Brazilian Journal of Microbiology 46, 3, 735-742 (2015)
ISSN 1678-4405
DOI: http://dx.doi.org/10.1590/S1517-838246320140541

Research Paper

Symbiotic potential and survival of native rhizobia kept on different carriers

Víctor Manuel Ruíz-Valdiviezo[1], Lucía María Cristina Ventura Canseco[1], Luis Antonio Castillo Suárez[1], Federico Antonio Gutiérrez-Miceli[1], Luc Dendooven[2], Reiner Rincón-Rosales[1]

[1]Laboratory of Biotechnology, Instituto Tecnológico de Tuxtla Gutiérrez, Chiapas, México.
[2]Laboratory of Soil Ecology, Abacus, Cinvestav, DF, México.

Submitted: October 23, 2013; Approved: August 15, 2014.

Abstract

Native rhizobia are ideal for use as commercial legume inoculants. The characteristics of the carrier used to store the inoculants are important for the survival and symbiotic potential of the rhizobia. The objective of this study was to investigate the effects of peat (PEAT), perlite sugarcane bagasse (PSB), carboxymethyl cellulose plus starch (CMCS), and yeast extract mannitol supplemented with mannitol (YEMM) on the survival, nodulation potential and N_2 fixation capacity of the native strains *Sinorhizobium mexicanum* ITTG $R7^T$ and *Rhizobium calliandrae* LBP2-1^T and of the reference strain *Rhizobium etli* CFN42^T. A factorial design (4 x 3) with four repetitions was used to determine the symbiotic potential of the rhizobial strains. The survival of the strains was higher for PEAT (46% for strain LBP2-1^T, 167% for strain CFN42^T and 219% for strain ITTG $R7^T$) than for the other carriers after 240 days, except for CFN42^T kept on CMCS (225%). All the strains kept on the different carriers effectively nodulated common bean, with the lowest number of nodules found (5 nodules) when CFN42^T was kept on CMCS and with the highest number of nodules found (28 nodules) when ITTG $R7^T$ was kept on PSB. The nitrogenase activity was the highest for ITTG $R7^T$ kept on PEAT (4911 μmol C_2H_4 per fresh weight nodule h^{-1}); however, no activity was found when the strains were kept on YEMM. Thus, the survival and symbiotic potential of the rhizobia depended on the carrier used to store them.

Key words: rhizobia, inoculants, nodulation, nitrogen fixation, carrier.

Introduction

The inoculation of the plant rhizosphere or seeds with rhizobia, *i.e.*, N_2-fixing bacteria, to stimulate plant growth has been used for a long time (Lopez-Lopez *et al.*, 2010). Symbiosis between the N_2-fixing bacteria and the plant reduces the need for inorganic N fertilizer applications. The use of native rhizobia has been recommended because these bacteria adapt easily to the specific environmental conditions, which facilitates their survival and the successful nodulation of the host plant (Romdhane *et al.*, 2008; Topre *et al.*, 2011).

The rhizobial inoculants are kept on a support or a carrier so that these bacteria can be stored for a long period and used when required (Albareda *et al.*, 2008). The material used as a carrier should allow the survival of the rhizobia and preserve their capacity to form nodules and to fix N_2. A high-grade carrier should have high water retention and stable pH, and it should be inexpensive, constitutive, nontoxic for the strain or environment and easy to sterilize (Swelim *et al.*, 2010). Peat is one of the most commonly used carriers (Albareda *et al.*, 2008), although other substrates have been used. For instance, sugarcane bagasse and perlite were tested as carriers for *Bradyrhizobium japonicum* strain CB1809 (Khavazi *et al.*, 2007). When the carrier was stored at 4 °C for six months, the bacterial inoculant survival was high, and a density of 10^9 cells g^{-1} was maintained. Mixtures of carboxymethyl cellulose and starch maintained a stable cellular concentration of *B. japonicum* strain BR3267 when stored at 20-26 °C for 180

Send correspondence to R. Rincón-Rosales. Laboratory of Biotechnology, Instituto Tecnológico de Tuxtla Gutiérrez, Chiapas, México. E-mail: reriro61@hotmail.com.

days (Fernandes-Júnior *et al.*, 2009). A stable rhizobia population of *Sinorhizobium fredii* SMH12 was also obtained when the cells where kept in yeast extract mannitol supplemented with 1% mannitol (YEMM) at 25 °C for 100 days (Albareda *et al.*, 2008).

While these carriers were able to maintain the rhizobia population, considering other factors, such as the stability and viability of the biofertilizer, which might vary between the strains used as biofertilizer, is necessary (Fernandes-Júnior *et al.*, 2012). Studying how the different carriers affect the biological activity of microorganisms, the infectivity of rhizobia inoculants and the symbiotic potential of the strains used as inoculants is also desirable (Hungria *et al.*, 2005). Therefore, the objective of the present study was to determine the effects of peat (PEAT) and perlite sugarcane bagasse (PSB) as solid carriers and of carboxymethyl cellulose plus starch (CMCS) and YEMM as liquid carriers on the survival, potential for nodulation of the common bean (*Phaseolus vulgaris* L.) and capacity for N_2 fixation of the native strains *Sinorhizobium mexicanum* ITTG $R7^T$ and *Rhizobium calliandrae* LBP2-1^T and of the reference strain *Rhizobium etli* $CFN42^T$.

Materials and Methods

Bacterial strains

The native rhizobia used in this study were *S. mexicanum* ITTG $R7^T$ (Lloret *et al.*, 2007) and *R. calliandrae* LBP2-1^T (Rincón-Rosales *et al.*, 2013) obtained from the *Instituto Tecnológico de Tuxtla Gutiérrez* (Chiapas, Mexico). These strains are characterized by rapid growth, high potentials for nitrogen fixation and activity under saline conditions and high aluminum concentrations. The reference strain *R. etli* $CFN42^T$ was provided by the *Centro de Ciencias Genómicas* (Cuernavaca, México). *R. etli* $CFN42^T$, which has been used commercially in Mexico as an inoculant, is characterized by a high nodulation potential (Martínez-Romero, 2003). The strains were grown in yeast extract mannitol (YEM) medium (Vincent, 1970) at 28 °C and then stored at 4 °C until use.

Preparation of the carriers

The carriers used in this study were 1) PSB and 2) PEAT as solid carriers and 3) CMCS and 4) YEMM as liquid carriers. *Sphagnum* peat (PROMIX 1100, Quebec, Canada) was used as a reference carrier (Albareda *et al.*, 2008). The carriers PSB and PEAT were first ground and then sieved (mesh size 100). The carrier PSB had a 4:1 (sugarcane bagasse:perlite) ratio (Khavazi *et al.*, 2007). The pH of the peat was adjusted to 6.7 using 35 g Na_2CO_3 for each 100 g material. The carriers PSB and PEAT were sterilized at 121 °C for 20 min.

The CMCS carrier was prepared by mixing carboxymethyl cellulose and starch (ratio 60:40 w/w) and amended with MgO at 1% to achieve a final concentration 1.28% w/v and a pH of 6.7 (Fernandes-Júnior *et al.*, 2009). The YEMM carrier was prepared with 1.0 g yeast extract, 0.1 g NaCl, 0.5 g K_2HPO_4, 0.2 g $MgSO_47H_2O$, and 20 g mannitol L^{-1} distilled water and adjusted to pH 6.7. Both carriers were sterilized at 121 °C for 15 min (Albareda *et al.*, 2008).

The physical characteristics of the carriers were determined each time a sample of the rhizobia was taken. The pH and water holding capacity (WHC) were determined for the solid carriers PEAT and PSB (Somasegaran and Hoben, 1985), and the pH and viscosity were determined for the CMCS and YEMM carriers (Fernandes-Júnior *et al.*, 2009).

Biofertilizer production

The biofertilizers combined the four carriers and the three strains ITTG $R7^T$, LBP2-1^T and $CFN42^T$. Erlenmeyer flasks (2 L) containing 800 mL YEM broth (Albareda *et al.*, 2008) were inoculated with 10% (v/v) of the corresponding microbial suspension and aired with 2 L min^{-1} sterile air for 27 h. All the carriers were inoculated with 1.5 mL inoculant g^{-1} under sterile conditions, *i.e.*, > 2 x 10^9 cells g^{-1}. Twenty-five grams solid and 25 mL liquid biofertilizer were added to sterilized polypropylene bags (20 cm x 30 cm) and stored at 25 °C until use.

Rhizobial survival determination and biofertilization test

The number of viable rhizobia was determined by serial dilution and plate counting on YEM agar (pH 6.7) after 14, 60, 120, 180 and 240 days (Vincent, 1970). Seeds of the common bean (*Phaseolus vulgaris* L.) cultivar Jamapa previously impregnated with 40% Arabic gum (Sigma-Aldrich, St. Louis, MO, USA) were inoculated with PSB or PEAT containing 8 g inoculant 100 g^{-1} seed. Biofertilizers formulated with CMCS or YEMM were directly applied to the seeds at 8 mL inoculant 100 g^{-1} seed. The treated seeds were sown in plastic pots containing sterilized vermiculite. Four replicate pots were used and arranged in a completely randomized design. The plants were grown in a climate chamber at 25 °C using photoperiods of 16 h light/8 h darkness (Rincón-Rosales *et al.*, 2009) and watered with a N-free nutritive Fahraeus solution (Fahraeus, 1957). The shoot dry weight, number of nodules and shoot N content were determined after 240 days. The N_2-fixing capacity was determined using the acetylene reduction assay. Ethylene was determined using a Varian-3300 (Pampa, TX, USA) gas chromatograph fitted with a flame ionization detector (FID) and a Porapak N column (300 x 0.1 cm) at 50-70 °C using nitrogen as carrier gas (50 mL min^{-1}) (Ruiz-Valdiviezo *et al.*, 2009).

Statistical analysis

Significant differences between the shoot dry weight, number of nodules, nitrogenase activity and shoot nitrogen

content of the legume *P. vulgaris* L. because of the application of the different rhizobia kept on different carriers were determined using analysis of variance (ANOVA) with Tukey's test. Relationships between physical characteristics of the carriers and strain viability were determined using the Pearson correlation coefficients. All analyses were performed using StatGraphics Centurion version XV.2 software (Warrenton, Virginia, USA).

Results and Discussion

Physical characteristics of the carriers

The pH of the peat used in this study was 3.8. This carrier normally has a pH ranging from 3.5 to 4.5 (Somasegaran and Hoben, 1985). The pH was adjusted to 6.7 with 35 g Na_2CO_3 so that a near neutral pH was obtained, which maintains the viability of the rhizobia (Vincent, 1970). The WHC of peat in this study was 282%, which is higher than the 120% reported by Somasegaran and Hoben (1985). The origin of the peat might have resulted in a higher WHC (Tittabutr *et al.*, 2007). The pH of the PSB was 7.7, which is appropriate for the growth of rhizobia (Albareda *et al.*, 2008). The WHC of the PSB was 512%, which is higher than the value reported for perlite (400%) (Khavazi *et al.*, 2007). The high WHC of both carriers favors the enzymatic processes involved in the degradation of the organic material that provide important nutrients such as phosphorus for the rhizobial bacteria.

The carrier CMCS, which is prepared by mixing carboxymethyl cellulose and starch, allowed the formation of a polymer with a viscosity of 414 cP. This viscosity favors the survival of the rhizobia. However, the initial pH was 10.8, which decreases the viability of the bacteria; thus, MgO was added. This addition adjusted the pH to 6.7, which is the pH recommended for rhizobia carriers (Fernandes-Júnior *et al.*, 2009). The YEMM liquid carrier (pH 6.7) had a viscosity of only 1.11 cP. This result suggested that this liquid carrier might decrease the survival of the rhizobia as reported by Tittabutr *et al.* (2007) and by Albareda *et al.* (2008).

Survival of rhizobia strains in different carriers

The survival of strains *S. mexicanum* ITTG $R7^T$, *R. calliandrae* LBP2-1^T and *R. etli* $CFN42^T$ was determined in two solid and two liquid carriers kept at 25 °C for 240 days (Figure 1). The survival of the rhizobia strains differed between the carriers. After 240 days, the survival of the rhizobia was higher in the solid carriers PEAT and PSB than in the liquid carriers (Figure 1a and 1b). In the PEAT and PSB carriers, the cellular concentration of all strains remained at 2 x 10^9 cells g^{-1}, which is recommended for the production of biofertilizers (Ben Rebah *et al.*, 2007). PSB and PEAT contain high concentrations of organic material and essential nutrients (Khavazi *et al.*, 2007), maintaining the viability of the rhizobia.

The cellular concentration of the *R. etli* $CFN42^T$ strain significantly decreased ($p < 0.05$) in the liquid carrier YEMM after 60 days (Figure 1c), while the cellular concentrations of the ITTG $R7^T$ and LBP2-1^T strains remained at > 10^8 cells g^{-1}. In the CMCS carrier, the strains maintained a viable population of > 10^8 bacteria g^{-1} until day 240 (Figure 1d), which is sufficient for use as biofertilizers (Fernandes-Júnior *et al.*, 2009). The high survival of the rhizobia in this carrier might be due to its rheological and chemical characteristics, *e.g.*, high viscosity and hygroscopicity. Flores da Silva *et al.* (2012) reported that survival of *Gluconacetobacter diazotrophicus* and *Azospirillum amazonense* was 10^9 cfu mL^{-1} in a medium of CMC-starch supplemented with MgO after 120 days.

Several researchers (*e.g.*, Deaker *et al.*, 2004; Fernandes-Júnior *et al.*, 2009) reported that different physical factors affect the viability and survival of rhizobia in the carrier. In the present study, the pH, WHC and viscosity were monitored to determine their possible effects on the survival and viability of the rhizobia studied (Table 1). After 240 days, the pH of the YEMM liquid carrier decreased from 6.7 to 4.9 when inoculated with the $CFN42^T$ strain; to 6.4, with the ITTG $R7^T$ strain; and to 4.8, with the LBP2-1^T strain. The viability of the $CFN42^T$ and ITTG $R7^T$ strains and their concentrations were negatively affected by a decrease in pH during storage, thus decreasing their symbiotic potentials.

Pearson correlation analysis showed that the pH and viscosity of the liquid carriers significantly affected the viability of strain LBP2-1^T and that only the WHC of the PSB affected viability ($p < 0.05$) (Table 2). The viability of ITTG $R7^T$ strain significantly correlated with the viscosity of the liquid carrier and with the pH of the PEAT carrier ($p < 0.05$). The viability of the $CFN42^T$ strain significantly correlated only with the pH of the YEMM carrier and with the viscosity of the CMCS carrier ($p < 0.05$).

Several strains of *Rhizobium* produce exopolysaccharides (EPSs), which facilitate symbiosis (Luque-Castellane *et al.*, 2014). In the present study, EPS production was higher for the $CFN42^T$ strain than for the native ITTG $R7^T$ and LBP2-1^T strains when kept in the YEMM carrier (data not shown). Rhizobial EPSs are composed of different types of monosaccharides and secreted into the environment. *Rhizobium leguminosarum* strains produce acidic EPSs that are composed of glucose, glucuronic acid and galactose, which may cause changes in the pH of the culture medium (Skorupska *et al.*, 2006). Our results indicated that the exopolysaccharides produced by the rhizobia strains used in the present study decreased the pH, thereby reducing the viability of the bacteria in the carrier.

When the CMCS support was inoculated with the $CFN42^T$, ITTG $R7^T$ or LBP2-1^T strain, the pH was maintained between 6.7 and 7.2 (Table 1), which is suitable for the growth of rhizobia cells. In the CMCS carrier, the viscosity decreased significantly from 190.5 cP to 3.1 cP for

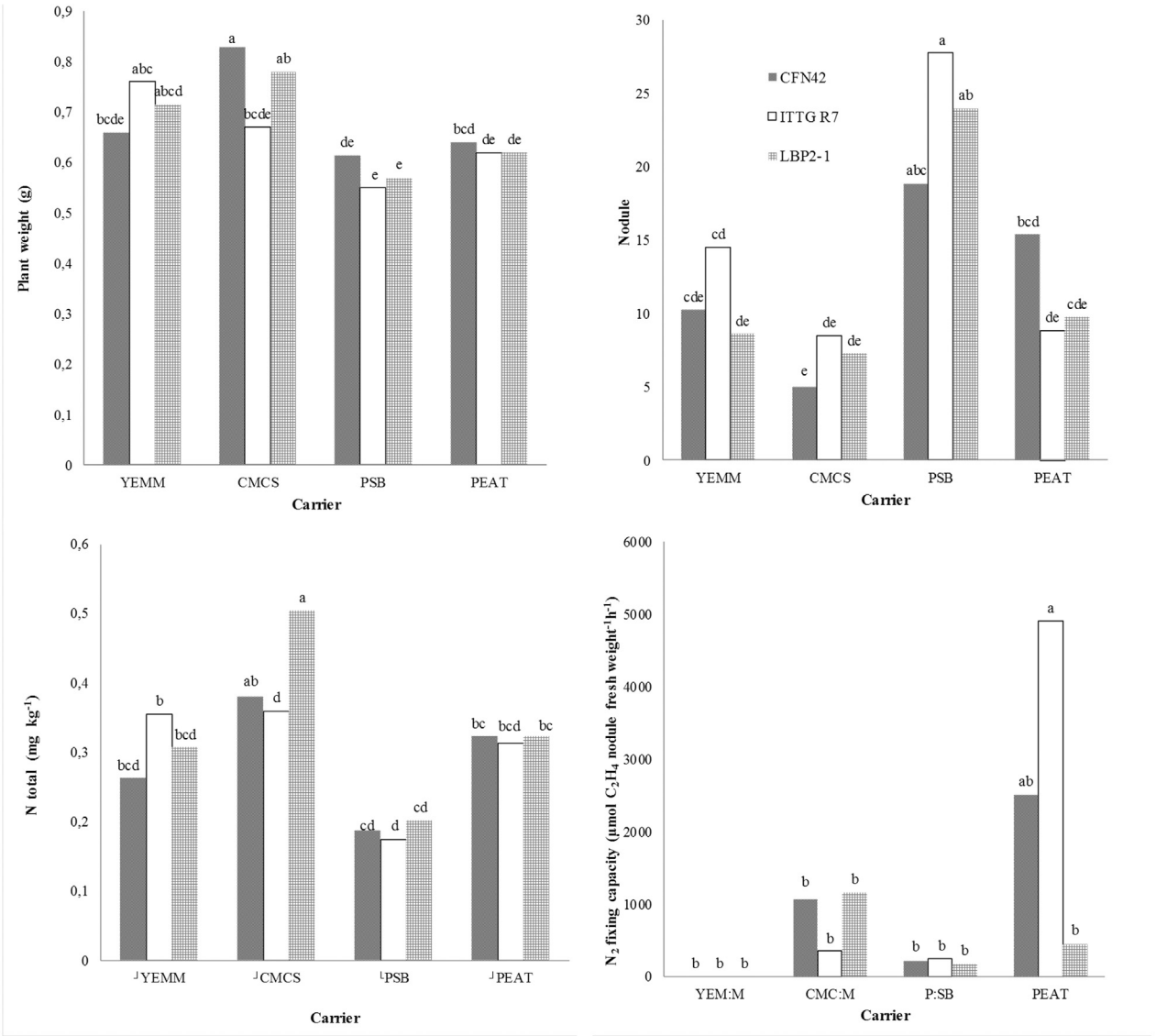

Figure 1 - Survival of the ITTG R7^T and LBP2-1^T strains and of the reference strain CFN42^T in the carriers a) PEAT, b) PSB, c) YEMM and d) CMCS stored at 25 °C for 240 days.

the CFN42^T strain, to 7.7 cP for ITTG R7^T and to 6.6 cP for LBP2-1^T ($p < 0.05$). These results indicated that the viscosity affected the viability of the ITTG R7 ^{T} and LBP2-1^T strains, which could be because the rhizobial cells used the carrier as a C source (Fernandes-Júnior *et al.*, 2009).

The WHC and pH are the factors that primarily affect the survival of rhizobia strains in carriers (Ben Rebah *et al.*, 2002). In the present study, the pH remained stable in the inoculated PSB carrier. However, the WHC decreased significantly from an initial value of 137% to 23% when the CFN42^T strain was added, to 58% when the ITTG R7^T strain was added and to 37% when the LBP2-1^T strain was added (Table 1). The high amount of water retained in the PSB carrier favored the enzymatic processes involved in the degradation of organic matter that generates nutrients required for bacterial growth (Ben Rebah *et al.*, 2007).

Peat is the most commonly used carrier to store rhizobia used as biofertilizer (Khavazi *et al.*, 2007; Kaira *et al.*, 2010). This carrier is excellent for storing microorganisms because peat has a high WHC, chemical and physical uniformity, a lack of toxic compounds and no environmental risk (Ferreira and Castro, 2005; Ferreira *et al.*, 2010). In the present study, the WHC values remained constant when peat was inoculated with the ITTG R7^T strain; the viability of this strain increased significantly compared with the other evaluated strains during 240 days of storage ($p < 0.05$) (Table 1).

Table 1 - Viscosity (cP), WHC and pH of the carrier and viability (%) of strains *R. etli* CFN42^T, *S. mexicanum* ITTG R7^T, and *R. calliandrae* LBP2-1^T kept on PEAT, PSB, CMCS, and YEMM for 240 days.

		Rhizobium etli CFN42^T			*Sinorhizobium mexicanum* ITTG R7^T			*Rhizobium calliandrae* LBP2-1^T		
Carrier	Days	pH	Viscosity	Viability	pH	Viscosity	Viability	pH	Viscosity	Viability
YEMM	0	6.7 A[a]	0.8 C	100 A	6.7 AB	0.8 A	100 A	6.6 A	0.8 A	100 A
	14	5.5 B	1.5 A	12 B	5.6 E	0.9 A	15 B	5.8 AB	1.1 A	36 B
	60	4.8 D	0.9 BC	5 C	6.2 D	1.1 A	15 B	5.7 AB	1.2 A	4 C
	120	4.8 D	0.8 C	0 D	6.5 BC	1.2 A	8 C	5.3 BC	1.2 A	3 C
	180	5.1 C	0.9 BC	0 D	6.7 A	1.5 A	1 D	6.1 A	1.6 A	1 C
	240	4.9 CD	1.0 B	0 D	6.4 CD	1.5 A	7 C	4.8 C	1.3 A	1 C
CMCS	0	6.7 C	190.5 A	100 C	6.8 B	190.5 A	100 A	6.7 E	190.5 A	100 A
	14	6.7 C	7.2 C	295 B	6.7 B	16.7 BC	30 B	7.4 C	24.3 BC	16 B
	60	6.7 C	38.7 B	269 B	7.4 A	33.2 B	27 B	7.7 B	34.6 B	20 B
	120	7.1 AB	9.0 C	219 B	7.6 A	30.5 B	6 C	7.9 A	30.6 BC	4 C
	180	7.4 A	4.9 C	507 A	6.6 B	7.7 C	2 C	7.7 AB	21.3 BC	5 C
	240	7.6 A	3.1 C	225 B	7.6 A	7.7 C	7 C	7.2 D	6.6 C	16 B
Carrier	Days	pH	WHC [b]	Viability	pH	WHC	Viability	pH	WHC	Viability
PSB	0	7.7 A	137 A	100 A	7.7 B	123 A	100 AB	7.8 A	134 A	100 A
	14	7.5 B	137 A	206 A	7.9 A	123 A	93 AB	7.7 AB	133 A	29 CD
	60	7.2 C	113 AB	106 A	7.3 C	150 A	122 A	7.0 D	110 AB	54 BC
	120	7.2 C	90 BC	153 A	7.3 C	103 A	114 AB	7.0 D	76 BC	67 B
	180	7.2 C	67 C	211 A	7.3 C	156 A	71 B	7.4 BC	60 C	37 CD
	240	7.2 C	23 D	139 A	7.3 C	58 A	21 C	7.3 CD	37 C	13 D
PEAT	0	6.7 B	120 A	100 C	6.7 D	117 A	100 C	6.7 BC	134 A	100 A
	14	6.8 B	120 A	413 AB	6.9 CD	117 A	116 C	7.0 B	134 A	10 C
	60	6.9 AB	140 A	492 A	7.2 AB	127 A	204 AB	6.7 C	102 A	37 BC
	120	6.9 AB	161 A	553 A	7.3 A	107 A	140 BC	7.2 A	113 A	47 B
	180	7.1 A	122 A	203 BC	7.1 AB	171 A	163 ABC	7.2 A	93 A	110 A
	240	7.1 A	102 A	167 C	7.1 BC	109 A	219 A	7.3 A	105 A	46 B

[a]Values with the same capital letter do not significantly differ over time, *i.e.*, within the column ($p < 0.05$), [b] WHC: Water holding capacity (%).

Table 2 - Pearson's correlation coefficient and p value between viability and viscosity, WHC or pH for the strains *R. etli* CFN42^T, *S. mexicanum* ITTG R7^T, and *R. calliandrae* LBP2-1^T kept on PEAT, PSB, CMCS, and YEMM for 240 days.

		Viability					
		R. etli CFN42^T		*S. mexicanum* ITTG R7^T		*R. calliandrae* LBP2-1^T	
Carrier		R value	p value	R value	p value	R value	p value
YEMM	pH	0.969	< 0.001	0.324	0.189	0.737	< 0.001
	Viscosity	-0.280	0.261	-0.560	0.019	-0.498	0.036
CMCS	pH	0.335	0.175	-0.198	0.432	-0.837	< 0.001
	Viscosity	-0.609	0.007	0.942	< 0.001	0.966	< 0.001
PSB	pH	-0.323	0.191	0.053	0.835	0.361	0.141
	WHC	-0.084	0.740	0.254	0.310	0.495	0.037
PEAT	pH	-0.140	0.579	0.626	0.006	0.159	0.526
	WHC	0.253	0.310	-0.097	0.702	-0.128	0.613

Plant growth, nodulation and nitrogen fixation of *Phaseolus vulgaris* inoculated with different biofertilizers

Plants treated with strain CFN42^T stored in the CMCS carrier had the highest dry weight (0.83 g) (Figure 2). The CMCS carrier is a polymer blend, which favors the survival of the bacteria and the nodulation of different legumes, while the starch served as a C substrate (Fernandes-Júnior, 2009). Aeron *et al.* (2012) reported that *Mucuna pruriens* plants inoculated with *Ensifer meliloti* RMP6 and *Bradyrhizobium* sp. BMP7^T strains kept in a CMC carrier significantly increased plant biomass, the number of nodules and other plant growth parameters. The *R. etli* strain CFN42^T, in combination with other diazotrophic bacteria, has a positive effect on the development of various legume and non-legume plants (Rosenblueth and Martinez-Romero, 2004).

All the strains kept on the different carriers effectively nodulated bean plants; however, the number of nodules formed was highest when inoculated with strain ITTG R7^T kept on the PSB carrier ($p < 0.05$). PSB is a carrier that provides stability to the bacterial cells and essential nutrients for symbiotic N_2 fixation while maintaining cell viability during infection and nodule formation on the host plant (Khavazi *et al.*, 2007). The N fixed by inoculated plants was significantly different between the treatments ($p < 0.05$). Plants inoculated with LBP2-1^T kept on CMCS fixed the largest amount of N (0.50 mg kg^{-1}). Rincón-Rosales *et al.* (2013) reported similar findings and found that the *R. calliandrae* strain LBP2-1^T isolated from the tropical legume *Calliandra grandiflora* grown in acidic

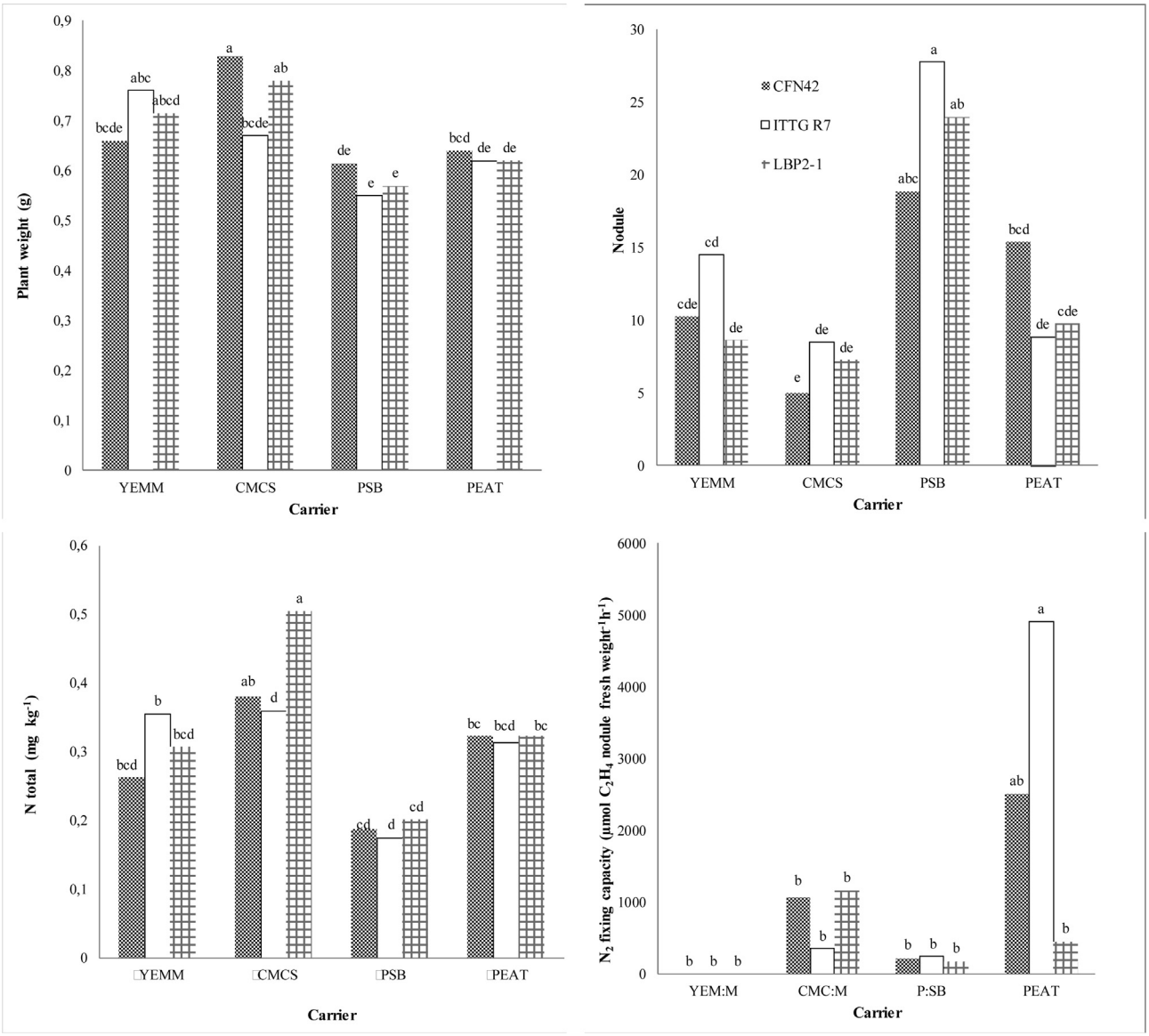

Figure 2 - Plant growth, nodulation and nitrogen fixation of *Phaseolus vulgaris* cv. Jamapa inoculated with different rhizobial strains.

soil and with high amounts of aluminum had a high potential for nodulation and nitrogen fixation.

The nitrogenase activity was higher in bean plants inoculated with the ITTG $R7^T$ strain kept on peat. As mentioned before, peat is a commonly used carrier due to its favorable characteristics (Khavazi *et al.*, 2007; Ferreira *et al.*, 2010). Lloret *et al.* (2007) reported that the strain ITTG $R7^T$ has high nitrogenase activity and nodulation capacity when used to inoculate *P. vulgaris* and other tropical legumes.

Conclusions

Peat and perlite sugarcane bagasse were shown to be the most appropriate carriers to store the rhizobial strains (2×10^9 cells g^{-1}) and to maintain their survival. Strains *Sinorhizobium mexicanum* ITTG $R7^T$, *R. calliandrae* LBP2-1^T and *R. etli* CFN42^T kept on perlite sugarcane bagasse induced the largest number of nodules in the common bean; however, their N_2 fixation capacity was lower than when the strains were kept on peat. Consequently, no direct relationship existed between nodule formation in the host plant and N_2 fixation capacity.

Acknowledgments

We thank M.A. Rogel-Hernández for technical assistance. This research was funded by Consejo Nacional de Ciencia y Tecnología (CONACyT, Mexico) and by Dirección General de Educación Superior Tecnológica (DGEST-SEP, Mexico). L.A.C.S. received grant-aided support from CONACyT.

References

Albareda M, Rodríguez-Navarro DN, Camacho M *et al.* (2008) Alternatives to peat as a carrier for rhizobia inoculants: Solid and liquid formulations. Soil Biol Biochem 40:2771-2779.

Aeron A, Khare E, Arora NK *et al.* (2012) Practical use of CMC-amended rhizobial inoculant for *Mucuna pruriens* cultivation to enhance the growth and protection against *Macrophomina phaseolina.* J Gen Appl Microbiol 58:121-127.

Ben Rebah F, Tyagi RD, Prevost D (2002) Wastewater sludge as a substrate for growth and carrier for rhizobia: the effect of storage conditions on survival of *Sinorhizobium meliloti*. Bioresour Technol 83:145-151.

Ben Rebah F, Prevost D, Yezza A *et al.* (2007) Agro-industrial waste materials and wastewater sludge for rhizobial inoculant production: a review. Bioresour Technol 98:3535-3546.

Deaker R, Roughley RJ, Kennedy IR (2004) Legume seed inoculation technology a review. Soil Biol Biochem 36:1275-1288.

Fahraeus G (1957) The infection of clover root hair by nodule bacteria studied by a single glass slide technique. J Gen Microbiol 16:374-381.

Fernandes-Júnior PI, Gusmão RT, Jansen de Oliveira P *et al.* (2009) Polymers as carriers for rhizobial inoculant formulations. Pesqui Agropecu Bras 44:1184-1190.

Fernandes-Júnior PI, da Silva Júnior EB, da Silva Júnior S *et al.* (2012) Performance of polymer compositions as carrier to cowpea rhizobial inoculant formulations: Survival of rhizobia in pre-inoculated seeds and field efficiency. Afr J Biotechnol 11:2945-2951.

Ferreira EM, Castro IV (2005) Residues of the cork industry as carriers for the production of legumes inoculants. Silva Lusitana 13:159-167.

Ferreira JS, Baldani JI, Baldani VLD (2010) Selection of peats inoculants with diazotrophic bacteria in two rice varieties. Acta Scientiarum Agronomy 32:179-185.

Flores da Silva M, de Souza AC, Jansen de Oliveira P *et al.* (2012) Survival of endophytic bacteria in polymer-based inoculants and efficiency of their application to sugarcane. Plant Soil 356:231-243.

Hungria M, Loureiro MF, Mendes JC *et al.* (2005) Inoculant preparation, production and application, *In*: Werner D, Newton WE (eds). Nitrogen Fixation in Agriculture, Forestry, Ecology and the Environment, Springer, Netherlands, 223-253.

Kaira A, Chandra M, Awasthi A *et al.* (2010) Natural compounds enhancing growth and survival of rhizobial inoculants in vermicompost-based formulations. Biol Fertil Soils 46:521-524.

Khavazi K, Rejali F, Seguin P *et al.* (2007) Effects of carrier, sterilization method, and incubation on survival of *Bradyrhizobium japonicum* in soybean (*Glycine max* L.) inoculants. Enzyme Microb Tech 41:780-784.

Lloret L, Ormeño-Orrillo E, Rincón R *et al.* (2007) *Ensifer mexicanum* sp. nov. a new species nodulating *Acacia angustissima* (Mill.) Kuntze in Mexico. Syst Appl Microbiol 30:280-290.

Lopez-Lopez A, Rogel MA, Ormeno-Orrillo E *et al.* (2010) *Phaseolus vulgaris* seed-borne endophytic community with novel bacterial species such as *Rhizobium endophyticum* sp. nov. Syst Appl Microbiol 33:322-327.

Luque-Castellane TC, Franco-Lemos MV, de Macedo-Lemos EG (2014). Evaluation of the biotechnological potential of *Rhizobium tropici* strains for exopolysaccharide production. Carbohydr Polym 111:191-197.

Martínez-Romero E (2003) Diversity of *Rhizobium-Phaseolus vulgaris* symbiosis: overview and perspectives. Plant Soil 252:11-23.

Rincón-Rosales R, Villalobos-Escobedo JM, Rogel MA *et al.* (2013) *Rhizobium calliandrae* sp. nov., *Rhizobium mayense* sp. nov. and *Rhizobium jaguaris* sp. nov., rhizobial species nodulating the medicinal legume *Calliandra grandiflora*. Int J Syst Evol Microbiol 63:3423-3429.

Rincón-Rosales R, Lloret L, Ponce RE *et al.* (2009) Rhizobia with different symbiotic efficiencies nodulate *Acaciella angustissima* in Mexico including *Sinorhizobium chiapanecum* sp. nov. that has common symbiotic genes with *S. mexicanum*. FEMS Microbiol Ecol 64:1-14.

Romdhane SB, Aovani ME, Trabelsi M *et al.* (2008) Selection of high nitrogen-fixing rhizobia nodulating chickpea (*Cicer arietinum*) for semi-arid Tunisia. J Agron Crop Sci 194:413-420.

Rosenblueth M, Martínez-Romero E (2004) *Rhizobium etli* maize populations and their competitiveness for root colonization. Arch Microbiol 181:337-344.

Ruiz-Valdiviezo VM, Ayora-Talavera TR, Gutiérrez-Miceli FA *et al.* (2009) Effects of inorganic fertilizers and rhizobial in-

oculation on growth, nodulation and tannin content of *Acaciella angustisima* (Mill.) Britton & Rose. Gayana Bot 66:206-217.

Skorupska A, Janczarek M, Marczak M *et al.* (2006) Rhizobial exopolysaccharides: genetic control and symbiotic functions. Microb Cell Fact 5:1-19

Somasegaran P, Hoben HJ (1985) Methods in legume-rhizobium technology. United States Agency for International Development, USA.

Swelim DM, Nassef MA, Elkhatib EI (2010) Survival and shelf life of leguminous trees rhizobia as affected by sterilization, culture dilution and maltose and trace elements enriched carrier. J Appl Sci Res 6:1366-1372.

Tittabutr P, Payakapong W, Teaumroong N *et al.* (2007) Growth, Survival and field performance of bradyrhizobial liquid inoculant formulations with polymeric additives. Sci Asia 33:69-77.

Topre SD, Panikar SS, Mahajani SU *et al.* (2011) Biofertilizer: A novel approach for agriculture. J Agric Biotech Sustain Dev 3:205-208.

Vincent JM (1970) A manual for the practical study of root nodule bacteria. Oxford: Blackwell Scientific Publications.

Associate Editor: Valeria Maia de Oliveira