

**ARTENISA CERQUEIRA RODRIGUES**

**INTERRELAÇÃO *Bradyrhizobium* - BPCP's - CAUPI: AVALIAÇÃO DA  
ATIVIDADE ENZIMÁTICA E PERFORMANCE SIMBIÓTICA**

**RECIFE-PE  
2012**

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ATIVIDADE ENZIMÁTICA E PERFORMANCE SIMBIÓTICA**

Tese apresentada ao programa de Pós-Graduação em Ciências do Solo da Universidade Federal Rural de Pernambuco, como parte dos requisitos para obtenção do título de Doutor em Ciências do Solo.

Orientadora: Márcia do Vale Barreto Figueiredo, D.Sc.  
Co-orientador: Joaquim Albenísio Gomes da Silveira, D.Sc.

**RECIFE-PE  
2012**

### Ficha Catalográfica

R696i Rodrigues, Artenisa Cerqueira  
Interrelação *Bradyrhizobium* - BPCP's - caupi: avaliação da atividade enzimática e performance simbiótica / Artenisa Cerqueira Rodrigues. – 2012.  
119 f.: il.

Orientadora: Márcia do Vale Barreto Figueiredo  
Tese (Doutorado em Agronomia – Ciência do Solo) – Universidade Federal Rural de Pernambuco, Departamento de Agronomia, Recife, 2012.

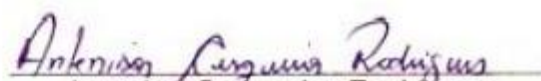
Inclui referências.

CDD 631.4

1. Simbiose
2. Metabolismo do Nitrogênio
3. Carbono
4. Senescência
5. Período de coleta
6. Espécies nativas de oxigênio
7. Antioxidante enzimático
8. Exopolissacarídeo
- I. Figueiredo, Márcia do Vale Barreto
- II. Título

Tese apresentada ao Programa de Pós-Graduação em Ciências do Solo como parte dos requisitos para obtenção do título de Doutor em Ciências do Solo.

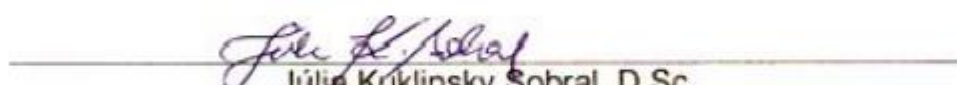
Tese aprovada em 21/06/2012

  
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Dedico

*A Deus, que me preparou emocionalmente, dando a força e os dons necessários para a conclusão deste trabalho; e a minha família, pelo amor, carinho e por acreditar na minha capacidade.*

“As coisas que não vemos são muito mais poderosas do que as que vemos. O que está embaixo do solo gera o que está em cima dele, o que é invisível cria o que é visível. Como seres humanos, não estamos acima da natureza, somos parte dela. Portanto, quando respeitamos as suas leis e cuidamos das nossas raízes a vida flui. É inútil concentrarmos a atenção nos frutos que já estão maduros. Não temos como mudar aqueles que já estão pendendo dos galhos, mas podemos modificar os que ainda vão nascer. Para isso, precisamos cavar a terra e reforçar as nossas raízes.”

Eker, T. H.  
*(Os Segredos da Mente Milionária - 2006)*

## **Agradecimentos**

A Deus pela vida, pelo amor supremo e por toda graça concedida.

Aos meus pais, pelo incentivo durante esta caminhada e por todos os valores que me ensinaram; aos meus irmãos Marinalva, Edvaldo, Edmilson e Jane, pelo apoio dado aos meus estudos, pelo carinho compreensão e pelas palavras de consolo nos momentos difíceis; e aos meus sobrinhos Caio, Heloyse, Lucas, Érick e Daniel por terem tornado os meus dias mais felizes.

À Profa. Dra. Márcia do Vale Barreto Figueiredo, pela orientação, apoio, sugestões, amizade, confiança, exemplo de vida, paciência e agradável convivência.

Ao Prof. Dr. Joaquim Albenísio Gomes da Silveira pela co-orientação e acolhimento em seu laboratório para parte das análises desenvolvidas.

Aos Drs. Antônio Félix da Costa, Aurenívia Bonifácio, Janete Magali de Araújo, José de Paula Oliveira, Maria Alice Gomes de Andrade Lima e Maria Luiza Ribeiro Bastos da Silva pelo apoio, amizade e incentivo.

As Dras. Claire Vendruscolo e Angelita Moreira, da Universidade Federal de Pelotas, pela colaboração e esclarecimentos prestados.

Aos amigos Alexandra Andrade, Benaia Barros, Carminha, Jadson Emanuel, Maria Vanilda, Nívia e Vívian pelas fundamentais e indispensáveis contribuições para a realização dos trabalhos.

A equipe do Laboratório de Biologia do Solo do IPA: Arthur Lira, Carolina Kropnicz, Emmanuella Vila Nova, Esmeralda, Maria do Carmo, Marília Malta, Marta Assunção, Rogério Portela e Rosa Moraes.

A Socorro (UFRPE) e Almira (IPA) pela amizade e colaboração.

Ao Sr. Arnaldo Sabóia e família pelo acolhimento e pela sincera amizade.

Aos colegas da pós-graduação pelo convívio e amizade durante o curso.

Ao Corpo Docente do Curso de Pós-Graduação em Ciências do Solo da Universidade Federal Rural de Pernambuco (UFRPE) pelos conhecimentos transmitidos.

Ao Instituto Agrônomo de Pernambuco (IPA), Laboratório de Biologia de Solo, onde foram desenvolvidos todos os experimentos e a Universidade Federal do Ceará (UFC), Laboratório de Metabolismo de Plantas, pela parceria nas análises laboratoriais e pelo intercâmbio científico.

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) pela concessão da bolsa.

Ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) pelo financiamento da pesquisa.

A Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco (FACEPE) pela concessão da bolsa-auxílio.

A todos que de alguma forma participaram deste trabalho e de minha vida acadêmica, o meu muito obrigado, e que Deus conceda a cada um, o dobro do bem que me fizeram.

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## RESUMO GERAL

Rodrigues, Artenisa Cerqueira; DSc. em Agronomia (Ciências do Solo); Universidade Federal Rural de Pernambuco; Junho/2012. **INTERRELAÇÃO *Bradyrhizobium* - BPCP's - CAUPI: AVALIAÇÃO DA ATIVIDADE ENZIMÁTICA E PERFORMANCE SIMBIÓTICA.** Márcia do Vale Barreto Figueiredo (Orientadora); Joaquim Albenísio Gomes da Silveira (co-orientador).

Para o incremento da fixação biológica do nitrogênio (FBN) tem sido recorrente o uso de bactérias promotoras de crescimento em plantas (BPCP's) em associação com o sistema simbiótico leguminosa-rizóbio. Desta forma, a busca da utilização de veículos alternativos que aumentem a qualidade e eficiência do inoculante levando a redução de custos e de possíveis impactos ambientais tem sido investigados. A FBN varia em resposta a fatores biológicos e ambientais e a sua efetividade pode ser mensurada através das concentrações dos compostos envolvidos neste processo, sendo um ponto relevante avaliar indicadores bioquímicos relacionados aos metabolismos do nitrogênio, do carbono e antioxidativo. Neste contexto, este trabalho teve como objetivos verificar a sobrevivência do caupi à colonização bacteriana, assim como avaliar a interrelação *Bradyrhizobium* sp. e BPCP's visando uma melhor performance simbiótica e desenvolvimento do caupi; otimizar a FBN e o desenvolvimento do caupi, inoculados e co-inoculados com *Bradyrhizobium* sp. e BPCP's, assim como avaliar as atividades de enzimas como indicadores de eficiência, senescência e de estresse/proteção oxidativo durante a após o estabelecimento da simbiose; e determinar a eficiência da inoculação e co-inoculação do caupi com *Bradyrhizobium* sp. e BPCP's utilizando variáveis de crescimento e produção e, juntamente, caracterizar o exopolissacarídeo utilizado como veículo de inoculação das sementes. Foram conduzidos três experimentos em casa de vegetação do Instituto Agrônomo de Pernambuco (IPA). A leguminosa utilizada foi caupi cv. "IPA 206" inoculada com *Bradyrhizobium* sp. (BR 3267) e co-inoculadas com diferentes estirpes de BPCP's. No experimento I, o delineamento experimental utilizado foi em blocos ao acaso com 24 tratamentos, sendo um com inoculação (BR 3267); 22 combinações (BR 3267 + BPCP's); uma testemunha absoluta (TA), com três repetições. No experimento II, o delineamento experimental adotado foi em blocos ao acaso com arranjo fatorial 2x4, dois períodos de coleta (PF, ponto de florescimento; IS, início de senescência) e quatro tratamentos (inoculados e co-inoculados) + TA, com quatro repetições. No experimento III, o delineamento experimental utilizado foi em blocos ao acaso com arranjo fatorial 2x6, dois períodos de colheita (PF, ponto de florescimento; e EG, enchimento de grãos) e seis tratamentos, sendo um com inoculação (BR 3267), e três com co-inoculações (BR 3267 + BPCP's); e duas testemunhas (TA e TN), com quatro repetições. Nos resultados constatou-se sinergismo entre BR 3267 e BPCP's em caupi principalmente na combinação de BR 3267 com *Paenibacillus graminis* (MC 04.21) e *P. durus* (C 04.50), que exibiram melhor resposta simbiótica. As estirpes de BPCP's pré-selecionadas foram combinadas com

BR 3267, formando dois pares simbióticos (BR 3267 + MC 04.21; BR 3267 + C 04.50) e uma tripartite (BR 3267+ MC 04.21 + C 04.50) onde os resultados mostraram diferenças significativas para as variáveis de crescimento bem como para os indicadores bioquímicos relacionados aos metabolismos do nitrogênio, carbono e antioxidativo. Houve aumento da concentração de proteínas solúveis totais nos nódulos das plantas co-inoculadas em relação às inoculadas isoladamente com a BR 3267. Ocorreu intensa atividade proteolítica e declínio na atividade das enzimas no IS, porém na co-inoculação tripartite houve retardo dos efeitos deletérios da senescência, o que permitiu uma melhor performance simbiótica na plantas de caupi. Pode-se sugerir o uso de inoculantes compostos com as bactérias estudadas visando incrementar a FBN e retardar a senescência dos nódulos assegurando a disponibilidade de nitrogênio por um maior período de tempo. Esta resposta ressalta a importância da combinação e compatibilidade de microrganismos introduzidos, em mistura, para promover uma melhor eficiência simbiótica do caupi, em especial para a co-inoculação em tripartite, com relação ao rizóbio nativo do solo, e esta resposta sinérgica resultou em melhoria nas variáveis de produção e eficiência da fixação de N<sub>2</sub>. Em relação às características químicas e reológicas do EPS, utilizado como veículo para inoculação, este se apresenta como um heteropolissacarídeo polianiónico com fluido pseudoplástico pouco viscoso, revelando-se pela técnica de calorimetria exploratória diferencial um pico endotérmico que representa uma característica favorável tendo em vista a possibilidade de seu uso em escala industrial.

**Palavras chave:** simbiose; metabolismo do nitrogênio; carbono; senescência; períodos de coleta; espécies reativas de oxigênio; antioxidante enzimático; exopolissacarídeo.



## GENERAL ABSTRACT

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It has been a recurrent the use of plant growth-promoting bacteria (PGPB) in association with the legume-rhizobia symbiotic system to increase the biological nitrogen fixation (BNF). Thus, the search for using alternative vehicles that increase quality and efficiency of inoculum leading to reduced costs and possible environmental impacts have been investigated. The BNF change in response to biological and environmental factors and their effectiveness can be measured by the concentrations of compounds involved in this process, being an important point to evaluate biochemical indicators related to the metabolisms of nitrogen, carbon and antioxidant. In this context, this study aimed at verifying the survival of cowpea to bacterial colonization and evaluating the interrelationship of *Bradyrhizobium* sp. and PGPB providing better symbiotic performance and cowpea development; optimizing the BNF and cowpea development, inoculated and co-inoculated with *Bradyrhizobium* sp. and PGPB, as well as evaluating the activities of enzymes as indicators of efficiency, senescence and stress/oxidative protection during and after the establishment of symbiosis; and determining the efficiency of inoculation and co-inoculation of cowpea with *Bradyrhizobium* sp. and PGPB using variables of growth and production and, jointly, characterizing the exopolysaccharide used as a vehicle for seed inoculation. Three experiments were conducted in greenhouse of the Agronomy Institute of Pernambuco (IPA). The legume used was cowpea cv. "IPA 206" inoculated with *Bradyrhizobium* sp. (BR 3267) and co-inoculated with different strains of PGPB. In the first experiment, the experimental design was randomized blocks with 24 treatments one inoculated (BR 3267), and 22 co-inoculated (BR 3267 + PGPB); and an absolute control (AC), with three replications. In the experiment II, the experimental design was randomized blocks with 2x4 factorial arrangement, two harvest periods (FP, flowering point; BS, beginning of senescence) and four treatments (inoculated and co-inoculated) + AC, with four repetitions. In the experiment III, the experimental design was a randomized block with 2x6 factorial arrangement, two harvest periods (FP, flowering point; GF, grain filling) and six treatments, one inoculated (BR 3267), and three co-inoculated (BR 3267 + PGPB), and two controls (AC and NC), with four replications. In the results was found synergism between BR 3267 and PGPB in cowpea especially in combination of BR 3267 with strains *Paenibacillus graminis* (MC 04.21) and *P. durus* (C 04.50), which exhibited better symbiotic response. Pre-selected strains of PGPB were combined with BR 3267, forming two symbiotic pairs (BR 3267 + MC 04.21; BR 3267 + C 04.50) and a tripartite (BR 3267 + MC 4.21 + C 04.50) where the

results showed significant differences for growth variables as well as the biochemical indicators related to the metabolisms of nitrogen, carbon and antioxidative. There was increased concentration of total soluble proteins in nodules of plants co-inoculated compared to those inoculated in isolation with BR 3267. There was intense proteolytic activity and a decline in enzyme activity in the BS, but there was delayed of the deleterious effects of aging in the tripartite co-inoculation, allowing better symbiotic performance in cowpea plants. It can be suggested the use of inoculating compounds with bacteria studied aiming at improving the BNF and delaying senescence of nodules ensuring the availability of nitrogen for a longer period of time. This response emphasizes the importance of the combination and compatibility of microorganisms introduced, in combination, to promote a better symbiotic efficiency of cowpea, especially for tripartite co-inoculation, with respect to *Rhizobium* native to soil, and this synergistic response resulted in improved variables of production and efficiency of N<sub>2</sub> fixation. With respect to chemical and rheological characteristics, the EPS used as vehicle for inoculation presents as a polyanionic heteropolysaccharide with low-viscosity fluid pseudoplastic, revealing an endothermal peak by the technique of differential scanning calorimetry that represents a favorable feature considering its use on industrial scale.

**Keywords:** symbiosis, nitrogen metabolism, carbon; senescence; harvest periods; reactive oxygen species; antioxidant enzymes; exopolysaccharide.

## 1. Introdução geral

Estudos visando os processos de nodulação e fixação biológica de nitrogênio (FBN) são de fundamental importância para o aumento da produtividade de diferentes espécies de leguminosas agronomicamente importantes, tais como soja [*Glycine max* (L.) Merr.], amendoim [*Arachis hypogaea* L.] e caupi [*Vigna unguiculata* (L.) Walp]. O caupi apresenta grande importância econômica e social e é crescente o número de estudos utilizando esta espécie como modelo em pesquisas abordando o metabolismo do nitrogênio. A FBN resulta da positiva interação entre bactérias e espécies vegetais e algumas pesquisas têm mostrado que certas propriedades atribuídas às plantas são muitas vezes atributos dos microrganismos que vivem no interior ou ao redor das raízes vegetais (Herridge et al., 2008).

Sabe-se que os microrganismos exercem muitas funções importantes para as espécies vegetais. As bactérias promotoras de crescimento em plantas (BPCP's) podem ser mais uma alternativa para aumentar a produtividade das culturas devido a sua influência no processo de FBN (Kuklinsky-Sobral et al., 2004). Tais bactérias podem efetivamente colonizar o hospedeiro e promover o crescimento da planta exercendo um efeito benéfico no desenvolvimento ou na sanidade do vegetal (Byrne et al., 2005). Assim, estudar o uso de microrganismos visando incrementar a FBN em espécies vegetais de importância agrônômica torna-se essencial e pode levar ao aumento da produtividade vegetal.

Uma adequada FBN é reflexo da ação coordenada de inúmeras enzimas envolvidas na manutenção de uma efetiva associação simbiótica leguminosa-rizóbio, principalmente as enzimas do metabolismo do carbono e do nitrogênio e as que conferem proteção contra danos oxidativos (Di Ciocco et al., 2008; Becana et al., 2010). O estresse oxidativo é resultante do desbalanço entre a produção e a eliminação das espécies reativas de oxigênio (EROs), que influenciam em processos vitais como a fotossíntese e a FBN (Becana et al., 2010). Diante disso, os níveis das EROs precisam ser mantidos sob controle e este é alcançado pela atuação conjunta de antioxidantes enzimáticos e não enzimáticos presentes no bacteróide (Chang et al., 2009; Silveira et al., 2011).

A associação efetiva entre microrganismos e o caupi resulta em uma maior FBN com conseqüente aumento da produtividade vegetal (Ferreira et al., 2011). Durante o processo de infecção e formação de nódulos, as bactérias

sintetizam polissacarídeos que são liberados ao meio extracelular (Castro et al., 2008). Estes polissacarídeos são capazes de proteger o nódulo de compostos tóxicos, tais como EROs, e evitar o estresse oxidativo (Soto et al., 2006; Chang et al., 2009). Além disso, podem ser utilizados eficientemente como veículos de inoculação, apresentando-se como uma alternativa viável para substituir a turfa e reduzir os custos do processo de inoculação (Albareda et al., 2008).

A busca por estirpes de microrganismos produtores de polissacarídeos em quantidades economicamente viáveis e que promovam a FBN é um desafio que vem sendo enfrentado por vários grupos de pesquisa e pode resultar em novos insumos biológicos capazes de promover grandes saltos qualitativos na pesquisa agrônômica. A importância dos polissacarídeos extracelulares sintetizados pelos microrganismos associados às plantas no processo da FBN para agricultura bem como os mecanismos de infecção e desenvolvimento simbiótico ainda não estão claros, o que resulta num contínuo interesse em elucidar as características reológicas bem como bioquímicas destes polissacarídeos extracelulares (Castro et al., 2008).

Neste contexto, este trabalho teve como objetivos: verificar a sobrevivência do caupi à colonização bacteriana, assim como avaliar a interrelação *Bradyrhizobium* sp. e BPCP's visando uma melhor performance simbiótica e desenvolvimento do caupi; otimizar a FBN e o desenvolvimento das plantas de caupi, inoculadas e co-inoculadas isoladamente e em mistura com *Bradyrhizobium* sp. e BPCP's, assim como avaliar as atividades de enzimas como indicadores de eficiência, senescência e de estresse/proteção oxidativo durante a após o estabelecimento da simbiose; e determinar a eficiência da inoculação e co-inoculação do caupi com *Bradyrhizobium* sp. e BPCP's utilizando variáveis de crescimento e produção e, concomitantemente, caracterizar o exopolissacarídeo utilizado como veículo de inoculação das sementes.

## **2. Fundamentação teórica**

### **2.1 A cultura do caupi**

O caupi [*Vigna unguiculata* (L.) Walp] é popularmente conhecido como feijão macassar, feijão-de-corda ou feijão verde (Freire Filho et al., 2005). Esta espécie pertence ao gênero *Vigna*, que está distribuído nas regiões tropicais e

subtropicais do globo e apresenta mais de 170 espécies, a grande maioria originária da África, sendo 66 consideradas endêmicas (Faris, 1965). O caupi é uma leguminosa rústica bem adaptada a solos de baixa fertilidade e a diferentes condições climáticas com relativa tolerância à seca, sendo amplamente cultivada nos trópicos e subtropicais. Tais características contribuíram decisivamente para a sua implantação no semiárido da região Nordeste do Brasil quando introduzido no século XVI (Freire Filho et al., 2006).

O caupi desempenha função de destaque sócio-econômico no Brasil e em mais 65 países devido a sua importância como alimento de elevado valor nutritivo (Freire Filho et al., 2006; Teófilo et al., 2008; Silva, 2009). Na região NE, segundo dados do “Melhoramento do feijão caupi para o semiárido brasileiro: situação atual e perspectiva” (em fase de elaboração)<sup>1</sup>, a área de cultivo do caupi é de aproximadamente 1,3 milhões de ha e a produção gira em torno de 558.000 toneladas do grão, entretanto a sua produtividade média (~256 kg ha<sup>-1</sup>) é baixa. Isso se dá pelo reduzido nível tecnológico empregado no cultivo, destacando-se a baixa disponibilidade hídrica, qualidade genética das sementes e, principalmente, a ausência de um programa de manejo de nutrientes com baixo ou escasso uso de fertilizantes e inoculantes microbianos (Cardoso & Ribeiro, 2006).

## 2.2 Fixação biológica do nitrogênio em leguminosas

O nitrogênio é o elemento mais abundante na atmosfera terrestre, presente principalmente na forma diatômica (N<sub>2</sub>) é considerado um macronutriente essencial para as espécies vegetais. Algumas bactérias possuem enzimas com a capacidade de reduzir o N<sub>2</sub> e transformá-lo em amônia, que posteriormente é utilizado na síntese de elementos essenciais, num processo denominado de fixação biológica do nitrogênio (FBN) (Hungria et al., 2007; Di Ciocco et al., 2008). A FBN pode ser simbiótica, quando ocorrem associações mutualísticas entre espécies vegetais e microrganismos fixadores, principalmente rizóbios, ou assimbiótica, quando é promovida por microrganismos fixadores de vida livre, tais como espécies dos gêneros *Azotobacter* e *Beijerinckia* (Freitas et al., 2007).

Os rizóbios são distribuídos em diferentes grupos taxonômicos de acordo com suas características morfológicas, fisiológicas, genéticas e

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<sup>1</sup>Capítulo de livro, de autoria de Antonio Felix da Costa, a ser editado pelo BNB (2012).

filogenéticas (Lindström et al., 2006). Atualmente, é possível classificá-los em  $\alpha$ - e  $\beta$ -rizóbios (Franche et al., 2009; Bomfeti et al., 2011). Os gêneros *Agrobacterium*, *Allorhizobium*, *Azorhizobium*, *Bradyrhizobium*, *Devosia*, *Mesorhizobium*, *Methylobacterium*, *Ochrobactrum*, *Phyllobacterium*, *Rhizobium* e *Sinorhizobium* pertencem ao grupo das  $\alpha$ -proteobactéria, enquanto as bactérias do gênero *Burkholderia*, *Cupriavidus* e *Herbaspirillum* são pertencentes ao grupo das  $\beta$ -proteobactérias (Weir, 2011; <http://edzna.ccg.unam.mx/rhizobial-taxonomy/node/4>).

Os rizóbios possuem habilidade para fixar nitrogênio em simbiose com leguminosas (Moreira & Siqueira, 2006). Tal associação apresenta considerável importância agrícola e é caracterizada pela formação de estruturas hipertróficas nas raízes denominadas de nódulos (Zilli et al., 2009). Quando ocorre limitação de nitrogênio, os rizóbios formam nódulos simbióticos nas raízes de leguminosas, e convertem o nitrogênio atmosférico em amônia que é utilizado pela planta. As leguminosas podem dispor de duas fontes de nitrogênio, sendo uma delas proveniente do solo e/ou de fertilizantes e a outra o nitrogênio fixado (Chianu et al., 2011).

Devido ao elevado preço dos fertilizantes nitrogenados, resultante do consumo de energia fóssil em sua fabricação, e dos graves problemas de poluição causados pelo uso intensivo desses adubos, a FBN apresenta-se como uma alternativa viável para suprir a necessidade de compostos nitrogenados das espécies vegetais. Desta forma, são necessários estudos que visem maximizar a FBN e, conseqüentemente, aumentar a disponibilidade de nitrogênio para a planta até o período de enchimento dos grãos e tornar mais eficiente a utilização de carboidratos pelos nódulos (Gualter et al., 2011).

O processo de FBN requer um suprimento contínuo de carboidratos, que fornecem tanto a energia para a redução do nitrogênio quanto os esqueletos de carbono, necessários à assimilação da amônia produzida. Durante os processos de infecção e desenvolvimento dos nódulos, que são perfeitamente interligados às plantas por meio de vasos xilemáticos e floemáticos, a energia necessária às divisões celulares é obtida da oxidação dos carboidratos produzidos na parte aérea da planta hospedeira (Silveira et al., 2011). Assim, o sistema simbiótico leguminosa-rizóbio está sujeito a variações hormonais, nutricionais e ambientais que podem afetar a assimilação, distribuição e utilização do carbono e do nitrogênio (Larrainzar et al., 2009).

A efetividade do sistema simbiótico leguminosa-rizóbio e o desenvolvimento dos nódulos resultam da troca de sinais químicos moleculares entre a planta e o simbionte (Zilli et al., 2009; 2011). As espécies nativas de bactérias fixadoras de nitrogênio realizam a FBN em baixo grau de eficiência e, portanto, é necessário obter estirpes de rizóbio de alta qualidade para que ocorra uma FBN eficiente. Tais estirpes devem ser capazes de competir pelos recursos necessários à sobrevivência, sobrevivência saprofítica no solo, exibir antagonismo, capacidade de predação e atuar sinergicamente com outros microrganismos (Figueiredo et al., 2008; Zilli et al., 2011).

### **2.3 Enzimas chaves envolvidas com a FBN e a senescência em nódulos**

A FBN requer a participação de inúmeras enzimas que são responsáveis em reduzir o  $N_2$  e assimilar a amônia formada ao final do processo (Di Ciocco et al., 2008). A amônia inicialmente é transferida do bacteróide para o citosol da planta hospedeira e então é incorporada em esqueletos de carbono gerando aminoácidos, principalmente glutamina e glutamato, em reações mediadas pelas enzimas sintetase da glutamina (GS) e sintase do glutamato (GOGAT) que atuam no ciclo GS/GOGAT (Hungria et al., 2007). As atividades de GS e GOGAT podem ser detectadas já na fase inicial de estabelecimento dos nódulos e aumentam à medida que os nódulos se desenvolvem (Larrainzar et al., 2009).

A enzima GS é considerada a enzima chave da FBN e, juntamente com GOGAT, converte amônia em aminoácidos prevenindo seu acúmulo no interior dos bacteróides, que poderia resultar em toxicidade e inibir a FBN (Prell & Poole, 2006; Fagan et al., 2007). Além destas, está presente nos nódulos a enzima desidrogenase do glutamato aminante (GDHa) que também pode assimilar amônia e, assim, é apontada como parte de um processo secundário de assimilação da amônia (Bernard & Habash, 2009). Essa reação tem sido proposta como sendo uma via complementar que atua em condições de alto suprimento de amônia ou de adversidade ambiental (Wang et al., 2007).

A conversão de amônia em aminoácidos durante a FBN requer quantidades consideráveis de carbono que são produzidos e fornecidos pelo vegetal hospedeiro (Frache et al., 2009). A sacarose é o principal carboidrato exportado para os nódulos onde é hidrolisada pela ação das enzimas sacarose sintase (SS) e/ou invertases ácida (SAI) e neutra (NI) em glicose e frutose

(Wienkoop et al., 2008). A SS é considerada a enzima mais importante do metabolismo de carbono nos nódulos sendo essencial para manutenção e regulação da FBN em condições normais e de senescência (Horst et al., 2007; Ben Salah et al., 2009).

O processo de senescência induz nos nódulos alterações visíveis, tais como a mudança de cor dos tecidos nodulares de vermelho para verde (Puppo et al., 2005) indicando degradação de leghemoglobina, e alterações não visíveis, que vão desde a reorganização de estruturas celulares e redução da concentração de proteínas solúveis como resposta ao aumento da atividade de enzimas proteolíticas (Naya et al., 2007). Devido ao aumento da proteólise, pode ocorrer o declínio da atividade de enzimas responsáveis pelo controle dos níveis de espécies reativas de oxigênio (EROs), especialmente superóxido dismutase (SOD), catalase (CAT) e peroxidase de ascorbato (APX). Os principais ROS são ânion superóxido ( $O_2^{\cdot-}$ ), peróxido de hidrogênio ( $H_2O_2$ ) e radical hidroxil ( $\cdot OH$ ) (Puppo et al., 2005; Ben Salah et al., 2011).

As EROs são produzidas naturalmente nos bacteróides devido ao forte ambiente redutor necessário para a FBN (Ben Salah et al., 2009) e podem interagir inespecificamente com vários componentes celulares resultando em danos às membranas e macromoléculas essenciais para o funcionamento adequado da célula (Puppo et al., 2005; Jebara et al., 2005). Para minimizar os danos e controlar níveis de EROs, a célula dispõe de um conjunto de enzimas antioxidativas e de compostos não enzimáticos, principalmente ascorbato e glutatona, que atuam de forma coordenada nos nódulos garantindo a eficiência do sistema simbiótico leguminosa-rizóbio e uma adequada FBN (Chang et al., 2009).

## **2.4 Polissacarídeos microbianos**

Polissacarídeos de origem microbiana, também conhecidos como gomas, biopolímeros ou exopolissacarídeos (EPS), têm a capacidade de formar géis e soluções viscosas em meio aquoso (Moreira et al., 2003; Berwanger et al., 2007). Devido às suas propriedades físico-químicas e biológicas e a sua capacidade de alterar a reologia de soluções, os biopolímeros apresentam grande importância na indústria farmacêutica, de alimentos, de tintas, petróleo, cosmética, têxtil, produtos agrícolas, dentre outras (Rinaudo, 2008). Além disso, podem representar uma alternativa válida



às gomas tradicionais por possuírem propriedades químicas específicas que os qualificam para o desenvolvimento de novos produtos.

Bactérias dos gêneros *Rhizobium*, *Beijerinckia* e *Azotobacter* são fixadoras de nitrogênio e conhecidas por produzirem quantidades abundantes de biopolímeros (Aranda-Selverio & Penna, 2010). Destas, merecem destaque as bactérias do gênero *Rhizobium* por estabelecerem uma relação simbiótica com leguminosas a fim de realizarem a fixação do nitrogênio com concomitante produção de grandes quantidades de polissacarídeos extracelulares, succinoglucanas e/ou galactoglucanas (Serrato et al., 2008; Aranda-Selverio & Penna, 2010). Tais moléculas podem atuar como sinais moleculares durante a infecção das raízes, levando a formação de estruturas celulares que auxiliam no processo de infecção e desenvolvimento do nódulo (Marczak et al., 2008).

Os biopolímeros desempenham inúmeros papéis, que vão desde proteger os microrganismos contra desidratação, servir de barreira para impedir que vírus e anticorpos se liguem a sítios específicos da parede celular, neutralizar toxinas carregadas ou íons metálicos tóxicos, atuar como fonte de carbono e ainda interagir com células de plantas em relações específicas, simbióticas ou patogênicas (Marczak et al., 2008; Rinaudi & Giordano, 2010). Considerando a importância dos biopolímeros microbianos, tem sido crescente o número de trabalhos visando aperfeiçoar as condições de cultivo para o aumento da sua produção e para isso é importante observar vários fatores envolvidos no processo de fermentação (Fernandes Júnior et al., 2010).

Durante o processo de fermentação, deve-se considerar desde o microrganismo que será utilizado, a composição e pH do meio de cultivo, o volume do inóculo, temperatura de incubação, velocidade de agitação, aeração e tempo de cultivo (Faria, 2002; Berwanger et al., 2007). Destes, o pH e a temperatura são os fatores que mais afetam o crescimento da bactéria e a síntese dos biopolímeros. Isso se dá, pois cada espécie bacteriana requer pH e temperatura ótimos a fim de manter a composição macromolecular da célula e o crescimento microbiano adequados (Duta et al., 2006). Considerando estes fatores, é possível obter rendimentos satisfatórios e um biopolímero de elevada qualidade (Souza & Garcia-Cruz, 2004; Badel et al., 2011).

Diferentemente do que acontece para as gomas tradicionais, a produção das gomas microbianas independem de condições climáticas, da contaminação por ambiente ou falha na colheita e são menos suscetíveis à variabilidade em

sua qualidade, pois sua produção pode ser cuidadosamente controlada. A fim de aperfeiçoar o processo de produção dos biopolímeros sintetizados por microrganismos, pesquisas estão sendo desenvolvidas enfocando principalmente a redução de meios de cultura e do tempo de cultivo. Tais estudos são fundamentais para determinar condições ótimas para os processos fermentativos que serão úteis na produção dos insumos microbiológicos a serem aplicados em sistemas agrícolas (Santos, 2010).

Além das bactérias fixadoras, microrganismos pertencentes ao domínio *Archaea* e habitantes de ambientes inóspitos, como as bactérias psicrófilas, termófilas, halófilas e acidófilas, produzem biopolímeros em quantidades consideráveis com funções diversas (Nicolaus et al., 2010). Nas psicrófilas, os biopolímeros produzidos podem promover estabilidade ao ambiente enquanto que nas termófilas, este pode servir como potenciador para a sobrevivência bacteriana (Quin et al., 2007; Nicolaus et al., 2010). Em bactérias halófilas e acidófilas, o biopolímero ajuda na manutenção da integridade da membrana (Arias et al., 2003). Segundo Poli et al. (2010), a análise das características químicas dos biopolímeros fornece o entendimento acerca do seu papel ecológico e ainda do seu potencial comercial.

Os biopolímeros produzidos por microrganismos de ambientes inóspitos despertam grande interesse comercial devido a sua vasta aplicabilidade, entretanto é necessário um processo de produção otimizado com adequada relação custo-benefício (Nichols et al., 2005; Nicolaus et al., 2010). Igualmente como ocorre em microrganismos produtores de biopolímeros do domínio *Bacteria*, a estrutura, composição e viscosidade dos biopolímeros produzidos por bactérias extremófilas dependem da composição do meio de cultivo e das condições de fermentação (Nicolaus et al., 2010). Assim, é possível atingir uma ampla produção e controlar as características destes biopolímeros através da manipulação das condições do processo de fermentação (Nichols et al., 2005).

## **2.5 Bactérias promotoras de crescimento em plantas**

A utilização de bactérias promotoras do crescimento de plantas (BPCP's) para o aumento da produção agrícola é provavelmente uma das táticas mais importantes para o mundo agrícola. Isso se deve à demanda emergente para a diminuição da dependência de fertilizantes químicos e a necessidade de desenvolvimento da agricultura sustentável (Moreira &

Siqueira, 2006). As BPCP's podem aumentar o crescimento de plantas por promoverem a mineralização de nutrientes, como por exemplo, a solubilização de fosfatos minerais, e ainda aumentam a produção de auxinas e giberelinas (Dobbelaere et al., 2003). Além do seu impacto positivo no crescimento vegetal, as BPCP's são importantes agentes de controle biológico de microrganismos patogênicos da rizosfera e agentes biorremediadores de áreas contaminadas, pois degradam substâncias xenobióticas (Coelho, 2006).

O uso de BPCP's resulta em aspectos ecologicamente benéficos que têm contribuído para alcançar a sustentabilidade no setor agrícola (Figueiredo et al., 2010). Vários gêneros bacterianos compõem o grupo das BPCP's, como *Arthrobacter*, *Bacillus*, *Azospirillum*, *Burkholderia*, *Herbaspirillum*, *Enterobacter*, *Gluconacetobacter*, *Klivosiella*, *Paenibacillus*, *Serratia* e *Pseudomonas*, sendo os gêneros *Pseudomonas* e *Bacillus* considerados os mais importantes (Spaepen et al., 2009). Em *Pseudomonas*, isso é devido à versatilidade nutricional e habilidade de crescer em ampla variedade de ambientes e substratos (Ferreira et al., 2009). Já *Bacillus* apresentam prolongado período de viabilidade e maior capacidade na produção de endósporos (Ciprino, 2009).

A interação entre as BPCP's e as espécies vegetais resulta em efeitos benéficos que vão desde o aumento da taxa de germinação, do crescimento das raízes, colmos e/ou caules, do número de folhas e da área foliar, do crescimento de tubérculos, do número de flores até um aumento do rendimento da produtividade vegetal (Compant et al. 2010). Além disso, as BPCP's inoculadas juntamente com rizóbios podem representar uma importante estratégia na promoção de crescimento vegetal e também atuar sinergisticamente resultando num aumento da fixação simbiótica do nitrogênio em raízes de leguminosas (Huergo et al., 2008; Figueiredo et al., 2010), sendo, neste caso, importante observar a combinação e compatibilidade dos isolados envolvidos.

É crescente o número de estudos visando aperfeiçoar o uso de BPCP's isoladas ou em combinação com outros microrganismos (Lima et al., 2011). A grande importância de se estudar tais interações se dá devido ao aumento da demanda por tecnologias limpas de adubação que minimizem o uso de fertilizantes químicos e estabeleçam uma política favorável para o desenvolvimento de uma agricultura sustentável (Moreira & Siqueira, 2006),

assim, é necessário que as pesquisas sejam aprofundadas para identificar inoculantes que aumentem a produtividade agrícola.

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## **Capítulo I**

**Interrelationship *Bradyrhizobium* sp. and plant-growth promoting bacteria  
in cowpea: survival and symbiotic performance**

*Artigo a ser submetido para publicação no periódico  
Current Microbiology*

## **Interrelationship *Bradyrhizobium* sp. and plant growth-promoting bacteria in cowpea: survival and symbiotic performance**

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**Abstract** The objective of this study was to evaluate the survival of cowpea to bacterial colonization and evaluate the interrelationship of the *Bradyrhizobium* sp. and plant growth-promoting bacteria (PGPB) as alternative to optimize the symbiotic performance and cowpea development. Two experiments were conducted and the legume used was cowpea cv. "IPA 206". In the first experiment, the seeds were disinfected, germinated and transferred to Gibson tubes, sterilized and containing nutritive solution free of nitrogen. The experimental design was randomized blocks with 24 treatments [*Bradyrhizobium* sp. (BR 3267); 22 PGPB; absolute control (AC)] with three repetitions. In the second experiment, seeds were disinfected, inoculated in according to the treatment and grown in Leonard jars containing washed and autoclaved sand. The experimental design was randomized blocks with 24 treatments [BR 3267; 22 BR 3267 + PGPB; AC] with three replications. The scanning electron microscopy analysis showed satisfactory colonization in roots of inoculated plants. It was found synergism between BR 3267 and PGPB in cowpea, mainly in BR 3267 + *P. graminis* (MC 04.21) and BR 3267 + *P. durus* (C 04.50), which showed better symbiotic performance and development of cowpea.

**Keywords:** PGPB, synergism, BNF, co-inoculation, colonization, *Vigna unguiculata* [L.] Walp.

### **Introduction**

The cowpea is grown in arid and semiarid regions of NE Brazil and having socio-economic highlight due to the high tolerance to adverse environmental conditions such as low rainfall and high concentration of salts in the soil [1]. Considering the nutritional requirements of cowpea, it has been sought alternatives that minimize the use of chemical fertilizers that cause negative impacts on the environment [19]. In this context, the use of nitrogen fixing bacteria in symbiosis with plant species have been shown to be a viable alternative to supply the demand for nitrogen and increase productivity [1]. The biological nitrogen fixation (BNF)

is known effective in cowpea and this, when well nodulated, can dispense other nitrogen sources and achieve high levels of productivity [28].

The use of microorganisms that enhance nodulation and BNF is of fundamental importance since it may contribute to increased plant productivity. The beneficial microorganisms to plants are of interest to the agricultural sector in view of their ecologically beneficial effects [9]. Among these microorganisms are plants growth-promoting bacteria (PGPB) that stimulate plant growth, increase productivity, reduce the incidence of pathogens and mitigate the deleterious effects of biotic and abiotic stresses [18]. The association of PGPB and nitrogen fixing bacteria can result in increased BNF; however, it is necessary a combination and compatibility of strains for increased productivity [12, 24].

The application of PGPB in association with fixing bacteria has been focus of numerous studies due to the search for strategies to increase agricultural productivity, including for cowpea [17]. However, the results on the effect of co-inoculation with *Bradyrhizobium* sp. and PGPB in improving the process of nodulation and BNF are still incipient. The present study was undertaken to evaluated the cowpea survival to bacterial colonization as well as interrelationship of *Bradyrhizobium* sp. and PGPB as alternative to optimize the symbiotic performance and cowpea development.

## **Material and Methods**

### **Multiplication and preparation of the inoculants**

It was used the standard strain for cowpea *Bradyrhizobium* sp. (BR 3267), from the collection of the Research National Center of Agrobiolgy (CNPAB, RJ-Brazil), and growth-promoting bacteria in plants (PGPB) *Bacillus*, *Paenibacillus* and *Brevibacillus* from the collections of the Federal University of Pernambuco (UFPE, Antibiotic Department) and Federal University of Rio de Janeiro (UFRJ, Microbiology Institute). To obtain the inoculant, the BR 3267 strain was grown in YM (Yeast-Mannitol) culture medium using a rotator shaker (200 rpm, 28 °C, 96 h), while PGPB strains were grown in TSB (Trypticase Soy Broth) culture medium using a rotator shaker (200 rpm, 32° C) for 24 h or 48 h according to the bacterial strain.

### **Cowpea survival to bacterial colonization**

In order to evaluate the cowpea survival to bacterial colonization, seeds of cowpea cv. "IPA 206" were disinfected [15], seeded in Petri dishes containing Germitest<sup>®</sup> paper and kept

in moist chamber until radicle protrusion. After germination, seeds were transferred to sterilized Gibson tubes and containing Hoagland and Arnon [14] nutritive solution modified by Silveira et al. [23] and free of nitrogen. For inoculation, 1.0 mL of culture medium containing BR 3267 was added ( $10^8$  CFU mL<sup>-1</sup>) or PGPB ( $10^7$  CFU mL<sup>-1</sup>). It was used an absolute control (AC). The experiment was conducted for 15 days and at harvest were measured the root length (RL) and root thickness (RT). To evaluate the efficiency of bacterial colonization, root fragments (~1-2 cm long) were fixed in modified Karnovksy solution and submitted to scanning electron microscopy (SEM). The experimental design was randomized blocks with 24 treatments, one BR 3267, 22 PGPB and one AC, with three replications. Data were subjected to analysis of variance (ANOVA), using the statistical program ASSISTAT version 7.6 beta, with 5% significance levels by the F test and means were compared by the Tukey's test ( $p < 0.05$ ).

### **Co-inoculation *Bradyrhizobium* sp. and PGPB in cowpea**

To evaluate the compatibility and efficiency of the interrelation between BR 3267 and PGPB, seeds of cowpea cv. "IPA 206" were desinfected [15], seeded and inoculated with 1.0 mL of culture medium containing BR 3267 ( $10^8$  CFU mL<sup>-1</sup>) or co-inoculated with 1.0 mL of culture medium containing PGPB ( $10^7$  CFU mL<sup>-1</sup>) followed by addition of 1.0 mL of culture medium containing BR 3267 in Leonard jars containing washed (pH 6.5) and autoclaved (120 °C, 101 KPa, 1 h) sand as substrate. After thinning the cowpeas at seven days, two plants were kept in each Leonard jar. The experiment was conducted in the greenhouse (of the Agronomical Institute of Pernambuco - IPA) at a temperatu range of 27-36 °C with 50-70% relative humidity. During the experimental period, plants were irrigated by capillary action with Hoagland and Arnon [14] nutritive solution modified according to Silveira et al. [23] and free of nitrogen. Uninoculated plants were used as absolute control (AC). The cowpeas were harvested 36 days after sowing. The following variables were evaluated: root length, nodules number and shoots (SDM), roots (RDM) and nodules dry matter, nodule size, nitrogen accumulated in the SDM, SDM/RDM ratio, absolute growth rate, nitrogen content in the SDM, nitrogen fixation efficiency and specific nodulation [7, 13, 6].

### **Stastitical design and analysis**

The experimental design was randomized blocks with 24 treatments, one BR 3267, 22 combinations between BR 3267 and PGPB and one AC, with three replications. Each variable

studied was subjected to analysis of variance (ANOVA) using the statistical program ASSISTAT version 7.6 beta at 5% significance levels by the F test and means were compared by the Tukey's test ( $p < 0.05$ ).

## Results and Discussion

### Cowpea survival to bacterial colonization

Bacterial colonization in the roots is an extremely complex process modulated by numerous biotic and abiotic factors, such as root exudation, humidity and luminosity [9]. To confirm the effectiveness of the bacterial colonization process in the roots of cowpea, root fragments were subjected to scanning electron microscopy (Fig. 1). It can be observed that all strains tested were able to colonize the roots, however, plants inoculated with *Brevibacillus brevis* (447) strain showed very low colonization (Fig. 1B).

Whereas the process of bacterial colonization in the cowpea roots was effective, it was proceeded the evaluation on the inoculation with bacterial strains, one BR 3267 and 22 PGPB, in terms of roots growth and thickness. The PGPB can lead to plant growth increased, since they promote the nutrients mineralization and hormones production such as auxins and gibberellins [11, 24]. In the present study were showed significant differences when the different strains were evaluated (Table 1). The association of cowpea plants with BR 3267 or *Paenibacillus kribbensis* (POC 115) showed in higher root length (RL) in relation to the others treatments.

The RL recorded in association of cowpea plants with BR 3267 may be a response resulting from nutrient increased promoted by nodulation. Plants with nodules on their roots as result of association with fixing bacteria show higher flow of nitrogen compounds due to nitrogen fixation that occurs in bacteroids [19, 28]. During fixation occur the conversion of atmospheric nitrogen in ammonia which is exported to the plant to be used in several metabolic reactions that can lead to increased plant growth [12, 8].

The increase in plant growth can be achieved either by inoculation with diazotrophic bacteria, as observed in *Bradyrhizobium* sp.-cowpea association, but also with the use of plant growth-promoting bacteria, as recorded for cowpea plants inoculated with *P. kribbensis* (POC 115). The association with *P. kribbensis* (POC 115) showed in plants with higher RL compared to other treatments with PGPB (Table 1). The species of the *Paenibacillus* genus are widely distributed in various environments and have as main characteristic the secretion of

extracellular compounds in the rhizosphere, such as amino acids and secondary metabolites, which can result in a favorable environment for plant development [27].

Plants inoculated with the ANBE 31 strain of the *Bacillus* sp. had lower RL compared to other strains analyzed (Table 1). This response may indicate that there was a lesser effect of the interaction of this strain with cowpea plants. In fact, the interactions in the rhizosphere play an important role in the transformation, mobilization and solubilization of the nutrients and their uptake by plants aiming at growth increasing [10]. Given that there was less interaction, the nutrients mobilization may be committed leading to restriction of root growth observed in these plants.

The presence of mucilaginous substances on the root surface, which act as lubricants, and even the root thickness can result in a bigger and better ability to exploit the rhizosphere looking for nutrients and water and consequently promoting increased in the root growth [5]. In this study, plants inoculated with the different bacterial strains resulted in significant changes in root thickness (Table 1). Plants inoculated with *P. brasilensis* (24) exhibited higher root thickness, whereas those inoculated with LMD 24.16 strain of bacterium *P. polymyxa* showed minor root thickness in comparison with other strains (Table 1).

The reduction of 27% observed in the root thickness of plants inoculated with *P. polymyxa* (LMD 24.16) compared to plants inoculated with *P. brasilensis* (24) may represent a smaller response by plant to the compounds synthesized by microorganisms in the rhizosphere. The PGPB can colonize internal and externally plants organs inducing beneficial or harmful effects to plant growth and this is due to synthesis of phytohormones in the root zone, which in high concentrations can impair root development [24].

### **Co-inoculation *Bradyrhizobium* sp. and PGPB in cowpea**

The synergistic responses of the plant-rhizobia-PGPB association can vary considerably depending on innumerable factors such as bacterial strains, plant species, inoculum density and environmental conditions [19]. In the present study, the cowpea plants were co-inoculated with BR 3267 and different PGPB in order to identify possible synergistic responses among microorganisms. Thus, were evaluated the absolute growth rate (AGR), the root length (CR), shoot (SDM) and root (RDM) dry matter and the SDM/RDM ratio of cowpea plants in response to different bacterial combinations (Table 2). The growth analysis allows evaluating the plant as a whole and also the contribution of different organs to total growth [6].

The growth rate of plants is a genetically controlled attribute and the determinant factors acting at the growth beginning have effect only on the exponential phase of growth of plants and decrease when plants enter in the establishment phase [6]. In this study, plants inoculated with BR 3267 and those co-inoculated with BR 3267 + PGPB showed no significant differences by the Tukey's test ( $p < 0.05$ ), except for the AC and BR 3267 + *P. durus* (C 04.50); BR 3267 + *B. pumilus* (455); BR 3267 + *B. pumilus* (444); BR 3267 + *P. durus* (V 22.32); BR 3267 + *P. kribbensis* (POC 115) and BR 3267 + *P. brasilensis* (24) treatments. Plants co-inoculated with BR 3267 + C 04.50 showed increase of the 5 times and of 32% in AGR compared to the AC plants and those inoculated only with BR 3267, respectively. The results reinforce the idea that the co-inoculation is not always effective and this inefficiency can result from increased production of phytohormones by PGPB that are released in the plant root surface and lead to inhibition and/or delay of plant growth [9].

The root development may indirectly contribute to the effective nodulation of roots and favor the BNF [26]. In this case, the cowpea plants were evaluated for root length (RL) in response to different co-inoculation (Table 2). The AC plants and those co-inoculated with the symbiotic pairs BR 3267 + *P. brasilensis* (24) and BR 3267 + *B. pumilus* (448) exhibited higher RL in relation to the other treatments (Table 2). The higher root growth in AC plants may represent a search for nutrients since these plants were grown with nitrogen-free nutrient solution. According Krapp et al. [16], plant species stimulate the growth and alter the root architecture with concomitant reduction of the shoot growth under conditions of nitrogen deprivation in seeking to acquire this nutrient.

The PGPB can promote increased plant growth by causing the nutrients mineralization, making them properly available to plant species [18]. Furthermore, PGPB strains can act in assymbiotic nitrogen fixation to be available to the plant and used in metabolic reactions that lead to plant growth and even lead to variable responses depending on the effectiveness of the association with the plant species [24]. This variability can be observed when comparing the plants co-inoculated with *P. brasilensis* (24) and with *P. polymyxa* (LMD 24.16) that showed higher and lower RL, respectively (Table 2). This response indicates differences in specificity and effectiveness of the bacteria-plant interaction as response to the intrinsic characteristics of each strain utilized, such as organic acids and plant hormones synthesized by these bacteria, or even the root exudates synthesized by plants that performing negative effects to the bacteria [11, 9].

The PGPB colonize different plant organs exerting several beneficial effects such as increased seed germination and root and leaves development [10, 24]. In this study, cowpea



plants showed significant differences ( $p < 0.05$ ) among treatments in terms of SDM, except for the AC. The co-inoculation of cowpea plants with the symbiotic pair BR 3267 + *P. graminis* (MC 04.21) resulted in higher SDM (Table 2). The presence of PGPB influences plants to produce more biomass in the shoot being this response variable depending on the plant species or strain used [3] and also for proper maintenance of the photosynthesis, process that produces carbon skeletons to be used in BNF [2].

The promotion of root growth is often considered a beneficial feature for improving water uptake by plants [21] and the induction of this process by the presence of PGPB roots is particularly important in field conditions, since it results in effective maintenance of water status, growth and productivity plant [4]. As shown in Table 2, cowpea plants co-inoculated with BR 3267 + *P. macerans* (LMD 10.24) exhibited higher RDM, but there was no significant difference ( $p < 0.05$ ). Furthermore, plants co-inoculated with BR 3267 + *B. pumilis* (444) showed higher SDM/RDM ratio though not differ statistically ( $p < 0.05$ ) according Tukey's test.

Bacteria of the genus *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium* and *Sinorhizobium* are part of the group of rhizobia able to induce the formation of nodules in several legumes species [25]. The cell-cell contact between the rhizobia and host plant is an important prerequisite for the formation of nodules, hypertrophic structures located in the roots where rhizobia fixes  $N_2$  converting it in ammonia [12, 28]. In the present study, inoculated and co-inoculated cowpea plants not showed significant difference ( $p < 0.05$ ) on the number, size and dry matter of cowpea nodules (Table 3).

The efficiency of nitrogen metabolism resulting from the BNF in bacteroids can be evaluated in terms of accumulate nitrogen ( $N_{ac}$ ), nitrogen content (N content), specific nodulation (SN) and the nitrogen fixation efficiency ( $N_2FE$ ) (Table 3). Cowpea plants inoculated with BR 3267 and with BR 3267 + *P. graminis* (MC 04.21) showed higher  $N_{ac}$  while the AC plants exhibited the lowest  $N_{ac}$  in comparison to other treatments. It is possible that the association between plant-*Bradyrhizobium* sp. and PGPB has, overall, induced a flow shift of the fixed nitrogen in bacteroids for the synthesis of nitrogenous compounds, mainly proteins responsive to PGPB, which remain in the nodules and not being translocated for plant tissues.

The fact of symbiotic association cowpea + BR 3267 + MC 04.21 has displayed  $N_{ac}$  similar to that obtained for BR 3267 may indicate a maintenance of nitrogen flow from rhizobia to the plant or yet a stimulus of BNF in the presence of PGPB, ensuring more fixed nitrogen to adequately supply the metabolism of the plant and rhizobia. The  $N_2FE$  in cowpea

plants was not differed significantly ( $p < 0.05$ ) when comparing plants inoculated separately with BR 3267 and the co-inoculated plants, except for plants co-inoculated with BR 3267 + *P. macerans* (LMD 10.24), BR 3267 + *B. megaterium* (462), BR 3267 + *B. pumilus* (445) and BR 3267 + *Bacillus* sp. (ANBE 31). SN did not differ statistically by the Tukey's test ( $p < 0.05$ ) among treatments.

Nitrogen limits growth and primary production of plant species and is present in minute concentrations in most terrestrial ecosystems in its biologically available form to the plants [22]. In order to minimize the nitrogen limitation, some plant species can form symbiotic associations with fixing bacteria and these can effectively convert atmospheric nitrogen in ammonia in the bacteroids [12] and release it to the plants in exchange of carbon skeletons [18]. The nodulation process in root plant induced by rhizobia represents an efficient way to obtain nitrogen and also an alternative to the use of nitrogen fertilizers.

## Conclusions

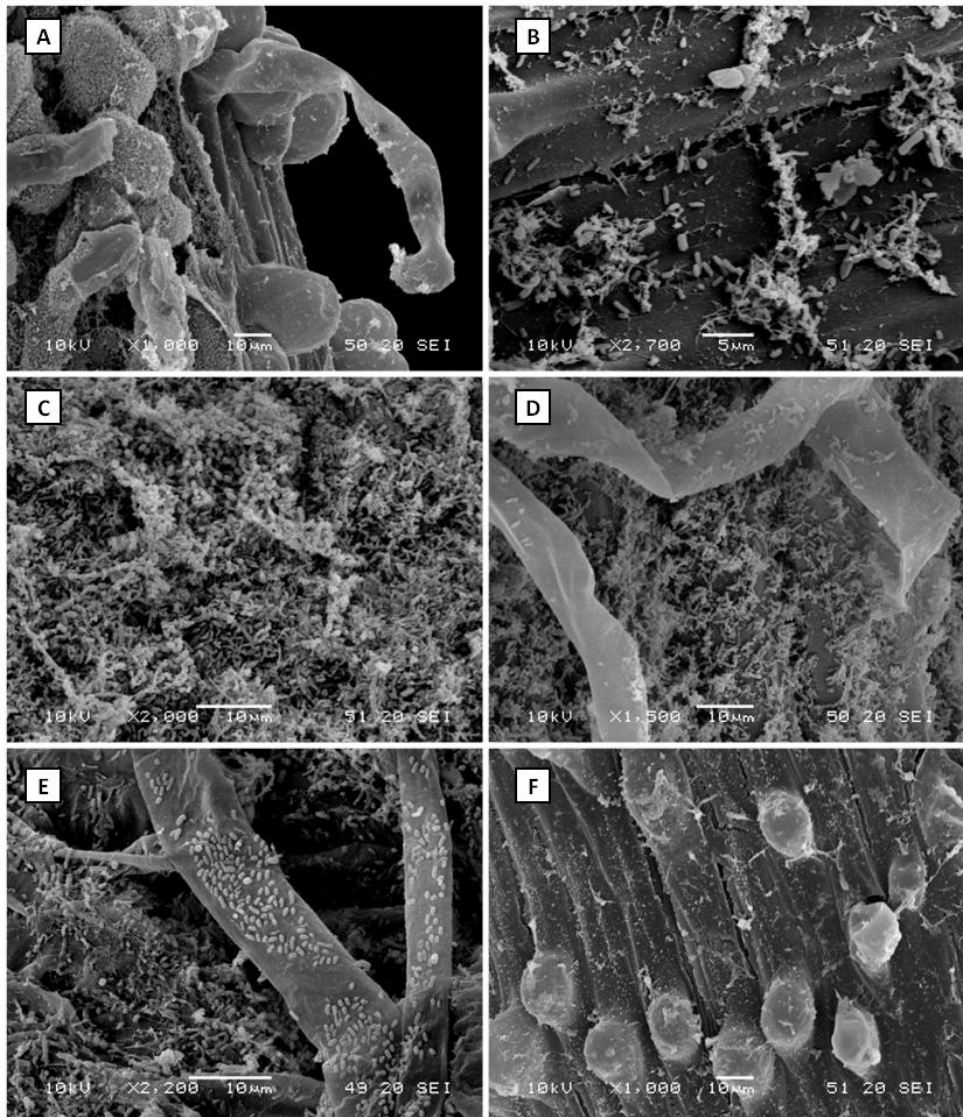
The analysis of scanning electron microscopy showed satisfactory colonization in the cowpea roots inoculated. There was synergism among *Bradyrhizobium* sp. (BR 3267) and PGPB (*Bacillus*, *Brevibacillus* and *Paenibacillus*) which promoted better growth and symbiotic performance in cowpea, mainly in those co-inoculated with the symbiotic pairs BR 3267 + *P. graminis* (MC 04.21) and BR 3267 + *P. durus* (C 04.50).

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**Fig. 1.** Images of the root fragments of the cowpea submitted to inoculation with *Bradyrhizobium* sp. and PGPB compared to absolute control obtained by scanning electron microscopy. (A) *Bradyrhizobium* sp. (BR 3267); (B) *Brevibacillus brevis* (447); (C) *Paenibacillus polymyxa* (LMD 24.16); (D) *Bacillus pumilus* (445); (E) *Paenibacillus polymyxa* (Loutit); (F) Absolute control (AC).

**Table 1.** Root length (RL) and thickness (RT) of the cowpea plants inoculated with *Bradyrhizobium* sp. (BR 3267) or plant growth-promoting bacteria (PGPB).

Treatments		RL	RT
		(cm)	(mm)
<i>Bradyrhizobium</i> sp.	BR 3267	23.50 a	2.51 ab
<i>Bacillus</i> sp.	ANBE 31	5.00 b	2.37 ab
<i>B. aubitilis</i>	441	7.66 ab	2.47 ab
<i>B. cereus</i>	440	11.00 ab	2.72 ab
<i>B. pumilus</i>	444	14.00 ab	2.16 ab
<i>B. pumilus</i>	445	15.33 ab	2.50 ab
<i>B. pumilus</i>	448	18.50 ab	2.68 ab
<i>Brevibacillus brevis</i>	447	17.16 ab	2.51 ab
<i>B. megaterium</i>	462	17.16 ab	2.55 ab
<i>B. subtilis</i>	455	16.00 ab	2.37 ab
<i>B. subtilis</i>	458	13.66 ab	2.40 ab
<i>Paenibacillus brasilensis</i>	24	11.50 ab	2.79 a
<i>P. durus</i>	CRIP 105	18.33 ab	2.33 ab
<i>P. durus</i>	V 22.32	17.50 ab	2.47 ab
<i>P. durus</i>	CRIL 156	15.66 ab	2.39 ab
<i>P. durus</i>	C 04.50	9.66 ab	2.55 ab
<i>P. graminis</i>	MC 22.13	10.16 ab	2.30 ab
<i>P. graminis</i>	MC 04.21	18.66 ab	2.24 ab
<i>P. kribbensis</i>	POC 115	23.26 a	2.64 ab
<i>P. macerans</i>	LMD 24.10	8.16 ab	2.58 ab
<i>P. polymyxa</i>	LMD 24.16	9.33 ab	2.05 b
<i>P. polymyxa</i>	PM 04.01	11.50 ab	2.52 ab
<i>P. polymyxa</i>	Loutit	11.00 ab	2.39 ab
Absolute control	AC	21.16 ab	2.39 ab
CV (%)	-	18.59	8.79

In each column, means (three replicates) followed by same letter do not differ statistically from each other at  $p < 0.05$  according to the Tukey' test.

**Table 2.** Absolute growth rate (AGT), root length (RL), shoots (SDM) and roots (RDM) dry matter and SDM/RDM ratio in cowpea plants inoculated with *Bradyrhizobium* sp. (BR 3267) and co-inoculated with BR 3267 and plant growth-promoting bacteria (PGPB).

Treatments	AGT (cm day <sup>-1</sup> )	RL (cm)	SDM (g jar <sup>-1</sup> )	RDM (g jar <sup>-1</sup> )	SDM/ RDM
<i>Bradyrhizobium</i> sp. (BR 3267)	3.67 ab	13.40 ab	4.15 abc	0.89 abc	4.85 a
BR 3267 + <i>Bacillus</i> sp. (ANBE 31)	2.28 ab	14.56 ab	2.91 c	1.06 abc	2.92 ab
BR 3267 + <i>B. cereus</i> (440)	3.38 ab	13.50 ab	4.33 ab	1.48 abc	3.32 ab
BR 3267 + <i>B. aubitilis</i> (441)	2.77 ab	15.30 ab	3.38 bc	1.02 abc	3.54 ab
BR 3267 + <i>B. pumilus</i> (444)	4.68 a	14.23 ab	3.52 abc	0.70 bc	5.02 a
BR 3267 + <i>B. pumilus</i> (445)	2.24 ab	12.96 ab	4.01 abc	1.30 abc	3.53 ab
BR 3267 + <i>B. pumilus</i> (448)	2.12 ab	16.63 ab	4.02 abc	1.69 ab	2.41 ab
BR 3267 + <i>Brevibacillus brevis</i> (447)	3.16 ab	13.70 ab	4.31 ab	1.09 abc	4.15 a
BR 3267 + <i>B. megaterium</i> (462)	2.48 ab	13.56 ab	4.04 abc	0.90 abc	4.61 a
BR 3267 + <i>B. subtilis</i> (455)	4.56 a	13.66 ab	4.18 abc	0.93 abc	4.45 a
BR 3267 + <i>B. subtilis</i> (458)	2.59 ab	14.23 ab	3.57 abc	1.11 abc	3.73 ab
BR 3267 + <i>Paenibacillus brasilensis</i> (24)	3.88 a	16.63 ab	4.09 abc	1.07 abc	3.85 a
BR 3267 + <i>P. durus</i> (CRIP 105)	2.79 ab	14.80 ab	4.10 abc	1.03 abc	4.31 a
BR 3267 + <i>P. durus</i> (V 22.32)	3.88 a	13.70 ab	4.28 abc	1.27 abc	3.78 a
BR 3267 + <i>P. durus</i> (CRIL 156)	3.25 ab	13.26 ab	3.84 abc	1.06 abc	3.65 ab
BR 3267 + <i>P. durus</i> (C 04.50)	4.84 a	14.23 ab	4.41 ab	1.19 abc	3.73 ab
BR 3267 + <i>P. graminis</i> (MC 22.13)	3.19 ab	13.73 ab	3.57 abc	0.98 abc	4.02 a
BR 3267 + <i>P. graminis</i> (MC 04.21)	3.50 ab	13.83 ab	4.86 a	1.20 abc	4.15 a
BR 3267 + <i>P. kribbensis</i> (POC 115)	3.89 a	13.10 ab	4.37 ab	1.18 abc	3.78 a
BR 3267 + <i>P. macerans</i> (LMD 24.10)	2.97 ab	13.16 ab	4.68 ab	1.91 a	2.44 ab
BR 3267 + <i>P. polymyxa</i> (LMD 24.16)	3.52 ab	12.53 b	3.77 abc	1.08 abc	3.60 ab
BR 3267 + <i>P. polymyxa</i> (PM 04.01)	3.56 ab	15.66 ab	4.68 ab	1.54 abc	3.91 a
BR 3267 + <i>P. polymyxa</i> (Loutit)	3.00 ab	13.23 ab	4.20 abc	1.34 abc	3.14 ab
Absolute control (AC)	0.71 b	18.40 a	0.29 d	0.40 c	0.73 b
CV (%)	31.17	12.19	11.25	32.02	26.19

In each column, means (three replicates) followed by same letter do not differ statistically from each other at  $p < 0.05$  according to the Tukey' test.

**Table 3.** Nodule number (NN), nodule size (NZ), nodule dry matter (NDM), nitrogen accumulated in the shoot dry matter ( $N_{ac}$ ), nitrogen fixation efficiency ( $N_2FE$ ) and specific nodulation (SN) in cowpea plants inoculated with *Bradyrhizobium* sp. (BR 3267) and co-inoculated with BR 3267 and plant growth-promoting bacteria (PGPB).

Treatments	NN (jar <sup>-1</sup> )	NZ (mg nodule <sup>-1</sup> )	MSN (g jar <sup>-1</sup> )	$N_{ac}$ (mg N jar <sup>-1</sup> )	$N_2FE$ (mg N g <sup>-1</sup> NDM)	SN (NN g <sup>-1</sup> RDM)
<i>Bradyrhizobium</i> sp. (BR 3267)	103 a	0.0045 a	0.46 a	170.54 a	380.18 a	122.64 a
BR 3267 + <i>Bacillus</i> sp. (ANBE 31)	101 a	0.0037 a	0.37 a	82.74 c	224.59 b	98.29 a
BR 3267 + <i>B. cereus</i> (440)	135 a	0.0043 a	0.59 a	138.01 ab	235.44 ab	105.44 a
BR 3267 + <i>B. aubitilis</i> (441)	135 a	0.0031 a	0.42 a	110.36 bc	270.29 ab	151.00 a
BR 3267 + <i>B. pumilus</i> (444)	119 a	0.0040 a	0.45 a	123.28 abc	273.17 ab	166.51 a
BR 3267 + <i>B. pumilus</i> (445)	137 a	0.0040 a	0.55 a	114.49 bc	209.09 b	120.57 a
BR 3267 + <i>B. pumilus</i> (448)	105 a	0.0046 a	0.47 a	113.30 bc	239.84 ab	62.39 a
BR 3267 + <i>Brevibacillus brevis</i> (447)	115 a	0.0039 a	0.45 a	134.89 ab	307.94 ab	111.13 a
BR 3267 + <i>B. megaterium</i> (462)	132 a	0.0039 a	0.52 a	115.06 bc	220.57 b	148.63 a
BR 3267 + <i>B. subtilis</i> (455)	120 a	0.0043 a	0.52 a	131.91 abc	249.09 ab	129.53 a
BR 3267 + <i>B. subtilis</i> (458)	89 a	0.0052 a	0.44 a	111.42 bc	271.23 ab	100.09 a
BR 3267 + <i>Paenibacillus brasilensis</i> (24)	120 a	0.0041 a	0.48 a	140.08 ab	290.53 ab	113.23 a
BR 3267 + <i>P. durus</i> (CRIP 105)	116 a	0.0047 a	0.53 a	150.56 ab	282.91 ab	115.27 a
BR 3267 + <i>P. durus</i> (V 22.32)	106 a	0.0052 a	0.55 a	137.00 ab	248.66 ab	92.03 a
BR 3267 + <i>P. durus</i> (CRIL 156)	114 a	0.0045 a	0.51 a	134.32 ab	262.67 ab	110.73 a
BR 3267 + <i>P. durus</i> (C 04.50)	143 a	0.0038 a	0.54 a	140.96 ab	261.44 ab	121.46 a
BR 3267 + <i>P. graminis</i> (MC 22.13)	134 a	0.0035 a	0.45 a	119.03 bc	259.99 ab	156.42 a
BR 3267 + <i>P. graminis</i> (MC 04.21)	134 a	0.0041 a	0.54 a	169.47 a	309.39 ab	118.24 a
BR 3267 + <i>P. kribbensis</i> (POC 115)	112 a	0.0050 a	0.56 a	151.64 ab	271.09 ab	95.73 a
BR 3267 + <i>P. macerans</i> (LMD 24.10)	121 a	0.0047 a	0.55 a	113.73 bc	203.46 b	63.59 a
BR 3267 + <i>P. polymyxa</i> (LMD 24.16)	143 a	0.0034 a	0.47 a	124.35 abc	271.25 ab	131.37 a
BR 3267 + <i>P. polymyxa</i> (PM 04.01)	114 a	0.0051 a	0.54 a	143.30 ab	263.57 ab	102.95 a
BR 3267 + <i>P. polymyxa</i> (Loutit)	127 a	0.0043 a	0.54 a	121.85 abc	236.86 ab	94.09 a
Absolute control (AC)	---	---	---	4.50 d	---	---
CV (%)	18.59	23.26	16.66	12.54	18.28	34.30

In each column, means (three replicates) followed by same letter do not differ statistically from each other at  $p < 0.05$  according to the Tukey' test.



## **Capítulo II**

**Metabolism of nitrogen and carbon: optimization of BNF and cowpea  
development (*Vigna unguiculata* [L.] Walp.)**

*Artigo submetido para publicação no periódico  
Soil Biology & Biochemistry*

**Title**

Metabolism of nitrogen and carbon: optimization of BNF and cowpea development  
(*Vigna unguiculata* [L.] Walp.)

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## **Abstract**

The rhizobia-legume symbiosis provides sucrose to the bacteroids and these are oxidized and used as energy source during biological nitrogen fixation (BNF), which requires the coordinated action of enzymes in the nitrogen and carbon metabolism. In this context, this study aimed at optimizing the BNF and the development of cowpea inoculated and co-inoculated with *Bradyrhizobium* sp. and different combinations of the plant growth-promoting bacteria (PGPB) as well as evaluating the biochemical indicators related to nitrogen and carbon metabolism during and after the symbiosis establishment. The experiment was conducted in greenhouse with cowpea plants cv. "IPA 206" inoculated with *Bradyrhizobium* sp. (BR 3267) and co-inoculated with BR 3267 + *Paenibacillus graminis* (MC 04.21); with BR 3267 + *P. durus* (C 04.50); with BR 3267 + MC 04.21 + C 04.50; and without inoculation, the absolute control (AC). Plants were grown in Leonard jars containing washed and autoclaved sand and the harvest was performed in two periods [flowering point (FP) and beginning of senescence (BS)]. There was significant difference in growth variables and biochemical indicators related to metabolism in different harvest periods of the cowpea. There was increase in the concentration of total soluble proteins in nodules of plants co-inoculated compared to those inoculated isolated with the standard strain (BR 3267). There was intense proteolytic activity and a decline in enzyme activity in the BS, but there was delay of the deleterious effects of nodules senescence in the co-inoculation BR 3267 + MC 04.21 + C 04.50, ensuring the nitrogen availability for a longer time period, which allowed better symbiotic performance in cowpea plants.

**Keywords:** biological nitrogen fixation, senescence, enzymatic activities, co-inoculation, harvesting periods, *Paenibacillus graminis*.

## 1. Introduction

Nitrogen is the most abundant element in Earth's atmosphere and its deficiency results in changes of roots formation, in photosynthesis, in production and translocation of the photoassimilates and in plant growth rate (Shridhar, 2012). According to Andrews et al. (2009), the nitrogen availability can occur in different ways according to plant species. This nutrient can be absorbed from the soil or obtained from atmospheric  $N_2$  through the process of biological nitrogen fixation (BNF). The BNF can be affected by physical, chemical and/or biological factors and it is more frequent in legumes in symbiosis with fixing bacteria (Fagan et al., 2007; Franche et al., 2009).

Due to the high price of the nitrogen fertilizers, resulting from the consumption of fossil energy in their manufacture, and the serious pollution problems caused by intensive use of fertilizers, BNF presents itself as a viable alternative to supply the need of nitrogen compounds to plant species. Thus, studies are needed aiming at maximizing the BNF and, consequently, increasing the availability of nitrogen to plant until the period of grains filling and making more efficient the carbohydrates use by nodules (Gualter et al., 2011), which are used as energy source during the BNF (Larrainzar et al., 2009).

During the course of the rhizobia-legume symbiosis, plants provide carbohydrates, mainly sucrose (Shridhar, 2012). Sucrose is derived from the phloem, released after the sucrose synthase enzyme activity, key enzyme regulatory of the carbon flux in nodules, and/or neutral invertase (Ben Salah et al., 2009; 2011) and oxidized in bacteroids in order to be used as energy source during the BNF (Larrainzar et al., 2009). Furthermore, carbohydrates may serve as carbon skeletons which are necessary for the assimilation of ammonia produced during the BNF (Andrews et al., 2009; Larrainzar et al., 2009; Shridhar, 2012).

For an efficient BNF it's necessary to select efficient and competitive strains of rhizobia, and that these present synergism with other microorganisms (Figueiredo et al., 2008; Lima et al., 2011; Zilli et al., 2011). This association can result in increased BNF being necessary to observe the combination and compatibility of strains in order to increase the agricultural productivity (Figueiredo et al., 2010). This is due to the plant growth-promoting bacteria (PGPB) beneficial effects to plant species ranging from improvements in growth and development of plants until the nodulation increase (Marino et al., 2009; Compant et al., 2010). The co-inoculation of these bacteria can result in positive effects by increasing the phytohormones production and nutrients mobilization, mainly nitrogen and carbon (Ott et al., 2005; Ladrera et al., 2007; Larrainzar et al., 2009).

The hypothesis that the interrelationship of rhizobia with PGPB can provide BNF optimization and growth promotion in legume species contributing to the senescence delay was tested and examined in this study. Cowpea plants inoculated with *Bradyrhizobium* sp (BR 3267) and co-inoculated with BR 3267 + *Paenibacillus graminis* (MC 04.21); BR 3267 + *P. durus* (C 04.50); BR 3267 + MC 04.21 + C 04.50 were evaluated for the biochemical indicators related to nitrogen and carbon metabolism during and after the establishment of symbiosis.

## **2. Material and Methods**

### **2.1 Multiplication, preparation and application of inoculants**

Before the inoculants preparation, the strains multiplication to be used in the experiment was carried out. The strain *Bradyrhizobium* sp. (BR 3267; standard strain) was grown in flasks containing YM (Yeast-Mannitol) culture medium using a rotator shaker under constant agitation (200 rpm; 28 °C) for 96 h. For strains of the plants growth-promoting bacteria (PGPB) *Paenibacillus graminis* (MC 04.21) and *P. durus* (C

04.50), it was used the TSB (Trypticase Soy Broth) culture medium using a rotator shaker under constant agitation (200 rpm, 32 °C) for 24 and 48 hours, respectively.

For the formulated inoculants preparation was used 2.0 g of the dry, sieved and sterilized (120° C, 101 kPa, 15 min) exopolysaccharide (EPS) synthesized by *Rhizobium tropici* (EI-6). After autoclaving, the medium-density polyethylene bags containing the EPS were cooled in cool and dry place and kept at room temperature. Subsequently, it was added 2.0 mL of distilled and autoclaved water, and the mixture was homogenized and incubated (30 h) until the pH reached the optimum range (6.8 to 7.0). After achieving the appropriate pH, the inoculation of the bacteria in the EPS (1.0 g of the EPS to 3.0 mL of inoculum final volume) was carried out in duplicate and therefore the formation of treatments.

The inoculation with the *Bradyrhizobium* sp. standard strain (BR 3267) was performed by adding 1.5 mL of YM culture medium containing bacteria ( $10^8$  CFU mL<sup>-1</sup>) followed by addition of 1.5 mL of sterile distilled water. For co-inoculation, it was added 1.5 mL of TSB culture medium containing MC 04.21 or C 04.50 at  $10^7$  CFU mL<sup>-1</sup> and then 1.5 mL of YM medium containing BR 3267 were added. For tripartite co-inoculation, it was added 0.75 mL of TSB culture medium containing MC 04.21 and 0.75 mL of TSB culture medium with C 04.50 and then 1.5 mL of YM culture medium containing BR 3267.

Once effected the inoculation on the EPS, the material was incubated at room temperature for 48 h for maturation. After this period, 1.0 g of inoculant was taken to make mixing and treatment formulations. Each mixture (treatment) was dissolved in saline solution (NaCl 0.85%) using a rotator shaker under constant agitation (300 rpm, 28 °C, 30 min) until homogeneity and subsequently used in the seeds inoculation process (2.0 mL inoculant seed<sup>-1</sup>). The plate count of the formulated inoculants was

made by the drop plate method in serial dilution ( $10^5$  to  $10^7$ ) and made the plating on YMA culture medium with congo red.

## **2.2 Experiment preparation, inoculation and planting**

The experiment was conducted in the greenhouse of the Agronomical Institute of Pernambuco - IPA at a temperature range of 27-36 °C with 50-70% relative humidity. The seeds of cowpea cv. "IPA 206" were disinfected (Hungria and Araujo, 1994) and sown in Leonard jars containing washed (pH 6.5) and autoclaved (120 °C, 101 KPa, 1 h) sand as substrate. The seeds were inoculated with *Bradyrhizobium* sp. (BR 3267); and co-inoculated with BR 3267 + *Paenibacillus graminis* (MC 04.21); BR 3267 + *P. durus* (C 04.50); BR 3267 + MC 04.21 + C 04.50; and an absolute control (AC) without inoculation. After thinning the cowpeas at seven days, two plants were kept in each Leonard jar.

During the experimental period, plants were irrigated by capillary with Hoagland and Arnon (1950) nutritive solution modified by Silveira et al. (1998) and free of nitrogen. Plants were harvested in two periods: (1) flowering point (FP), period with higher nitrogen fixation (at 36 days) and (2) beginning of senescence (BS), period of decline in nitrogen fixation (at 56 days). During the harvest, in the respective periods, roots were collected and the nodules removed, weighed, frozen in liquid N<sub>2</sub> and stored in a freezer at -80 °C until the determinations.

## **2.3 Biochemical determinations**

### *2.3.1 Metabolites concentration*

Samples of cowpea nodules were extracted with specific reagents, and measured the concentration of ammonia (Weatherburn, 1967); total soluble proteins (Bradford, 1976); total free amino acids (Yemm and Cocking, 1955); leghemoglobin (Smagghe et

al., 2009); total nitrogen (Baethgen and Alley, 1989); ureides (Young and Conway, 1942); sucrose (van Handel, 1968); total soluble carbohydrates (Dubois et al., 1956); and starch (McCready et al., 1950). The reducing sugars concentration was estimated by subtracting the concentration of sucrose from that obtained for total soluble carbohydrates.

### 2.3.3 Enzymatic activities

To obtain the extract used in the enzymatic activity, fresh nodules were extracted with appropriated buffer and the supernatant was collected and used to determine the activity of glutamine synthetase (GS; EC 6.3.1.2) in according to Elliott (1955); glutamate synthase (GOGAT; EC 1.4.1.14) based on the method proposed by Suzuki et al. (1994); aminant glutamate dehydrogenase (GDHa; EC 1.4.1.2) according to the methodology proposed by Coombs and Hall (1982); phenylalanine ammonia-lyase (PAL; EC 4.3.1.5) based on the conversion of L-phenylalanine into trans-cinnamic acid (Zucker, 1965; El-Shora, 2002); neutral invertase (NI; EC 3.2.1.26) according to the method described by Zhu et al. (1997); and sucrose synthase (SS; EC 2.4.1.13) following the method proposed by Hubbard et al. (1989).

## 2.4 Statistical design and analysis

The experimental design was a randomized block with 2x4 factorial arrangement, two harvest periods (FP and BS) and four treatments, one inoculation (*Bradyrhizobium* sp.) and three co-inoculation of *Bradyrhizobium* sp. + PGPB, with four replications. Each variable we studied was subjected to analysis of variance (ANOVA) using the statistical software ASSISTAT version 7.6 beta by the F test and means were compared by the Tukey's test, both at 5% probability.



### 3. Results and discussion

Changes in growth variables in the shoot of cowpea plants were observed in response to the harvest periods (Fig. 1). When comparing plants inoculated with *Bradyrhizobium* sp. (BR 3267) and co-inoculated with plants growth-promoting bacteria (PGPB) in tripartite [BR 3267 + *Paenibacillus graminis* (MC 04.21) + *P. durus* (C 04.50)] during the flowering point (FP), there was no significant difference in mean shoot dry matter (SDM) and the absolute growth rate (AGR) by the Tukey's test ( $p < 0.05$ ). Since the plants co-inoculated with the tripartite showed higher SDM accumulation in beginning of senescence (BS), it can be observed that the presence of these PGPB induces a beneficial effect on plant development to maintain the photosynthetic process in order to ensure the production of photoassimilates in the shoots of the host plant that will be translocated to the nodules and used as energy source for cell divisions that occur during development (Ladrera et al., 2007; Liu et al., 2011).

The plants were co-inoculated with symbiotic pairs (BR 3267 + MC 04.21 and BR 3267 + C 04.50) did not exhibit the same effect recorded for the tripartite in SDM and AGR (Fig. 1) during the BS, which may be due to microbial combinations being less effective when formulated in pairs. Moreover, in plants co-inoculated with the tripartite were observed the best average in growth-related variables (SDM and AGR) during the BS. During the senescence process occurring physiological changes that can lead to loss of efficiency of BNF (Franche et al., 2009), however these changes were probably minimized in plants co-inoculated with the tripartite. This may be due to changes in the carbon and nitrogen flow between the plant and bacteroids in response to compounds released by promoting bacteria (Shridhar, 2012).

There were changes in variables related to nitrogen in the shoot of cowpea in response to different treatments (Fig. 2). Plants inoculated with the tripartite showed nitrogen accumulated ( $N_{ac}$ ) in shoots higher in comparison to the plants inoculated only with BR 3267 (Fig. 2A) and this may represent a response to the beneficial substances released by PGPB for cowpea plants (Lima et al., 2011). The effective association between rhizobia and PGPB can influence the BNF process (Kuklinsky-Sobral et al., 2004). In addition, a positive interaction between bacteria and plant species results in plant productivity and BNF increased (Herridge et al., 2008).

The nitrogen content did not differ significantly among treatments during the FP; however, there was difference in this variable in the BS (Fig. 2B). In BS, the plants inoculated with the tripartite stood out for having nitrogen content average significantly higher than the other treatments (Fig. 2B). This result reinforces the idea that co-inoculation with the tripartite was more effective compared to other combinations and/or interactions between fixing bacteria and those growth-promoting. According to Dawson et al. (2008), the BNF process is influenced by the symbiont genotypic characteristics and responds differently in relation to host range, specificity and symbiotic efficiency.

There were no significant differences ( $p < 0.05$ ) in fresh matter of nodules (FMN), however it was significant differences in mean FMN values comparing to the two collection periods (Fig. 3A). For nodule total nitrogen, plants inoculated with tripartite showed higher values (~70%) than those inoculated only with BR 3267 (Fig. 3B). Although the nodules had begun the senescence process, the results indicate that the energetic flow directed to the nodule production and growth in this treatment was maintained suggesting a higher efficiency of BNF process.

The BNF varies in according to biological and environmental factors and their effectiveness can be measured by the concentrations of the compounds involved in the process (Liu et al., 2011) and, in this sense, it was evaluated the nitrogen compounds concentration present in the nodules (Fig. 4). There were no significant differences in the total free amino acids (TFAA) concentration by the Tukey's test ( $p < 0.05$ ) during the FP. In the BS, the combination of plants with BR 3267 resulted in 39% increase in nodule TFAA concentration, and in plants co-inoculated with BR 3267 + MC 04.21 there was 27% accumulation in the TFAA content (Fig. 4A). This result may be a stimulus in the proteins catabolism and also a reduction in the amino acids exportation produced in the bacteroids to the host plant (Sarma and Emerich, 2005; Dawson et al., 2008).

The amino acids and ammonia exchange between the bacteria and host plant is important for the continuing the BNF process (Salavati et al., 2011). In the present study, it was observed an increased in free ammonia concentration in response to different treatments and harvest periods (Fig. 4B). Plants co-inoculated with the symbiotic pair BR 3267 + MC 04.21 and those co-inoculated with the tripartite had higher mean values of free ammonia in the nodules than those recorded for the other treatments during the FP (Fig. 4B). In BS, plants inoculated with BR 3267 showed lower ammonia accumulation in their nodules statistically differing in relation to the other treatments. The ammonia accumulation may be a reflection of the frequent protein turnover process which provides the amino acid reuse and maintains the appropriate nodule metabolism (Cheng et al., 2010).

Ammonia is produced during the BNF and, after a series of reactions, it is incorporated into amines or exported via xylem to the host plant as ureides (King and Purcell, 2005; Ladrera et al., 2007). According to King and Purcell (2005), the ureides,

as well as asparagine, are the main means of nitrogen transport from nodules to the plants shoot. The ureides concentration in plant nodules harvested in the FP was not significantly different ( $p < 0.05$ ); however, differences were observed in plants harvested in the BS (Fig. 4C). It has been proposed that high concentrations of nitrogen compounds, such as amino acids and ureides, function as indicator molecules of BNF decline (Larrainzar et al., 2009). Plants inoculated with the tripartite showed few differences in nitrogen compounds (TFAA, ammonia and ureides) levels when comparing FP and BS (Fig. 4) indicating that the interaction among the three bacteria in cowpea was more favorable and minimized the nodules senescence compared to other treatments.

For the proper nodule functioning is necessary adequate proteins content and their synthesis may be increased due to the need for specific proteins that act in the interaction process between plant and bacteria (Sarma and Emerich, 2005, Cheng et al., 2010). The concentration of total soluble protein (TSP) in cowpea nodules showed significant difference ( $p < 0.05$ ) in response to the different treatments and harvest periods (Fig. 4D). During the FP, it can be observed that the plants co-inoculated with the association between BR 3267 + PGPB showed higher TSP values than those inoculated only with BR 3267. There was observed a reduction in the TSP concentration in the nodules during the BS compared with FP, whereas plants co-inoculated with the tripartite had higher TSP content than the other treatments.

The establishment of an effective symbiosis between plants and bacteria is a complex process which involves increasing the synthesis of proteins related to different signaling and recognition pathways (Herridge et al., 2008; Compant et al., 2010). This process may have been stimulated by the PGPB presence, particularly in plants co-inoculated with a tripartite, since there was increased TSP concentration in nodules of

co-inoculated plants in relation to those inoculated with BR 3267 (Fig. 4D). In this study, the mixture of strains again favored this response probably successfully colonizing the root system.

Were recorded changes in the enzymes activity involved in the ammonia metabolism and consequently in the BNF in the bacteroids (Fig. 5), such as glutamine synthetase (GS), glutamate synthase (GOGAT), aminant glutamate dehydrogenase (GDHa) and phenylalanine ammonia-lyase (PAL) in response to the different treatments and harvest periods, except for the PAL activity, enzyme that releases ammonia in its reaction, which showed no changes (Fig. 5B). During the FP, only plants co-inoculated with BR 3267 + MC 04.21 showed reduction in the GS (Fig. 5A), GOGAT (Fig. 5C) and GDHa (Fig. 5D) activity in comparison to other treatments. These enzymes can have their activity differentially modulated in response to numerous endogenous and environmental stimuli (Franche et al., 2009; Larrainzar et al., 2009).

The GS/GOGAT system activity increases as the nodules develop, being GS considered the key enzyme of BNF (Larrainzar et al., 2009). Together with GOGAT, the GS converts ammonia into amino acids avoiding its excessive accumulation within the bacteroids, which can result in toxicity and BNF inhibition (Prell and Poole, 2006; Fagan et al., 2007). Besides these enzymes, GDHa can also metabolize ammonia, especially in conditions where there is excess; however, some authors highlight that this enzyme has less importance than the others (Patriarca et al., 2002; Bernard and Habash, 2009). The GS, GOGAT and GDHa activity were measured at the BS; however, only GS activity was detected in nodules of plants nodulated with BR 3267 and in the co-inoculated with BR 3267 + PGPB (Fig. 5). The partial GS inhibition, combined with the absence of GDHa and GOGAT enzymatic activity, seems to be a direct response to the start of the senescence process, whereas this phase of nodule development there is

reduction of TSP concentration and increase of ammonia content in bacteroids, results that corroborate with those found by Patriarca et al. (2002).

The nodule growth and the BNF in bacteroids consume considerable amounts of the carbon. This is due to the need for energy and specific metabolites to supply the BNF that are provided after the respiratory catabolism of carbohydrates produced by plants (Franche et al., 2009). In this study, the concentration of total soluble carbohydrates (TSC), sucrose and reducing sugars (RS) were significantly reduced in nodules of cowpea plants co-inoculated with BR 3267 + C 04.50 and in those co-inoculated in tripartite compared to plants inoculated only with BR 3267 during the FP (Fig. 6A-C). Plants co-inoculated in tripartite had lower starch content compared to other treatments during the FP (Fig. 6D). This response may represent a higher energy demand for cell divisions that occur during the PGPB colonization in the host plant (Prell and Poole, 2006; Larrainzar et al., 2009).

During the BS, plants inoculated only with BR 3267 showed reduction in sucrose concentration (~85%) while the TSC, starch and reduced sugars (RS) contents, were not significantly altered compared to the FP (Fig. 6). In plants co-inoculated in tripartite there was increase in starch concentration (~45%) and the TCS, sucrose and RS content did not differ significantly by the Tukey's test ( $p < 0.05$ ). The photosynthates is imported into the nodule and used as carbon skeletons in the ammonia assimilation produced by bacteria within the nodules (Larrainzar et al., 2009) and when they are not metabolized, due to partial or complete blockage of BNF, it can occur accumulation as starch (Ben Salah et al., 2009).

The appropriate BNF in bacteroids can be achieved by maintaining of the photoassimilates levels, mainly sucrose (Ben Salah et al., 2011). Sucrose is formed in the leaves by photosynthesis and translocated to the roots; this process being regulated

by sucrose synthase (SS). The SS activity decrease may not necessarily be offset by both neutral (NI) and soluble acid (SAI) invertase activities (Ben Salah et al., 2009). Thus, the SS is considered the key enzyme regulating the carbon metabolism and crucial for adequate BNF in nodules (Marino et al., 2009).

In this study, the enzymes of carbon metabolism - sucrose synthase (SS) and soluble acid invertase (SAI) - were evaluated in the nodules of cowpea plants inoculated with BR 3267 and in co-inoculated with BR 3267 + PGPB in the different treatments and harvest periods (Fig. 7). The results showed that the SS activity in the FP remained higher in nodules of plants co-inoculated with the PGPB compared to plants inoculated with BR 3267 (Fig. 7A). For the SAI activity during the FP, there was no significant difference by the Tukey's test ( $p < 0.05$ ) among the different treatments (Fig. 7B). Although the invertase can cleave sucrose, SS is the major enzyme conducting this process (Duncan et al., 2006). Thus, it is possible that the high SS activity in plants inoculated with the PGPB has been inhibited the SAI activity in FP.

The SS activity was reduced while the SAI was significantly increased in the BS (Fig. 7). It is likely that the increased SAI activity recorded in the BS has compensated for the SS reduction and maintained the sucrose supply at optimal levels to prevent the starch synthesis inhibition, which is necessary when the photosynthates consumption in the nodules is probably reduced due to a BNF decline in the bacteroids (Duncan et al., 2006). In plants co-inoculated with the tripartite, the increased SAI activity (~121%) was more pronounced than the other treatments and this response must have been crucial to keep unchanged the carbon skeletons concentration avoiding changes in the BNF ability in cowpea nodules.

#### 4. Conclusions

There was increase in the total soluble proteins concentration in nodules of plants co-inoculated compared to those inoculated isolated with the standard strain (BR 3267). There was intense proteolytic activity and enzymatic activity decline in the beginning of senescence (BS), but there was delay of the deleterious effects of nodules senescence in the co-inoculation with the tripartite (BR 3267 + MC 04.21 + C 04.50) ensuring the nitrogen availability for a longer time period, which allowed better symbiotic performance in cowpea plants.

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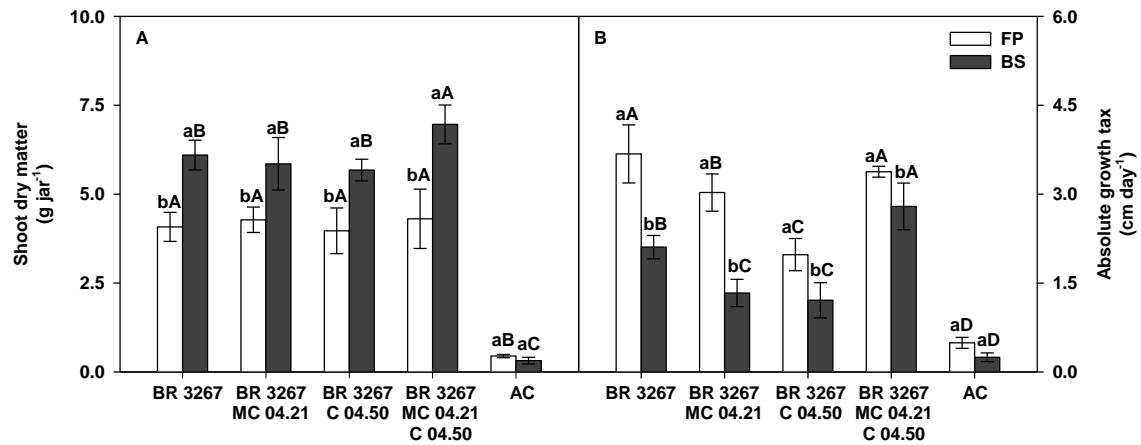
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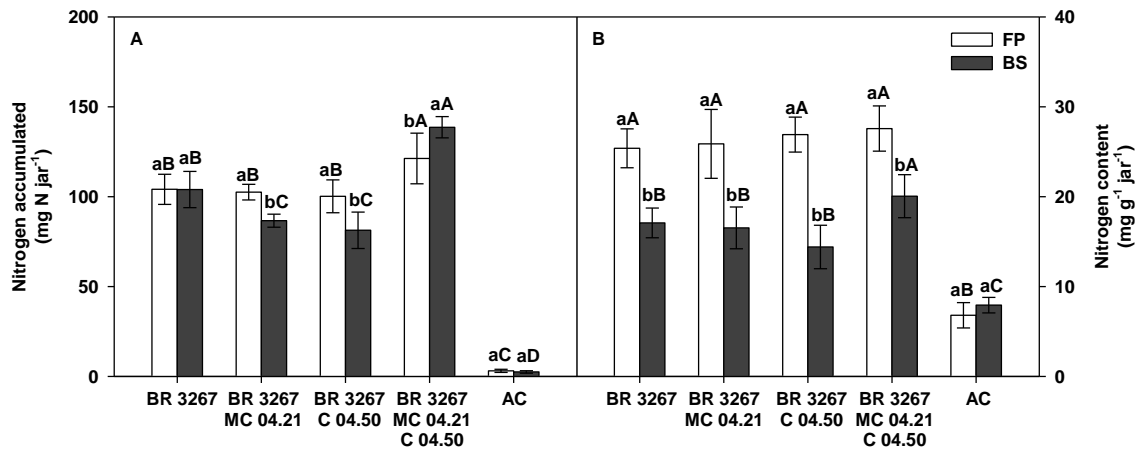
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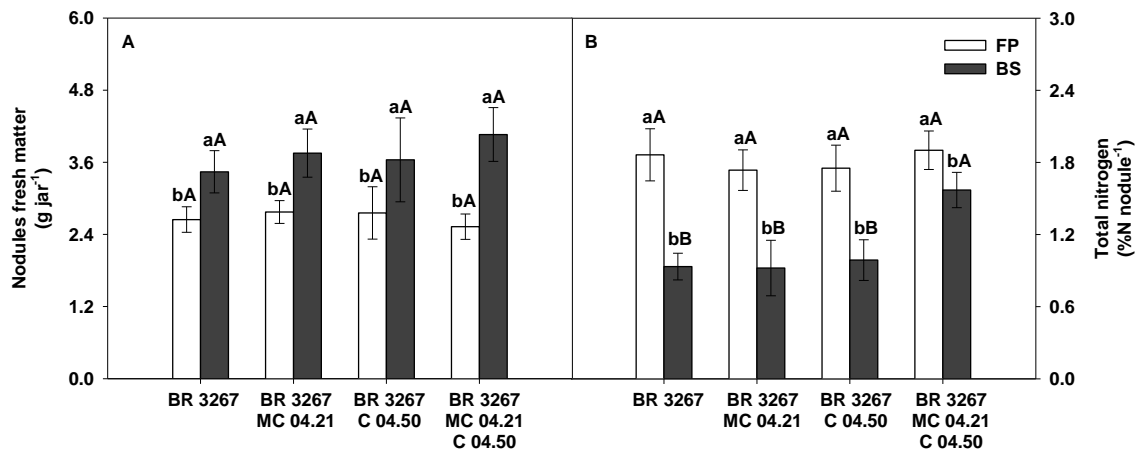
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**Fig. 1.** Characterization of the cowpea plants. (A) Shoot dry matter (SDM; CV<sup>\*</sup>=11.06%) and (B) absolute growth tax (AGT; CV=14.10%) in cowpea plants inoculated with *Bradyrhizobium* sp. (BR 3267); co-inoculated with BR 3267 + *Paenibacillus graminis* (MC 04.21); with BR 3267 + *P. durus* (C 04.50); with BR 3267 + MC 04.21 + C 04.50; and an absolute control (AC) without inoculation. Means (four replicates) followed by the same letter [lower (harvest periods (FP, flowering point; BS, beginning of senescence) and capital (treatments) letters] not differ statistically ( $p < 0.05$ ) according to Tukey's test. \* variation's coefficient.

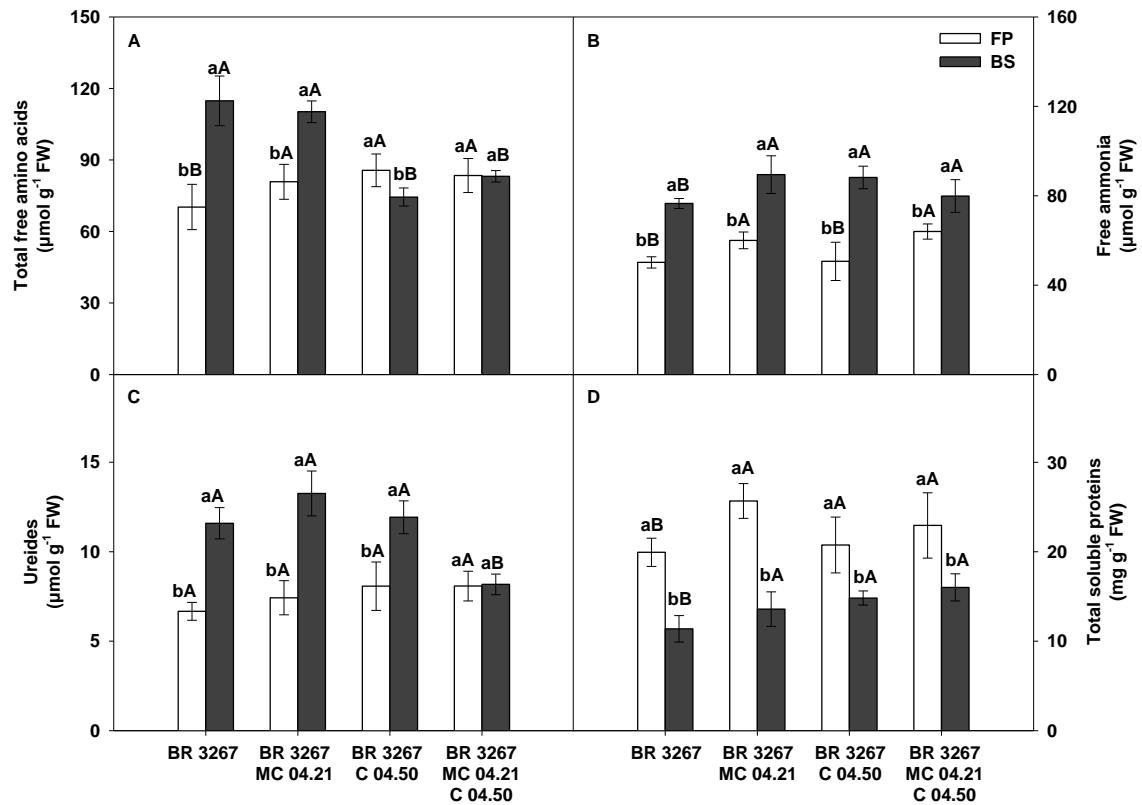


**Fig. 2.** Characterization of the cowpea plants. (A) Nitrogen accumulated ( $N_{ac}$ ;  $CV^*=9.20\%$ ) and (B) Nitrogen content ( $CV=11.58\%$ ) in cowpea plants inoculated with *Bradyrhizobium* sp. (BR 3267); co-inoculated with BR 3267 + *Paenibacillus graminis* (MC 04.21); with BR 3267 + *P. durus* (C 04.50); with BR 3267 + MC 04.21 + C 04.50; and an absolute control (AC) without inoculation. Means (four replicates) followed by the same letter [lower (harvest periods (FP, flowering point; BS, beginning of senescence) and capital (treatments) letters] not differ statistically ( $p < 0.05$ ) according to Tukey's test. \* variation's coefficient.

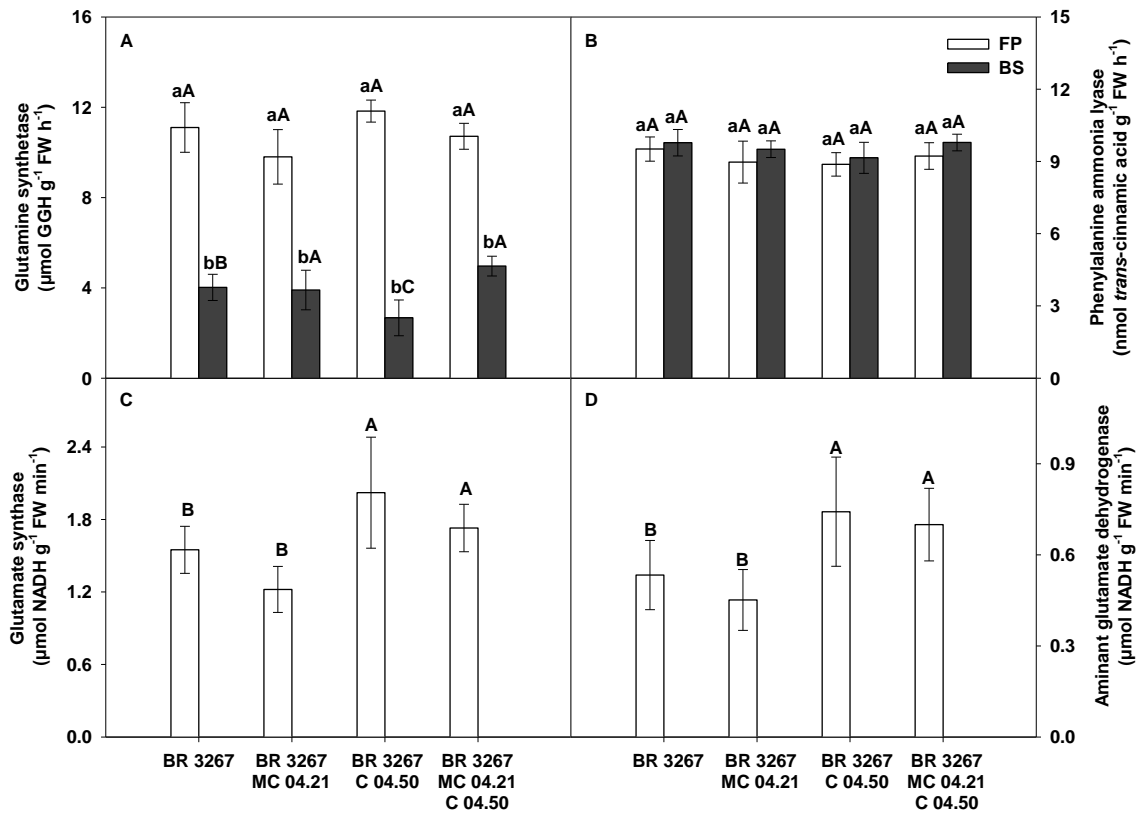


**Fig. 3.** Characterization of the nodules. (A) nodules fresh matter ( $CV^*=12.64\%$ ) and (B) total nitrogen ( $CV=11.81\%$ ) in cowpea plants inoculated with *Bradyrhizobium* sp. (BR 3267); co-inoculated with BR 3267 + *Paenibacillus graminis* (MC 04.21); with BR 3267 + *P. durus* (C 04.50); with BR 3267 + MC 04.21 + C 04.50. Means (four replicates) followed by the same letter [lower (harvest periods (FP, flowering point; BS, beginning of senescence) and capital (treatments) letters] not differ statistically ( $p<0.05$ ) according to Tukey's test. \* variation's coefficient.

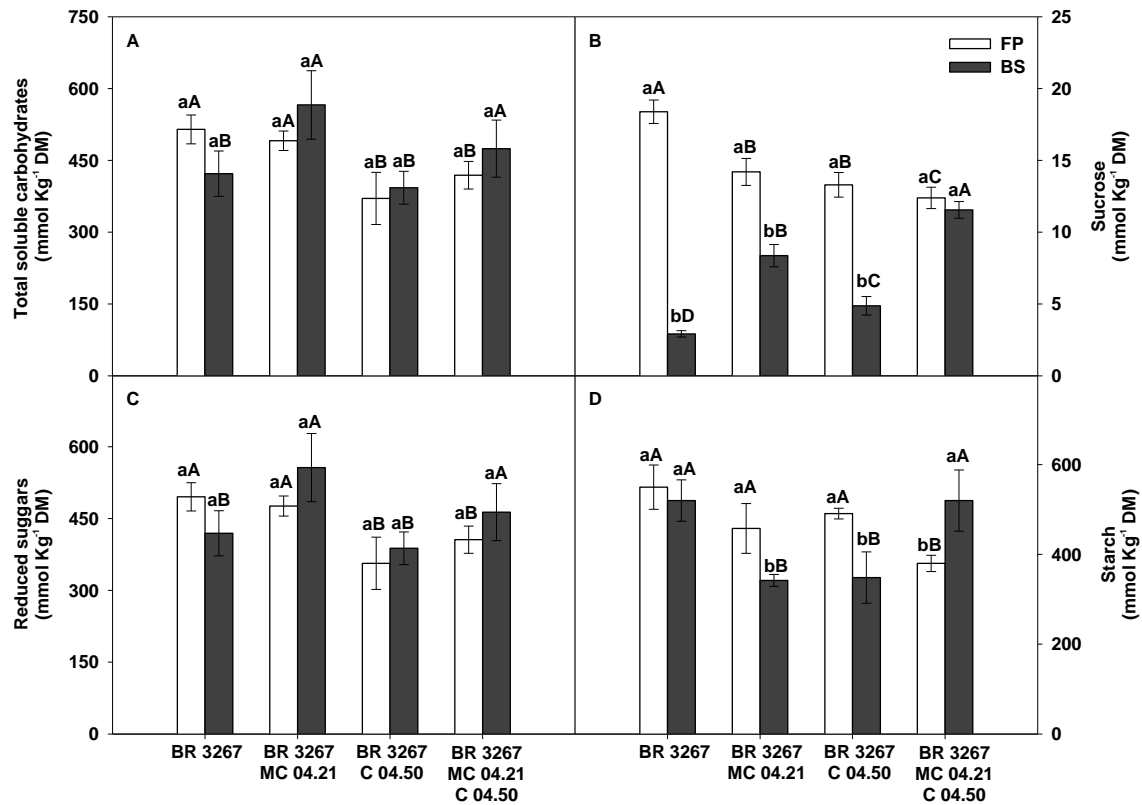




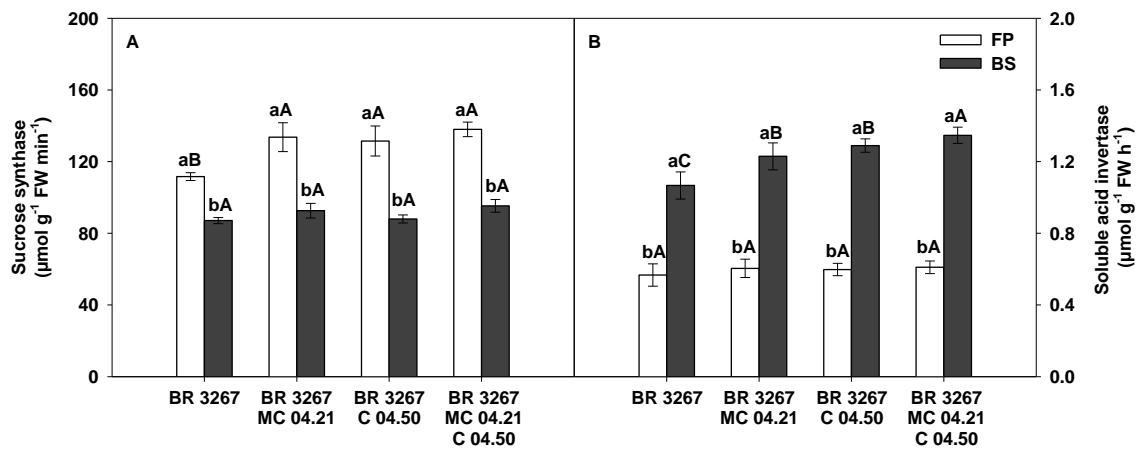
**Fig. 4.** Nitrogen metabolism compounds. (A) Total free amino acids ( $CV^* = 7.96\%$ ), (B) free ammonia ( $CV = 7.52\%$ ), (C) ureides ( $CV = 10.57\%$ ) and (D) total soluble proteins ( $CV = 12.04\%$ ) in cowpea plants inoculated with *Bradyrhizobium* sp. (BR 3267); co-inoculated with BR 3267 + *Paenibacillus graminis* (MC 04.21); with BR 3267 + *P. durus* (C 04.50); with BR 3267 + MC 04.21 + C 04.50. Means (four replicates) followed by the same letter [lower (harvest periods (FP, flowering point; BS, beginning of senescence) and capital (treatments) letters] not differ statistically ( $p < 0.05$ ) according to Tukey's test. \* variation's coefficient.



**Fig. 5.** Enzymes involved in nitrogen metabolism. (A) Glutamine synthetase (GS;  $CV^*=10.95\%$ ), (B) phenylalanine ammonia-lyase (PAL;  $CV=6.00\%$ ), (C) glutamate synthase (GOGAT;  $CV=17.47\%$ ) and (D) aminant glutamate dehydrogenase (GDHa;  $CV=21.76\%$ ) in cowpea plants inoculated with *Bradyrhizobium* sp. (BR 3267); co-inoculated with BR 3267 + *Paenibacillus graminis* (MC 04.21); with BR 3267 + *P. durus* (C 04.50); with BR 3267 + MC 04.21 + C 04.50. Means (four replicates) followed by the same letter [lower (harvest periods (FP, flowering point; BS, beginning of senescence) and capital (treatments) letters] not differ statistically ( $p < 0.05$ ) according to Tukey's test. \* variation's coefficient.



**Fig. 6.** Carbon metabolism compounds. (A) total soluble carbohydrates (TSC; CV<sup>\*</sup>=10.73%), (B) sucrose (CV=6.91%), (C) reduced sugars (RS; CV=10.97%) and (D) starch (CV=10.20%) in cowpea plants inoculated with *Bradyrhizobium* sp. (BR 3267); co-inoculated with BR 3267 + *Paenibacillus graminis* (MC 04.21); with BR 3267 + *P. durus* (C 04.50); with BR 3267 + MC 04.21 + C 04.50. Means (four replicates) followed by the same letter [lower (harvest periods (FP, flowering point; BS, beginning of senescence) and capital (treatments) letters] not differ statistically ( $p < 0.05$ ) according to Tukey's test. \* variation's coefficient.



**Fig. 7.** Enzymes involved in carbon metabolism. (A) Sucrose synthase (SS; CV<sup>\*</sup>=5.68%) and (B) soluble acid invertase (SAI; CV=5.91%) in cowpea plants inoculated with *Bradyrhizobium* sp. (BR 3267); co-inoculated with BR 3267 + *Paenibacillus graminis* (MC 04.21); with BR 3267 + *P. durus* (C 04.50); with BR 3267 + MC 04.21 + C 04.50. Means (four replicates) followed by the same letter [lower (harvest periods (FP, flowering point; BS, beginning of senescence) and capital (treatments) letters] not differ statistically (p<0.05) according to Tukey's test. \*variation's coefficient.

## **Capítulo III**

**Minimization of oxidative stress in cowpea nodules by interrelationship**

***Bradyrhizobium* sp. and plant growth-promoting bacteria**

*Artigo submetido para publicação no periódico  
Applied Soil Ecology*

## **Title**

Minimization of oxidative stress in cowpea nodules by interrelationship *Bradyrhizobium* sp. and plant growth-promoting bacteria

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## Abstract

Oxidative damage can result in various degenerative processes, including membrane lipid peroxidation that is a process promoted by chain reactions initiated by reactive oxygen species and can quickly affect many lipid molecules resulting in damage to cellular structures. This study aimed to evaluate the symbiotic performance of cowpea plants inoculated and co-inoculated with *Bradyrhizobium* sp. and different combinations of the plant growth-promoting bacteria (PGPB) based on biochemical variables related to protection/oxidative stress and senescence during and after the symbiosis establishment. The experiment was conducted in greenhouse using cowpea cv. "IPA 206". Seeds were disinfected and inoculated with *Bradyrhizobium* sp. (BR 3267), co-inoculated with BR 3267 + *Paenibacillus graminis* (MC 04.21); with BR 3267 + *P. durus* (C 04.50); with BR 3267 + MC 04.21 + MC 04.50 (tripartite); and an absolute control, without inoculation; and grown in Leonard jars containing washed and autoclaved sand. The harvest of cowpea nodules was performed at flowering point (FP) and beginning of senescence (BS). The results show significant differences in variables related to antioxidant metabolism in response to treatments and harvest periods. Although there has been less ascorbate peroxidase (APX), catalase (CAT) and phenols peroxidase (POX) activities in plants co-inoculated with BR 3267 + MC 04.21 and BR 3267 + C 04.50 in FP, it was not registered hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) accumulation or increase in lipid peroxidation, indicating that in these plants the enzymatic activity was effective in controlling possible oxidative damage. The cowpea plants co-inoculated with PGPB, particularly those co-inoculated with the tripartite, there was a delay of the deleterious effects of senescence and therefore a better symbiotic performance.

**Keywords:** PGPB, metabolism, senescence, enzymatic antioxidant, N<sub>2</sub> fixation, reactive oxygen species (ROS).

## 1. Introduction

Rhizobia can interact positively with plant growth-promoting bacteria (PGPB) and they have the ability to promote plant growth in legume species contributing to increase of the crop productivity (Lima et al., 2011). Furthermore, the PGPB may act in the biological control, promote improvements in plants growth and development and provide increased nodulation (Marino et al., 2009; Compant et al., 2010). The co-inoculation of these bacteria can result in positive effects by increasing the phytohormones production and nutrients mobilization, mainly nitrogen and carbon (Ott et al., 2005; Ladrera et al., 2007; Larrainzar et al., 2009).

During legume with fixing bacteria symbiosis, plants provide sugars to bacteroids that are oxidized and used as energy source to sustain the process of biological nitrogen fixation (BNF) (Larrainzar et al., 2009). During the BNF, due to the highly reducing environment may be formed reactive oxygen species (ROS), such as superoxide radical ( $O_2^{\bullet-}$ ) and hydrogen peroxide ( $H_2O_2$ ), which can cause oxidative damage (Puppo et al., 2005, Møller et al., 2007). In addition, ROS can be produced by leghemoglobin autoxidation and some proteins with strong reducing potential (Chang et al., 2009) and act as signaling molecules (Marino et al., 2009).

Control systems of the ROS levels in the cell are needed to avoid possible oxidative damage and promote adequate redox balance (Møller et al., 2007; Becana et al., 2010). Such redox balance is result of the equilibrium between protective and reparative antioxidant systems and these are formed by non-enzymatic antioxidants, such as ascorbate and glutathione, and enzymatic, mainly catalase, superoxide dismutase and ascorbate peroxidase (Puppo et al., 2005). An efficient protection against oxidative stress is required to maintain adequate BNF and delay the senescence (Marino et al., 2009).



We undertook this study as a first step to evaluate if inoculation and co-inoculation of cowpea with *Bradyrhizobium* and *Paenibacillus* could keep adequate BNF during and after the symbiosis establishment, based in the determinations of the compounds and enzymes indicative of senescence and/or oxidative protection.

## **2. Material and Methods**

### **2.1 Preparation and application of inoculants formulated with exopolysaccharide (EPS) synthesized by rhizobia**

Before the inoculants preparation, it was proceeded the strains multiplication to be used in the experiment. The strain of *Bradyrhizobium* sp. (BR 3267; standard strain) was grown in flasks containing YM (Yeast-Mannitol) culture medium using a rotator shaker under constant agitation (200 rpm; 28 °C) for 96 h. For strains of the plants growth-promoting bacteria in (PGPB) *Paenibacillus graminis* (MC 04.21) and *P. durus* (C 04.50), it was used the TSB (Trypticase Soy Broth) culture medium using a rotator shaker under constant agitation (200 rpm, 32 °C) for 24 and 48 hours, respectively.

For the preparation of formulated inoculants were used 2.0 g of dry, sieved and autoclaved (120 °C; 101 kPa; 15 min) exopolysaccharide (EPS) synthesized from *Rhizobium tropici* (EI-6). After autoclaving, the medium-density polyethylene bags containing the EPS were cooled in a fresh and dry place and kept at room temperature. Subsequently, it was added 2.0 mL of autoclaved and distilled water and the mixture was homogenized and incubated (30 h) until the pH reached the optimum range (6.8 to 7.0). After achieving the appropriate pH, the bacteria inoculation was carried out in duplicate in the EPS (1.0 g EPS to 3.0 mL inoculums final volume) and therefore forming the treatments.

The inoculation with standard strain was performed by adding 1.5 mL YM culture medium containing BR 3267 ( $10^8$  CFU mL<sup>-1</sup>) followed by addition of 1.5 mL of sterile and distilled water. For co-inoculation, it was added 1.5 mL of TSB culture medium containing MC 04.21 or C 04.50 at  $10^7$  CFU mL<sup>-1</sup> and then it was added 1.5 mL YM culture medium containing BR 3267. For co-inoculation in tripartite, it was added 0.75 mL TSB culture medium containing MC 04.21 and 0.75 mL TSB medium with C 04.50 and then 1.5 mL YM culture medium containing BR 3267 were added.

Once the EPS was inoculated, the material was incubated at room temperature for 48 h for maturation. After this period, 1.0 g of inoculant was taken to make the mixture and treatments formulation. Each mixture (treatment) was dissolved in saline solution (0.85% NaCl) and stirred until homogeneity. Then, the material was kept in agitation (300 rpm; 28 °C; 30 min) and subsequently used in the process of seed inoculation (2.0 mL inoculant per seed). The formulated inoculants count in plate was made by the drop plate method in serial dilution and plating of dilutions ( $10^5$  to  $10^7$ ) in YMA culture medium with congo red.

## **2.2 Experiment preparation, inoculation, and planting**

The experiment was conducted in greenhouse of the Agronomical Institute of Pernambuco - IPA at a temperature range of 27-36 °C with 50-70% relative humidity. The seeds of cowpea cv. "IPA-206" were disinfected (Hungria and Araujo, 1994) and sown in Leonard jars containing washed (pH 6.5) and autoclaved (120 °C, 101 KPa, 1 h) sand as substrate. The seeds were inoculated with *Bradyrhizobium* sp. (BR 3267); and co-inoculated with BR 3267 + *Paenibacillus graminis* (MC 04.21); with BR 3267 + *P. durus* (C 04.50); with BR 3267 + MC 04.21 + C 04.50; and an absolute control (AC)

without inoculation. After thinning the cowpeas at seven days, two plants were kept in each Leonard jar.

During the experimental period, plants were irrigated by capillary with Hoagland and Arnon (1950) nutritive solution modified by Silveira et al. (1998) and free nitrogen. Plants were collected in two periods: (1) flowering point (FP), period with higher nitrogen fixation (at 36 days) and (2) beginning of senescence (BS), period of nitrogen fixation decline (at 56 days). During the harvest, in the respective periods, roots were collected and nodules removed, weighed, frozen in liquid N<sub>2</sub> and stored in a freezer at -80 °C until the determinations.

## **2.3 Biochemical determinations**

### *2.3.1 Leghemoglobin concentration (LHb)*

The extraction and determination of LHb concentration in cowpea nodules was performed using the Drabkin's reagent following the method described by Smagghe et al. (2009) and the data expressed in mg g<sup>-1</sup> FW.

### *2.3.2 Determination of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and lipid peroxidation*

To obtain the extract used in determining the H<sub>2</sub>O<sub>2</sub> concentration and lipid peroxidation, nodules were extracted with 5% TCA and the supernatant collected and used for determinations. The H<sub>2</sub>O<sub>2</sub> concentration was determined as described by Brennan and Frenkel (1977) and data expressed in μmol g<sup>-1</sup> FW. The lipid peroxidation was determined in according to Heath and Packer (1968) and data expressed as nmol MDA-TBA g<sup>-1</sup> FW.

### 2.3.3 Enzymatic activities

To obtain the extract used in the enzymatic activity, fresh nodules were extracted with 100 mM buffer potassium phosphate (pH 7.0) and the supernatant was collected and used to determine the activity of lipoxygenase (LOX; EC 1.13.11.12) according to Axelrod et al. (1981); proteases (EC 3.4) according to the method proposed by Ainouz and Freitas (1991); Uricase (EC 1.7.3.3) according to Schubert (1981); catalase (CAT; EC 1.11.1.6) according to Havir and McHale (1987); ascorbate peroxidase (APX; EC 1.11.1.1), according to the method described by Nakano and Asada (1981); phenols peroxidase (POX; EC 1.11.1.7) by the method of Amako et al. (1994); and superoxide dismutase (SOD; EC 1.15.1.1) as described by Gianopolitis and Ries (1977).

### 2.4 Statistical design and analysis

The experimental design was a randomized block with 2x4 factorial arrangement, two harvest periods (FP and BS) and four treatments, one with inoculation (*Bradyrhizobium* sp.) and three co-inoculation of *Bradyrhizobium* sp. + BPCP's, with four replications. Each variable studied was subjected to analysis of variance (ANOVA) using the statistical software ASSISTAT version 7.6 beta by the F test and means were compared by the Tukey test, both at 5% probability.

## 3. Results and discussion

The overproduction of reactive oxygen species (ROS) or oxidative stress is a secondary response normally stimulated under stress conditions or metabolic decline, such as nodules senescence (Becana et al., 2010; Ben Salah et al., 2011). ROS, mainly hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and superoxide radical (O<sub>2</sub><sup>•-</sup>), can react with innumerable molecules present in cells, such as amino acids, proteins, lipids and until DNA, causing

oxidative damage (Møller et al. 2007) that can break off the normal cell operation (Silveira et al., 2011).

The H<sub>2</sub>O<sub>2</sub>, besides being capable of causing damage to cellular structures, it has been highlighted as a powerful signaling to several cellular events (Møller et al., 2007) and has a central role in oxidative metabolism being detected during infection, development and senescence of bacteroids (Becana et al., 2010). As shown in fig. 1A, plants co-inoculated with BR 3267 + *Paenibacillus graminis* (MC 04.21) and with BR 3267 + *P. durus* (C 04.50) during the FP had lower H<sub>2</sub>O<sub>2</sub> content in nodules than plants inoculated with BR 3267 and with the BR 3267 + MC 04.21 + C 04.50 (tripartite). In order to maintain normal function of the nodule, it is necessary an efficient control of ROS levels which is widely distributed in bacteroids, acting coordinately to promote oxidative protection (Silveira et al., 2011).

It can be observed that plants inoculated with BR 3267 isolated had higher H<sub>2</sub>O<sub>2</sub> levels than the other treatments in beginning of senescence (BS) (Tukey's test, p<0.05), while those co-inoculated with the tripartite the H<sub>2</sub>O<sub>2</sub> concentration was unchanged in the nodules compared to flowering point (FP) and BS (Fig. 1A). This response may suggest that plants inoculated with BR 3267 lacked an adequate control system of the H<sub>2</sub>O<sub>2</sub> levels, which has its production naturally increased when nodules enter the senescence process (Becana et al., 2010). It is possible that plants co-inoculated with the tripartite have stimulated the antioxidant defense systems during the FP and this may have triggered the most favorable response registered in plants in the BS avoiding possible degenerative processes, such as lipid peroxidation, are triggered (Salavati et al., 2011).

In this study, as shown in fig. 1B, it can be noted that the level of lipid peroxidation was changed in response to different treatments evaluated. The lipid

peroxidation is a process that occurs by chain reactions initiated by ROS and can quickly affect many lipid molecules resulting in damage to cellular structures (Puppo et al., 2005; Salavati et al., 2011). The means of this variable was increased in nodules of plants inoculated isolated with BR 3267 compared to other treatments in both FP and BS and this response may indicate lower efficiency of the antioxidant system in the nodules of these plants.

Plants co-inoculated with the tripartite exhibited increase in lipid peroxidation in nodules from 15% when comparing the two harvest periods, while in the other treatments this increase was in around 30% (Fig. 1B). This response of plants co-inoculated with the tripartite corroborates those presented by Ben Salah et al. (2011) working on two *Medicago* species and may be related to a more efficient antioxidant system that minimized the harmful effects of oxidative stress and delayed the nodules senescence of these plants.

The lipid peroxidation may be a response to the increase in lipoxygenase (LOX) activity rate that is located in lipid bodies, peroxisomes and plastids present in nodule cells (Feussner and Wasternack, 2002; Becana et al., 2010). The LOX catalyzes the O<sub>2</sub> addition to the pentadiene system of polyunsaturated fatty acids forming the corresponding hydroperoxides of fatty acids (Michalak, 2006). Plants co-inoculated with BR 3267 + MC 04.21; with BR 3267 + C 04.50; and in tripartite showed significantly greater LOX activity during FP in the Tukey's test with (p<0.05) compared to plants nodulated only with BR 3267 (Fig. 2). Plants co-inoculated with BR 3267 + C 04.50 and those co-inoculated in tripartite showed lower and higher LOX activity, respectively, during the beginning of the senescence process in the nodules (Fig. 2).

During the senescence process in the nodules, it is natural that occur an reduction in leghemoglobin concentration (LHB) (Ott et al., 2005; Ben Salah et al.,

2011; Liu et al., 2011), as observed in the nodules of plants co-inoculated with BR 3267 + PGPB when collected in the FP compared to plants inoculated only with BR 3267 (Fig. 3). This response seems to be related to the beneficial compounds synthesized by PGPB and released in the roots surface. Such favorable response was also observed in nodules of co-inoculated plants, mainly in those co-inoculated with the tripartite, and it has not been registered for plants inoculated only with BR 3267, which showed an LHB reduction superior to 50% compared to FP (Fig. 3). The LHB is a protein responsible for creating a barrier oxygen diffusion making it the ideal environment for the bacteroids development (Ma et al., 2011) and their concentrations can be reduced during senescence by the action of compounds related to oxidative stress and also by the increased proteolysis via proteases action present in bacteroid (Ott et al., 2005).

According to Cheng et al. (2010), proteases are enzymes that hydrolyze peptide links of proteins releasing small peptides and/or amino acids and in this study these enzymes are strongly induced in the plant nodules in the BS unlike that recorded during the FP, where no significant difference was showed for the different treatments (Fig. 4A). Such induction of proteolytic activity in cowpea nodules in the BS may have been responsible by not detect the uricase activity in the BS (Fig. 4B). In FP, as shown in fig. 4B, the uricase activity in nodules did not differ significantly ( $p < 0.05$ ). Uricase is an enzyme present in nodules that acts synthesizing ureides from uric acid and this reaction results in the  $H_2O_2$  production (Werner and Witte, 2011).

The ascorbate peroxidase (APX) and catalase (CAT) enzymes remove  $H_2O_2$  by means of different mechanisms which result equally in water (Moller et al., 2007; Ben Salah et al., 2011). As observed in fig. 5A and 5B, there was reduction in the CAT and APX activity in plant nodules co-inoculated with BR 3267 + MC 04.21 and with BR 3267 + C 04.50 during FP; while in the tripartite APX and CAT activities remained

equal to the obtained for those inoculated only with BR 3267 (Fig. 5A and 5B). In the BS, overall, the APX and CAT activities were reduced, though in plants inoculated with the tripartite the activity of these enzymes has remained high compared to the other treatments (Fig. 5A and 5B). The reduction in the rate of CAT activity in bacteroids can reduce symbiotic efficiency according to results of Jebara et al. (2005), corroborating the data presented in this paper.

The APX activity protects the leghemoglobin and other proteins from the  $H_2O_2$  action and may be involved in maintaining the oxygen diffusion barrier present in the bacteroids (Marino et al., 2009; Becana et al., 2010). In addition to APX and CAT activities, phenols peroxidase (POX) can degrade  $H_2O_2$  using phenolic compounds or ascorbate as electron donors (Michalak, 2006). It was observed in FP an increased POX activity in plant nodules inoculated only with BR 3267 compared to other treatments. In BS, the POX activity did not differ statistically according to the Tukey's test ( $p < 0.05$ ) among treatments (Fig. 5C). Although there has been less activity of APX, CAT and POX in plants co-inoculated with BR 3267 + MC 04.21 and BR 3267 + C 04.50 in FP was not registered  $H_2O_2$  accumulation or increase in lipid peroxidation, indicating that the activity rate of these enzymes was effective in controlling possible oxidative damage in these plants.

Besides of the APX, CAT and POX activities, the enzyme superoxide dismutase (SOD) operates in the enzymatic antioxidant system of detoxification of ROS excess (Puppo et al., 2005; Michalak, 2006) can act on the control of several metabolic pathways since they can control the gene expression related to oxidative stress (Puppo et al., 2005; Salavati et al., 2011). The SOD activity in plant nodules co-inoculated in the FP was greater than that presented in plants inoculated only with BR 3267 according to Tukey's test ( $p < 0.05$ ) (Fig. 5D) and this result may account for the  $H_2O_2$



accumulation recorded in this treatment (see Fig. 1A). In BS, the SOD activity in plants co-inoculated with the tripartite was lower (~15%) than that reported for other treatments (Fig. 5D). According to Chang et al. (2009), the presence of SOD enzyme in bacteroids has a critical protective role to nodulation and BNF efficient and still acts in the delay of senescence processes.

#### **4. Conclusions**

Although there was less ascorbate peroxidase, catalase and phenols peroxidase activity in plants co-inoculated with BR 3267 + MC 04.21 and with BR 3267 + C 04.50 at the flowering point, there was no H<sub>2</sub>O<sub>2</sub> accumulation or increase in lipid peroxidation, indicating that the activity rate of these enzymes was effective in controlling possible oxidative damage in these plants. Plants co-inoculated with BR 3267 + *Paenibacillus graminis* (MC 04.21); with BR 3267 + *P. durus* (C 04.50), and mainly with BR 3267 + MC 04.21 + MC 04.50 (tripartite) showed better results on the biochemical indicators related to antioxidant metabolism and thus, it can be suggested that the co-inoculation with PGPB with the tripartite in cowpea resulted in delay of the deleterious effects of senescence.

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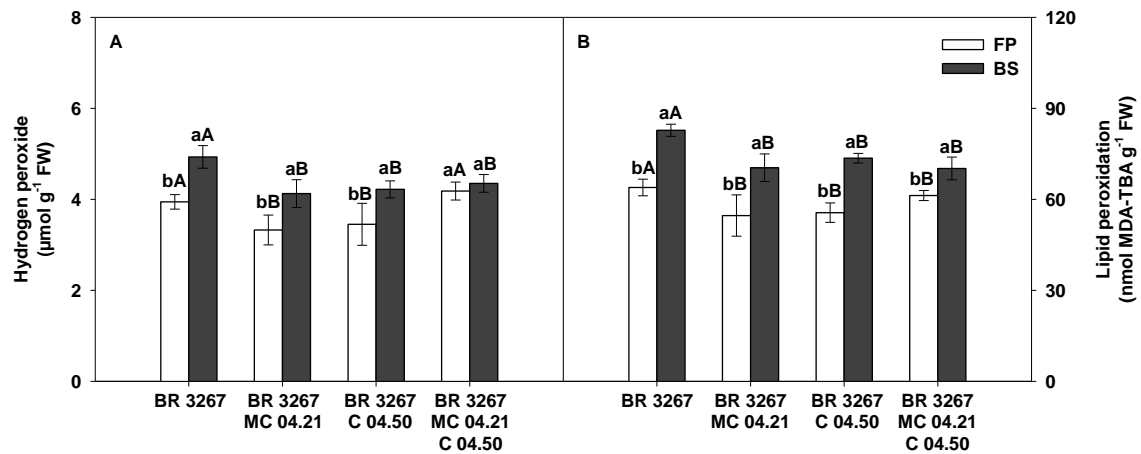
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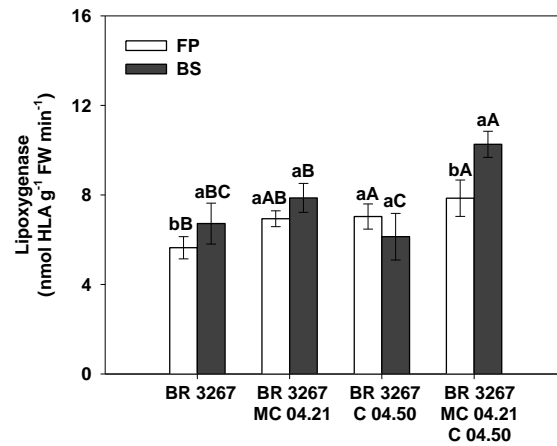
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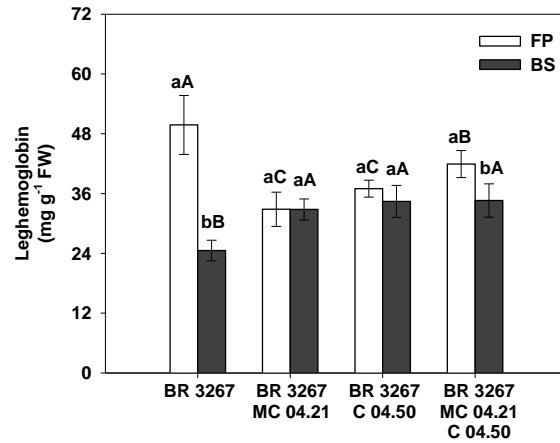
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**Fig. 1.** Indicators of oxidative stress. (A) Hydrogen peroxide ( $CV^*=6.08\%$ ) and (B) lipid peroxidation ( $CV=5.65\%$ ) in cowpea plants inoculated with *Bradyrhizobium* sp. (BR 3267); co-inoculated with BR 3267 + *Paenibacillus graminis* (MC 04.21); with BR 3267 + *P. durus* (C 04.50); with BR 3267 + MC 04.21 + C 04.50. Means (four replicates) followed by the same letter [lower (harvest periods (FP, flowering point; BS, beginning of senescence) and capital (treatments) letters] not differ statistically ( $p<0.05$ ) according to Tukey's test. \* variation's coefficient.

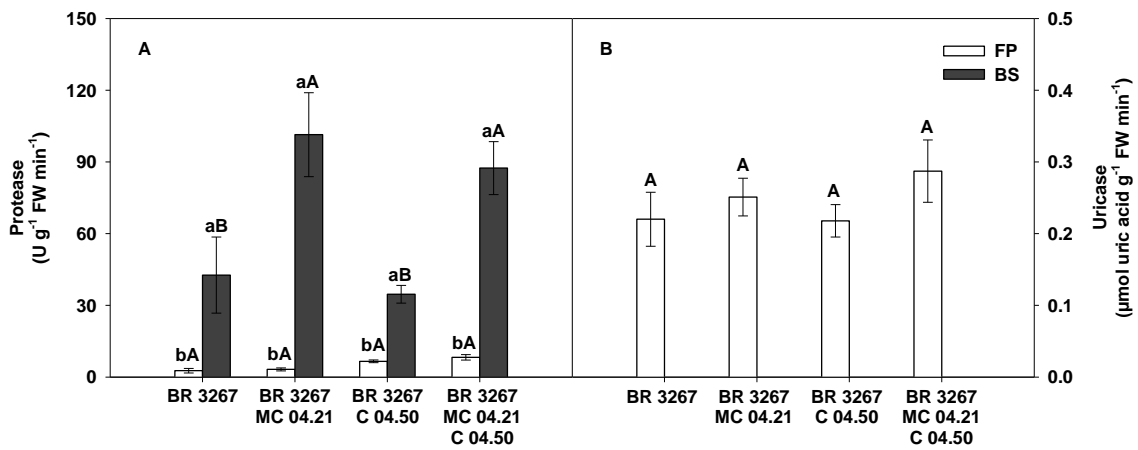


**Fig. 2.** Lipoxygenase activity enzyme ( $CV^*=9.72\%$ ) in cowpea plants inoculated with *Bradyrhizobium* sp. (BR 3267); co-inoculated with BR 3267 + *Paenibacillus graminis* (MC 04.21); with BR 3267 + *P. durus* (C 04.50); with BR 3267 + MC 04.21 + C 04.50. Means (four replicates) followed by the same letter [lower (harvest periods (FP, flowering point; BS, beginning of senescence) and capital (treatments) letters] not differ statistically ( $p < 0.05$ ) according to Tukey's test. \* variation's coefficient.

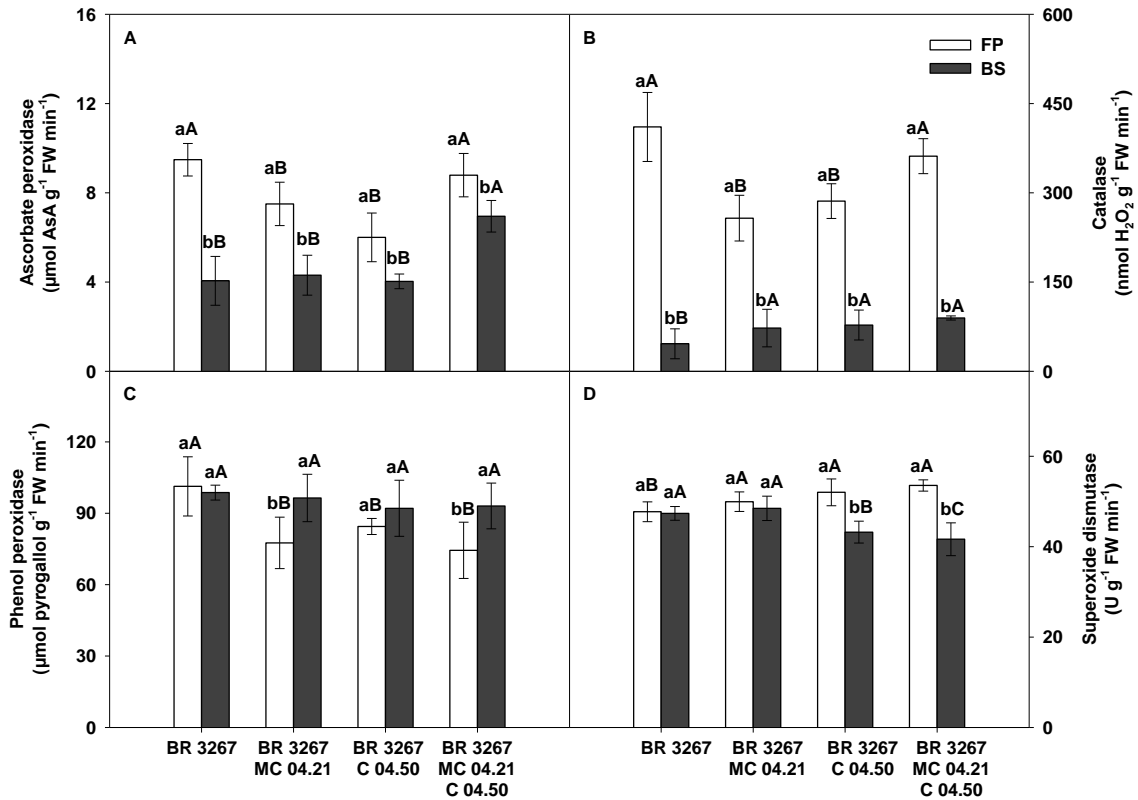


**Fig. 3.** Leghemoglobin concentration ( $CV^*=8.99\%$ ) in cowpea plants inoculated with *Bradyrhizobium* sp. (BR 3267); co-inoculated with BR 3267 + *Paenibacillus graminis* (MC 04.21); with BR 3267 + *P. durus* (C 04.50); with BR 3267 + MC 04.21 + C 04.50. Means (four replicates) followed by the same letter [lower (harvest periods (FP, flowering point; BS, beginning of senescence) and capital (treatments) letters] not differ statistically ( $p < 0.05$ ) according to Tukey's test. \* variation's coefficient.





**Fig. 4.** Activity of (A) protease (CV<sup>\*</sup>=26.11%) and (B) uricase (CV=13.47%) in cowpea plants inoculated with *Bradyrhizobium* sp. (BR 3267); co-inoculated with BR 3267 + *Paenibacillus graminis* (MC 04.21); with BR 3267 + *P. durus* (C 04.50); with BR 3267 + MC 04.21 + C 04.50. Means (four replicates) followed by the same letter [lower (harvest periods (FP, flowering point; BS, beginning of senescence) and capital (treatments) letters] not differ statistically (p<0.05) according to Tukey's test. \*variation's coefficient.



**Fig. 5.** Enzymes of the antioxidative metabolism degrading H<sub>2</sub>O<sub>2</sub>. (A) Ascorbate peroxidase (CV<sup>\*</sup>=14.71%), (B) catalase (CV=17.44%), (C) phenols peroxidase (CV=11.19%) and (D) superoxide dismutase (CV=5.13%) in cowpea plants inoculated with *Bradyrhizobium* sp. (BR 3267); co-inoculated with BR 3267 + *Paenibacillus graminis* (MC 04.21); with BR 3267 + *P. durus* (C 04.50); with BR 3267 + MC 04.21 + C 04.50. Means (four replicates) followed by the same letter [lower (harvest periods (FP, flowering point; BS, beginning of senescence) and capital (treatments) letters] not differ statistically (p<0.05) according to Tukey's test. \* variation's coefficient.

## **Capítulo IV**

**Polissacarídeo microbiano sintetizado por *Rhizobium tropici* inoculado e  
co-inoculado com *Bradyrhizobium* sp. e BPCP's em caupi no  
Espodossolo**

*Artigo a ser submetido para publicação no periódico  
Revista Brasileira de Ciência do Solo*

## Polissacarídeo microbiano sintetizado por *Rhizobium tropici* inoculado e co-inoculado com *Bradyrhizobium* sp. e BPCP's em caupi no Espodossolo<sup>2</sup>

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### RESUMO

Pesquisa visando à aplicação agroindustrial dos exopolissacarídeos (EPS) está concentrada nos polissacarídeos microbianos extracelulares, por ser mais simples de extrair e purificar. A fixação biológica de nitrogênio (FBN) é reconhecidamente eficiente em caupi, porém é necessária a utilização de novas tecnologias, visto que a baixa produtividade desta cultura esta associada ao baixo nível tecnológico empregado. Neste contexto, o trabalho objetivou avaliar a efetividade da inoculação e co-inoculação com *Bradyrhizobium* sp. e diferentes BPCP's, no caupi, avaliadas no ponto de florescimento (PF) e no enchimento dos grãos (EG), assim como caracterizar o EPS produzido pelo *Rhizobium tropici* (EI-6) que foi utilizado como veículo de inoculação. O experimento foi conduzido em casa de vegetação do Instituto Agrônomo de Pernambuco (IPA). As sementes de caupi cv. "IPA 206" foram desinfestadas e inoculadas com *Bradyrhizobium* sp. (BR 3267); co-inoculadas com a BR 3267 + BPCP's [BR 3267 + *Paenibacillus graminis* (MC 04.21); BR 3267 + *P. durus* (C 04.50)]; e com a tripartite (BR 3267 + MC 04.21 + C 04.50). Foram utilizadas duas testemunhas, sendo uma absoluta (TA) e outra nitrogenada (TN), como controles. O solo utilizado foi Espodossolo oriundo da Estação Experimental de Itapirema (IPA). As variáveis estudadas foram avaliadas em dois períodos [ponto de florescimento (PF) e enchimento de grãos (EG)]. As plantas co-inoculadas com BR 3267 + BPCP's apresentaram os melhores resultados em comprimento de raiz, matéria seca de raiz, número de nódulos e nodulação específica do que nas plantas inoculadas apenas com BR 3267. As plantas co-inoculadas com a tripartite apresentaram diferença significativa nas variáveis de eficiência da fixação de nitrogênio e produtividade. O EPS apresentou-se como um heteropolissacarídeo polianiónico com fluido pseudoplástico pouco viscoso, revelando-se pela técnica de calorimetria exploratória diferencial um pico endotérmico que representa uma característica favorável tendo em vista a possibilidade de seu uso em escala industrial.

**Termos de indexação:** Exopolissacarídeo, *Vigna unguiculata*, temperatura de fusão, simbiose, períodos de colheita, produtividade.

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<sup>2</sup>Parte da tese de doutorado da primeira autora. Recebido para publicação em // .

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Microbial polysaccharides synthesized by *Rhizobium tropici* inoculated and co-inoculated with *Bradyrhizobium* sp. and PGPB in cowpea in the Spodosol

## SUMMARY

Research aiming at the agroindustrial application of exopolysaccharides (EPS) is concentrated in the extracellular microbial polysaccharides by being easier to extract and purify. The biological nitrogen fixation (BNF) in cowpea is admittedly efficient, but it requires the use of new technologies, since the low productivity of this culture is associated with low level of technology employed. In this context, the aim of this study was evaluated the effectiveness of the inoculation and co-inoculation with *Bradyrhizobium* sp. and different PGPB's in cowpea as well as characterized the EPS produced by *Rhizobium tropici* (EI-6) and used as vehicle of inoculation. The experiment was conducted in the greenhouse of the Agronomical Institute of Pernambuco (IPA). The seeds of cowpea cv. "IPA 206" were disinfected and inoculated with *Bradyrhizobium* sp. (BR 3267); co-inoculated with BR 3267 + PGPB's [BR 3267 + *Paenibacillus graminis* (MC 04.21) and BR 3267 + *P. durus* (C 04.50); and with tripartite (BR 3267 + MC 04.21 + C 04.50)]. Were used the absolute (TA) and nitrogen (TN) controls. The soil utilized was a Spodosol from the Experimental Station Itapirema (IPA). The variables evaluated were evaluated in two periods [point of flowering (PF) and grain filling (GF)]. Plants co-inoculated with BR 3267 + BPCP's showed the best results in root length, root dry matter, nodule number and specific nodulation than plants inoculated only with BR 3267. Plants co-inoculated with tripartite showed significant differences in variables of the nitrogen fixation efficiency and productivity. The EPS presented as a polyanionic heteropolysaccharide with slightly viscous pseudoplastic fluid, revealing an endothermic peak by the technique of differential scanning calorimetry that represents a favorable feature in view of the possibility of its use in industry scale.

**Index terms:** Exopolysaccharide, *Vigna unguiculata*, melting temperature, symbiosis, harvest periods, productivity.

## INTRODUÇÃO

Os exopolissacarídeos (EPS) são polímeros sintetizados e liberados ao meio extracelular por bactérias e que podem formar géis e/ou soluções viscosas (Badel et al., 2011; Freitas et al., 2011). As bactérias dos gêneros *Rhizobium*, *Beijerinckia* e *Azotobacter* são fixadoras de nitrogênio e conhecidas por produzirem quantidades abundantes de EPS (Serrato et al., 2008; Aranda-Selverio & Penna, 2010) que pode atuar como sinais moleculares durante a infecção das raízes e levar a formação de estruturas celulares que auxiliam no processo de infecção e desenvolvimento dos nódulos (Marczak et al., 2008; Rinaudi & Giordano, 2010).

A inoculação de sementes de leguminosas com bactérias fixadoras é prática bem estabelecida e contribui significativamente para o aumento dos rendimentos na produção de grãos (Rumjanek et al., 2005). Veículos de inoculação alternativos têm sido estudados visando aumentar a qualidade e eficiência do inoculante, reduzir custos e possíveis impactos ambientais (Ben Rebah et

al., 2007; Albareda et al., 2008). Os EPS sintetizados por bactérias fixadoras podem representar uma alternativa viável, como inoculantes, para substituir a turfa e ainda reduzir os custos de produção (Fernandes Júnior et al., 2009). Além disso, estes desempenham papéis importantes durante os eventos de pré-infecção na rizosfera, podendo prevenir danos ambientais e manter a viabilidade do rizóbio no campo (Schuh, 2005; Fernandes Júnior et al., 2009).

Durante a interação leguminosa-rizóbio, o processo de fixação biológica de nitrogênio (FBN) pode contribuir para a produção vegetal e apresenta-se como eficiente fonte de nitrogênio para a planta (Rinnofner et al., 2008; Larrainzar et al., 2009). Algumas combinações de microrganismos são efetivas em aumentar a disponibilidade de nutrientes às plantas, a qualidade do sistema radicular e incrementar a FBN e a produtividade (Rumjanek et al., 2005). Assim, o uso de mistura de microrganismos através da interação rizóbios e bactérias promotoras de crescimento em plantas (BPCP's) têm apresentado aumento na nodulação e fixação do N<sub>2</sub> dependendo da combinação e compatibilidade (Figueiredo et al., 2010).

Considerando a importância do uso de polissacarídeos microbianos no processo de inoculação e a necessidade de otimização dos processos de nodulação e FBN através do uso de microrganismos e ainda a potencialidade e necessidade de incrementar a produtividade do caupi, este trabalho objetivou avaliar a efetividade da inoculação e co-inoculação com *Bradyrhizobium* sp. e diferentes BPCP's nos diferentes períodos de coleta, ponto de florescimento (PF) e enchimento de grãos (EG), em plantas de caupi utilizando variáveis de crescimento, desenvolvimento e produção assim como caracterizar o EPS produzido pelo *Rhizobium tropici* (EI-6) que foi utilizado como veículo alternativo de inoculação.

## **MATERIAL E MÉTODOS**

### **Extração e caracterização do EPS de *Rhizobium tropici***

O EPS utilizado foi extraído da estirpe EI-6 de *Rhizobium tropici* (Oliveira, 2011) isolado de nódulos da raiz de *Vigna unguiculata* [L.] Walp. (Figueiredo et al., 1999). Para extração, o EI-6 foi multiplicado em meio de cultura YMA (manitol, extrato de levedura e ágar) por 72 h, inoculado em frascos contendo meio de cultura YM (manitol e extrato de levedura) e incubado em agitação constante (200 rpm; 28 °C; 72 h). Transcorrido esse período, o mosto foi colocado em banho-maria (80 °C; 20 min), resfriado, recuperado com precipitação em álcool etílico (1:3) e então colhido e seco em estufa (30 °C; 72 h). Ao final, o precipitado foi moído e peneirado manualmente, acondicionado em frascos de vidro e armazenado em local fresco e seco. Para caracterização do EPS, amostras foram avaliadas quanto ao comportamento térmico, através da análise de calorimetria exploratória diferencial (DSC); quanto à viscosidade e viscoelasticidade em reômetro

(Haake®; modelo RS 150); e quanto a composição de monossacarídeos e ácidos derivados por cromatografia de camada delgada comparativa (CCDC) segundo Moreira et al. (1998).

### **Multiplicação, preparação e aplicação dos inoculantes formulados com EPS**

Procedeu-se inicialmente a multiplicação das estirpes a serem utilizadas nos tratamentos experimentais. A estirpe BR 3267 de *Bradyrhizobium* sp. (estirpe padrão) foi multiplicada em frascos contendo meio de cultura YM em agitação constante (200 rpm; 28 °C) por 96 h. Para as estirpes de bactérias promotoras de crescimento em plantas (BPCP's) *Paenibacillus graminis* (MC 04.21) e *P. durus* (C 04.50), utilizou-se meio de cultura TSB (Caldo Tripticaseína de Soja) que foi mantido em agitação constante (200 rpm; 32 °C) por 24 e 48 horas, respectivamente.

Para o preparo dos inoculantes, inicialmente sacos de polietileno de média densidade contendo 2,0 g do EPS foram autoclavados (120 °C; 101 Kpa; 15 min), resfriados e adicionou-se 2,0 mL de água destilada e autoclavada e a mistura foi homogeneizada e incubada até o pH atingir a faixa ideal (6,8 a 7,0) (Santos, 2010). A posteriori, procedeu-se a inoculação das bactérias no EPS (1,0 g do EPS para 3,0 mL do volume final do inóculo) e, por conseguinte, a formação dos tratamentos. A inoculação com o BR 3267 foi realizada pela adição de 1,5 mL do meio de cultura YM contendo a bactéria ( $10^8$  UFC mL<sup>-1</sup>) seguido da adição de 1,5 mL de água destilada e esterilizada. Para a co-inoculação, adicionou-se 1,5 mL do meio de cultura TSB contendo MC 04.21 ou C 04.50 ( $10^7$  UFC mL<sup>-1</sup>) e, em seguida, acrescentou-se 1,5 mL do meio de cultura YM contendo BR 3267. Para a co-inoculação em tripartite (BR 3267 + MC 04.21 + C 04.50), adicionou-se 0,75 mL do meio de cultura TSB contendo MC 04.21; 0,75 mL do meio de cultura TSB com C 04.50; e, finalmente, 1,5 mL do meio de cultura YM contendo a BR 3267.

Feita a inoculação no EPS, o material foi incubado para a maturação por 48 h à temperatura ambiente. Posteriormente, retirou-se 1,0 g do inoculante que foi dissolvido em solução salina (0,85% NaCl) e, então, efetuou-se a mistura para formulação dos tratamentos. O material foi mantido em agitação (300 rpm; 28 °C; 30 min) e, então, utilizado no processo de inoculação da semente (2,0 mL do inoculante semente<sup>-1</sup>). A contagem em placas dos inoculantes líquido foi efetuada pelo método da gota ("Drop-plate") em diluição seriada ( $10^5$  a  $10^7$ ) e efetuado o plaqueamento em meio de cultura YMA com vermelho congo.

### **Condução do experimento**

O experimento foi conduzido em casa de vegetação do Instituto Agronômico de Pernambuco (IPA; Recife-PE). As sementes de caupi cv. "IPA 206" foram desinfestadas (Hungria & Araújo, 1994) e semeadas em vasos contendo 6 kg de Espodossolo não esterilizado, onde se efetuou

calagem com  $\text{CaCO}_3$  e suplementação com  $\text{P}_2\text{O}_5$  e  $\text{K}_2\text{O}$  seguindo recomendações do IPA (2008) e os resultados da análise química do solo (Tabela 1).

Para instalação dos tratamentos, as sementes foram inoculadas com o BR 3267; co-inoculadas com a BR 3267 + BPCP's (BR 3267 + MC 04.21; BR 3267 + C 04.50); e com a tripartite (BR 3267 + MC 04.21 + C 04.50). Uma testemunha absoluta (TA), sem inoculação, e uma testemunha nitrogenada (TN), sem inoculação e suplementada com  $(\text{NH}_4)_2\text{SO}_4$  aos 7, 14 e 21 dias após o desbaste seguindo recomendações da análise de solo, foram utilizadas como controle. Sete dias após a semeadura, realizou-se o desbaste e mantiveram-se duas plantas por vaso (parcela experimental). Durante o período experimental, as plantas de caupi, em seus respectivos tratamentos, foram irrigadas com solução nutritiva de Hoagland & Arnon (1950) modificada por Silveira et al. (1998) e isenta de nitrogênio.

A coleta do experimento foi realizada em dois períodos: (1) ponto de florescimento (PF), período de maior fixação de nitrogênio (aos 36 dias) e (2) enchimento de grãos (EG), período em que todas as vagens encontravam-se cheias e maduras (aos 103 dias). Foram avaliadas as seguintes variáveis: comprimento da raiz, matéria seca de nódulos, da parte aérea (MSPA) e da raiz (MSR) e relação MSPA/MSR, eficiência e eficácia (Faria & Franco, 2002); o nitrogênio acumulado na MSPA ( $N_{ac}$ ) e teor de nitrogênio na MSPA (Teor de N) (Bremner, 1965); a eficiência da fixação de  $\text{N}_2$  e a nodulação específica (Gulden & Vessey, 1998). A taxa de crescimento absoluto foi calculada com base na altura das plantas que foram avaliadas a cada sete dias até a primeira coleta (Benincasa, 2003). Após a primeira coleta, optou-se por descontinuar a mensuração da altura das plantas devido ao início da formação de vagens que desvia o fluxo energético para enchimento de grãos com concomitante redução na taxa de crescimento. O número de nódulos foi avaliado apenas nas plantas da primeira coleta (PF) devido à presença de nódulos senescentes, nas raízes das plantas, da segunda coleta (EG). Foram ainda obtidas variáveis relacionadas com a produção: número de vagens por vaso, comprimento médio da vagem por vaso, peso da vagem por vaso, número de grãos por vaso e peso total de sementes por vaso.

O delineamento experimental adotado foi em blocos ao acaso com arranjo fatorial  $2 \times 6$ , dois períodos de coleta (PF e EG) e seis tratamentos, sendo um com inoculação (*Bradyrhizobium* sp.; BR 3267), três com co-inoculação de BR 3267 + BPCP's; e duas testemunhas (TA e TN), com quatro repetições. Os dados foram submetidos à análise de variância (ANOVA) utilizando o programa estatístico ASSISTAT versão 7.6 beta pelo teste F e as médias comparadas pelo teste de Tukey, ambos a 5% de probabilidade.



## RESULTADOS E DISCUSSÃO

### Caracterização do EPS de *Rhizobium tropici*

Análise do EPS produzido pelo *R. tropici* (EI-6) revelou um pico endotérmico bem definido a partir do qual foi possível definir a temperatura de fusão ( $T_m$ ) da amostra como sendo de 178 °C (Figura 1). O  $T_m$  da amostra do EPS semelhante às  $T_m$  dos polímeros bacterianos obtidos de estirpes de *Pseudomonas* e *Beijerinckia*, que variam de 162 a 170 °C, e de *Xanthomonas arboricola* pv. *pruni*, que oscila entre 120 a 185 °C (Borges et al., 2009). A existência de “ombros” na curva de DSC, antes da  $T_m$ , pode ser relacionada a algum subproduto originário do complexo processo produtivo do EPS. A tolerância do EPS a temperaturas superiores a 100 °C representa uma característica favorável (Poli et al., 2010) e desta forma é possível utilizá-lo em escala industrial.

A avaliação da viscosidade do EPS bacteriano é de fundamental importância para determinar sua qualidade e possível aplicabilidade industrial e/ou comercial (Toneli et al., 2005). No presente estudo, o EPS foi definido como um fluido não-Newtoniano, pouco viscoso e pseudoplástico. Além disso, a análise da viscoelasticidade do EPS permitiu defini-lo como viscoso até 6 Hz de frequência e sem comportamento de gel verdadeiro, devido ao comportamento elástico registrado acima de 6 Hz (Figura 2). Fluidos pseudoplásticos podem ter uma ampla faixa de utilização, indo desde a aplicação na indústria de alimentos e fármacos como também na agricultura e perfuração de poços de petróleo (Toneli et al., 2005; Farias et al., 2009).

Em relação aos monossacarídeos (Figura 3), o EPS foi classificado como um heteropolissacarídeo devido à presença de glicose e galactose e ausência de ácidos urônicos (ácidos glicurônico e galacturônico). As bactérias fixadoras de nitrogênio, principalmente espécies do gênero *Rhizobium*, produzem EPS que são excretados para o ambiente extracelular (Serrato et al., 2008; Aranda-Selverio & Penna, 2010). Tais biopolímeros bacterianos apresentam inúmeras propriedades físico-químicas podendo ser classificados em homo ou heteropolissacarídeos (Badel et al., 2011; Freitas et al., 2011). Os homopolissacarídeos são os biopolímeros constituídos por unidades repetidas de apenas um tipo simples de monossacarídeos, enquanto que os heteropolissacarídeos são constituídos de diferentes monossacarídeos (Donot et al., 2012).

### Caracterização do caupi inoculado e co-inoculado com *Bradyrhizobium* sp. e BPCP's

As espécies vegetais apresentam diferenças na dinâmica de crescimento de acordo com o suprimento de nitrogênio e carbono (Ghaley et al., 2005). Estas diferenças foram observadas em termos de crescimento das raízes nas plantas de caupi associadas com *Bradyrhizobium* sp. e BPCP's nos diferentes períodos de coleta, ponto de florescimento (PF) e enchimento de grãos (EG) (Figura 4). No PF, o comprimento da raiz (CR) foi maior nas plantas inoculadas com o par simbiótico

BR 3267 + *Paenibacillus graminis* (MC 04.21) enquanto as plantas controle (TA) apresentaram os menores valores médios de CR em relação aos demais tratamentos, pelo teste de Tukey ( $p < 0,05$ ) (Figura 4). As baixas concentrações de nitrogênio na solução do solo podem resultar em alterações no crescimento radicular (Krapp et al., 2011), como observado nas plantas do grupo TA. As plantas de caupi co-inoculadas com a tripartite [(BR 3267 + MC 04.21 + *P. durus* (C 04.50))] apresentaram comprimento da raiz maior no EG do que no PF indicando que a co-inoculação em tripartite foi capaz de manter a continuidade do processo de crescimento da raiz e da parte aérea (Figura 4).

A avaliação do crescimento e desenvolvimento das plantas é muito complexa por envolver o efeito de fatores externos, bióticos e abióticos, sobre os processos fisiológicos nas plantas (Dey et al., 2004). A análise das variáveis de crescimento permite inferir sobre a capacidade dos vegetais em translocar adequadamente os fotoassimilados produzidos nos sítios de fixação através da fotossíntese para os locais de crescimento e diferenciação dos órgãos vegetais (Benincasa, 2003). Além disso, a expansão da área foliar é uma estratégia que permite ao vegetal expor as folhas a maior intensidade luminosa e assegurar um maior aproveitamento da luminosidade (Martuscello et al., 2009).

Durante o EG, as plantas co-inoculadas com o par simbiótico BR 3267 + C 04.50 e com a tripartite apresentaram maior matéria seca de nódulos (MSN) em comparação às plantas inoculadas apenas com a BR 3267 (Figura 5). Pode-se sugerir que esta resposta está relacionada à presença da estirpe C 04.50 da bactéria *Paenibacillus durus*, que pode secretar compostos extracelulares – particulares desta espécie bacteriana – na rizosfera, como aminoácidos, fitohormônios e metabólitos secundários, promovendo um ambiente favorável ao desenvolvimento vegetal (Yoon et al., 2003). Plantas inoculadas apenas com a BR 3267 apresentaram menor MSN em comparação aos demais tratamentos avaliados no EG, fato possivelmente correlacionado ao processo de senescência que provavelmente foi retardado pela presença das BPCP's.

Os valores médios de matéria seca da parte aérea (MSPA) mensurados nas plantas no PF e no EG não apresentaram diferença significativa entre os diferentes tratamentos pelo teste de Tukey ( $p < 0,05$ ) (Figura 6A), entretanto para a matéria seca de raiz (MSR) foram registradas diferenças significativas durante o EG, não tendo ocorrido o mesmo no PF (Figura 6B). No EG, as plantas co-inoculadas com BR 3267 + BPCP's apresentaram as médias mais elevadas de MSR em relação aos demais tratamentos, enquanto as plantas inoculadas apenas com BR 3267 apresentaram o menor valor médio de MSR (Figura 6B). Esta resposta pode estar associada aos fitohormônios produzidos e liberados pelas BPCP's na superfície radicular que pode utilizar os fotoassimilados produzidos na parte aérea para sustentar energeticamente as suas divisões celulares garantindo seu crescimento (Larrainzar et al., 2009).

A não alteração da matéria seca da parte aérea com concomitante aumento da matéria seca da raiz registrado nas plantas co-inoculadas com a BR 3267 + BPCP's pode indicar que houve um particionamento do suprimento de carbono mais efetivo nestas plantas do que nas inoculadas apenas com a BR 3267, fato corroborado pela relação MSPA/MSR. A relação MSPA/MSR não sofreu alterações significativas durante o PF entre todos os tratamentos avaliados, entretanto no EG houve redução na relação MSPA/MSR nas plantas co-inoculadas com BR 3267 + BPCP's em relação às inoculadas apenas com a BR 3267 (Figura 6C). Essa resposta tem relação direta com a variação de MSR e reforça a ideia de que o fluxo de carbono fixado fotossinteticamente foi direcionado para sustentar o crescimento das raízes e dos nódulos.

Foram registradas alterações nas variáveis de crescimento avaliados em resposta a inoculação e co-inoculação do caupi com *Bradyrhizobium* sp. e BPCP's. A taxa de crescimento absoluto (TCA) foi mensurada nas plantas submetidas às diferentes combinações de microrganismos até o PF (Tabela 2), tendo em vista que o fluxo metabólico nas plantas após o PF encontra-se direcionado para o enchimento de grãos. No geral, os resultados mostraram que as plantas TA e TN apresentaram TCA média similar ao observado para as plantas inoculadas com a tripartite, enquanto que as plantas inoculadas com os pares simbióticos BR 3267 + MC 04.21 e BR 3267 + C 04.50 apresentaram médias inferiores ao registrado para as plantas inoculadas apenas com a BR 3267, pelo teste de Tukey a 5% de probabilidade (Tabela 2).

O crescimento dos nódulos interage fortemente com a parte aérea devido à competição pelos fotoassimilados produzidos pelos vegetais (Voisin et al., 2010). Durante a fase vegetativa, a taxa fotossintética tem efeito maior na massa seca total e declina drasticamente à medida que as vagens começam a se desenvolver e isto provavelmente pode estar relacionado com a queda do conteúdo de nitrogênio total, da atividade da Rubisco (enzima responsável por fixar o CO<sub>2</sub> durante a fotossíntese) e na integridade dos nódulos (Antolín et al., 2010; Ben Salah et al., 2011). Esta afirmativa é corroborada pelo declínio no número de nódulos viáveis nas plantas coletadas no EG, provavelmente uma resposta ao processo natural de senescência, e assim a quantidade dos nódulos na zona radicular, e consequentemente a nodulação específica (NE) e a eficiência da fixação de nitrogênio (EFN<sub>2</sub>) das plantas de caupi foram mensuradas apenas no PF (Tabela 2).

O uso de BPCP's promove inúmeros efeitos benéficos tanto para as espécies vegetais como também para a bactéria fixadora que levam igualmente ao incremento da FBN e da produtividade vegetal (Dey et al., 2004; Compant et al., 2010). No presente estudo, foi possível registrar aumento do número de nódulos (NN), nodulação específica (NE) e EFN<sub>2</sub> em resposta a presença das BPCP's (Tabela 2). As plantas co-inoculadas com a tripartite apresentaram o NN, a NE e a EFN<sub>2</sub> mais elevados em comparação com as plantas inoculadas apenas com BR 3267, pelo teste de Tukey a 5%

de probabilidade. Além disso, não foram registradas diferenças significativas no teor de nitrogênio e no nitrogênio acumulado na matéria seca da parte aérea ( $N_{ac}$ ), variáveis indicadoras do fluxo de nitrogênio do rizóbio para planta.

No teor de nitrogênio e o nitrogênio acumulado (Tabela 3) não foram registradas diferença significativa ( $p < 0,05$ ) e esta resposta pode estar relacionada com a não alteração na matéria seca dos nódulos (MSN), como apresentado na figura 5. Segundo Dey et al. (2004), a quantidade de nitrogênio fixado simbioticamente pela associação rizóbio-planta pode estar relacionada com a biomassa dos nódulos. Pode-se notar uma forte relação nas variáveis teor de nitrogênio,  $N_{ac}$  e MSN, que não apresentaram diferença significativa ( $p < 0,05$ ). Para o teor de nitrogênio, observou-se redução de 50% no EG em relação ao PF (Tabela 3) e esta resposta indica um possível redirecionamento do fluxo metabólico do nitrogênio fixado nos nódulos para a formação de vagens e/ou o enchimento de grãos. Estes resultados corroboram com Ferreira et al. (2011) em seu estudo com feijão-caupi.

Calculou-se a eficiência e eficácia (Tabela 4) em comparação com as testemunhas absoluta (TA) e nitrogenada (TN), respectivamente, nos dois períodos de coleta (PF e EG). As plantas inoculadas apenas com a BR 3267 apresentaram um percentual médio de eficiência no PF menor que o registrado para as plantas co-inoculadas com BR 3267 + BPCP's (Tabela 4). No EG, as plantas co-inoculadas com a BR 3267 + C 04.50 e co-inoculadas em tripartite mantiveram uma alta eficiência em comparação aos demais tratamentos (Tabela 4). Segundo a classificação de Date & Norris (1979), plantas com valores de eficiência acima de 85% são consideradas como muito eficientes. As plantas co-inoculadas com BR 3267 + BPCP's apresentaram eficácia maior que o registrado para as plantas inoculadas apenas com BR 3267 no PF (Tabela 4). A eficácia das plantas co-inoculadas com a BR 3267 + C 04.50 e co-inoculadas em tripartite foi claramente aumentada no EG em relação ao PF. A maior eficiência e eficácia registrada nas plantas co-inoculadas com a BR 3267 + BPCP's pode indicar que a presença das BPCP's induziu uma melhor capacidade de fixar o nitrogênio e fornecê-lo para o desenvolvimento vegetativo das plantas (Larrainzar et al., 2009; Figueiredo et al., 2010).

Características relacionadas com a produção das plantas submetidas aos diferentes combinações de BR 3267 + BPCP's foram avaliadas e foram encontradas diferenças significativas pelo teste de Tukey ( $p < 0,05$ ) em todas as diferentes variáveis analisadas [número de vagens por vaso (NV), o comprimento médio da vagem por vaso (CMV), peso da vagem por vaso (PV), número de grãos por vaso (NG) e peso total de semente por vaso (PTS)] (Tabela 5). Nas plantas co-inoculadas com a tripartite registrou-se o aumento de NV (87%), CMV (37%), PV (75%), NG (68%) e PTS (49%) em comparação com plantas TA, pelo teste de Tukey ( $p < 0,05$ ). Comparando-se

as plantas co-inoculadas com a tripartite com plantas inoculadas apenas com BR 3267 houve também aumento de NV (22%), CMV (5%), PV (22%), NG (4%) e PTS (12%).

Segundo Martins et al. (2003), a estirpe BR 3267 de *Bradyrhizobium* sp. é capaz de estabelecer uma eficiente simbiose com caupi resultando em boa taxa de produção de grãos em comparação com o tratamento nitrogenado. De fato, neste estudo, os dados obtidos para as plantas noduladas apenas com a BR 3267 foram estatisticamente semelhantes pelo teste de Tukey ( $p < 0,05$ ) com os obtidos para TN, corroborando com Martins et al. (2003). Além disso, observou-se que a co-inoculação das plantas de caupi com a tripartite promoveu um maior número de grãos e vagens, além de incremento nas demais variáveis de produção analisadas. Estes resultados indicam que houve neste tratamento uma manutenção da fixação e do transporte do nitrogênio fixado durante o período de formação das vagens (Campo et al., 2009) e, portanto, ressaltam a importância de uma combinação ideal de microrganismos que promova aumento da produtividade.

## CONCLUSÕES

O polissacarídeo microbiano extracelular apresenta-se como um heteropolissacarídeo polianiónico com fluído pseudoplástico pouco viscoso, revelando-se pela técnica de calorimetria exploratória diferencial um pico endotérmico que representa uma característica favorável tendo em vista a possibilidade de seu uso em escala industrial. O seu uso como veículo de inoculação foi efetivo, e a combinação e compatibilidade de microrganismos introduzidos, em mistura, promoveu uma melhor performance simbiótica, crescimento e produtividade no caupi no Espodossolo. As plantas co-inoculadas com *Bradyrhizobium* sp. (BR 3267) + *Paenibacillus graminis* (MC 04.21) + *P. durus* (C 04.50) foram superiores nas variáveis relacionadas com a produção e na eficiência da fixação de  $N_2$ .

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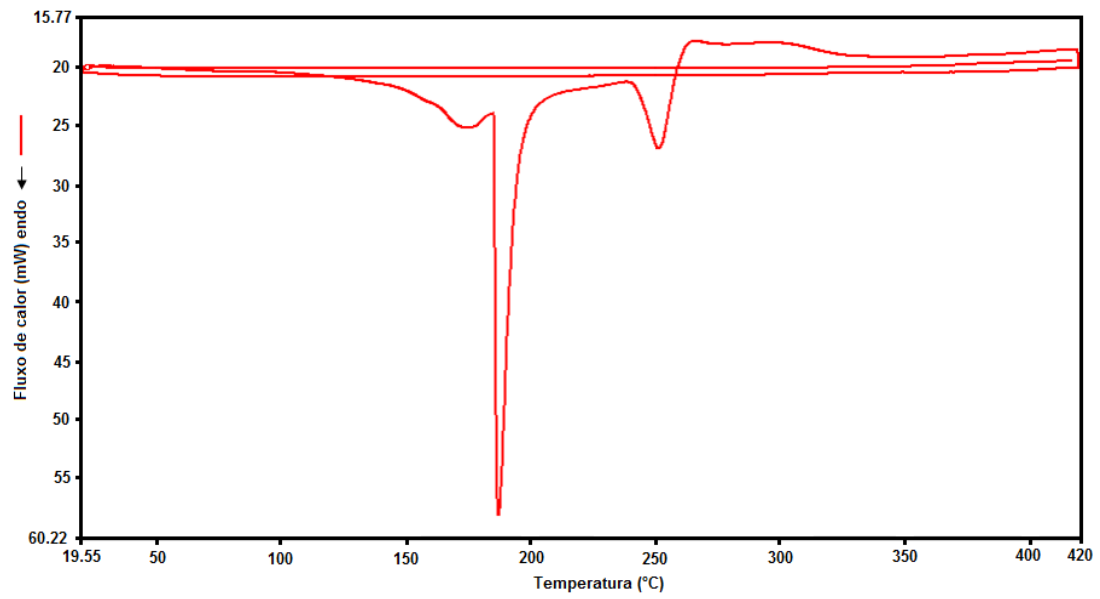
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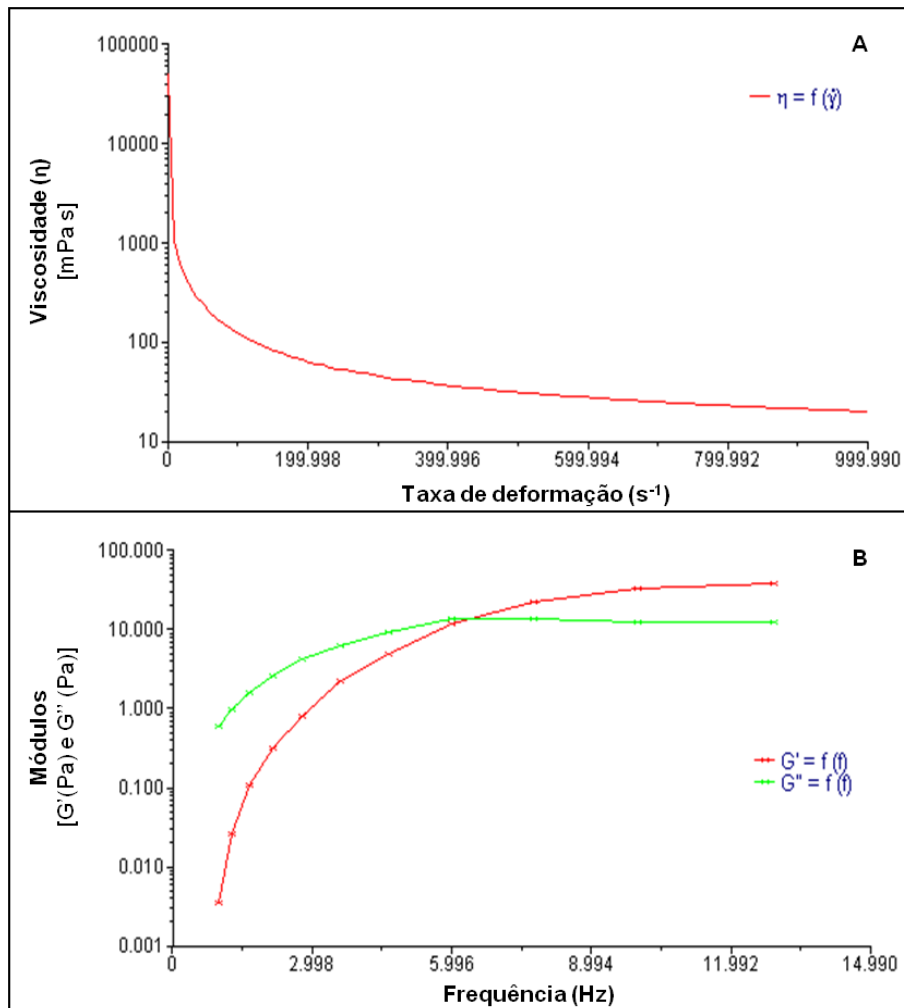
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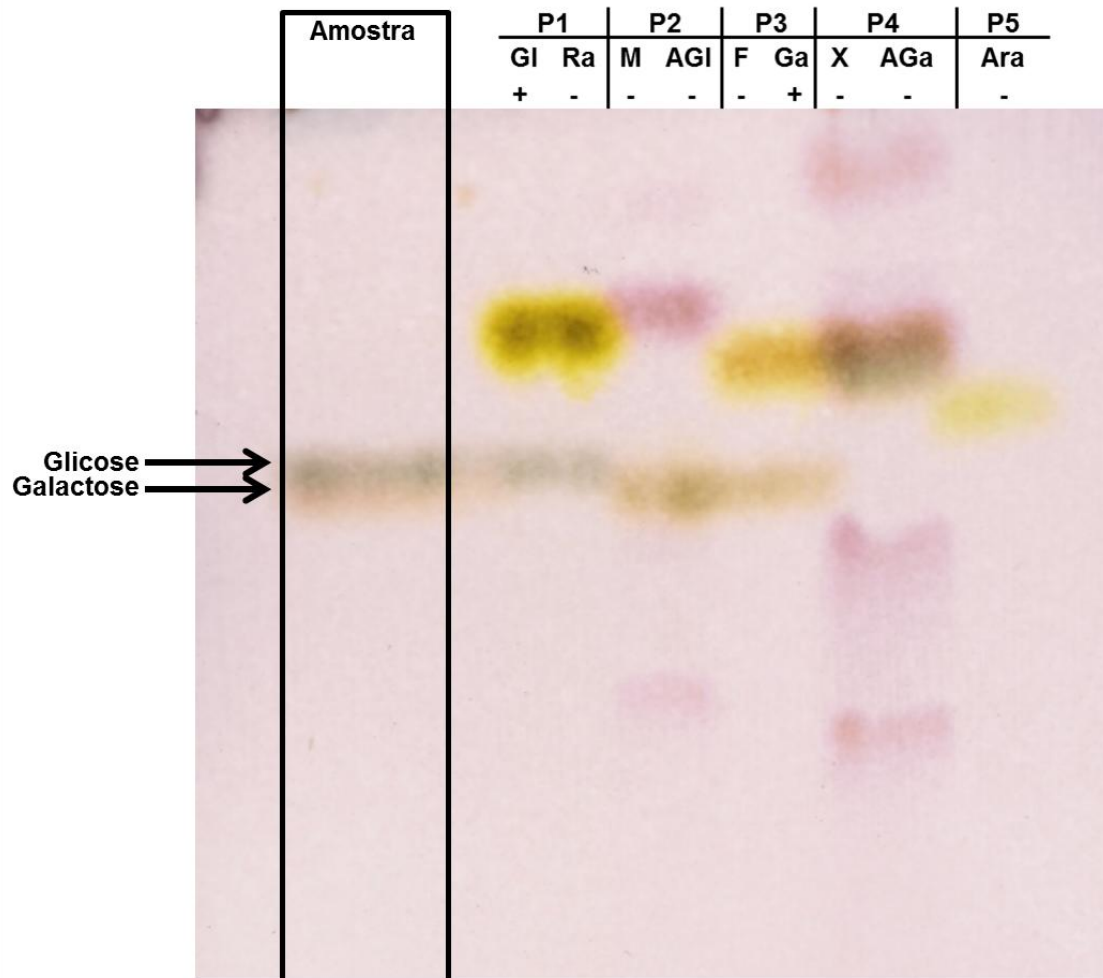




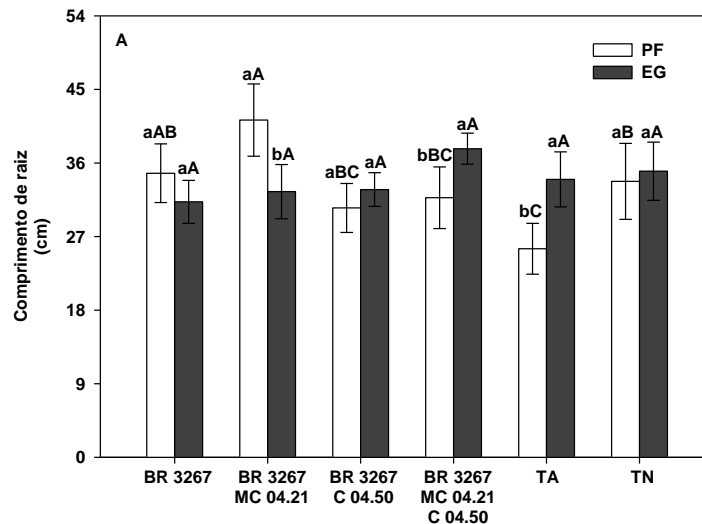
**Figura 1.** Análise térmica das amostras do EPS produzido pelo *Rhizobium tropici* (EI-6) por calorimetria exploratória diferencial (DSC).



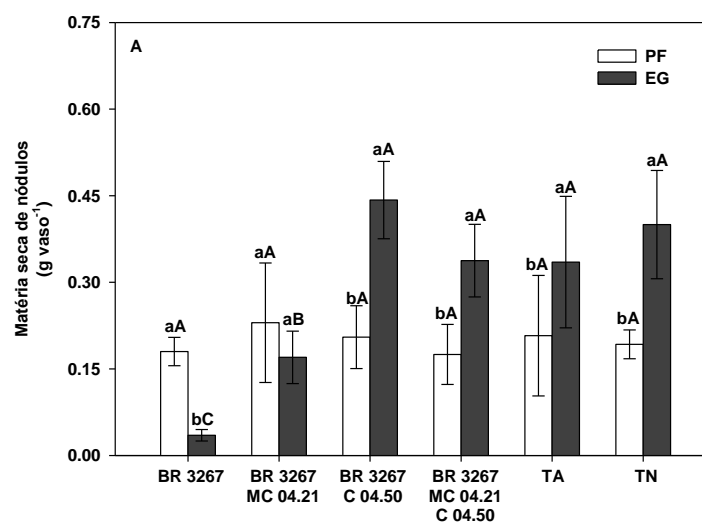
**Figura 2.** Avaliação da solução aquosa do EPS produzido pelo *Rhizobium tropici* a 1% (m/v) a 25 °C. (A) Viscosidade ( $\eta$ ) em função da variação da taxa de deformação (0,01 a 1000 s<sup>-1</sup>) e (B) Comportamento do módulo elástico ( $G'$ ) e viscoso ( $G''$ ) em função da frequência (0,1 a 15 Hz).



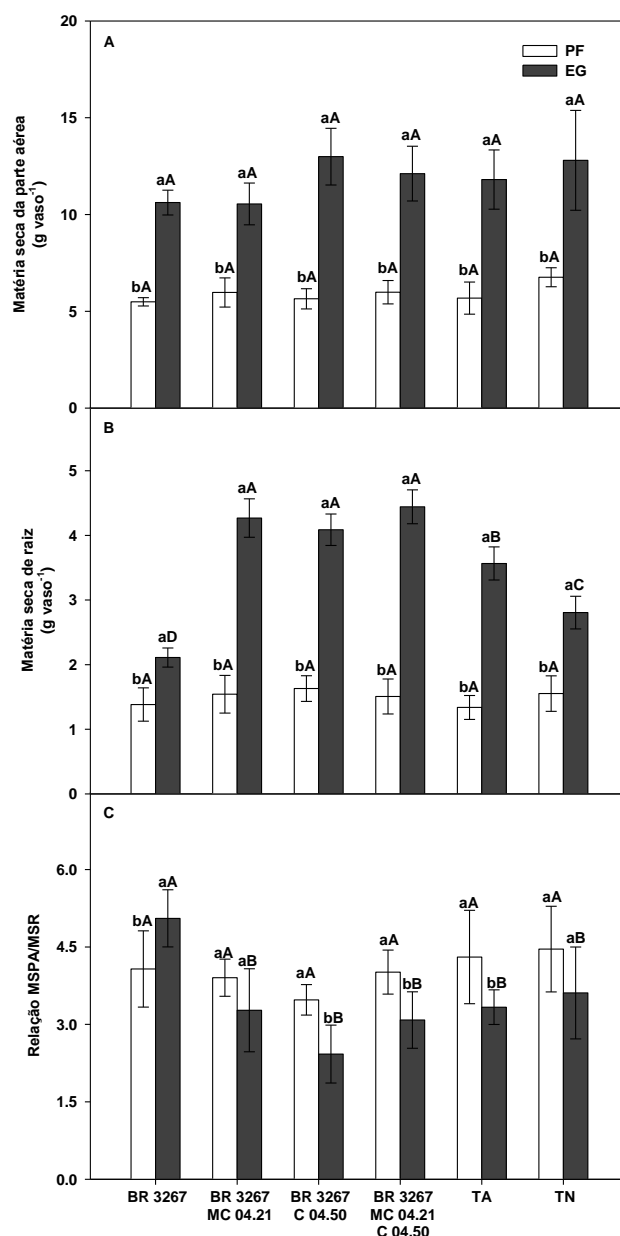
**Figura 3.** Monossacarídeos e ácidos derivados presentes no EPS de *Rhizobium tropici* (EI-6) identificados através da cromatografia de camada delgada comparativa (CCDC). Gl = glicose; Ra = ramnose; M = manose; AGI = ácido glicurônico; F = fucose; Ga = galactose; X = xilose; AGa = ácido galacturônico. Ara = arabnose; “+” = presença; “-” = ausência.



**Figura 4.** Comprimento de raiz (CV=10,39%) de plantas de caupi inoculadas com *Bradyrhizobium* sp. (BR 3267); co-inoculadas com BR 3267 + *Paenibacillus graminis* (MC 04.21); com BR 3267 + *P. durus* (C 04.50); com BR 3267 + MC 04.21 + C 04.50; e sem inoculação, testemunhas absoluta (TA) e nitrogenada (TN). Letras minúsculas iguais entre os tempos de coleta (PF, ponto de florescimento; EG, enchimento de grãos) e maiúsculas iguais entre os tratamentos não diferem entre si pelo teste de Tukey ( $p < 0,05$ ).



**Figura 5.** Matéria seca de nódulos (MSN; CV=28,19%) de caupi inoculadas com *Bradyrhizobium* sp. (BR 3267); co-inoculadas com BR 3267 + *Paenibacillus graminis* (MC 04.21); com BR 3267 + *P. durus* (C 04.50); com BR 3267 + MC 04.21 + C 04.50; e sem inoculação, testemunhas absoluta (TA) e nitrogenada (TN). Letras minúsculas iguais entre os períodos de coleta (PF, ponto de florescimento; EG, enchimento de grãos) e maiúsculas iguais entre os tratamentos não diferem entre si pelo teste de Tukey a 5% de probabilidade.



**Figura 6.** Valores da (A) matéria seca da parte aérea (MSPA; CV=15,48%), (B) matéria seca da raiz (MSR; CV=16,08%) e (C) relação MSPA/MSR (CV=16,97%) em plantas de caupi inoculadas com *Bradyrhizobium* sp. (BR 3267); co-inoculadas com BR 3267 + *Paenibacillus graminis* (MC 04.21); com BR 3267 + *P. durus* (C 04.50); com BR 3267 + MC 04.21 + C 04.50; e sem inoculação, testemunhas absoluta (TA) e nitrogenada (TN). Letras minúsculas iguais entre os períodos de coleta (PF, ponto de florescimento; EG, enchimento de grãos) e maiúsculas iguais entre os tratamentos não diferem entre si pelo teste de Tukey ( $p < 0,05$ ).

**Tabela 1.** Caracterização química do Espodossolo (0-20 cm) utilizado no experimento.

pH	P	Ca <sup>+2</sup>	Mg <sup>+2</sup>	Na <sup>+</sup>	K <sup>+</sup>	Al <sup>+2</sup>	H <sup>+</sup>	S	CTC	V
(água)	mg dm <sup>-3</sup>	cmolc dm <sup>-3</sup>								%
6,2	10	2,15	0,45	0,01	0,02	0,00	1,65	2,6	4,3	61

**Tabela 2.** Taxa de crescimento absoluto (TCA), número de nódulos (NN), nodulação específica (NE) e eficiência da fixação de nitrogênio (EFN<sub>2</sub>) em caupi inoculado com *Bradyrhizobium* sp. (BR 3267); co-inoculadas com BR 3267 + *Paenibacillus graminis* (MC 04.21); com BR 3267 + *P. durus* (C 04.50); com BR 3267 + MC 04.21 + C 04.50; e sem inoculação, testemunhas absoluta (TA) e nitrogenada (TN).

Tratamentos	TCA (cm dia <sup>-1</sup> )	NN (vaso <sup>-1</sup> )	NE (NN g <sup>-1</sup> MSR)	EFN <sub>2</sub> (mg N g <sup>-1</sup> MSN)
BR 3267	2,65 b	187,50 c	139,82 c	1036,61 b
BR 3267 + MC 04.21	1,83 c	273,50 a	180,93 a	833,91 b
BR 3267 + C 04.50	2,28 c	302,50 a	187,33 a	916,84 b
BR 3267 + MC 04.21 + C 04.50	3,76 a	210,00 b	140,56 b	1256,59 a
TA	4,40 a	157,75 d	119,27 d	792,21 c
TN	3,88 a	161,25 d	108,39 d	1033,06 b
<b>CV(%)</b>	<b>15,33</b>	<b>8,97</b>	<b>16,19</b>	<b>7,86</b>

Na coluna, as médias seguidas da mesma letra não diferem estatisticamente entre si pelo teste de Tukey (p<0,05).

**Tabela 3.** Teor de nitrogênio (Teor de N) e nitrogênio acumulado ( $N_{ac}$ ) na parte aérea de plantas de caupi inoculadas com *Bradyrhizobium* sp. (BR 3267); co-inoculadas com BR 3267 + *Paenibacillus graminis* (MC 04.21); com BR 3267 + *P. durus* (C 04.50); com BR 3267 + MC 04.21 + C 04.50; e sem inoculação, testemunhas absoluta (TA) e nitrogenada (TN) nos diferentes períodos de coleta (PF, ponto de florescimento; EG, enchimento de grãos).

Tratamentos	Teor de N		$N_{ac}$	
	PF	EG	PF	EG
BR 3267	34,02 aA	13,55 bA	186,95 aA	143,22 aA
BR 3267 + MC 04.21	29,12 aA	16,35 bA	174,44 aA	172,71 aA
BR 3267 + C 04.50	32,52 aA	15,52 bA	184,28 aA	202,20 aA
BR 3267 + MC 04.21 + C 04.50	32,20 aA	15,15 bA	192,43 aA	182,51 aA
TA	31,15 aA	14,67 bA	178,19 aA	174,43 aA
TN	30,10 aA	16,60 bA	203,65 aA	213,52 aA
<b>CV (%)</b>	<b>11,15</b>		<b>18,47</b>	

Nas linhas (letras minúsculas) e nas colunas (letras maiúsculas), médias seguidas de mesma letra não diferem estatisticamente, entre si, pelo teste de Tukey ( $p < 0,05$ ).

**Tabela 4.** Eficiência e eficácia em caupi inoculado com *Bradyrhizobium* sp. (BR 3267); co-inoculadas com BR 3267 + *Paenibacillus graminis* (MC 04.21); com BR 3267 + *P. durus* (C 04.50); com BR 3267 + MC 04.21 + C 04.50 nos diferentes períodos de coleta (PF, ponto de florescimento; EG, enchimento de grãos).

Tratamentos	Eficiência (%) <sup>1</sup>		Eficácia (%) <sup>2</sup>	
	PF	EG	PF	EG
BR 3267	99	92	82	87
BR 3267 + MC 04.21	106	90	88	86
BR 3267 + C 04.50	102	111	84	104
BR 3267 + MC 04.21 + C 04.50	108	105	89	98

<sup>1</sup>Eficiência = (matéria seca da parte aérea (MSPA) dos tratamentos inoculados ÷ MSPA da testemunha absoluta) x 100; <sup>2</sup>Eficácia = (matéria seca da parte aérea (MSPA) dos tratamentos inoculados ÷ MSPA da testemunha nitrogenada) x 100.

**Tabela 5.** Número de vagens por vaso (NV), comprimento médio da vagem por vaso (CMV), peso da vagem por vaso (PV), número de grãos por vaso (NG) e peso total de semente por vaso (PTS) de plantas de caupi inoculadas com *Bradyrhizobium* sp. (BR 3267); co-inoculadas com BR 3267 + *Paenibacillus graminis* (MC 04.21); com BR 3267 + *P. durus* (C 04.50); com BR 3267 + MC 04.21 + C 04.50; e sem inoculação, testemunha absoluta (TA) e nitrogenada (TN).

<b>Tratamentos</b>	<b>NV</b> (und. vaso <sup>-1</sup> )	<b>CMV</b> (cm vaso <sup>-1</sup> )	<b>PV</b> (g vaso <sup>-1</sup> )	<b>NG</b> (und. vaso <sup>-1</sup> )	<b>PTS</b> (g vaso <sup>-1</sup> )
BR 3267	5,75 ab	18,92 ab	14,38 ab	56,25 ab	10,76 ab
BR 3267 + MC 04.21	5,50 abc	19,48 ab	13,87 abc	49,50 ab	10,61 ab
BR 3267 + C 04.50	6,00 ab	18,34 ab	14,10 abc	46,50 ab	10,50 ab
BR 3267 + MC 04.21 + C 04.50	7,00 a	19,84 a	17,59 a	58,50 a	12,06 a
TA	3,75 c	14,52 b	10,08 c	34,75 b	8,08 b
TN	4,75 bc	17,17 ab	12,51 bc	48,25 ab	9,46 ab
<b>CV(%)</b>	<b>14,03</b>	<b>12,15</b>	<b>13,25</b>	<b>20,32</b>	<b>12,19</b>

Nas colunas, as médias seguidas da mesma letra não diferem estatisticamente entre si pelo teste de Tukey (p<0,05).