, Araujo RS, Hungria M (2015) Maize growth promotion by inoculation with *Azospirillum brasilense* and metabolites of *Rhizobium tropici* enriched on lipochitooligosaccharides (LCOs). AMB Express 5:1–11

Matsumura EE, Secco VA, Stolf-Moreira R, Santos OJAP, Hungria M, Oliveira ALM (2015) Composition and activity of endophytic bacterial communities in field-grown maize plants inoculated with *Azospirillum brasilense*. Ann Microbiol 65:2187–2200

Mitrus M, Moscicki L (2014) Extrusion-cooking of starch protective loose-fill foams. Chem Eng Res Des 92:778–783

Moad G (2011) Chemical modification of starch by reactive extrusion. Prog Polym Sci 36:218–237

Niba LL, Hoffman J (2003) Resistant starch and β-glucan levels in grain sorghum (*Sorghum bicolor* M.) are influenced by soaking and autoclaving. Food Chem 81:113–118

Okon Y, Itzigsohn R (1992) Poly-β-hydroxybutyrate metabolism in *Azospirillum brasilense* and the ecological role of PHB in the rhizosphere. FEMS Microbiol Rev 103:131–140

Okon Y, Labandera-Gonzales C, Lage M, Lage P (2015) Agronomic applications of *Azospirillum* and other PGPR. In: de Bruijn FJ (ed) Biological nitrogen fixation. Wiley Blackwell, Hoboken, pp. 925–936

Pankievicz VCS, Amaral FP, Santos KFDN, Agtuca B, Xu Y, Schueller MJ, Arisi AC, Steffens MBR, Souza EM, Pedrosa FO, Stacey G, Ferrieri RA (2015) Robust biological nitrogen fixation in a model grass-bacterial association. Plant J 81:907–919

Pii Y, Mimmo T, Tomasi N, Terzano R, Cesco S, Crecchio C (2015) Microbial interactions in the rhizosphere: beneficial influences of

plant growth-promoting rhizobacteria on nutrient acquisition process. Biol Fertil Soils 51:403–415

Polat S, Uslu MK, Aygün A, Certel M (2013) The effects of the addition of corn husk fibre, kaolin and beeswax on cross-linked corn starch foam. J Food Eng 116:267–276

Rathore S, Desai PM, Liew CV, Chan LW, Heng PWS (2013) Microencapsulation of microbial cells. J Food Eng 116:369–381

Rockland LB (1960) Saturated salt solutions for static control of relative humidity between 5° and 40 °C. Anal Chem 32:1375–1376

Salgado PR, Schmidt VC, Ortiz SEM, Mauri AN, Laurindo JB (2008) Biodegradable foams based on cassava starch, sunflower proteins, and cellulose fibers obtained by a baking process. J Food Eng 85: 435–443

Schoebitz M, López MD, Roldán A (2013) Bioencapsulation of microbial inoculants for better soil-plant fertilization. A review. Agron Sustain Dev 33:751–765

Shogren RL, Lawton JW, Tiefenbacher KF, Chen L (1998) Starch–poly(-vinyl alcohol) foamed articles prepared by a baking process. J Appl Polym Sci 68:2129–2140

Silva MF, Antônio CS, Oliveira PJ, Xavier GR, Rumjanek NG, Soares LHB, Reis VM (2012) Survival of endophytic bacteria in polymer-based inoculants and efficiency of their application to sugarcane. Plant Soil 356:231–243

Sjöqvist M, Boldizar A, Rigdahl M (2010) Processing and water absorption behaviour of foamed potato starch. J Cell Plast 46:497–517

Streeter JG (2003) Effect of trehalose on survival of *Bradyrhizobium japonicum* during desiccation. J Appl Microbiol 95:484–491

Tal S, Okon Y (1985) Production of the reserve material poly-β-hydroxybutyrate and its function in *Azospirillum brasilense*. Can J Microbiol 31:608–613

Trujillo-Roldán MA, Valdez-Cruz NA, Gonzalez-Monterrubio CF, Acevedo-Sánchez EV, Martínez-Salinas C, García-Cabrera RI, Gamboa-Suasnavart RA, Marín-Palacio LD, Villegas J, Blancas-Cabrera A (2013) Scale-up from shake flasks to pilot-scale production of the plant growth-promoting bacterium *Azospirillum brasilense* for preparing a liquid inoculant formulation. Appl Microbiol Biotechnol 97:9665–9674

Vercelheze AES, Oliveira ALM, Rezende MI, Muller CMO, Yamashita F, Mali S (2013) Physical properties, photo- and bio-degradation of baked foams based on cassava starch, sugarcane bagasse fibers and montmorillonite. J Polym Environ 21:266–274

Xu YX, Dzenis Y, Hanna MA (2005) Water solubility, thermal characteristics and biodegradability of extruded starch acetate foams. Ind Crop Prod 21:361–368

Yu L, Dean K, Li L (2006) Polymer blends and composites from renewable sources. Prog Polym Sci 31:576–602