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Full Length Research Paper

Performance of polymer compositions as carrier to cowpea rhizobial inoculant formulations: Survival of rhizobia in pre-inoculated seeds and field efficiency

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Rhizobial-legume association is a symbiosis that is exploited in agriculture by inoculant production. Efficient inoculant formulations are needed to increase the biological nitrogen fixation rates in agroecosystems. The aim of this work was to evaluate inoculant formulations to cowpea (*Vigna unguiculata* L. Walp) containing polymers as carrier materials and rhizobial strains. To assess the efficiency of the new inoculants formulations, the rhizobial survival in inoculated and stored seeds and the field performance of a selected formulation were evaluated. The rhizobial survival was higher in cowpea seeds inoculated with MgO compatibilized polymeric formulations, compared to standard peat-based inoculant and compatibilized polymeric formulations compatibilized with ZnO. Seeds inoculated with IPC 2.2 formulation presented more than 10^6 cells per seed through 35 days of storage with low contamination levels. The field performance of the polymeric compositions was the same as that observed in the peat-based inoculant treatments. Among the three rhizobial strains isolated, two of them were able to provide the same shoot nitrogen content and productivity in comparison to the recommended strain. These results indicate the feasibility of inoculants with the polymer blends as carrier materials and the efficiency of the new fast growing rhizobia from Brazilian semi-arid.

Key words: Inoculant technology, strain selection, biological nitrogen fixation.

INTRODUCTION

The biological nitrogen fixation (BNF) is a natural process carried out exclusively by bacteria known as diazotrophs. Several of them can establish associations with plants and provide high amounts of fixed nitrogen (N) to the host. The association between the diazotrophic-group rhizobia and plants belonging to legume (Fabaceae) family is the most studied and well characterized symbiosis (Xavier et al., 2010). The rhizobia-legume association is exploited in agriculture and forestry by

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Abbreviations: BNF, Biological nitrogen fixation; **CFU,** colony forming units.

rhizobial inoculants production. The rhizobial inoculant is a cheap and non polluting source of bacteria that can provide high rates of nitrogen to the legume, and the use of improved inoculants is a strategy to increase the BNF rates in agroecosystems (Deaker et al., 2004; Xavier et al., 2010; Araújo et al., 2011).

The inoculation of Brazilian soybean is the most successful example of the rhizobial inoculants application. More than 30 million hectares in Brazil are cropped with soybean without any nitrogen fertilizer application and all nitrogen required by the crop is provided by BNF (Hungria and Campo, 2007). To other legume species, for example, cowpea (*Vigna unguiculata* L. Walp), the selection of strains and the development of new inoculant formulations had been carried out at the last few years and the available results already show the potential of this crop to associate with efficient rhizobia mainly in Brazilian dry lands (Martins et al., 2003; Lacerda et al., 2004; Zilli et al., 2009a). In addition, the increased BNF rate in Brazilian agroecosystems is a government requirement to reduce the emission of greenhouse gases, according to the Brazilian Politics to Climate Change (Brasil, 2009).

Cowpea presents several uses and it has being applied mainly to grain production and green manure (Singh et al., 2003; Freire Filho et al., 2005; Guedes et al., 2010). It is produced in different regions of Brazil, highlighting the north, northeast and, more recently, the middle-west region (Freire Filho et al., 2005; Zilli et al., 2009b). At the semi-arid region in Brazilian northeast, cowpea is a crop very important economically and socially. In spite of its importance, the productivity at the region is lower than other Brazilian regions (Freire Filho et al., 2005). The application of adequate inoculants containing efficient strains and carriers adapted to the edaphoclimatic conditions of the region is an important tool to increase the cowpea productivity in Brazilian semi-arid and to other semi-arid regions around the world (Araújo et al., 2011).

To ensure the efficiency of the rhizobial inoculant at the field conditions, the strain selection must be carried out concomitantly with the evaluation of a feasible carrier material. Peat is the most common material used as carrier to cowpea inoculants in Brazil (Fernandes Júnior et al., 2009). However, peat is not the most suitable material to produce rhizobial inoculants and studies have been carried out focusing alternative carriers. Among the alternative carriers evaluated, polymeric materials stands out because of its low costs, microbial compatibility and easy field application (Denardin and Freire, 2002; Fernandes Júnior et al., 2009; Silva et al., 2009). The carboxymethylcellulose (CMC) / starch blend is a polymer composition that has shown the ability to sustain a high rhizobial concentration up to six months of storage, and the inoculant produced with these carriers shows the same performance to peat inoculants in greenhouse conditions (Fernandes Júnior et al., 2009). These blends are also feasible to produce inoculants containing associative diazotrophic bacteria to important grasses such as sugarcane (Silva et al., 2009). These results allowed the patent registration of the inoculant produced with theses polymeric blends in Brazil (PI0506338-8), United States (US2007/0068072) and Argentina (AR051081). In this context, the aim of this study was to evaluate selected CMC/starch polymeric blends as carriers to cowpea inoculants and to evaluate the field performance of recently isolated rhizobial strains.

MATERIALS AND METHODS

In the first experiment, the survival of the *Bradyrhizobium* sp. strain BR 3267 (Martins et al., 2003) inoculated in cowpea seeds cv. "Mauá", with different inoculant formulations was evaluated through a period of 35 days. The polymeric inoculants were prepared using the polymer blends described in Table 1. The performance of peat-based inoculants was also evaluated. The rhizobial strain BR 3267 (*Bradyrhizobium* sp.) was obtained from the culture collection of diazotrophic bacteria of Embrapa Agrobiologia (Seropédica-RJ, Brazil). The bacteria was grown in 250 ml flasks containing 100 ml of yeast mannitol (YM) liquid media in an orbital shaker with constant stirring of 150 rpm at 28°C (Vincent, 1970), for five days. The culture broth was centrifuged (8000 *g* for 10 min) and the supernatant discarded. The pellet was re-suspended with distilled sterile water and the inoculant was prepared with the addition of the cell suspension at the carrier (1:3 v/v) and homogenized manually (Fernandes Júnior et al., 2009).

The inoculation was carried out following the recommendations of the Brazilian Ministry of Agriculture, where 10 g (or ml) of inoculant where used to inoculate 1 kg of seeds. The inoculated seeds where transferred to Petri dishes and stored at 10°C. To obtain the bacteria adhered to seed surface, the procedure described by Temprano et al. (2002) was adapted. Briefly, ten seeds where used for each count and the seeds were transferred to flasks containing 100 ml of NaCl solution which were stirred in orbital shaker for 60 min. To quantify the rhizobial cells in the solution, a cell suspension aliquot (1 ml) was diluted serially and the dilutions from 10^{-2} to 10^{-8} were plated in yeast mannitol agar (YMA) medium (Vincent, 1970) by the drop plate method (Miles and Misra, 1938). The experiment was carried out with a completely randomized design with three independent replications. The data was evaluated by linear regression analysis, observing the significance of the equations terms and the simple correlation coefficient (Steel and Torrie, 1980).

For the second experiment, the polymer blend IPC 2.2 (described at the Table 1) was selected as carrier material. In this experiment, the seeds were not superficially disinfected as described previously. The inoculant preparation and the inoculation procedure were done as aforementioned. In addition to the rhizobial concentration at the seed surface, the presence of non-rhizobial microorganisms at the media potato dextrose agar (PDA) and agar nutrient was also evaluated according to the determinations of Brazilian laws (Brasil, 2011). The data were evaluated with an ANOVA using the software SISVAR (Ferreira, 2008), applying the Scott-Knott mean test ($p<0.05$).

To evaluate the field performance of rhizobial inoculants, a field trial was implemented at the semi-arid region of Pernambuco State, at Pesqueira municipality (S 8°21'28" and W 36°41'45"), in a Haplic Vertisol. The experiment was carried out between April to October, 2009. The precipitation accumulated during the experiment conduction was around 450 mm. The soil presented the following characteristics: pH (H_2O): 6.3; Ca^{2+}: 0.104; Al^{3+}: not detected; Mg^{2+}: 0.034; Na^+: 0.145; K^+: 0.246 ($cmol_c.dm^{-3}$); P 28.625 $mg. dm^{-3}$,

Table 1. Bacterial strains and polymer blends used in this study.

Bacterial strain	Bacterial strain characterization				
	Growth time (days)	**pH reaction**	**Mucus production**	**Colony size (mm)**	**Reference**
BR 3267	5	alkaline	medium	3 to 4	Martins et al., (2003)
A2B3 (IIR3)	1	neutral	low	2 to 3	This study
S2V4 (R2II)	1	neutral	high	< 1	
S2V4 (R2III)	2	neutral	high	< 1	

Polymer blends characterization

Polymer blend	CMC* (g L^{-1})	Starch** (g L^{-1})	Compatibilizing agent	
IP 2.0	1.28	1.28	None	Fernandes Júnior et al. (2009)
IPD 2.0	1.28	1.28	ZnO* 1% w/w	
IPC 2.0	1.28	1.28	MgO* 1% w/w	
IP 2.2	1.54	1.02	None	
IPD 2.2	1.54	1.02	ZnO 1% w/w	
IPC 2.2	1.54	1.02	MgO 1% w/w	

*Vetec Química Fina, Duque de Caxias, RJ, Brazil; ** Quimesp LTDA, Guarulhos, SP, Brazil

assessed according to Claessen et al. (1997). A basal dose of phosphorus and potassium, corresponding to 20 kg of P_2O_5 and 20 kg of K_2O ha^{-1} was uniformly broadcasted before planting, according to the technical recommendation for the region.

The inoculants were prepared as follows, using the polymeric composition IPC 2.2 and peat as carrier material. For inoculation, 5 kg of cowpea seeds (cv. IPA 206) where transferred to plastic bags and 50 ml of each polymeric inoculant were added and handy homogenized. To the peat-based formulation inoculant, 30 mL of sucrose solution (10% w/v) was also added as sticking agent. The assay was conducted in randomized blocks with six replications, performed in a factorial design 2 (carriers) x 4 (rhizobial strains) with two additional treatments (control without N fertilization and N fertilized control with 80 $kg.ha^{-1}$ of urea during sowing). Each plot was composed of 5 rows of 6 m, spaced by 0.5 m. At the harvest, 1 m at each row edge was discarded in addition to the both border rows, resulting in a useful area of 15 m^2.

Two harvests where carried out. At 35 days after sowing (DAS), during the beginning of the flowering stage, the shoot and nodule dry mass and the shoot nitrogen content were evaluated through the semi-micro Kjeldahl method (Liao, 1981). At the end of the genotype cycle (70 DAS), the second harvest was performed to evaluate pods dry weight and grain yield; parameters related to grain production. To analyze the data, eight inoculated independent treatments (four bacteria x two carriers) and two controls, totaling ten treatments, were considered ignoring the factorial design aimed to include the controls at the F test and the means comparison test (Zilli et al., 2010a). To evaluate the performance of the rhizobial strains and the carriers, another statistical analysis was carried out ignoring the control treatments and evaluating the effect of the two factors (carrier and bacterial strain). The variance analysis and means test (Scott-Knott; p<0.05) were assessed by the software SISVAR (Ferreira, 2008).

RESULTS

Rhizobial survival on pre-inoculated cowpea seeds

The seeds inoculated with the six evaluated polymer blends showed high rhizobial survival, principally throughout the first three weeks of storage (Figure 1). Cowpea seeds inoculated with the inoculants produced with the polymeric carriers non-compatibilized or compatibilized with MgO, showed higher cell survival in comparison to seeds inoculated with the ZnO compatibilized compositions or the peat inoculants. The significance of all terms of the linear equations and the correlation index (R) enabled us to evaluate comparatively, the behavior of all treatments during the experiment. The lower (more negative) linear equation angular coefficients observed at the regression analysis on treatments inoculated with the peat-based inoculants and the polymeric inoculants compatibilized with ZnO, showed that the seeds pre-inoculated with these formulations present a faster decrease of the rhizobial cell number on their surface. On the other hand, inoculation with MgO compatibilized compositions presents a higher maintainability of rhizobial cells on stored seeds, highlighting the inoculant which the carrier material was the IPC 2.2 polymer composition (Figure 1). This blend is also the only one able to maintain the cell concentration above the 10^6 cells per seed, as determined by the Brazilian inoculant legislation (Brasil, 2011).

The second experiment was performed to evaluate the cell survival in conditions different from those evaluated in the first experiment. The inoculated seeds were stored at room temperature and the presence of non rhizobial microorganisms and the seed germination was also evaluated. The results showed that cell count on cowpea seeds where higher than 10^7 cells per seed (Table 2). The contamination analysis showed the absence of non rhizobial colonies and fungal contamination was observed only in one sample in a concentration above that allowed by Brazilian inoculant legislation (Brasil, 2011). Also, it

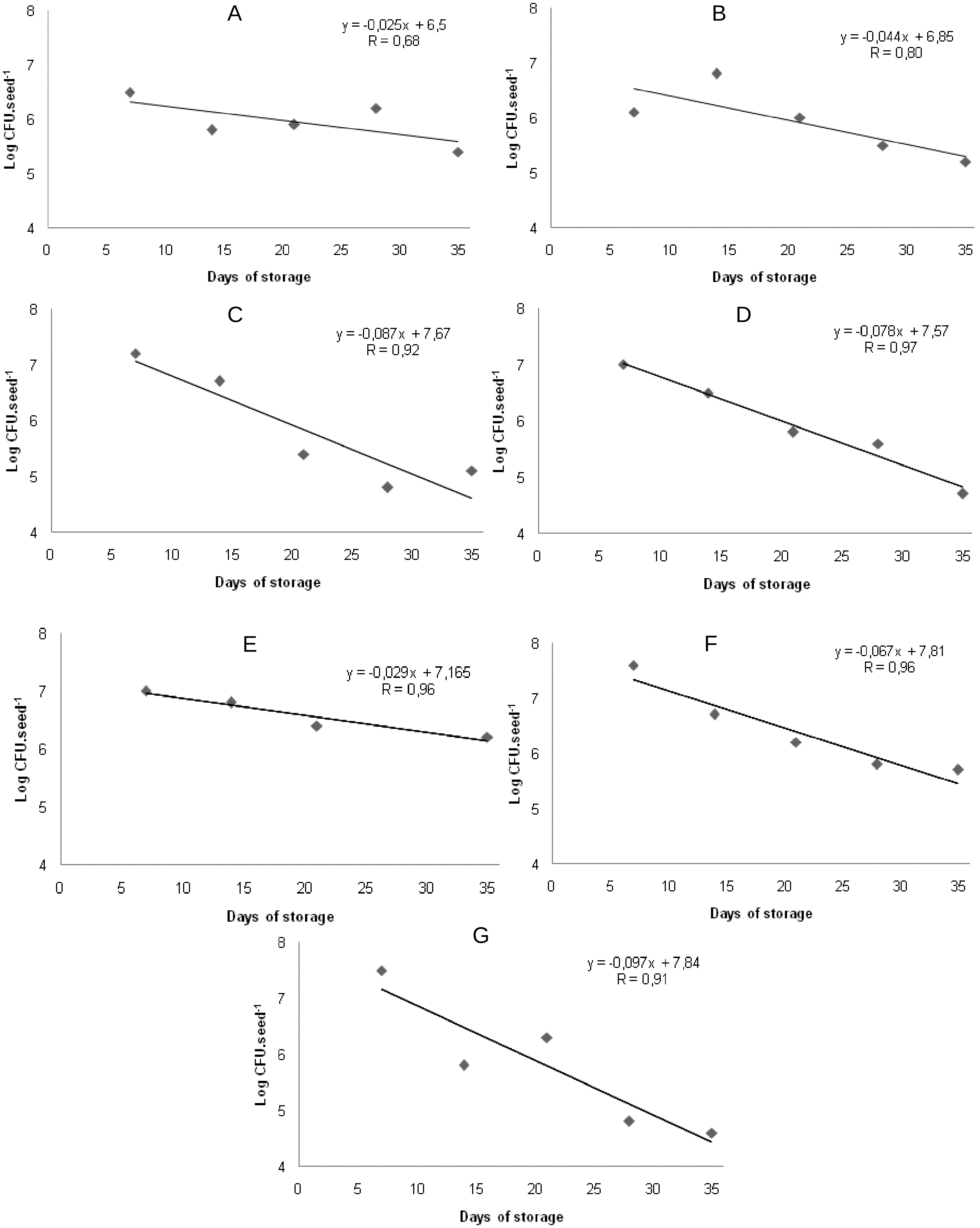

Figure 1. Survival of rhizobial strain BR 3267 in pre-inoculated cowpea seeds using inoculant formulations with different carriers: IP 2.0 (A); IP 2.2 (B); IPC 2.0 (C); IPD 2.0 (D); IPC 2.2 (E); IPD 2.2 (F) and peat (G).

Table 2. Survival of rhizobial cell inoculated on cowpea seeds with polymer based inoculants containing the polymer composition IPC 2.2 and BR 3267 strain.

Parameter	Days of storage				
	7	14	21	25	35
Log cfu.seed^{-1}	7.15^{a}	7.19^{a}	7.33^{a}	7.40^{a}	7.34^{a}
Bacterial contamination	None	None	None	None	None
Fungal contamination	None	None	10^{-4}*	None	None
Seed germination (%)	100^{a}	100^{a}	98^{a}	100^{a}	98^{a}

was possible to observe that the inoculation and storage did not affect the cowpea germination (Table 2).

Field performance of inoculant formulations

The field trial showed no differences for the inoculation with the recommended strain (BR 3267), presenting the same nodulation applying either the polymer based inoculant and the peat based inoculant (Table 3). The nodulation of the strains BR 3267 and A2B3 (IIR3) inoculated with the polymeric inoculant was higher than the other treatments (Table 3). Also, the A2B3 (IIR3) strain showed higher nodulation at the treatments inoculated with the polymer blend IPC 2.2 as carrier, while plants inoculated with the strains S2V4 (RIII) and S2V4 (RII) presented higher nodulation when the peat-based inoculant was applied (Table 3). The treatments inoculated with the polymer based inoculants showed higher shoot dry weight for strains BR 3267 and S2V4 (R2II), while the treatments inoculated with the strains S2V4 (RIII) and A2B3 (IIR3) showed no difference for the shoot dry weight comparing both carriers evaluated. Only the absolute control and the S4V4 (RII) with peat inoculant treatments showed lower shoot nitrogen content than the other inoculated treatments and the nitrogen-fertilized control.

Evaluating the production results, it is possible to verify that the strains S2V4 (R2III) and S2V4 (R2II) showed the same pod weight per plant comparing both inoculant formulations. The grain yield showed no differences among the majority of inoculated treatments and N fertilized control. Only the treatments inoculated with the polymeric inoculant with the recommended BR 3267 strain, and the peat-based inoculant containing the strain A2B3 (IIR3) showed productivity lower than the other inoculated treatments. However, both treatments also presented higher production than the absolute control (Table 3).

DISCUSSION

The survival of the strain BR 3267 in CMC/Starch compositions compatibilized with MgO was already observed in previous studies (Fernandes Júnior et al., 2009). This polymer blend is also able to maintain high concentrations of bacterial cells of the associative diazotrophic bacteria *Herbaspirillum* sp., *Azospirillum* sp., *Burkholderia* sp. and *Gluconacetobacter* sp. (Silva et al., 2009). The efficiency evaluation of the pre-inoculation of legume seeds using different inoculants formulations showed that some formulations are able to maintain a great amount of rhizobial cells in the seeds, principally when natural polymers are used as sticking agent (Temprano et al., 2002). Polymers must act as protectors when cells are under the reduction of the water activity (Deaker et al., 2007), as observed during the storage of pre-inoculated seeds. Zilli et al. (2010b) recently showed the feasibility of the pre-inoculation technology to soybean using the peat-based inoculants. Previous results had also indicated the reduction of the cell concentration in pre-inoculated seeds generally during the storage, as what can compromise the field performance of the technology (Rice et al., 2001; Temprano et al., 2002). The reduction of the cell concentration in pre-inoculated seeds using the peat-based formulations as inoculant was also observed in this study.

Previous research evaluating the effectiveness of the CMC/starch polymer blends as carrier to cowpea inoculants was already carried out and among several blends, the blend IPC 2.2 compatibilized with MgO showed the better performance (Fernandes Júnior et al., 2009). The polymeric inoculant evaluated in this study showed similar performance than the standard (peat based) inoculants, resulting in similar pods and grain productivity (Table 3). Other studies evaluating the field performance of polymeric inoculants also showed similarity among the standard and alternative formulations (Jung and Mungnier, 1982; Kostov and Lynch, 1998; Silva et al., 2009), indicating that the polymers and the polymer blends can represent a feasible alternative to inoculant production. In addition to its performance, as showed in this study, the polymeric inoculants have low environmental impact to production, low variability on its characteristics and low costs, compared to the peat-based inoculants (Deaker et al., 2007; Fernandes Júnior et al., 2009).

The field performance of the rhizobial strains evaluated in this study also showed that the strains recently isolated also stand out, mainly the strains A2B3 (IIR3) and S2V4(R2III), which in spite of the lower nodulation in comparison with the strain BR 3267, presented the same

Table 3. Nodule, shoot and pod dry weight, grain yield and shoot nitrogen content of cowpea (cv. IPA 206) inoculated with three rhizobial strains using two different carriers materials at Brazilian semi-arid region (Pesqueira, Pernambuco State).

Nitrogen source	Carrier material	Nodule dry weight (g per plant)	Shoot dry weight (g per plant)	Pod dry weight (g per plant)	Grain yield (kg per ha)	Shoot nitrogen content (mg per plant)
BR3267	IPC 2.2	0.33^{a}*	5.69^{a}	21.65^{b}	993^{b}	17.19^{a}
	Peat	0.35^{a}	4.48^{b}	32.75^{a}	1314^{a}	17.51^{a}
A2B3 (IIR3)	IPC 2.2	0.33^{a}	4.89^{a}	17.48^{b}	1456^{a}	17.47^{a}
	Peat	0.26^{b}	5.06^{a}	20.87^{b}	978^{b}	16.99^{a}
S2V4 (R2II)	IPC 2.2	0.16^{c}	4.46^{b}	39.63^{a}	1547^{a}	13.85^{a}
	Peat	0.31^{b}	2.44^{c}	25.57^{a}	1433^{a}	8.85^{b}
S2V4 (R2III)	IPC 2.2	0.21^{c}	4.70^{b}	32.92^{a}	1770^{a}	17.95^{a}
	Peat	0.25^{b}	4.21^{b}	31.02^{a}	1615^{a}	15.95^{a}
Absolute control		0.19^{c}	2.61^{c}	13.23^{c}	541^{c}	10.23^{b}
N fertilized control		0.09^{d}	2.70^{c}	22.43^{b}	1858^{a}	13.46^{a}
CV** (%)		7.2	9.8	22.9	14.5	17.3
Carrier comparison						
Peat		0.26^{b}	4.05^{b}	27.95^{a}	1335^{a}	14.83^{a}
IPC 2.2		0.30^{a}	4.93^{a}	27.55^{a}	1441^{a}	16.62^{a}
Bacterial strain comparison						
BR3267		0.34^{a}	5.09^{a}	27.20^{a}	1154^{b}	17.35^{a}
A2B3 (IIR3)		0.30^{b}	4.97^{a}	19.18^{b}	1217^{b}	17.23^{a}
S2V4 (R2II)		0.23^{c}	4.46^{b}	31.97^{a}	1490^{a}	11.35^{b}
S2V4 (R2III)		0.24^{c}	3.45^{c}	32.60^{a}	1693^{a}	16.95^{a}

*Means followed by the same letters do not differ by the Scott-Knott test at 5% of probability; **Coefficient of variation.

N amount accumulated in the shoot, thus indicating high nodule activity upon N fixation. The strain BR 3267 was isolated from soils collected at the semi-arid region of Pernambuco State and it is very well adapted to the semi-arid edaphoclimatic conditions (Martins at al., 2003). Differing from BR 3267 strain that belongs to *Bradyrhizobium* genera, the strains A2B3 (IIR3) and S2V4 (R2III) are able to grow fast without altering the culture media pH and its characteristics are not compatible to that presented by common Bradyrhizobial strains. Several fast growing rhizobia have been isolated from cowpea (Thies et al., 1991; Zilli et al., 1999; Leite et al., 2009), and some of them have been evaluated regarding its efficiency, revealing that some of Brazilian fast growing cowpea strains also present the same efficiency showed by the traditional inoculant strains (Zilli et al., 1999; Gualter et al., 2011). Previous studies evaluating the genetic and phenotypical diversity of fast growing strains isolated from cowpea in the Brazilian semi-arid region indicate that this crop harbors rhizobia different from that already described and well characterized (Leite et al., 2009), opening new perspectives to studies on rhizobial ecology, taxonomy and technology, since the inoculant manufacturers are desirable to obtain efficient fast growing rhizobia due to the minor time required to produce the inoculants applying the standard methods used in Brazil (Fernandes Júnior and Reis, 2008). The strains evaluated in this study will be evaluated in network experiments as candidates for recommendation to inoculant production in Brazil. Further studies are necessary to evaluate the compatibility of the CMC/ Starch blends with rhizobial strains from other legume crops and to investigate the taxonomic relationship of the strains evaluated. This is the first report evaluating at the same time the efficiency of new inoculant formulations using polymeric compositions as carrier and new isolates obtained from cowpea to Brazilian semi-arid region. In addition, the present study reports for the first time, the feasibility of the CMC/starch polymeric compositions as carrier material to pre-inoculated seeds.

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