Biol Fertil Soils (2011) 47:963–969
DOI 10.1007/s00374-011-0555-3

SHORT COMMUNICATION

Development of two culture media for mass cultivation of *Azospirillum* spp. and for production of inoculants to enhance plant growth

Yoav Bashan · Adan Trejo · Luz E. de-Bashan

Received: 25 November 2010 / Revised: 25 February 2011 / Accepted: 3 March 2011 / Published online: 7 April 2011

Abstract High yield culture medium is fundamental for production of inoculants for plant growth-promoting bacteria. Based on substitution of glucose in tryptone–yeast extract–glucose medium by Na-gluconate or glycerol, two new culture media were developed for mass cultivation of the commonly used plant growth-promoting bacterium *Azospirillum* sp. After 18 h of incubation, these modifications increased populations of different strains of *Azospirillum* (to ~10^{11} cells ml^{-1} [single cell count] and ~5×10^{9} CFU ml^{-1} [plate count method]), significantly reduced generation time, and were also suitable for production of common synthetic inoculants.

Keywords *Azospirillum* · Biofertilizer · Culture medium · Inoculant · Mass propagation · Plant growth-promoting bacteria

Introduction

The end goal and economic justification of any long-term investigation of the effect of plant growth-promoting bacteria (PGPB and PGPR) on plant growth and productivity is practical application of inoculants by growers. To that end, inoculants are made of viable cultures with high numbers of the desired PGPB embedded, suspended, or mixed in a carrier of choice. The latter can be either solid or liquid. A fundamental requirement of such technology is the production of cultures of PGPB of high cell number, usually in fermenters using the cheapest, yet efficient, culture medium for growth and simple procedures for growing conditions (Bashan 1998).

Azospirillum spp. is one of the most studied PGPB, apart from symbiotic rhizobia (Bashan and de-Bashan 2010; Bashan et al. 2004). It has been commercially used on a relative large scale in Argentina, Mexico, Europe, South Africa, and India, mainly on cereals though it has also been used on other crops (Díaz-Zorita and Fernández-Canigia 2009; Fuentes-Ramirez and Caballero-Mellado 2005; Hartmann and Bashan 2009) where many, mostly small-to-medium companies and governmental agencies, are involved in inoculant production (Fuentes-Ramirez and Caballero-Mellado 2005). Most published literature on *Azospirillum* spp. used general microbiological media or some specific nitrogen-free media for cultivation (Bashan et al. 1993; Bashan et al. 2004). Most media used for the production of commercial inoculants are considered proprietary and are therefore not revealed.

The hypothesis of this technological development is based on the assumption that, because carbon metabolism of *Azospirillum* spp. is well-known (Hartmann and Zimmer 1994), it is possible to develop culture medium for mass cultivation of this PGPB by replacing or supplementing the carbon sources in contemporary media (Bashan et al. 1993) by other preferred but less common carbon sources of *Azospirillum* spp. The purpose of this work was to produce *Azospirillum* cultures of high cell numbers suitable for production of inoculants that can sustain the shear and tear

This study is dedicated to the memory of Dr. Jesus Caballero-Mellado (1953–2010) of Centro de Ciencias Genómicas de la UNAM, the pioneer of *Azospirillum* research in Mexico.

Y. Bashan (✉) · A. Trejo · L. E. de-Bashan
Environmental Microbiology Group,
Northwestern Center for Biological Research (CIBNOR),
Mar Bermejo 195, Col. Playa Palo de Santa Rita,
La Paz, Baja California Sur 23090, Mexico
e-mail: bashan@cals.arizona.edu

Y. Bashan · L. E. de-Bashan
The Bashan Foundation,
3740 NW Harrison Blvd.,
Corvallis, OR 97330, USA

of the formulation process. This has been done by improving the performance of existing mass production culture media using available industrial sources of gluconate and glycerol. Preliminary in vitro studies indicated that, in complex media, the bacterium prefers these compounds (unpublished data) as supplements over the organic acids (malate and succinate), which are commonly used for cultivation in specific media for this genus (Bashan et al. 1993; Okon et al. 1977).

Materials and methods

Bacterial strains

Three strains, commonly used as inoculants of *Azospirillum* sp., were employed as models to develop the media: *Azospirillum brasilense* Cd (wild-type strain of *A. brasilense* that originated in the USA, DSM 1843, Braunschweig, Germany); *A. brasilense* Sp6 (wild-type strain of *A. brasilense* that originated in Italy; Barbieri and Galli 1993); and *Azospirillum lipoferum* JA4, wild-type rhizosphere isolate that originated in Brazil (Castellanos et al. 1997).

Culture media

Five culture media were compared. All chemicals were of analytical grade (Sigma-Aldrich, St. Louis, MO), unless specifically noted. Control media were: (1) nutrient broth, (2) OAB medium containing solution A [(g/l): DL-malic acid, 5; NaOH, 3; $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2$, 0.02; NaCl, 0.1; NH_4Cl, 1; yeast extract, 0.1; $FeCl_3$, 0.01; (mg/l): $NaMoO_4 \cdot 2H_2O$, 2; $MnSO_4$, 2.1; H_3BO_3, 2.8; $Cu(NO_3)2 \cdot 3H_2O$, 0.04; $ZnSO_4 \cdot 7H_2O$, 0.24; 900 ml distilled water] and solution B [(g/1): K_2HPO_4, 6; KH_2PO_4, 4; 100 ml distilled water]. After autoclaving and cooling, the two solutions should be mixed. The Ph of the medium is 6.8 (Okon et al. 1977). (3) Modified tryptone–yeast extract and glucose (TYG) medium containing [(g/l): tryptone, 5 (Difco); yeast extract, 5, D-glucose, 5; NaCl, 1.2; $MgSO_4 \cdot 7H_2O$, 0.25; K_2HPO_4 0.13; $CaCl_2$, 0.22; K_2SO_4, 0.17; Na_2SO_4, 2.4; $NaHCO_3$, 0.5; Na_2CO_3, 0.09; Fe(III) EDTA, 0.07]; the pH was adjusted to 7.0 after sterilization (Bashan et al. 2002). The two tested media contained the modified TYG medium where the glucose was replaced by (4) 5 g/l Na-gluconate or (5) 8 ml/l glycerol.

All cultures started from single colonies. Each single colony was grown in nutrient broth medium at 36±1°C for 24 h at 120 rpm in 100 ml Erlenmeyer flasks. Cells were harvested by centrifugation at 4,000×*g*, rinsed twice in sterile 0.85% NaCl solution, and OD_{540} was adjusted to 1.0, corresponding to 10^9 CFU/ml (Bashan and Levanony 1985). Two milliliters of this suspension was used as an inoculum into each medium containing 120 ml in unbaffled 500 ml Erlenmeyer flasks. Cultures were cultivated as described above for 18 h.

Measuring populations of *Azospirillum* spp.

Optical density at 540 nm was measured at 6 h intervals at 1, 6, 12, and 18 h after inoculation. Counts of bacteria were done by two independent methods: (1) by image analyzer and then verified by (2) the plate count method. For image analysis, bacteria were counted using a modification of the fluorescein diacetate method (Chrzanowski et al. 1984). Five hundred microliters of each culture were homogenized (vortex, 2 min, three times) and centrifuged at 12,800×*g* for 3 min. The pellet was suspended in 50 µl fluorescein diacetate (20 mg in 10 ml acetone at 4°C) in 0.002 M phosphate buffer at pH 7.2. Twenty microliters of this suspension were placed on glass slide, and the number of cells was counted by the image analysis system (Image ProPlus 4.5, Media Cybernetics, Silver Spring, MD) connected to a epifluorescent microscope (Olympus BX41, Tokyo, Japan) with a green filter (excitation 460–490 nm with maximum emission of light at 520 nm). One suspension from each replicate was analyzed. Fifty individual fields were automatically counted from each of the four slides (n=200) that represented one culture. Counting was made immediately after inoculation and again 18 h later; counts were expressed as number of cells per milliliter. Similarly, conventional plate counting on Nutrient Agar was done and expressed as CFU ml^{-1}. Growth rate (μ) was calculated using the equation: $\mu = (\ln Nt_1 - \ln Nt_0)/(t_1 - t_0)$, where Nt_1 is the number of cells at sampling time and Nt_0 is the number of cells at the beginning of the experiment. Generation time (g) was calculated using the equation $g = \ln 2/\mu$.

Fluorescence in situ hybridization

Identification of the three strains was done by fluorescence in situ hybridization at the end of the experiment on cultures of TY-gluconate cultures according to the technique described by Stoffels et al. (2001), but with numerous small modifications: Hybridization was performed at 35% formamide stringency at 46°C for 2 h. The final concentration of the probe was 3 ng μl^{-1}. Samples were then washed at 48°C for 5 min with 50 ml of pre-warmed washing buffer. The slides were rinsed for a few seconds with ice-cold, deionized water and then air dried. Slides were stored at −20°C in the dark until observation. Three types of probes were used: an equimolar mixture of probes EUB-338 I (Amann et al. 1990), II, and III (Daims et al. 1999) that cover the domain of bacteria. An *A. brasilense* specific probe (Abras 1420, Stoffels et al. 2001) was used

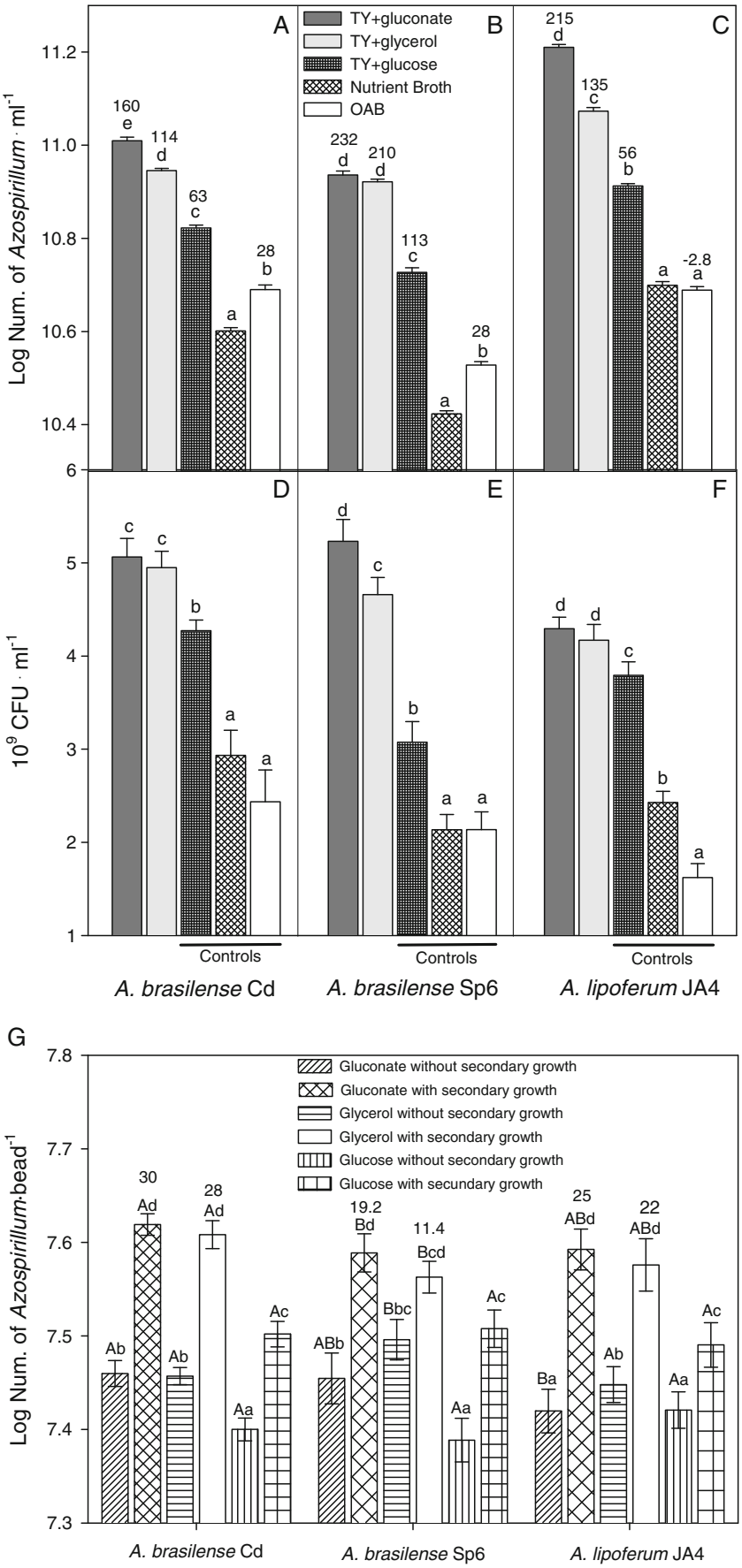

Fig. 1 Populations of three strains of *Azospirillum* spp. growing in five different culture media. **a–c** Cell numbers counted by image analyzer technique. **d–f** Colony forming unit (CFU) counted by agar plating technique. **g** Population size of three strains of *Azospirillum* spp. after immobilization in alginate beads, with or without secondary incubation in nutrient broth. *Columns denoted with a different lower case letter* at each subfigure differ significantly by one-way ANOVA and by Tukey's post hoc analysis at $P<0.05$. In **a–c**, *number above each column* represents percentage (%) of increase in growth for each medium, compare with the growth in nutrient broth control, a general purpose medium. In **g**, for each bacterial strain, *columns denoted with different lower case letter* differ significantly at $P<0.05$ by one-way ANOVA and Tukey's post hoc analyses. For each bacterial strain, *numbers above columns* represent percentage (%) of increase in growth in treatments supported by gluconate and glycerol (after secondary multiplication), compared with control supported with glucose. *Bars* represent standard error (SE)

for the two *A. brasilense* strains and Alila1113 (Stoffels et al. 2001) for *A. lipoferum*. The probes Abras 1420 and Alila 1113 were labeled with the fluorochrome FITC (green) and the mix of EUB I, II, and III was labeled with the fluorochrome Cy3 (red). All fluorescent-labeled probes were purchased from IDT (Coraville, IA). Before visualization, the slides were mounted in AF1 anti-fading reagent (Citifluor, Electron Microscopy Sciences, Hatfield, PA). Visualization was done with an epifluorescent microscope (Olympus) with two filters, the Cy3 filter (maximum excitation at 552 nm with maximum emission of light at 565 nm, red fluorescence, Olympus America, Melville, NY) and the FITC filter (maximum excitation at 490 nm with maximum emission of light at 520 nm, green fluorescence, Olympus America, Melville, NY).

Production of synthetic inoculant

To validate the capacity of the cultures to form inoculants, the standard solid alginate bead inoculant formulation (Bashan 1986) was used for the three strains. Beads (2–3 mm in diameter) were automatically produced in a 2% $CaCl_2$ solidification solution (de-Bashan and Bashan 2010). After formation, half of the inoculant was stored under saline conditions for 24 h. Then, beads were dissolved in 2% sodium bicarbonate for 2 h under constant agitation and the released bacteria were counted by image analysis. Because immobilization normally reduces the number of *Azospirillum* cells in the beads (Bashan 1986), the other half of the inoculant underwent a second overnight incubation (secondary multiplication) in diluted (10% of full strength) nutrient broth, incubated in saline solution, dissolved, and the number of cells present was counted, as described earlier.

Experimental design and statistical analysis

Each medium was tested in four replicates where a single Erlenmeyer flask serves as a replicate. The experiments were repeated twice. Data were analyzed by one-way ANOVA and then by Tukey's post hoc analysis set at $P<0.05$, using statistical software (Statistica 6.0, StatSoft, Tulsa, OK).

Results

Even though the undefined TYG control medium supports high cell number of two strains of *A. brasilense* and one of *A. lipoferum* (Fig. 1), addition of gluconate or glycerol significantly enhanced bacterial growth. This was confirmed either when single cells were counted (Fig. 1a–c) or CFU were counted (Fig. 1d–e), where obviously, values of CFU are smaller. When compared to a common laboratory medium (NB), the enhancing effect was significantly larger. The enhancement over NB medium was in the range of 115–230% or when compared to specific medium for *Azospirillum* (OAB), the enhancement was in a range of 85–200%, depending on the strain (Fig. 1a–c). Although growth with supplementary Na-gluconate (BTB-glu; BTB-1) usually resulted in media with a higher number of bacterial cells than growth with supplemental glycerol (BTB-gly; BTB-2), the general effect was similar. Generation time in media with the two supplements was the shortest, and the growth rate was the highest (Table 1), where generation time for the three strains in the five media was: BTB-1<BTB-2<TYG<NB<OAB. No reduction in lag period or more vigorous start of the log phase was observed (data not shown).

Formulation of the three strains as a common alginate bead inoculant showed that significant numbers of each strain (>10^7 cells/bead) survive the encapsulation process without any need for a secondary multiplication process common to formulations of *Azospirillum* strains in alginate (Fig. 1g, compare values without secondary growth). When secondary multiplication process was done, as expected, each inoculant contained significantly higher number of cells where inoculants incubated with gluconate or glycerol yielded the highest populations compared to medium with glucose only, by a range of ~10–30% (Fig. 1g). Using fluorescence in situ hybridization to identify the strains in the bead inoculant showed that inoculants contained only

Table 1 Growth rates (μ) and generation time (g) of *Azospirillum* spp. in different culturing media

Bacterial species	Medium/supplement	μ (h^{-1})	g (h)
A. brasilense Cd	Gluconate	0.49	1.41a
	Glycerol	0.48	1.44a
	Glucose	0.46	1.50b
	OAB	0.45	1.54b
	Nutrient broth	0.43	1.61c
A. brasilense Sp6	Gluconate	0.48	1.44a
	Glycerol	0.47	1.47a
	Glucose	0.45	1.54b
	OAB	0.42	1.65c
	Nutrient broth	0.41	1.69c
A. lipoferum JA4	Gluconate	0.51	1.36a
	Glycerol	0.49	1.41a
	Glucose	0.47	1.47ab
	OAB	0.45	1.54b
	Nutrient broth	0.45	1.54b

Gluconate (BTB-1 medium), glycerol (BTB-2 medium), and glucose are supplements for tryptone–yeast extract medium (TYG, Prabhu et al. 2000). For each bacterial strain, values denoted with different lower case letter differ significantly at $P<0.05$ by one-way ANOVA and Tukey's post hoc analyses

the intended strain (Fig. 2). These inoculants could be stored for at least 1 year without significant reduction in the number of the cells (data not shown), as is common for alginate inoculants (Bashan and Gonzalez 1999). When these new inoculants were compared to routinely produced alginate inoculants based on TYG medium for their effect on plant growth, they performed identically in the greenhouse and in the field as was published before for wheat, sorghum, and mesquite plants (Bashan et al. 2002, 2009), and therefore, this data is not presented.

Discussion

The plant-associated, rhizosphere-dwelling, diazotrophic PGPB *Azospirillum* spp. was re-discovered in the 1970s in studies using semi-solid, buffer-free, nitrogen-free medium (NFb) that was based on organic acids, mainly malate and succinate, the preferred carbon sources of this bacterium in situ (Döbereiner and Day 1976). This medium, with several minor modifications, even though it produces relatively small populations, is still currently very common in laboratory studies (Cassán et al. 2009). This medium was further improved by increasing its buffering capacity over the original medium and adding microelements, a limited amount of NH_4Cl to initiate aerobic growth, and a small amount of yeast extract to shorten the lag phase and aid vigorous growth (OAB; Okon et al. 1977). This is currently one of the most common and useful medium for research on *Azospirillum* spp. (de-Bashan et al. 2008). This medium was later supplemented with Congo red stain to increase selectivity for the bacterium (NFb-Congo red and BL; Bashan and Levanony 1985; Rodriguez-Caceres 1982). While these media were well suited for laboratory studies, they were not suitable for mass cultivation in inoculant production at larger scales, larger than bench experiments. Consequently, general purpose bacterial media such as nutrient broth, Luria–Bertani broth, and a few others are commonly used, all with less than optimal yields of cells (personal communication with sources in the inoculant industry). As a breakthrough to overcome the problems with these general microbiological media, a complex, undefined medium was proposed, based on TYG that allowed more massive cultivation of many PGPB/PGPR (Prabhu et al.

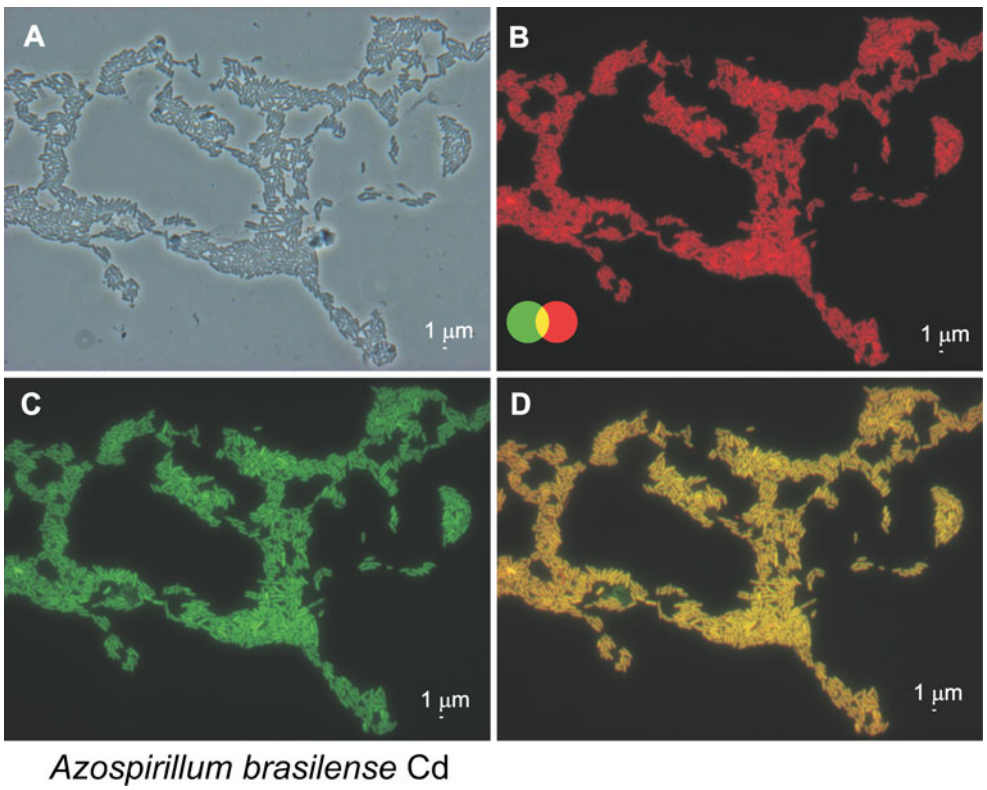

Fig. 2 Fluorescence in situ hybridization (FISH) photomicrographs of *Azospirillum brasilense* Cd (**a**–**d**) after formulation and release from the alginate inoculant. Two types of probes were used: an equimolar mixture of probes EUB-338 I, II, and III that covers the domain of bacteria. An *Azospirillum brasilense* specific probe (Abras 1420) was used. The probe Abras 1420 were labeled with the fluorochrome FITC (*green*). The mix of EUB I, II, and III was labeled with the fluorochrome Cy3 (*red*). Positive fluorescent signals that identify the bacteria are therefore a combination of *red* and *green* that yields a *green-yellow-orange tone*, depending on the intensities of the individual color channels. *Bars* represent 1 µm

2000). This medium was further developed to enhance the growth of *Azospirillum* by supplementing the buffer capacity and micronutrients of OAB medium that were specially designed for this species (Bashan et al. 2002). Yet, it has a major deficiency because glucose is not used by some species of *Azospirillum*, such as the most common species used as an inoculant, *A. brasilense*, and is not a preferred carbon source for this genus (Hartmann and Zimmer 1994).

From its beginning, all studies of the interaction of *Azospirillum* with plants indicated its high affinity for organic acids, mainly malate and succinate, as carbon sources (Okon et al. 1977; Umali-Garcia et al. 1980). Both compounds are common ingredient of root exudates of many plants (Jones 1998). These organic acids were incorporated into the traditional initial media for this species, as mentioned earlier. Later physiological studies indicated that this bacterium is highly versatile in meeting its carbon requirements (Hartmann and Zimmer 1994), some that are not normally found in the rhizosphere. Even though these compounds can potentially support growth of *Azospirillum*, they were not used for preparing media. We evaluated two of those substances, gluconic acid, which is found in some root exudates (Leyva and Bashan 2008), and glycerol, which is a common by-product of transesterification of lipids and fats and was evaluated earlier for growth of *Bradyrhizobium* for inoculants (Jain et al. 2000), on which *Azospirillum* grows well (Hartmann and Zimmer 1994). Both compounds have a history of many applications in the food, cosmetics, and pharmaceutical industries (Ramachandran et al. 2006; Robergs and Griffin 1998). They were used as simple amendments and substitutions for glucose to enhance the performance of TYG medium. The objectives were to increase production of cells more than what was obtained in TYG medium in the shortest period of time.

In the original TYG medium (Prabhu et al. 2000), glucose served as an immediately available carbon source for the bacteria where the more complex tryptone and yeast extract in the medium were used as the main carbon sources. In our study, the two substitutions for glucose, gluconate and glycerol, outperform glucose as an immediately available carbon source by increasing growth rates and reducing generation time of each strain. This indicates that gluconate and glycerol enhance growth either by increasing populations during the logarithmic period or serve as additional carbon source when the main sources are reduced. In either case, the populations present after only 18 h were far higher than populations in the richest known medium for cultivating *Azospirillum*, the TYG medium. In our study, generation time of all cultures were within the range known for this species, about 1–2 h (Mukherjee and Ghosh 1987; Okon et al. 1977), although in most cases, generation time for this genus ranges from 2.8 to 11.2 h in different minimal media enriched with different N and C sources (Kadouri et al. 2003; Madkour et al. 1990; Nur et al. 1982; Westby et al. 1983). Although gluconate and glycerol can be used by *Azospirillum* spp. for growth (Hartmann and Zimmer 1994), why they outperformed established organic acids preferred by *Azospirillum* spp. require further comparative physiological study.

Inoculants can be made in a variety of carriers. We choose the alginate polymer for three reasons: (1) It is the most common polymer used to encapsulate microorganisms for industrial use (Prasad and Kadokawa 2009); (2) It has been used as an experimental inoculant for *Azospirillum* spp. for over two decades (Bashan 1986; Bashan et al. 2009) and does not need further proof of its efficacy; and (3) Polymerized inoculants produce the strongest stress on bacterial cells during formulation. The encapsulation process usually reduces the population in comparison to formulations when the culture is used as is or when it is incorporated into traditional peat inoculant as is usually the case of industry applications (Bashan 1998; Díaz-Zorita and Fernández-Canigia 2009). Additionally, a second, short multiplication period in culture medium is needed in previous formulations of alginate inoculants (Bashan 1986; Bashan et al. 2002). Our success with alginate formulations demonstrated that the proposed culture media may also be useful in easier formulations, such as liquid or inert material inoculants. We have shown that when producing very high cell number cultures of *A. brasilense* and *A. lipoferum* in the new media, even the evitable decline of populations did not require a secondary multiplication phase for creating a formulation with a sufficient number of cells. This points out the potential of these media with other inoculant formulation. One limitation of the two proposed media for inoculant production is that, in some countries, tryptone is relatively expensive and cannot be used for large-scale production of cheap inoculants. In summary, we propose two culture media (BTB-1 with gluconate and BTB-2 with glycerol) for mass cultivation of *A. brasilense* and *A. lipoferum* that are highly suitable for production of inoculants.

Acknowledgments This study was supported by Secretaria de Medio Ambiente y Recursos Naturales of Mexico (SEMARNAT contract 23510) and the time for writing by The Bashan Foundation, USA. Ira Fogel of CIBNOR provided useful editorial comments. A.T. is a recipient of a fellowship from the graduate school at CIBNOR, with additional funding from the Bashan Foundation.

References

Amann RI, Binder BJ, Olson RJ, Chisholm SW, Devereux R, Stahl DA (1990) Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl Environ Microbiol 56:1919–1925

Barbieri P, Galli E (1993) Effect on wheat root development of inoculation with an *Azospirillum brasilense* mutant with altered indole-3-acetic acid production. Res Microbiol 144:69–75

Bashan Y (1986) Alginate beads as synthetic inoculant carriers for the 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, de-Bashan LE (2010) How the plant growth-promoting bacterium *Azospirillum* promotes plant growth-a critical assessment. Adv Agron 108:77–136

Bashan Y, Gonzalez LE (1999) Long-term survival of the plant-growth-promoting bacteria *Azospirillum brasilense* and *Pseudomonas fluorescens* in dry alginate inoculant. Appl Microbiol Biotechnol 51:262–266

Bashan Y, Levanony H (1985) An improved selection technique and medium for the isolation and enumeration of *Azospirillum brasilense*. Can J Microbiol 31:947–952

Bashan Y, Holguin G, Lifshitz R (1993) Isolation and characterization of plant growth-promoting rhizobacteria. In: Glick BR, Thompson JE (eds) Methods in plant molecular biology and biotechnology. CRC Press, Boca Raton, pp 331–345

Bashan Y, Hernandez JP, Leyva LA, Bacilio M (2002) Alginate microbeads as inoculant carrier for plant growth-promoting bacteria. Biol Fertil Soils 35:359–368

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

Bashan Y, Salazar B, Puente ME, Bacilio M, Linderman RG (2009) Enhanced establishment and growth of giant cardon cactus in an eroded field in the Sonoran Desert using native legume trees as nurse plants aided by plant growth-promoting microorganisms and compost. Biol Fertil Soils 45:585–594

Cassán F, Maiale S, Masciarelli O, Vidal A, Luna V, Ruiz O (2009) Cadaverine production by *Azospirillum brasilense* and its possible role in plant growth promotion and osmotic stress mitigation. Eur J Soil Biol 45:12–19

Castellanos T, Ascensio F, Bashan Y (1997) Cell-surface hydrophobicity and cell-surface charges of *Azospirillum* spp. FEMS Microbiol Ecol 24:159–172

Chrzanowski TH, Crotty RD, Hubbard JG, Welch RP (1984) Applicability of the fluorescein diacetate method of detecting active bacteria in freshwater. Microb Ecol 10:179–185

Daims H, Brühl A, Amann R, Schleifer KH, Wagner M (1999) The domain-specific probe EUB338 is insufficient for the detection of all bacteria: development and evaluation of a more comprehensive probe set. Syst Appl Microbiol 22:434–444

de-Bashan LE, Bashan Y (2010) Immobilized microalgae for removing pollutants: review of practical aspects. Bioresour Technol 101:1611–1627

de-Bashan LE, Antoun H, Bashan Y (2008) Involvement of indole-3-acetic-acid produced by the growth-promoting bacterium *Azospirillum* spp. in promoting growth of *Chlorella vulgaris*. J Phycol 44:938–947

Díaz-Zorita M, Fernández-Canigia MV (2009) Field performance of a liquid formulation of *Azospirillum brasilense* on dryland wheat productivity. Eur J Soil Biol 45:3–11

Döbereiner J, Day JM (1976) Associative symbiosis in tropical grasses: characterization of microorganisms and dinitrogen fixing sites. In: Newton WE, Nyman CJ (eds) Proceedings of the 1st international symposium on nitrogen fixation, vol 2. Washington State University Press, Pullman, pp 518–538

Fuentes-Ramirez LE, Caballero-Mellado J (2005) Bacterial biofertilizers. In: Siddiqui ZA (ed) PGPR: biocontrol and biofertilization. Springer, Dordrecht, pp 143–172

Hartmann A, Bashan Y (2009) Ecology and application of *Azospirillum* and other plant growth-promoting bacteria (PGPB)—special issue. Eur J Soil Biol 45:1–2

Hartmann A, Zimmer W (1994) Physiology of *Azospirillum*. In: Okon Y (ed) *Azospirillum*/plant associations. CRC, Boca Raton, pp 15–39

Jain SK, Pathak DV, Sharma HR (2000) Alternate carbon substrate for mass production of *Rhizobium* inoculants. Haryana Agric Univ J Res 30:1–6

Jones DL (1998) Organic acids in the rhizosphere—a critical review. Plant Soil 205:25–44

Kadouri D, Jurkevitch E, Okon Y (2003) Poly-β-hydroxybutyrate depolymerase (PhaZ) in *Azospirillum brasilense* y characterization of a *phaZ* mutant. Arch Microbiol 180:309–318

Leyva LA, Bashan Y (2008) Activity of two catabolic enzymes of the phosphogluconate pathway in mesquite roots inoculated with *Azospirillum brasilense* Cd. Plant Physiol Biochem 46:898–904

Madkour MA, Tombas Smith L, Smith GM (1990) Preferential osmolyte accumulation: a mechanism of osmotic stress adaptation in diazotrophic bacteria. Appl Environ Microbiol 56:2876–2881

Mukherjee A, Ghosh S (1987) Regulation of fructose uptake and catabolism by succinate in *Azospirillum brasilense*. J Bacteriol 169:4361–4367

Nur I, Okon Y, Henis Y (1982) Effect of dissolved oxygen tension on production of carotenoids, poly-β-hydroxybutyrate, succinate oxidase and superoxide dismutase by *Azospirillum brasilense* Cd grown in continuous culture. J Gen Microbiol 128:2937–2943

Okon Y, Albrecht SL, Burris RH (1977) Methods for growing *Spirillum lipoferum* and for counting it in pure culture and in association with plants. Appl Environ Microbiol 33:85–88

Prabhu SR, Thomas GV, Nierzwicki-Bauer SA, Prasad TG (2000) GA-like substances produced by endophytic Gram-positive bacteria are associated with coconut palm (*Cocos nucifera*). In: Rajagopal V, Naresh Kumar S, Upadhyay A, Niral V (eds) National Seminar on Recent Advances in Plant Biology, Central Plantation Crops Research Institute, Kasaragod, India, pp 141–142

Prasad K, Kadokawa JI (2009) Alginate-based blends and nano/microbeads. Microbiol Monographs 13:175–210

Ramachandran S, Fontanille P, Pandey A, Larroche C (2006) Gluconic acid: a review. Food Technol Biotechnol 44:185–195

Robergs RA, Griffin SE (1998) Glycerol: biochemistry, pharmacokinetics and clinical and practical applications. Sports Med 26:145–167

Rodriguez-Caceres EA (1982) Improved medium for isolation of *Azospirillum* spp. Appl Environ Microbiol 44:990–991

Stoffels M, Castellanos T, Hartmann A (2001) Design and application of new 16S rRNA-targeted oligonucleotide probes for the *Azospirillum–Skermanella–Rhodocista*-cluster. Syst Appl Microbiol 24:83–97

Umali-Garcia M, Hubbell DH, Gaskins MH, Dazzo FB (1980) Association of *Azospirillum* with grass roots. Appl Environ Microbiol 39:219–226

Westby CA, Cutshall DS, Vigil G (1983) Metabolism of various carbon sources by *Azospirillum brasilense*. J Bacteriol 156:1369–1372