Universidade Federal de Pernambuco
Centro de Biociências (CB)
Programade Pós-Graduação em Ciências Biológicas

MARIA DO CARMO SILVA BARRETO

BIOPROSPECÇÃO DA INTERAÇÃO PLANTA/BACTÉRIAS DIAZOTRÓFICAS ASSOCIADAS A DUAS VARIEDADES DE CANA-DE-AÇÚCAR EM MUDAS MICROPROPAGADAS

Recife
2017

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Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas, Área de Concentração Biotecnologia, da Universidade Federal de Pernambuco, comorequisito a obtenção do título de doutor em Ciências Biológicas.

Orientadora: Profª Drª Vera Lúcia Lima Menezes

Coorientadora: Profª Drª Márcia do Vale Barreto Figueiredo

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Aprovada em: 21/02/2017

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RESUMO GERAL

A FBN por bactérias diazotróficas associadas a plantas não leguminosas pode contribuir com parte do suprimento de N para as plantas, reduzindo os custos de produção e danos ambientais. O uso dessas bactérias tem sido foco de inúmeras pesquisas, por ser uma alternativa para reduzir o uso de fertilizantes químicos nitrogenados. Neste contexto o trabalho teve como objetivos avaliar a combinação de bactérias diazotróficas endofíticas regionais visando à introdução comercial da prática da inoculação destas bactérias em biorreatores em plantas micropropagadas de cana-de-açúcar na variedade RB92579; isolar, identificar e verificar a população de bactérias diazotróficas associadas à cana-de-açúcar variedade RB867515, em diferentes Estados do Nordeste do Brasil assim como avaliar os efeitos de diferentes estirpes de bactérias endofíticas oriundas da cana-de-açúcar RB867515 em sua fase inicial de crescimento (45 e 150 Dias Após Inoculação), com e sem inoculação e adubação nitrogenada. As variáveis analisadas foram: massa seca da parte aérea e raiz, perfilho e teor de N acumulada na planta. Amostras de colmos e raízes de cana-de-açúcar foram coletadas em três Estados do Nordeste do Brasil: Paraíba, Pernambuco e Alagoas. As raízes e colmos foram submetidas à desinfestação superficial e utilizou-se os meios semi-seletivos NFb, JMV, LGI-P e JNFb para contagem e isolamento, e em seguida procedeu-se a caracterização fisiológica e molecular dos isolados bacterianos. Foram isoladas 52 bactérias endofíticas nativas as quais foram avaliadas à capacidade de fixar nitrogênio, a atividade de redução do acetileno (ARA), capacidade de solubilizar fosfato inorgânico e produção de ácido indol acético (AIA). Foram realizados três ensaios em casa de vegetação em delineamento inteiramente ao acaso, em tubete com substrato estéril e em vasos com solo. No ensaio com a variedade RB92579 em vasos com solo, a inoculação mista promoveu um aumento significativo da massa seca da parte aérea das plantas sem adição do adubo nitrogenado, enquanto que o acúmulo de N-total nos tecidos não apresentou diferença significativa entre os tratamentos com e sem adubação. Quanto ao potencial biotecnológico da comunidade bacteriana, 57% do total de isolados apresentaram capacidade de fixar N_2*in vitro*, 69% apresentaram halo de solubilização, com índice de solubilização (IS) variando de 1,4 a 3,3 em meio de cultura com fosfato inorgânico e que praticamente todos os isolados foram capazes de produzir AIA. A inoculação de bactérias fixadoras de nitrogênio nativas da região Nordeste em mudas micropropagadas de cana-de-açúcar na variedade RB867515 aos 45 DAI não houve diferença significativa entre as plantas inoculadas e o controle não inoculado. Em vasos aos 150 DAI, a inoculação promoveu o desenvolvimento e também apresentaram performance semelhante ao tratamento nitrogenado. Os resultados mostram que a resposta da inoculação na micropropagação em biorreator de imersão temporária (BIT) é possível, e sugere um grande potencial na resposta à inoculação e otimização do processo em escala comercial, assim como a aplicação de estirpes homólogas pode ter contribuído para uma melhor resposta pela interação bactérias endofítica vs variedades de cana-de-açúcar (RB92579 e RB867515).

Palavras-chave: FBN. Endofíticos. Micropropagação. BIT. Inoculação. *Saccharum officinarum* L.

GENERAL ABSTRACT

BNF by diazotrophic bacteria associated with non-leguminous plants may contribute with part of the N supply to the plants, reducing production costs and environmental damage. The use of these bacteria has been the focus of numerous researches, since it is an attractive alternative to reduce the use of chemical nitrogen fertilizers. In this context, the work had as objectives evaluate the combination of regional endophytic diazotrophic bacteria aiming at the commercial introduction of the inoculation of these bacteria in bioreactors in micropropagated plants of sugarcane variety RB92579; isolated, identify and verify the sugarcane-associated diazotrophic bacteria population in the sugarcane variety (RB867515), in different states of northeastern Brazil and evaluate the effects of different strains of endophytic bacteria from the sugarcane variety RB867515, on its growth at the initial growth stage (45 and 150 Days After Inoculation), with and without inoculated and nitrogen fertilization equivalent to 80 kg of N ha^{-1}. The variables analyzed were: dry mass of shoots and roots, tillering and N content accumulated in the plant. Samples of stems and roots of sugarcane were collected in three States of the northeast of Brazil: Paraíba, Pernambuco and Alagoas. Roots and stems were submitted to surface disinfestation and the semi-selective media NFb, JMV, LGI-P and JNFb were used for counting and isolation, and then the molecular and physiological characterization of the bacterial isolates. Fifty-two native endophytic bacteria were isolated and characterized, being evaluated for their nitrogen fixation ability, acetylene reduction activity (ARA), inorganic phosphate solubilization and indole acetic acid (IAA) production. Were carried out in a greenhouse in a completely randomized design, in a tube with sterile commercial substrate and another in pots with soil. In the soil pot experimente with variety RB92579, the mixed inoculation promoted a significant increase in the dry mass of shoots of the plants without nitrogen fertilizer addition, whereas the total N accumulation in the tissues did not present significant difference between the treatments with and without fertilization. Concerning the biotechnological potential of the bacterial community, 57% of the total isolates showed *in vitro* N_2-fixation ability, 69% presented solubilization halo, with solubilization index (SI) ranging from 1.4 to 3.3 in culture medium with inorganic phosphate and virtually all isolates were able to produce IAA. The inoculation of nitrogen-fixing bacteria native to the northeast region in micropropagated seedlings of sugarcane variety RB867515 at 45 days after inoculation, there was no significant difference between the inoculated plants and the uninoculated control. In pots, at 150 DAI, the inoculation promoted plant development and also presented similar performance to the nitrogen treatment.The results show that inoculation response in micropropagation in temporary immersion bioreactor (BIT) is possible and suggests a great potential in response to inoculation and optimization of the process in commercial scale, as the application of homologous strains may have contributed to a better response by interaction between endophytic bacteria and sugarcane varieties (RB92579 and RB867515).

Keywords: BNF.Endophytic.Micropropagation.Inoculation.*Saccharum officinarum* L.

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1 INTRODUÇÃO

O nitrogênio (N) é um dos elementos requeridos em maior quantidade e um dos mais importantes nutrientespara o desenvolvimento das plantas. Sendo o N_2 (dinitrogênio)o principal reservatório de nitrogênio no ciclo biogeoquímico do nitrogênio e, portanto, um fator verdadeiramente ilimitado para proporcionar crescimento organísmico (SPATZAL, 2015). Este nutriente é encontrado em apenas 1% da massa seca total da planta, mas sua deficiência causa redução na síntese de clorofila, de aminoácidos essenciais e da energia necessária à produção de carboidratos e esqueletos carbônicos, refletindo diretamente no desenvolvimento e rendimento da cultura (MALAVOLTA, 2006; ROBERTSON; VITOUSEK, 2009).

O único processo biológico de obtenção de N, através da conversão do dinitrogênio (N_2) em amônia (NH_3), e que pode beneficiar as plantas, é a fixação biológica de nitrogênio atmosférico (FBN). O processo de FBN é realizado por um grupo restrito de organismos chamados diazotróficos, por meio da enzima nitrogenase(NOVAKOWISKI, et al., 2011; SPATZAL, 2015). O maior exemplo de sucesso de sua aplicação biotecnológica no Brasil é a inoculação da soja, exemplo de simbiose destes organismos com plantas.

Culturas economicamente importantes, tais como Poaceae, podem obter uma parte substancial do seu N de associações FBN, com bactérias diazotróficas endofíticas e associativas. Grandes aumentos de rendimento por meio do N fixado foram relatados no campo (BHATTACHARYYA; JHA, 2012). Assim, este tipo particular de associação planta-bactéria consiste em um sistema benéfico natural a ser explorado.

No Brasil, devido à falta de subsídios para fertilizantes, os adubos minerais nitrogenados comerciais são usados normalmente em doses menores que um quinto das aplicadas nos países industrializados. Segundo Urquiaga et al. (2012),os canaviais no Brasil são fertilizados com 60-70 kg N ha^{-1} ano^{-1}.

O Brasil se destaca no cenário atual como o maior produtor mundial de cana-de-açúcar, e é a terceira maior atividade agrícola em termos de área de produção e de valor bruto produzido, sendo que soja e milho são as maiores culturas do país. Os maiores produtores de cana-de-açúcar são os estados de São Paulo e Paraná, o Triângulo Mineiro e a zona da Mata Nordestina (PANTOJA et al., 2016). A cultura da cana-de-açúcar foi introduzida no Brasil no período colonial e desde então se transformou em uma das principais atividades da economia brasileira (REZENDE; RICHARDSON, 2015).

A inoculação com bactérias promotoras de crescimento em cana-de-açúcar pode ser considerada uma alternativa capaz de contribuir para a sustentabilidade desse setor, uma vez que a atuação de mecanismos de promoção de crescimento e o suprimento do N via FBN permitem ganhos de produtividade e reduzem a utilização de insumos de alto custo, desonerando o sistema de produção da cana-de-açúcar (CHAVESet al.,2015).

As bactérias diazotróficas associativas e endofíticas são geneticamente diversas. Foram identificados vários gêneros de Proteobactérias alfa, beta e gama, incluindo *Azospirillum*, *Azorhizobium*, *Azoarcus*, *Burkholderia*, *Citrobacter*, *Enterobacter*, *Gluconacetobacter*, *Herbaspirillum*, *Klebsiella*, *Pseudomonas* e *Rhizobium* (MAGNANIet al., 2010;SANTI et al., 2013). O uso dessas bactérias, principalmente em plantas não leguminosas, tem sido foco de inúmeras pesquisas (VIDEIRAet al., 2011;URQUIAGA et al., 2012;SCHULTZ et al., 2012, 2014; TAULÉ et al., 2012; PEREIRA et al., 2013; COSTA et al., 2013;CASTANHEIRA et al., 2014;RODRIGUEZ-ANDRADE, et al., 2015), uma vez que é uma alternativa bastante atrativa para reduzir o uso de fertilizantes químicos nitrogenados.Essa associação planta-bactéria pode promover o desenvolvimento da planta por outros benefícios, independentemente da FBN, como, por exemplo, pela produção de fitohormônios e controle de patógenos.

A micropropagação de plantas por sua vez é um método de propagação vegetativa amplamente estudado em diversas espécies vegetais, sendo a modalidade dentro da cultura de tecidos que mais se tem difundido e encontrado aplicações práticas comprovadas. Entre as vantagens de sua utilização estãoaredução do tempo e da área necessária à propagação da espécie; melhores condições sanitárias por meio do cultivo de meristemas previamente tratados por termoterapia, livres de doenças e pragas; com elevada qualidade genética, geralmente com fidelidade durante a multiplicação e a propagação vegetativa de espécies difíceis de serem propagadas por outros métodos. Por meio dessa técnica, é possível produzir grandes quantidades de plantas uniformes ao longo de todo o ano, sob condições controladas, sem a influência das variações climáticas (ROCHA, 2009).

Espera-se que a aplicação desta forma alternativa de adubo associadaà micropropagação *in vitro* reduza a necessidade de N fertilizante para a cultura da cana-de-açúcar.

O objetivo deste trabalho foi selecionar bactérias diazotróficas nativas de diferentes ambientes do Nordeste eficientes na associação com as variedades RB92579 e RB867515, avaliando o efeito da promoção de crescimento e otimização desta associação com o uso de plantas micropropagadas.Portanto, a hipótese deste trabalho propõe que as bactérias diazotróficas inseridas na micropropagação *in vitro,* utilizando micro-organismos nativos da região Nordeste, possam

suprir total ou parcialmente as necessidades de N para a cultura da cana-de-açúcar, melhorando a produção verticalmente, o que é uma necessidade para os produtores desta cultura, além disso, pode ser possível manipular as condições de crescimento das plantas, e assim, viabilizar trabalhos com a finalidade de analisar a contribuição do nitrogênio fixado biologicamente.

2 OBJETIVOS

2.1 OBJETIVO GERAL

Seleção de bactérias diazotróficas nativas de diferentes ambientes do Nordeste (Pernambuco, Paraiba e Alagoas) eficientes na associação com duas variedades de cana-de-açúcar (RB92579 e RB867515), testando o efeito da promoção de crescimento e otimização desta associação com o uso de plantas micropropagadas.

2.2 OBJETIVOS ESPECÍFICOS

- ✓ Coletar e isolar bactérias endofíticas das variedades RB92579 e RB867515 de elevado grau de cultivo no Nordeste, amostras de colmo e raízes para isolamento e identificação de bactérias diazotróficas fixadoras de nitrogênio e promotoras de crescimento;
- ✓ Avaliar a produção de substâncias reguladores de crescimento vegetal (AIA) e solubilizadoras de fosfato inorgânico por bactérias diazotróficas endofíticas;
- ✓ Identificar e analisar filogeneticamente os isolados bacterianos pelo estudo do gene 16S rRNA;
- ✓ Identificar o potencial de resposta à inoculação na fase "*in vitro*";
- ✓ Aplicar bactérias diazotróficas no cultivo de mudas micropropagadas;
- ✓ Testar inoculante através da seleção de bactérias diazotróficas capazes de promover o crescimento da planta e que reduzam parcialmente a demanda de N na cana-de-açúcar.

3 REVISÃO BIBLIOGÁFICA

3.1 A cultura da cana-de-açúcar (*Saccharum* spp.)

A cana-de-açúcar sempre teve um papel importante na economia brasileira, desde o período dos engenhos coloniais. As primeiras mudas da planta chegaram ao Brasil por volta de 1515, vindas da Ilha da Madeira (Portugal), tendo sido o primeiro engenho de açúcar construído em 1532, na capitania de São Vicente. Mas foi no Nordeste, especialmente nas capitanias de Pernambuco e da Bahia, que os engenhos de açúcar se multiplicaram(CIB, 2009).

Historicamente, a cana-de-açúcar sempre foi um dos principais produtos agrícolas do Brasil e, hoje, o País é o maior produtor mundial de cana-de-açúcar, de acordo com o Ministério da Agricultura, Pecuária e Abastecimento (BRASIL, 2015), e de seus principais derivados: o açúcar corresponde a mais da metade do que é comercializado no mundo e o etanol está em crescente expansão por conta do uso do biocombustível em veículos automotores e como alternativa energética (REZENDE; RICHARDSON, 2015; BORTOLETTO; ALCARDE, 2015), seguido pela Índia, China, Tailândia, Paquistão e México (AHMEDet al., 2014).

A cana-de-açúcar (*Saccharum* sp.) é uma gramínea,considerada uma planta de metabolismo C4 com ciclo de vida longo e perene (GÓMEZ-MERINO et al., 2014), com dois ciclos básicos de produção no campo: o ciclo de cana-planta, que se inicia no plantio e se encerra com a primeira colheita; e os ciclos de cana-soca, que se iniciam após as colheitas e continuam sucessivamente até a reforma da área, que ocorre após quatro a cinco colheitas, em média (CHEAVEGATTI-GIANOTTO et al., 2011) (Figura 1). Esse termo provém de “gramina”, nome usado pela primeira vez por Lineu, significando plantas semelhantes à grama. Segundo a classificação taxonômica (CRONQUIST, 1981), a cana-de-açúcar pertence à divisão *Magnoliophyta*, classe *Liliopsida*, subclasse *Commilinidae*, ordem *Cyperales* e família *Poaceae*.

Figura 1- Cana-de-açúcar (*Saccharum* sp.)

As principais variedades de cana-de-açúcar cultivadas comercialmente pertencem ao gênero *Saccharum*, que compreende seis espécies: *Saccharum officinarum, S. robustum, S. spontaneum, S. sinense, S. barberi* e *S. edule* (PERIN, 2007). As características ambientais e a competitividade exigem produtividade, redução de custos e dos impactos no meio ambiente, sendo necessários investimentos para seu cultivo, o que já está sendo realizado no país.

A primeira variedade de cana-de-açúcar cultivada comercialmente era conhecida como "cana criola", resultado do cruzamento de variedades do grupo mungo (*S. barberi*) e uma cana nobre (*S. officinarum*), importada para a América por Cristóvão Colombo, tendo sido cultivada por mais de 250 anos. No Brasil, a variedade Caiana foi amplamente cultivada, sendo responsável pela produção mundial do açúcar durante quase um século (ANDRADE, 1985).

No início da década de 70, o Estado de São Paulo assumiu a liderança na produção e tecnologia de cana-de-açúcar no país. Neste período, novas opções de variedades para o plantio foram proporcionadas pelos programas de melhoramento genético da cana-de-açúcar: IAC (Instituto Agronômico de Campinas), RB (PLANALSUCAR) e as SPs (COPERSUCAR). Posteriormente, o censo varietal de 1996 destacava as variedades dos programas de melhoramento das séries RB (República do Brasil) e São Paulo como as variedades mais importantes no cenário sucroalcooleiro.

Uma das variedades de cana-de-açúcar mais cultivadas no Brasil,a RB867515 foi lançada oficialmente em março de 1997 como variedade comercial pela Universidade Federal de Viçosa, por meio da Rede Interuniversitária de Desenvolvimento do Setor Sucroalcooleiro (RIDESA), esta variedade apresenta crecimento rápido,melhor desempenho em solos de textura leve e fertilidade

média, possui médio perfilhamento, boa brotação e alto teor de sacarose.É uma variedade de boa produtividade, apresentando em resultados experimentais 117,25 toneladas de cana por hectare (RIDESA, 2010), e é bastante difundida no Nordeste e resistente a fitopatógenos.

Uma outra variedade que foi liberada para o cultivo em 2003 pela Universidade Federal de Alagoas, também filiada à RIDESA, é a variedade RB92579. Esta variedade possuicrescimento lento, alto perfilhamento, boa brotação, alto teor de sacarose, excelente produtividade agrícola e apresenta boa recuperação após períodos de seca. Sob irrigação plena apresenta elevada produtividade, com média acima de 140 toneladas de cana por hectare (RIDESA, 2010).

A cana-de-açúcar foi responsável pelo primeiro ciclo econômico brasileiro, o que a torna, historicamente, um dos principais produtos agrícolas do país. Como tal planta é matéria-prima de grande flexibilidade, pode ser empregada na forma de forragem para a alimentação animal e na fabricação de aguardente, açúcar e álcool. Seus resíduos podem ser utilizados como fertilizantes e o bagaço possui potencial para a produção de energia (Figura 2). Além disso, é possível obter etanol, por meio da fermentação do caldo extraído do colmo (SRIVASTAVA; RAI, 2012); etanol de segunda geração, por meio da utilização de enzimas que promovem a degradação lignocelulósica do bagaço e palhada (OLIVEIRAet al., 2013; PRICE et al., 2014); energia elétrica, pela queima do bagaço e da palhada (LEAL et al., 2013), além de outros subprodutos como a compostagem e a vinhaça para uso na adubação de culturas (TRIVELINet al., 2013; FUESS; GARCIA, 2014).

O Programa Nacional do Álcool Combustível (Proálcool) foi lançado em 1975 e expandiu a cultura canavieira no Brasil, representando o marco na evolução do etanol no mercado brasileiro (LIMAet al., 2013) para produzir etanol a partir da cana-de-açúcar objetivando o aumento da produção de álcool para substituir os combustíveis derivados de petróleo, em especial a gasolina, sem prejuízo na fabricação de açúcar (DUARTE JR., 2006; PERIN, 2007). Esse programa cresceu rapidamente, assim como a cultura canavieirano país (BODDEY et al., 2003). Foi criado o Programa Nacional de Melhoramento da cana-de-açúcar (PLANALSUCAR), visando desenvolver processos e métodos ligados ao cultivo da cana-de-açúcar e à produção de açúcar e álcool. Essas instituições desenvolveram e substituíram as antigas variedades de cana-de-açúcar pelas novas variedades CB (Campos, Brasil), PB (Pernambuco, Brasil) e IAC (Instituto Agronômico de Campinas), sendo mais adaptadas ao tipo de solo, clima e sistema de corte (manual ou mecânico), bem como apresentando resistência a pragas e maior concentração de sacarose (PERIN, 2007).

Figura 2 - Produtos obtidos da cana-de-açúcar. (a) Forragem, (b) açúcar, (c) Álcool, (d) bagaço.

Dentro do agronegócio, a cana é uma das culturas que mais gera renda. A Companhia Nacional de Abastecimento (CONAB) acompanha a produção de cana-deaçúcar no Brasil. Em seu relatório técnico divulgado em agosto de 2016,apresentou dados da safra passada, e expectativas para a safra em andamento. Segundo a CONAB, a área plantada na safra 2015/2016foi em torno de8.7 milhões de hectares, distribuídos em todos os estados produtores conforme suas características. O estado de São Paulo é o maior produtor com 56% (4.668,2 mil hectares), seguido por Goiás com 10,4% (930,9 mil hectares), Minas Gerais com 9% (862,5 mil hectares), Mato Grosso do Sul com 7,1% (615 mil hectares), Paraná com 6,7% (623,6 mil hectares), Alagoas com 2,7% (340,5 mil hectares), Pernambuco com 2,1% (267,7mil hectares) e Mato Grosso com 2,6% (230,2 mil hectares) (Figura 3). Estes oito estados são responsáveis por 94,5% da produção nacional. Os outros 14 estados produtores possuem áreas menores, totalizando 5,5% da área total do país (CONAB, 2016).

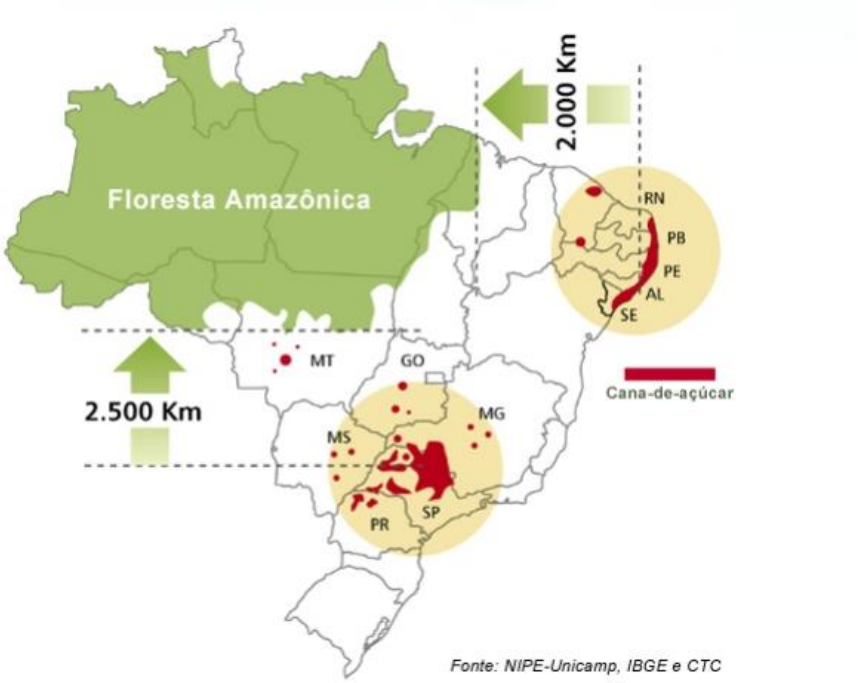


Figura 3 - Mapa de produção da cana-de-açúcar no Brasil. Área destacada em vermelhoconcentra as plantações e usinas produtoras de açúcar, etanol e bioeletricidade. (www.unica.com.br).

A produção de cana-de-açúcar estimada para a safra 2016/17 é de 684,77 milhões de toneladas. O crescimento está previsto em 3,8% em relação à safra anterior. A área colhida destinada à atividade sucroalcoleira deverá ser de 8.973,2 mil hectares, aumento de 3,7%, se comparada com a safra 2015/16.No Nordeste, os maiores produtores, situados na Zona da Mata, apresentam números positivos, com aumento de 26,9% em Pernambuco e 12,6% em Alagoas. Espera-se melhorias de produtividade motivada pelas chuvas, condição climática favorável à cultura. Somam-se ainda as boas expectativas de mercado para o açúcar e para o etanol (CONAB, 2016).

3.2 Micropropagação de cana-de-açúcar (*Saccharum* spp.)

O mercado da micropropagação movimenta bilhões de dólares em todo o mundo, notadamente na Alemanha, Holanda, Inglaterra, Índia, Estados Unidos, entre outros países (CID, 2010).No Brasil, uma das principais limitações para o maior acesso dos produtores às mudas micropropagadas é o elevado custo desses materiais, ainda muito superior ao das mudas obtidas pelos métodos convencionais (ROCHA, 2009).

A propagação convencional para cana-de-açúcar é realizada a partir de segmentos de colmos provenientes de plantas do campo, após o primeiro ou segundo ano de plantio (propagação vegetativa). A propagação vegetativa ano após ano resulta em acúmulo de infecção patogênica sistêmica, levando ao declínio varietal, o que coloca um sério problema na multiplicação de um genótipo elite. Porém, novas variedades estão continuamente sendo desenvolvidas e sua disponibilização pode ser acelerada por meio da biotecnologia, via micropropagação, sendo um método eficiente de propagação vegetativa na cana-de-açúcar (OLIVEIRA et al., 2010; KAUR;KAPOOR, 2016).

Segundo Alveset al. (2008), o princípio da cultura de tecidos baseia-se na teoria da totipotência, segundo a qual os seres vivos têm a capacidade de regenerar organismos inteiros, idênticos à matriz doadora, a partir de células únicas. Os mesmos autores afirmam que a micropropagação é a propagação fiel de um genótipo, por meio das técnicas da cultura *in vitro*. Essa técnica é empregada em locais que garantem controle de esterilidade e rastreabilidade, chamados Biofábrica.

Um sistema eficiente para produção de mudas de cana-de-açúcar de qualidade e em larga escala é a micropropagação, já estabelecida como rotina em muitos países (MELOet al., 214). No Brasil, alguns laboratórios ou biofábricas produzem mudas micropropagadas de diversas espécies

incluindo variedades elite de cana-de-açúcar. E este sistema foi potencializada com a utilização de biorreatores de imersão temporária (BIT), os quais aumentam significativamente as taxas de crescimento e multiplicação e melhoram a qualidade das plantas produzidas (GERALD; LEE, 2011).

Micropropagação (ou propagação vegetativa *in vitro*), constitui uma das principais aplicações da cultura de tecidos vegetais, permite a multiplicação de plantas oriundas de cultura de meristemas livres de doenças. Possibilita o controle das condições ambientais durante o processo de propagação, garantindo o desenvolvimento apropriado e eventuais ajustes. Outra vantagem é a possibilidade de grandes quantidades de plantas uniformese sadias ao longo de todo o ano, em curto espaço de tempo, utilizando uma área relativamente pequena quando comparada com o sistema convencional (ROCHA, 2009) (Figura 4).

Figura 4 - Mudasmicropropagadas de cana-de-açúcar.

Segundo Winkelmann et al. (2006), os primeiros laboratórios de produção comercial de mudas micropropagadas foram implantados na Europa Ocidental e na América do Norte, nas décadas de 1970 e 1980. De acordo com Liu; Liu (2010), na China, a tecnologia da cultura de tecidos de plantas teve início na década de 1970, desenvolvendo-se rapidamente. Na Alemanha, desde a década de 1980, a cultura de tecidos vem sendo empregada como uma importante ferramenta para a propagação de plantas de interesse econômico e, a partir de 1995, a produção comercial de mudas *in vitro* tem sido registrada com a criação da Associação Alemã dos Laboratórios de Cultura *In Vitro* (ADVK), (WINKELMANNet al., 2006).

O termo “biofábrica” é empregado para as empresas que produzem e comercializam mudas micropropagadas. Segundo Gerald &Lee (2011), uma biofábrica de plantas pode ser definida como um laboratório de cultura de tecidos vegetais que produza maciçamente *in vitro* determinada quantidade de mudas e cujo processo de produção esteja bem definido.

Para que variedades sejam micropropagadas eficientemente faz-se necessário, primeiramente, o estabelecimento de protocolos de desinfestação dos explantes, a elaboração de meios nutritivos específicos e demais condições ideais de cultivo *in vitro*. Para a cultura da cana-de-açúcar, o explante inicial a ser micropropagado é o meristema apical, que depois de isolado e inoculado em meio de cultura apropriado se desenvolve, dando origem às plântulas que serão então multiplicadas, enraizadas e aclimatizadas. A técnica de propagação desta espécie por meio de meristema apical é considerada uma alternativa vantajosa para a multiplicação de diversas variedades, devido à economia de tempo em relação às técnicas convencionais, além da obtenção de mudas de excelente qualidade fitossanitária e geneticamente idênticas ao material de origem (VIEIRA et al., 2009).

A primeira etapa do processo de micropropagação é o cultivo do explante *in vitro* com concentrações controladas de reguladores de crescimento (LEE, 1987). A origem e a sanidade das matrizes a serem multiplicadas, devem ser cuidadosamente controladas. O genótipo (matriz utilizada), a fonte de explante (raiz, caule, folhas, etc.) e a condição de cultivo (luz, temperatura, meio de cultura, recipiente) constituem fatores capazes de exercer grande influência sobre o processo de regeneração dos explantes. No entanto, segundo Toledo (2011), a primeira etapa se inicia no jardim de matrizes, onde as plantas são indexadas para as principais doenças da cana-de-açúcar e depois, cultivadas. A seguir, os colmos são selecionados das matrizes, coletados e cortados em mini-toletes, que serão tratados termicamente, plantados e acondicionados numa estrutura denominada (Unidade de Termoterapia Intensiva – UTI) à 35ºC, para brotamento rápido das gemas. Em seguida, as brotações são retiradas dos mini-toletes, cortadas (explantes) e desinfectadas.

Murashige (1974) apresentou o conceito de três estádios de desenvolvimento no processo de propagação *in vitro*. No primeiro, ocorrem a seleção dos explantes (pequenos fragmentos de tecido vivo), desinfestação e cultura em meio nutritivo, sob condições assépticas, enquanto no segundo ocorre a multiplicação dos propágulos, mediante sucessivas subculturas em meio próprio para multiplicação e o terceiro é caracterizado pela transferência das partes aéreas produzidas para meio de enraizamento e subseqüente transplante das plantas obtidas para substrato ou solo. Nesta fase, a planta fica mais susceptível ao estresse hídrico e ainda passa de uma existência heterotrófica para um estado autotrófico. (EMBRAPA ALGODÃO, 2006). O objetivo é obter uma nova planta idêntica à original, ou seja, realizar uma clonagem vegetal.

Diferentes protocolos de micropropagação de cana-de-açúcar mostram a viabilidade desta técnica em meio semi-sólido (GARCIA et al., 2007), líquido estacionário e por imersão temporal (LORENZO et al., 2001; ARENCIBA et al., 2008). Cidade et al. (2006) relataram diferenças entre estes métodos de cultivo na morfogênese *in vitro* de cana-de-açúcar. Estes autores verificaram que o

cultivo de brotos de cana-de-açúcar em meio líquido,sob agitação, possibilita aumentos de até 100% na produção de plantas, em comparação com o cultivo em meio semissólido.

A micropropagação é realizada com rigorosa assepsia, principalmente devido ao fato de que uma parte dos micro-organismos, ao entrar em contato com o meio de cultura, encontrará as condições necessárias para se desenvolver, inviabilizando a cultura. O alto grau de contaminação e a localização sistêmica de micro-organismos são responsáveis, em alguns casos, pelo insucesso da implantação de culturas *in vitro*. Existem três fontes básicas de contaminação por micro-organismos; o meio de cultura, o explante e o ambiente (PASQUAL, 2001).

A cana-de-açúcar é o único membro da Poaceae pertencente ao gênero *Saccharum* onde protocolos da propagação *in vitro* foram padronizados e comercialmente viáveis (KAUR; KAPOOR, 2016). No entanto, apesar de alguns protocolos já estarem bem estabelecidos, estes não se aplicam universalmente a todas as variedades de cana-de-açúcar, uma vez que diversos fatores influenciam a regeneração e a estabilidade genética das plantas *in vitro*, tais como genótipo, tipo de explante, condições de cultivo e combinação destes (SNYMANet al., 2011).

A cana-de-açúcar foi uma das primeiras culturas industriais a ser micropropagada em escala comercial (WORLD SUGAR STATISTICS, 2005), eocupa o quarto lugar entre as grandes culturas micropropagadas (CARVALHOet al., 2012). Em 2008, apenas três entidades possuíam registro no Renasem (Registro Nacional de Sementes de Mudas) para a produção de mudas dessa espécie, em unidade de propagação *in vitro*. Já, em 2012, esse número saltou para sete; portanto, um aumento de 133,3%. Dessas sete, quatro são biofábricas especializadas na produção unicamente de mudas de cana-de-açúcar. Provavelmente, a significativa ampliação do número de biofábricas pode estar relacionada com a posição de destaque mundial que o Brasil vem apresentando no setor canavieiro, como maior produtor e exportador de açúcar e de álcool biocombustível veicular, oriundos dessa cultura (CARVALHO et al., 2012).

Estudos evidenciam que as mudas produzidas *in vitro* podem aumentar a produtividade da cultura de 10% a 30% e a longevidade dos canaviais em até 30% (LEE et al., 2007), uma vez que possibilitam melhor padrão fitossanitário, controle das condições ambientais do processo, produção durante todo o ano e otimização da área utilizada, quando comparadas com mudas produzidas pelos métodos convencionais (CRUZ et al., 2009).

Garcia et al. (2007) enfatizam a utilização de plantas de cana-de-açúcar previamente estabelecidas *in vitro* como fonte preferencial de explante. Pois, ao contrário das plantas oriundas do campo, estas apresentam uniformidade fisiológica e constante disponibilidade de material, não requerem desinfestação e usualmente liberam menor quantidade de compostos fenólicos.

A multiplicação *in vitro* de cana-de-açúcar tem recebido considerável atenção em pesquisa. Atualmente, a aplicação da micropropagação tem sido empregadana produção de cana-de-açúcar, que além de atuar na melhoria da qualidade do produto, possibilita a propagação de plantas isentas de viroses e outras doenças, promove a manutenção das características da planta matriz e a otimização da produtividade, proporcionando a multiplicação rápida e em grande escala das mudas, a fim de acelerar o processo de criação e comercialização da cana-de-açúcar (TOLEDO, 2011).

A comercialização de plantas micropropagadas de cana-de-açúcar foi introduzida no Brasil a partir da década de 80, com o objetivo de produzir mudas com características genéticas originais, excelente grau de fitossanidade e produzidas de variedades adaptadas, permitindo cada vez mais o aumento da produção e da competitividade do produtor, sem a necessidade de avançar sobre áreas de preservação ambiental; além disso, por terem maior potencial de crescimento, as mudas micropropagadas exercem um melhor controle da vegetação invasora, reduzindo os custos de produção com os tratos culturais(CRUZ et al., 2009).

O uso de mudas com alto padrão de qualidade pode contribuir para expressivos ganhos da produtividade agrícola que são essenciais para manter o Brasil como maior produtor e exportador de açúcar e álcool (etanol) oriundos da cana-de-açúcar. Entretanto, além de eliminar os micro-organismos patogênicos, a micropropagação também promove a eliminação das bactérias diazotróficas endofíticas.

3.3 Fixação biológica de nitrogênio em gramíneas

Um dos principais macronutrientes requeridos pelos vegetais é o nitrogênio (N), considerado o elemento mais abundante na atmosfera terrestre (em torno de 79%), está presente principalmente na forma diatômica (N_2). O dinitrogênio (N_2) é a modificação mais abundante do elemento na terra e crucial para todo o organismo vivo sintetizar compostos biológicos essenciais, além de ser o principal reservatório de nitrogênio no ciclo biogeoquímico do nitrogênio (SPATIZAL, 2015).Sua disponibilidade é um fator importante que limita a produção vegetal tanto em ambientes naturais como ambientes agrícolas (KRAISERet al., 2011) e representa um dos mais altos custos para o agricultor. O seu fornecimento para a planta depende da disponibilidade do elemento no solo, fornecimento via adubação (solo ou foliar) e da fixação biológica de nitrogênio (FBN).

Além da FBN, quase 50% do nitrogênio global atualmente é produzido por um processo industrial que surgiu no início do século 20, o processo Haber-Bosch.Enquanto o sistema enzimático realiza a redução de nitrogênio em condições ambientais, o processo industrial Haber-Bosch requer altas temperaturas de reação (350-550 °C) e pressões (250-350 atm), consumindo

quase 2% da produção de energia global de hoje. Além disso, a eficiência de absorção de nitrogênio de fertilizantes artificiais é baixa. Apenas cerca de 60% do nitrogênio fixado é incorporado nas culturas, enquanto 40% do nitrogênio fertilizante sofre desnitrificação, correlacionando-se com um enorme desperdício de energia e levando a uma significativa "poluição nitrogenada" do solo (SPATZAL, 2015).

As doses de feritilizante nitrogenado, utilizadas nas áreas de cana-de-açúcar no Brasil, são, em média, de 40 Kg ha $^{-1}$ de N na cana-planta e de 80 Kg ha $^{-1}$ de N nas soqueiras (NUNUS JUNIOR et al., 2005), chegando a 40% de aproveitamento na cana-planta e 70% na cana-soca (FRANCO et al., 2011). Estima-se que a contribuição da FBN para a nutrição nitrogenada da cana-de-açúcar no Brasil pode contribuir com pelo menos 40 kg ha^{-1} de N (URQUIAGAet al., 2012). Podendo o genótipo da planta e as condições da fertilidade do solo, está relacionado a esta variação, porém, em se tratando de uma cultura que ocupa milhões de hectares, o suprimento de cerca de 30 % da demanda de N pela FBN certamente trará benefícios ambientais e econômicos para essa cultura (CHAVESet al., 2015).

A FBN é um dos processos naturais mais importantes do planeta, assim como a fotossíntese. A maior parte do nitrogênio fixado no ambiente terrestre é originada da FBN. É uma alternativa para um manejo sustentável dos solos realizado por alguns micro-organismos que possui habilidade em reduzir o nitrogênio atmosférico (N_2) a amônia (NH_3), por intermédio da enzima nitrogenase, segundo a reação:

$$N_2 + 8H^+ + 8e^- + 16ATP \xrightarrow{\text{Nitrogenase}} 2NH_3 + H_2 + 16ADP + 16Pi$$

Na agriculturarepresenta um substituto promissor para os fertilizantes químicos (CARVALHO et al., 2014; WEZEL et al., 2014).

A FBNé realizada por micro-organismos conhecidos por fixadores de N_2 ou diazotróficos. Estas bactérias possuem o complexo enzimático da nitrogenase, capaz de catalisar a fixação do nitrogênio atmosférico (SPATZEL, 2015; NOVAKOWISKIet al., 2011). A maioria tem sido observada em associação com plantas de arroz, milho, sorgo, trigo, soja, feijão, tomate, algodão e cana-de-açúcar (BALDANI et al., 2000).

O Brasil tornou-se o único país do mundo a obter, com absolutamente nenhuma aplicação de N, altos rendimentos de soja, que se tornou o maior produto de exportação do país (HUNGRIAet al., 2006). Outras culturas economicamente importantes, especialmente monocotiledóneas, tais como Poaceae, podem obter uma parte substancial do seu N de associações FBN com bactérias

diazotróficas endofíticas e associativas. Embora a quantidade de N fixo não seja tão grande quanto a medida em endossimbiose, grandes aumentos de rendimento foram relatados no campo (BHATTACHARYYA; JHA, 2012).

Utilizando a técnica de diluição isotópica de ^{15}N, Boddey e colaboradores verificaram que 25 a 60% do N assimilado pela planta de cana-de-açúcar era proveniente da FBN. Os dados de literatura mostram que a contribuição de FBN associadas às plantas de cana-de-açúcar cultivadas no campo e sem inoculação varia de zero a 70%, conforme demonstrado pelas medições realizadas pela da técnica de abundância natural de delta ^{15}N e da técnica de diluição isotrópica de ^{15}N(URQUIAGAet al., 1992; BODDEY et al., 2001,2003; SCHULTZ et al., 2014).

A baixa resposta da maioria das variedades a altas doses de N-fertilizante que se observa no Brasil, comparada a outros países produtores, tem sido associada à significativa contribuição da FBN na nutrição nitrogenada da cultura.

A viabilidade da aplicação tecnológica de bactérias que promovem o crescimento em gramíneas encontra respaldo em muitos trabalhos científicos, que apontam diversos benefícios para a cultura, visando obter respostas quanto à contribuição da FBN.Oliveira e colaboradores em 2002 efetuaram a inoculação de consórcios bacterianos, e demonstraram não haver aumento superior a 30% na concentração de N, sugerindo que novos estudos deveriam ser realizados para que se pudesse melhor explorar o potencial das bactérias diazotróficas para culturas agrícolas. Uma das possíveis causas dessa baixa eficiência foi reportada no estudo realizado por Oliveira et al. (2009), utilizando a técnica de grupos específicos de bactérias diazotróficas colonizando a cana-de-açúcar, ocorrendo dessa forma uma competição entre as bactérias nativas e as inoculadas, levando a uma menor eficiência do processo de FBN.Pereira et al. (2013) constataram que algumas variedades, quando inoculadas, chegam a acumular mais matéria seca do que em tratamentos com uso de fertilizante nitrogenado. Gosal et al. (2012), no entanto, relataram que o uso de inoculante na espécie permite maior acúmulo de biomassa apenas quando combinado com a adubação nitrogenada. Segundo Pedraza (2008), além de contribuir na FBN, a associação com bactérias promotoras de crescimento em cana-de-açúcar pode reduzir o uso de fertilizantes na cultura, por solubilizar fosfatos e zinco (ESTRADA et al., 2013) e produzir sideróforos e reguladores de crescimento, como auxinas, giberelinas e citocininas (LIN et al., 2012; SANTI et al., 2013). Alguns gêneros, como *Azospirillum*, favorecem o crescimento vegetal, principalmente pela síntese de auxinas (SANTIet al., 2013). Assim como na adubação nitrogenada, as respostas à inoculação dependem da variedade utilizada (SCHULTZet al., 2012; URQUIAGA et al., 2012) e costumam ser mais frequentes em solos de média e baixa fertilidade (OLIVEIRA et al., 2006; GOSAL et al., 2012). A inoculação de

bactérias fixadoras de nitrogênio proporcionou significativa contribuição da FBN para a variedade de cana-de-açúcar SP70-1143, em solos de baixa fertilidade, e promoveu produtividades similares de áreas que receberam fortilizante nitrogenado (OLIVEIRAet al., 2006). Pesquisas realizadas com a cultura de trigo indicam que a inoculação de bactérias diazotróficas não substitui os fertilizantes nitrogenados, porém promove melhor absorção e utilização do N disponível no solo (ROESCHet al., 2005). Neste sentido, Suman et al.(2008) mostraram que variedades de cana-de-açúcar, com maior número de bactérias diazotróficas, não somente apresentam maior potencial de FBN, porém, quando submetidas à metade da dose recomendada de fertilizante nitrogenado, atingiram níveis de produtividade similares aos de plantas com a dose completa. A inoculação com bactérias diazotróficas também pode aumentar a velocidade de brotação das gemas e emissão de raízes em colmos de cana-de-açúcar utilizados para o plantio (LANDELLet al., 2012). Em trabalhos realizados em condições de campo, a inoculação mista com bactérias diazotróficas proporcionou aumentos de produtividade de colmose matéria seca total similares ao efeito da adubação com 120 kg ha^{-1} de N (SCHULTZ et al., 2012; 2014).

As variedades de cana-de-açúcar apresentam potenciais diferenciados com relação à FBN. Por ser um genoma complexo, cada variedade deve ser testada quanto a sua resposta à inoculação, sendo que há indícios bem concretos que as variedades mais responsivas a adição de fertilizantes nitrogenados é que são as mais prováveis de responder ao inoculante.

Como visto, os resultados de pesquisas focadas na inoculação de diazotróficos e aumento da eficiência da FBN em gramíneas é um desafio, e a interação planta-bactéria deve ser bem explorada (OLIVEIRA et al., 2003).

3.4 Bactérias diazotróficas endofíticas associadas a plantas não leguminosas

Um dos grupos mais estudados de diazótrofos compreende microrganismos que vivem em estreita associação com as plantas, e que têm o potencial de transferir o nitrogêniofixado para o hospedeiro. Os diazótrofos endofíticos são capazes de colonizar os tecidos internos da planta sem danificar o hospedeiro (CHUBATSU, et al., 2012).

No sistema natural são encontrados diversos micro-organismos que contém o complexo enzimático da nitrogenase, portanto, fixadores de N e conhecidos como bactérias diazotróficas(SPATZEL, 2015). Estão presentes nos mais diversos ecossistemas como o solo, as plantas, a água, etc., ocorrendo de forma livre ou em interações de diversas formas como a realizada de forma associativa, promovendo o crescimento das plantas e melhorando a tolerância ao estresse

(SESSITSCH; PUSCHENREITER, 2008). Além disso, os metabólitos sintetizados por bactérias endofíticas podem induzir resistência a patógenos de plantas com potencial para uso em aplicações biotecnológicas. As bactérias diazotróficas endofíticas podem ser detectadas dentro de plantas esterilizadas superficialmente, e uma de suas características é que elas estão localizadas dentro da planta e não causam nenhum efeito nocivo visível (MONTEIROet al., 2012) (Figura 5). Acredita-se que estas sejam as principais responsáveis pelo ganho de N através da FBN observado em diversas culturas.

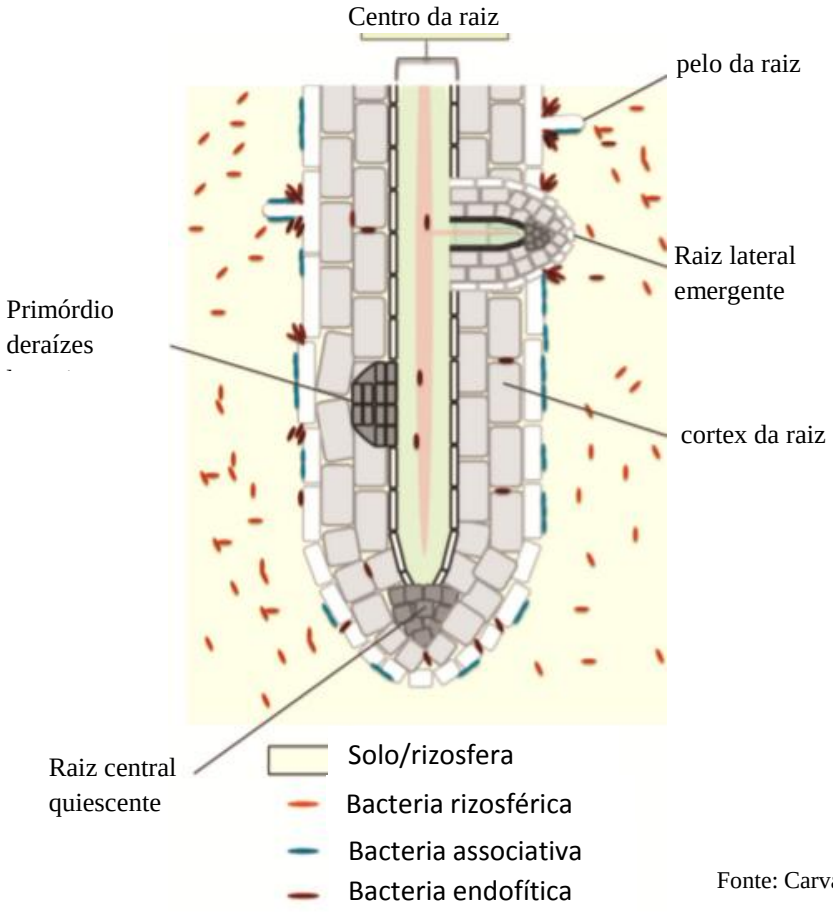
Estudos sobre associação de bactérias diazotróficas com gramíneas e cereais se iniciaram na década de 1960. Nestas plantas, não ocorre a formação de nódulos, mas sim a colonização da superfície e/ou interior das raízes e folhas por bactérias do solo que fixam nitrogênio do ar e disponibilizam às plantas.

Vários trabalhos têm dado especial atenção à estrutura das comunidades microbianas associadas à cana-de-açúcar, buscando a promoção de crescimento vegetal e a aplicação biotecnológica por meio dos processos de fixação biológica de N, solubilização de fosfato inorgânico, antagonismo a fitopatógenos, reguladores de crescimento vegetal e produção de enzimas de interesse industrial, entre outros (COMPANTet al., 2010; LUVIZOTTO et al., 2010; MENDES et al., 2011).

Um dos pioneiros e relevantes trabalhos sobre a contribuição da FBN para gramíneas foi realizado por Urquiaga e colaboradores (1992), em que os autores descobriram por meio de técnicas isotópicas de ^{15}N que algumas variedades de cana-de-açúcar poderiam obter cerca de 60% do N necessário para seu desenvolvimento por meio da associação com bactérias endofíticas.

Tais diazotróficos ocupam preferencialmente ointerior das plantas, localizando-se em nichos protegidos do oxigênio que,juntamente com outros fatores, os tornam os mais promissores grupos de diazotróficos associados às plantas não leguminosas, e, como resultado dessa associação, a planta recebe benefícios ecológicos da presença do simbionte como controle de fitopatógenos ou promoção do crescimento vegetal (RYAN et al., 2008).

A maior limitação para a FBN em sistemas não simbióticos é a disponibilidade de fontes de carbono para a bactéria e, consequentemente, para obtenção de energia, uma vez que o processo demanda grande quantidade de ATP. Essa limitação tenta ser compensada pelo diazotrófico com a sua localização mais próxima da planta, ou seja, ao redor ou dentro das raízes, como endófitos (TILAKet al, 2005). Tais endófitos, se estabelecem em nichos menos competitivos que apresentam melhores condições de fixação e assimilação de N fixado pela planta (REINHOLD HUREK; HUREK, 2011).



Fonte: Carvalho et al. (2014)

Figura 5 - Nichos radiculares colonizados por bactérias diazotróficas. As bactérias endófitas (glóbulos vermelhos escuros) colonizam qualquer região dentro da epiderme da raiz da planta, podendo residir nos espaços intercelulares apoplásicos e no vaso do xilema apoplasto. Em geral, os endófitos invadem os tecidos vegetais internos através de sítios de lesão na epiderme, as pontas radiculares e as rachaduras radiculares formadas nos locais de emergência das raízes laterais.

As espécies de poáceas são capazes de se associar com diversas espécies de bactérias diazotróficas que colonizam desde as raízes até as folhas, na região da rizosfera até o interior do tecido vegetal (endofíticas). Bactérias endofíticas podem oferecer vários benefícios à planta hospedeira, particularmente a promoção de crescimento e a proteção contra agentes patogênicos, além do mais,em condições ambientais diversas bacterias são capazes de comunicar e interagir com a planta de forma mais eficiente do que as bactérias rizosféricas (ALI et al., 2012; COUTINHO et al., 2015).

Nos últimos anos, foram descobertas novas espécies de bactérias fixadoras de N_2 que vivem numa associação menos perfeita nas raízes das gramíneas, várias destas bactérias foram descobertas no Brasil (MOREIRA; SIQUEIRA, 2006; FIGUEIREDO et al., 2008). As bactérias diazotróficas associativas e endofíticas são geneticamente diversas. Foram identificados vários gêneros de

Proteobactérias alfa, beta e gama, incluindo *Azospirillum*, *Azorhizobium*, *Azoarcus*, *Burkholderia*, *Citrobacter*, *Enterobacter*, *Gluconacetobacter diazotrophicus*, *Herbaspirillum*, *Klebsiella*, *Serratia,Pseudomonas* e *Rhizobium* (MAGNANI et al., 2010, SANTI et al., 2013). Na maioria desses micro-organismos tem sido encontrada uma variedade de genes que codificam vários traços de crescimento-promoção de plantas nos seus genomas, incluindo genes que codificam a fixação de nitrogenio, atividade desaminase ACC, biossíntese de sideróforos, produção de fitohormônios (AIA, acetoin, 2,3-butanodiol), produção e síntese de compostos antimicrobianos (SANTOYOet al., 2016).

A descoberta de bactérias endofíticas como *Gluconacetobacter diazotrophicus*, *Herbaspirillum* e *Burkholderia*colonizando a cana-de-açúcare suas interações foram bastante estudadas (SANTI et al., 2013). Estudos recentes demonstraram que as enterobactérias endofíticas fixadoras de N_2 estão predominantemente associadas a plantas de cana-de-açúcar em todo o mundo (LINet al., 2012; MAGNANI et al., 2010; TAULÉ et al., 2012). Além disso, alguns *Enterobacter* spp. (LINet al., 2012) e *Klebsiella* spp. (GOVINDARAJANet al., 2007) têm sido demonstrado como fixadores de N_2 além de promoverem o crescimento da cana-de-açúcar.

Alguns estudos relatam em detalhes todas as etapas de invasão e colonização de plantas por bactérias diazotróficas associativas e endofíticas (COMPANTet al., 2010). A interação planta-bactéria começa na rizosfera e é induzida por exsudatos radiculares que atraem as bactérias diazotróficas. Mecanismos de quimiotaxia envolvidos na migração bacteriana para as raízes das plantas incluem a presença de flagelos que permitem que as bactérias entrem em contato com as raízes. A colonização radicular também depende da adesão e ancoragem das bactérias no sistema radicular, bem como da proliferação microbiana e da formação de estruturas de biofilme na superfície radicular (CARVALHOet al., 2014). O local preferido de fixação e entrada subsequente é a zona apical da raiz, tal como a zona de alongamento de células e a zona de pêlos da raiz com pequenas rachaduras causadas pelo surgimento de raízes laterais. Para colonização endofítica, os pontos de emergência das raízes laterais e as zonas de diferenciação e alongamento próximas à ponta da raiz, onde os tecidos ligeiramente alterados ou não completamente diferenciados podem facilitar a penetração, são considerados locais de colonização primária nas raízes e preferenciais para a colonização bacteriana (RAVENet al., 2009; REINHOLD-HUREK; HUREK, 2011). Nos locais de penetração passiva bactérias formam biofilmes (REINHOLD-HUREK; HUREK, 2011; MALFANOVAet al., 2013). Os exopolissacarídeos e lipopolissacarídeos de superfície bacteriana (LPSs) estão envolvidos na adesão e colonização de raízes (ROSENBLUETH; MARTÍNEZ-ROMERO, 2006;REINHOLD-HUREK; HUREK, 2011). Depois de atravessar a barreira exodermal, os endófitos podem permanecer no local de entradaou mover-se mais para dentro e

ocupar o espaço intercelular do córtex (GASSERet al., 2011; MALFANOVA et al., 2013). Para a penetração, as bactérias têm de produzir enzimas celulolíticas necessárias para hidrolisar as paredes exodérmicas, tais como endoglucanases e endopolygalacturoni-dases. Estas enzimas também parecem ser importantes para se espalhar pelo espaço intercelular do córtex radicular e além (REINHOLD-HUREK et al., 2006; HARDOIM et al., 2008).

A capacidade de colonização dos tecidos internos das plantas por algumas bactérias pode conferir a estas uma vantagem ecológica sobre as bactérias que podem apenas colonizar plantas epifiticamente, que as tornam os mais promissores grupos de diazotróficos associados às plantas não leguminosas (RYANet al., 2008). Os tecidos internos das plantas proporcionam um ambiente mais uniforme e protegido para os micro-organismos que a superfície onde estão expostas a condições ambientais extremas como temperatura, potencial osmótico, radiação ultravioleta e competição microbiana, que são os fatores mais limitantes à sobrevivência das bactérias ao longo do tempo (SANTOYO et al., 2016). Diante destas diversas vantagens, as bactérias de vida livre e rizosféricas perderam importância e a maior parte das pesquisas está direcionada aos endofíticos.

Além de fixar N, essas bactérias também podem produzir hormônios de crescimento de plantas, e algumas espécies são relatadas para melhorar a absorção de nutrientes e aumentar a tolerância da planta contra stresses bióticos e abióticos (CARVALHOet al., 2014).Além do mencionado, outros efeitos benéficos para o crescimento das plantas são atribuídos aos micro-organismos endofíticos, que incluem o ajuste osmótico, regulação de estômatos, modificação morfológica da raiz, o reforço na absorção de minerais e alteração do metabolismo da planta (COMPANTet al., 2005).

A vantagem da aplicação de micro-organismos em comparação com produtos químicos é que os micro-organismos são muito mais eficientes na sua aplicação de compostos ativos. Eles praticamente produzem seus metabólitos secundários somente na superfície da planta ou dentro da planta, enquanto uma grande parte dos produtos químicos aplicados externamente nem sequer entrar em contato com a planta e, portanto, só polui o meio ambiente.

A reintrodução de bactérias diazotróficas endofíticas em plantas micropropagadas de cana-de-açúcar tem auxiliado os estudos da associação entre as plantas e as bactérias diazotróficas, e tem permitido avaliar o potencial de FBN e de promoção de crescimento devidos à inoculação debactérias diazotróficas endofíititcas (OLIVEIRA et al., 2002).

A aplicação da FBNna agricultura em plantas da família *Fabaceae*, por meio da inoculação de estirpes selecionadas de bactérias, é utilizada extensivamente em culturas como soja, amendoim, ervilha e alfafa (HERRIDGE te al., 2008). No entanto, entre as culturascomo cana-de-açúcar,

milho, arroz e trigo, pertencentes à família *Poaceae,*a aplicação prática da FBN é uma possibilidade recente (HUNGRIA, 2011).

O potencial de FBN por bactérias diazotróficas nativas em associação com poáceas tem grande amplitude (valores superiores a 100 Kg N $há^{-1}$ ano^{-1}), devido à variação entre as diferentes espécies dessa família (HERRIDGE et al., 2008). A cana-de-açúcar é uma das espécies nas quais este potencial é maior, como demonstrado por Boddey et al. (2003), que em média 37% do N seria proveniente da FBN, em 11 diferentes canaviais (nos Estados de SP, MG, RJ e PE). Os autores comentam que esse efeito pode estar relacionado às bactérias encontradas na cana-de-açúcar – *Gluconacetobacter diazotrophicus, Herbaspirillum* sp. *e Burkholderia* sp. – terem como nicho de colonização principal a endosfera, demostrando que os micro-organismos endofíticos seriam mais eficientes em fornecer N para as plantas.

Estudos recentes de inoculação de plantas micropropagadas de cana-de-açúcar com uma mistura deestirpes de espécies diferentes mostraram contribuições ao redor de 30% (OLIVEIRA et al., 2002).Assim, este tipo particular de associação planta-bactéria consiste em um sistema benéfico natural a ser explorado. Desta forma, a seleção de bactérias diazotróficas endofíticas com alto potencial de associação e contribuição para a FBN, pode ser um fator diferencial para diminuir a dependência da cultura pelo N derivado do solo ou da adubação nitrogenada sem que haja perda de produção de cana-de-açúcar.

4 CAPÍTULO I

BIOTECHNOLOGICAL POTENTIAL OF ENDOPHYTIC BACTERIA TO IMPROVE THE SEEDLING MICROPROPAGATED OF VARIETY RB92579 SUGARCANE (*SACCHARUM OFFICINARUM* L.)

(Submetido ao periódico Microbiological Research)

Biotechnological potential of endophytic bacteria to improve the seedling micropropagated of variety RB92579 sugarcane (*Saccharum officinarum* L.)

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Running title: Biotechnological potential of endophytic bacteria in micropropagated sugarcane seedlings.

Abstract

Endophytic bacteria may influence agricultural production in several ways, including promoting plant growth. Two experiments were conducted in order to evaluate the combination of endophytic bacteria from the Brazilian Northeast region aiming the commercial introduction of the inoculation of these bacteria in micropropagated sugarcane plants using a temporary immersion bioreactor. One experiment was done in tubes with sterile commercial substrate, and the other was done in pots with soil; both were installed in a greenhouse. A mixed inoculation was performed in six inoculated endophytic diazotrophic bacteria in micropropagated sugarcane plants, variety RB92579. In the experiment with soil, the mixed inoculation significantly increased the shoot dry matter of plants without the addition of nitrogen fertilizer. However, the accumulation of total-N in the tissues showed no significant differences between treatments with and without nitrogen fertilization. The

evaluation of micropropagated seedlings showed no increases in the parameters tested. The results showed that the response of inoculation in temporary immersion bioreactor micropropagation is possible, and that the application of homologous strains may have contributed to a better response by the interaction of endophytic bacteria with sugarcane RB92579. Further studies should be conducted to improve the methodology, which indicates a great potential to optimize this process on a commercial scale.

Keywords: Biological nitrogen fixation; meristem culture; diazotrophic bacteria; endophytes; micropropagation.

1 Introduction

Brazil is the largest producer of sugarcane, with a planted area of approximately eight and a half million hectares and an estimated productivity for the current season of the 2016/2017 harvest of 76,313 kg/ha (CONAB, 2016). The production is concentrated in the South-Central and Northeast regions. This culture demands a high amount of nitrogen, the most limiting macronutrient for crop productivity. It is one of the highest costs for farmers. Since fertilizer is not subsidized in Brazil, most commercially used genotypes were chosen aiming to obtain a high productivity with low levels of soil N, favoring, even indirectly, the selection of varieties that are capable of covering part of the need for N by the association with diazotrophic bacteria (Boddey et al., 2001).

The biological process of converting dinitrogen to ammonia is called nitrogen fixation (BNF), and is performed exclusively by the enzyme nitrogenase, so that it is a great support for the increase in productivity. Moreover, it is also an ecological and more economical alternative (Spatzel, 2015). Research has shown that the key to the success of BNF processes lies in the selection of diazotrophic bacteria that can associate more efficiently. Therefore, a more detailed study on the community of diazotrophic bacteria during plant growth cycles is necessary. Studies on inoculation of micropropagated sugarcane plants with a mixture of five strains from different species showed contributions of around 30% (Oliveira et al., 2002).Nitrogen fixation has a profound agronomic, economic, and ecological impact owing to the fact that the availability of fixed nitrogen represents the factor that most frequently limits agricultural production throughout the world (Hoff man et al., 2014).

Micropropagationis a practice widely used in many countries in Europe, Asia, United States and Brazil. This method is based on the production of more uniform and healthy plants and on a much higher growth speed within a limited physical space (Baldotto et al.,2010). However, endophytic microorganisms have been mentioned in several studies as contamination sources to micropropagation. Others consider their presence as a positive factor, arguing that they are able to

assist plants, since they live inside their tissues without causing symptoms of their presence, and in the case of *in vitro* cultivation they can favor osmotic adjustment, production of phytohormones and absorption of nutrients (Almeida et al.,2009; Abreu-Tarazi et al., 2010). The reintroduction of diazotrophic endophytic bacteria in micropropagated sugarcane plants has helped studies on the association between plants and diazotrophic bacteria, allowing us to evaluate the potential of BNF and growth promotion (Oliveira et al., 2002; Canuto et al., 2003). In this context, the present study was performed to evaluate the micropropagated sugarcane seedlings using a temporary immersion bioreactor system aiming the commercial introduction of bacterial inoculation as well as the consequent benefit for the culture through a BNF process and/or other mechanisms for promoting plant growth.

2 Material and Methods

2.1 Culture media

The culture media used for isolation of bacteria were LGI-P, JNFb, NFb and JMV according to Döbereiner et al. (1995). The medium DYGS (Rodrigues Neto et al., 1986) was used for the growth of strains and for DNA extraction.

2.2 Isolation and quantification of bacteria

Triplicate samples of roots and stems of the sugarcane variety RB92579 were obtained from a field in the state of Paraíba (06° 57' 25.6877" S latitude and 35º 07' 06.1412" W longitude), Brazil. The culms were disinfected with a pre-wash of the surface using soap and water, and then scrubbed with cotton-soaked 70% alcohol. The roots were disinfected with 70% alcohol for 30 seconds, then washed with sodium hypochlorite (2.5%) for 1 minute under agitation and with sterile water for five times during 5 minutes. After disinfestation, the roots were ground in 90 mL of a saline solution, thus characterizing a dilution of 10^{-1}, with three replications (Döbereiner et al., 1995). Then, they were diluted serially in 0.1 mL of suspension and inoculated in vials containing 5 mL of semisolid free-N, LGI-P, NFb, JNFb and JMV (Döbereiner et al., 1995). After 72-96 h of incubation, the pots with a white film on the surface were also replicated for a new source medium.

2.3 DNA extraction

DNA bacterial extraction was conducted using phenol-chloroform. Endophytic bacterium isolates were grown in 5 mL DYGS (Rodrigues Neto et al., 1986) for 24 h at 28°C. A 400 µL aliquot of the solution was transferred to a microtube and 400 µL saturated phenol solution was added. The mixture was shaken in a vortex apparatus and subjected to centrifugation at 16,000 *g* for

5 min. The supernatant (aqueous layer) was transferred to a new microtube and the phenolic step was repeated. After centrifugation the supernatant was again transferred to a new microtube and 400 μL chloroform was added. The microtube was shaken in a vortex and centrifuged for 5 min at 16,000 *g*. The aqueous layer was transferred to another microtube, to which 1 mL cold ethanol was added. To complete the process of extracting the DNA, the microtube was centrifuged for 3 min at 16,000 *g*, the ethanol discarded, and the tubes incubated at 37°C for 30 min to evaporate residual solvent. The extracted material was resuspended in 15 μL Mili-Q sterile water.

2.4 Sequencing of gene 16S rDNA and Gyrase β

The 16S rDNA gene was amplified as per using universal primers fD1 (5´-AGAGTTTGATCCTGGCTCAG-3´) e rD1 (5´-AAGGAGGTGATCCAGCC-3´) (Weisburg et al., 1991). Gyrase β gene was amplified using gyrB3F (5'- TCCGGCGGTCTGCACGGCGT-3') e gyrB14R (5´- TTGTCCGGGTTGTACTCGTC-3´) gene and PCR product for the isolates was sequenced in both directions.

PCR reactions (25 uL) contained 10X PCR reaction buffer, 10 mM dNTPs, 50 mM $MgCl_2$, 10 pmol primer, 15 ng DNA and 2.5 units *Taq* DNA-polimerase (Invitrogen). The temperature profile consisted of 5 min initial denaturation at 95°C followed by 30 cycles of 94°C for 45 sec, 54°C for 45 sec, and 72°C for 2 min followed by a final extension at 72°C for 5 min. PCR product was purified using PureLink PCR Purification Kit (Invitrogen) according to the instructions. Sequencing was performed on an ABI PRISM 9700 capillary sequencer using the ABI Prism Big Dye Terminator Cycle sequencing kit (Applied Biosystems).

The 16S rDNA and Gyrase β gene sequences were compared with the GenBank database (http://www.ncbi.nlm.nih.gov/) using BLAST. For local alignment, was used to BLASTn tool (NCBI-www.ncbi.nih.gov) and Multiple alignments were performed with CLUSTAL W (Thompson et al., 1997). A phylogenetic tree was constructed using MEGA 6 (version 6) (Tamura et al., 2013).

2.5 Micropropagation of sugarcane

Endophytic diazotrophic bacteria were used in the mixture to inoculate sugarcane plants (Table 1).

Table 1 - Bacteria used for mixing the inoculant, isolated plant tissue and sugarcane variety.

Genus of Bacteria	Code	Plant tissue
Enterobacter	1b	Culm
Enterobacter	5	Root
Enterobacter	10	Root
Enterobacter	25	Root
Gluconacetobacter	30	Culm
Enterobacter/Pantoea	22	Root

The experiment was conducted at the Northeast Strategic Technologies Center (CETENE), Recife/PE state, Brazil. Micropropagated sugarcane plants, commercial variety RB92579, were used to evaluate the effects of inoculation of diazotrophic bacteria. Micropropagated plants in a TIB that did not present contamination at the rooting phase received a mixed bacterial inoculum. For the inoculum, bacteria were grown for 48 hours in a liquid medium under agitationin a Dygs medium (Döbereiner et al., 1995), at 30°C. An aliquot of 6.7 mL of mixed inoculum with an optical density (OD 540 = 0.05), containing approximately 10^4 cells/mL, was added to TIB pots with a capacity of 5,000 mL, containing 2,000 mL of MS medium modified by Reis et al. (1999) and micropropagated already rooted plants.

Two experiments were conducted at the CETENE greenhouse. One used plastic tubes filled with the sterilized commercial substrate Basaplant® and the other used pots (8 L) with non-sterile forest soil under greenhouse conditions.

In the first experiment, the mixed inoculation was evaluated with the followingtreatments:

- One level of inoculation: A combination of previously identified diazotrophic bacteria: *Enterobacter* + *Gluconacetobacter diazotrophicus*.

- Two levels of nitrogen fertilizer: Recommended dose (80 kg of N ha) 1), and without nitrogen fertilization.

- One non-inoculated control with nitrogen fertilization, following the recommended dose.

The experimental design was randomized blocks with four replications. The plants were kept in plastic tubes (acclimatized) for 45 days and fertilized with a nutrient solution. The effects of inoculation considering fertilization was evaluated by determining the accumulation of shoot and root dry mass. Height was also evaluated.

For the second experiment, the remainder of the seedlings that did not undergo evaluation during the first experiment were transplanted into 8-liter pots containing non-sterile soil. From this soil, several single samples were collected to form a composite sample, which was then analyzed (Table 2), obtaining the fertilization recommendation. The experimental design was randomized blocks with four replications. After 120 days after planting, evaluations of accumulation of root and shoot dry mass and the determination of total nitrogen accumulated in plant tissues were made using the Kjeldahl method (Alves et al.,1994).

Table 2 - Chemical characteristics of the soil sample used in the conduction of the second experiment (pots).

mg/dm^3					$cmol_c/dm^3$								
Fe	Cu	Zn	Mn	P	pH	K	Na	Al	Ca	Mg	H	S.B	CTC
44,20	0,60	8,30	7,10	5	6,3	0,10	0,06	0,0	4,50	0,90	2,60	5,55	8,15

2.6 Statistical Analysis

Each variable studied was subjected to analysis of variance (ANOVA), F test, and Tukey's test, at 5% significance levels using the statistical software ASSISTAT version 7.7 (Silva and Azevedo, 2016).

3 Results and Discussion

3.1 Isolation of diazotrophic bacteria

Populations of nitrogen-fixing bacteria were higher in samples of roots compared with samples of culms. Previously conducted tests indicated that the bacteria used in this experiment have an ability to fix N_2 *in vitro*, which becomes a potential tool for the production of IAA and for inorganic phosphate solubilization.

3.2 Molecular phylogeny of bacterial isolates

The total DNA of the six bacterial isolates was purified and used as a model for PCR (polymerase chain reaction) in order to amplify their 16S rRNA genes. The sequencing of the 16S rRNA gene allows an accurate identification of the genera of endophytic bacteria from various

species of plants, including sugarcane, corn, rice and medicinal plants (Ratón et al., 2011; Szilagyi-Zecchin et al., 2014; Rhoden et al., 2015). However, it is necessary to analyze other genes, such as *gyr*A and gyrβ, to obtain a precise definition of the species and even subspecies (Hurtle et al., 2004). Using a molecular approach, the occurrence of phylogenetic types of organisms and their distribution in natural communities may be studied directly from the environment. Since 1988, when only twelve phyla of bacteria were reported, the number of phyla increased due to cultivation activities, especially by the research on rRNA genes on the environment. Currently, over 70 phyla of bacteria are recorded in public databases (Pace, 2009; Youssef et al., 2015). The sequencing of rRNA genes is an efficient method of choice for phylogenetic reconstruction based on the detection of nucleic acid and the quantification of microbial diversity.

Based on the sequence of the 16S rRNA gene, the endophytic bacterial isolates in this study were identified as *Enterobacteria, Gluconacetobacter, Rhizobium, Pantoea* and non-cultivable bacteria (Figure 1). The results of phylogenetic analyses allowed grouping the endophytic sugarcane isolates into two groups, with similarities with sequences in the GenBank public database ranging from 99% to 100% (Figure 2). Group I was composed by the isolates 1, 5, 10, 22 and 25, which are related to *Pantoea sp*., *Rhizobium sp*., *Enterobacter sp*., *E. asburiae*, *E. ludwigii* and *E. cloacae*. This result was confirmed by the sequencing of the gene gyrβ, which enabled identifying the isolates at the species level, such as *E. cloacae* (Table 3). *Pantoea* was found in sugarcane and soybeans (Magnani et al. 2010) e em soja (Kuklinsky-Sobral et al., 2004). Studies have shown the potential of *Pantoea* sp. to induce a systemic resistance and protection against pathogenic microorganisms. Additionally, these bacteria may induce the growth of plants, increasing the supply of nitrogen in non-symbiotic associations, solubilizing phosphorus and stimulating the production of phytochromes (Quecine et al., 2012).

Although Rhizobia infect naturally legumes as host plants, some strains may form symbiotic relations with non-legume species. Besides fixing N_2, they are also capable of contributing to the promotion of growth of these species.

Group II included the isolated 30, which is related to *Gluconacetobacter diazotrophicus*. *G. diazotrophicus* is considered the main diazotrophic endophyte in sugarcane and has been isolated from leaves, stems and roots of sugarcane plants and other economically important grasses (Magnani et al., 2010). Several studies have shown that such endophytes colonize their hosts in vast numbers and cause an increase in production (Hardoim et al., 2008; Rashid et al., 2012; Santoyo et al., 2016). The possibility of replacing fertilized nitrogen for biological nitrogen fixation is a very important economic and environmental factor (Jha et al., 2013).

The results obtained in this study are essential to provide the necessary knowledge on the analysis of endophytic bacteria in micropropagated sugarcane plants and to indicate the potential for future applications of endophytes that promote plant growth.

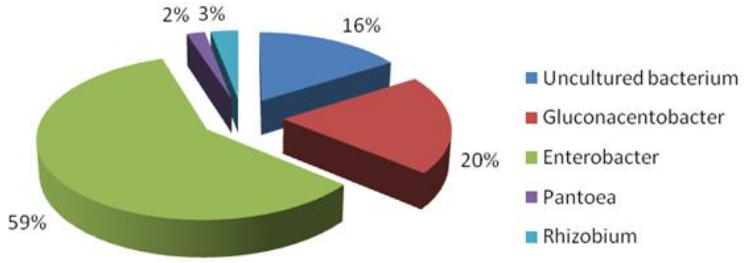

Figure 1 - Abundance of each genus identified among endophytic bacterial isolates from RB 92579 sugarcane cultivar.

Table 3 - Isolated bacterial endophytes identified with relationship to species by sequencing of the *gyrβ* gene and the identity percentage found in the National Center for Biotechnology Information database.

Isolates	Species	Query value	E value	Identity	Accession
1	*Enterobacter clocae*	100%	0.0	97%	AB972391.1
5	*Enterobacter clocae*	99%	0.0	94%	AB084016.1
10	*Enterobacter clocae*	100%	0.0	98%	AB972391.1
22	*Enterobacter clocae*	100%	0.0	97%	AB972391.1
25	*Enterobacter clocae*	100%	0.0	94%	AB084013.1

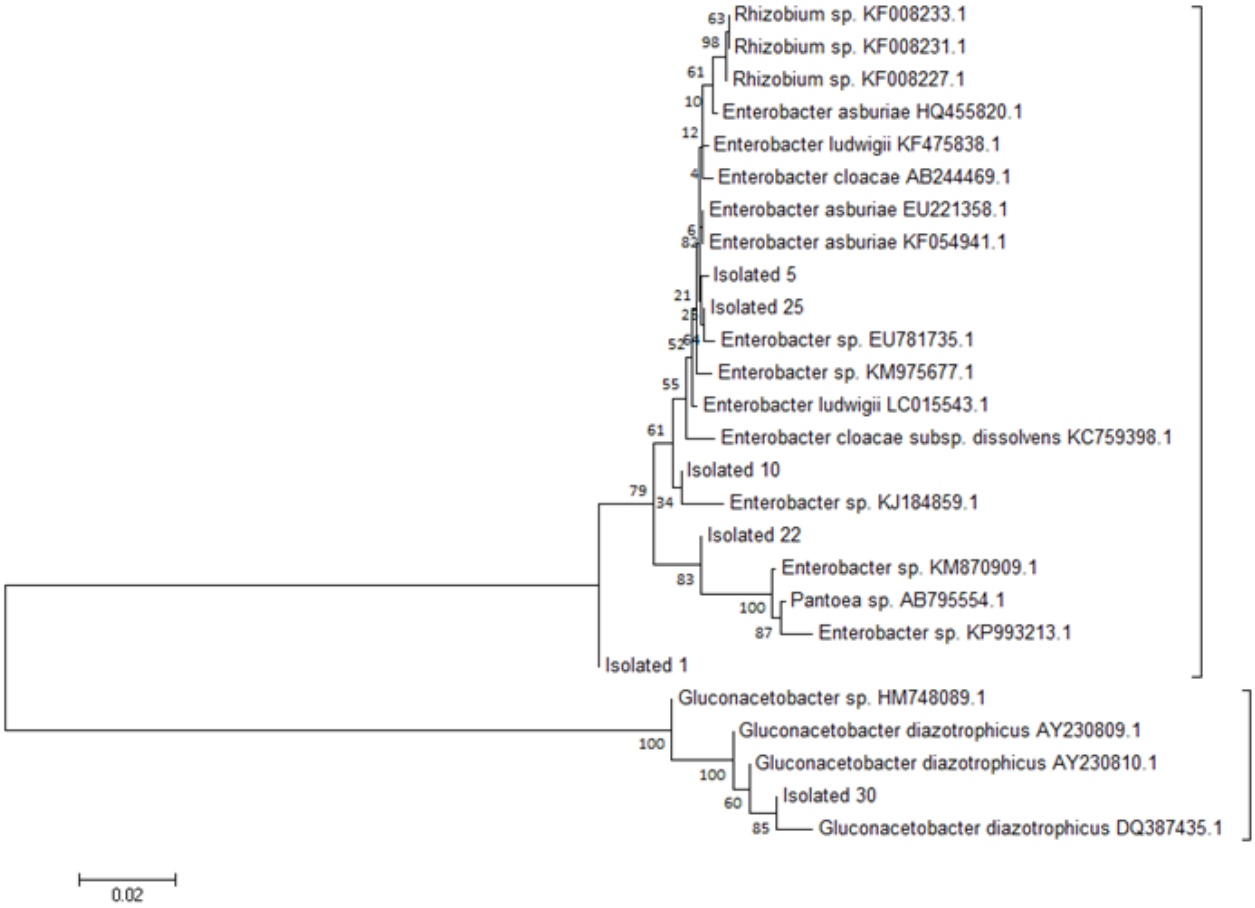

Figure 2 - Phylogenetic tree constructed with sequences of the 16S rRNA regions of endophytic bacteria isolated from sugarcane and sequences from GenBank (indicated by accession number), using the neighbor-joining method and utilizing Tamura-Nei for nucleotides, with the pairwise gap deletion option. Numbers indicate frequency of each branch from bootstrap analyses of 10,000 replicates.

In addition to the common ability to fix N_2, associative and endophytic bacteria are genetically diverse. They were identified among various genera: alpha, beta and gamma-proteobacteria, including *Azospirillum*, *Azorhizobium*, *Azoarcus*, *Burkholderia*, *Citrobacter*, *Enterobacter*, *Gluconacetobacter*, *Herbaspirillum*, *Klebsiella*, *Pseudomonas* and *Rhizobium* (Vessey, 2003; Magnani et al., 2010; Santi et al., 2013).

3.3 Evaluation of micropropagated sugarcane seedlings

The results of this experiment showed no significant statistical differences by Tukey test ($p<0.05$) among treatments inoculated with the mixture of diazotrophic bacteria (with and without nitrogen fertilization) and the control. There was a tendency for increase for the control treatment at this micropropagated phase (Table 4).

Table 4 - Effect of inoculation of bacteria from micropropagated sugarcane plants (RB92579) at the 45th day.

Treatment	Height (cm)	RDM (g)	SDM(g)
Control - Nitrogen fertilization	70,05 a	0,16 a	0,54 a
Inoculant - Nitrogen fertilization	64,45 ab	0,09 b	0,37 b
Inoculant - Fertilization without Nitrogen	61,85 b	0,12 ab	0,43 ab
CV%	11,84	32,00	37,30

RDM = root dry matter; SDM = shoot dry matter. Means followed by the same letters do not differ by Tukey test ($p\leq0.05$).

Results similar to those obtained in this study were found by Canuto et al. (2003) upon analyzing the effect of inoculation by determining the accumulation of root and shoot dry mass of sugarcane seedlings, variety SP701143, at the 65th day. In this study, no statistically significant differences were observed among treatments inoculated with 44 strains of diazotrophic bacteria and the non-inoculated control. Suggesting that at this stage the plant does not respond adequately to inoculation.

3.4 Evaluation of sugarcane in pots

The height of plants in pots showed no significant differences by Tukey test ($p<0.05$) among treatments inoculated with the mixture of diazotrophic bacteria (with and without nitrogen fertilization) and the control. The addition of nitrogen fertilizer to the inoculated treatment resulted in a slight decrease in RDM (14.14 g). It did not differ statistically from the control treatment with the addition of nitrogen fertilizer (16.38 g), evidencing that N is a limiting factor in this experiment (Table 5). However, the inoculation without nitrogen fertilizer promoted a greater accumulation of root dry matter compared to the control treatment.

The evaluation of the SDM accumulation showed that the mixed inoculation promoted a positive effect on plant development without the addition of nitrogen fertilizer (41.7 g), differing from the other treatments (Table 5).

Table 5 - Effect of inoculation of bacteria from micropropagated sugarcane plants (RB 92579) in pots (at the 120th day).

Treatment	Height (cm)	RDM (g)	SDM (g)	Leaf N (g/Kg dry wt $^{-1}$)
Control - Nitrogen fertilization	1,76 a	16,38 ab	35,28 b	10,39 a
Inoculant - Nitrogen fertilization	1,76 a	14,14 b	31,71 b	9,90 a
Inoculant - Fertilization without Nitrogen	1,82 a	17,77 a	41,7 a	10,08 a
CV%	7,73	25,57	20,35	17,81

RDM = root dry matter; SDM = shoot dry matter. Means followed by the same letters do not differ by Tukey test ($p \leq 0.05$).

The results suggest that the inoculation used with the commercial variety RB92579 affected the interaction with inoculated bacteria. A better understanding of the plant-bacteria interaction, the selection of diazotrophic endophyte strains and the variety of cane needs to be further studied aiming a maximum benefit of BNF.

Microbial inoculants are an alternative method to increase crop productivity and may reduce the use of chemical fertilizers, which is one of the agricultural practices that affect the environment (Ambrosini et al., 2016). The positive effects of inoculating some bacteria during plant growth may be associated to the BNF process and the synthesis of growth hormones produced by bacteria. Among the effects associated with this synthesis of hormones, the growth of lateral and adventitious roots, the stimulus to cell division and the elongation of roots and stems are mentioned (Teale et al., 2006). This may explain the accumulation of root dry matter.

Similar results were observed by some authors. Lin et al. (2012), upon inoculating two strains of *Enterobacter* spp., observed that both strains increased the biomass content and the nitrogen of micropropagated sugarcane seedlings grown with a nitrogen fertilizer equivalent to 180 kg of urea ha^{-1}, a recommended nitrogen fertilization dose for the cultivation of the sugarcane ROC22 at the seedling stage. Oliveira et al. (2002), upon inoculating in micropropagated sugarcane plants different species of diazotrophic bacteria, isolated and in mixtures, observed that *Herbaspirillum* sp., *A. amazonense* and a combination of five strains of different bacteria showed a significant increase in the accumulation of fresh mass in the culms of plants, evidencing a 30%

contribution of BNF. However, the individual inoculation of *G. diazotrophicus* promoted a negative effect on the accumulation of fresh mass of culms compared to the non-inoculated control. The results presented by Canuto et al. (2003) showed that the inoculation response in micropropagated SP 701143 seedlings at the rooting stage showed variations that may have been dependent on several factors, including plant genotype and the environment. In this study, the inoculation with the strains PAL3 and CBAmC caused a significant increase in the accumulation of culm dry matter compared to the non-inoculated control treatment. However, the accumulation of N in plant tissues grown in pots after 180 days of growth showed that plants inoculated with a mixture of the strains PAL5 and HCC103 and the individual strains HRC54, Z94 and CBAmC showed a higher nitrogen content in the tissues. Govindarajan et al. (2006), studying potted micropropagated seedlings, observed that the total biomass increased due to inoculation with one strain or a combination of strains without nitrogen fertilization. Muthukumarasamy et al. (1999) inoculated a mixture of diazotrophic bacteria and mycorrhizal fungi in micropropagated sugarcane plants and obtained an effect equivalent to half the recommended dose of nitrogen fertilizers for potted plants. Oliveira et al. (2002) demonstrated that the combined inoculation of associative and endophytic bacteria promotes a synergistic effect compared to the individual inoculation of bacteria in micropropagated sugarcane plants. Increases of 30% in the accumulation of N in plants via BNF were observed for these plants.

3.5 Effects on growth

The contribution of endophytic bacteria for the nutrition of legume plants by BNF is well known. Among non-legume species, BNF is still subject of much discussion. The contributions observed are varied and depend on specific interactions among bacterial and plant genotypes (Chaves et al., 2015).

The inoculation of micropropagated sugarcane plants has already been performed and resulted in interesting effects on the outcome of plants. Preliminary inoculation experiments with *G. diazotrophicus* in micropropagated sugarcane plants showed increases of up to 28% in shoot fresh matter. Promising results were also obtained when micropropagated sugarcane plants were inoculated with the strain PAL-5 associated with small doses of nitrogen, as shown by Moraes and Tauk-Tornisielo (1997).

When the colonization of the plant is established, one result arising from the association is the promotion of plant growth by direct and indirect mechanisms. In addition to fixing N, endophytic diazotrophic bacteria produce plant growth hormones such as auxin and gibberellic acid

(Cassan et al., 2009; Verma et al., 2013), Im provements in nutrient absorption are also reported (Richardson et al., 2009; Saha et al., 2013).

Several experiments demonstrated that endophytic bacteria may indirectly benefit the development of the plant, increasing the plant's tolerance to biotic and abiotic stresses (Arencibia et al, 2006; Rosenblueth and Martínez-Romero, 2006; Yasuda et al., 2009). Beneficial results from such associations in sugarcane plants include a significant increase in plant height and biomass, root length and production of dry matter (Oliveira et al., 2003; Suman et al.,2005).

Current evidence indicates that the BNF process performed by diazotrophic bacteria may contribute up to 60% to the sugarcane's N uptake (Boddey et al.,2001), and that it depends on the plant genotype and on its interaction with various associative bacteria genera (Reis et al., 2007).

Quantitative analyses of BNF and the promotion of plant growth evidenced that plant and bacterial genotypes are important factors to the control of association efficiency (Carvalho et al., 2011). In this context, the determination of the best combination between diazotrophic bacteria and plant varieties to obtain the maximum benefit of such association in agriculture is a challenge in this area.

4 Conclusions

This study showed that inoculation using a temporary immersion bioreactor (TIB) is possible. This is the first inoculation report for seedlings using this system. The use of homologous strains may also have contributed to the benefit of the interaction with the plant (sugarcane variety RB92579). The results suggest a high response potential to inoculation and optimization of the process on a commercial scale.

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5 CAPÍTULO II

CHARACTERIZATION OF ENDOPHYTIC DIAZOTROPHIC BACTERIA ISOLATED FROM SUGARCANE (*Saccharum officinarum*sp.) VARIETY RB867515 IN DIFFERENT REGIONS OF NORTHEASTERN BRAZIL

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Characterization of endophytic diazotrophic bacteria isolated from sugarcane (*Saccharum officinarum*sp.) variety RB867515 in different regions of northeastern Brazil

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ABSTRACT

Biological nitrogen fixation by diazotrophic bacteria associated with non-leguminous plants can contribute with part of the N supply to the plants, reducing production costs and environmental damage. The objective of this study was to verify the population of diazotrophic bacteria associated with the sugarcane variety RB867515 from different regions of northeastern Brazil and characterize the isolated bacteria. Samples of stems and roots of sugarcane were collected between April and May 2014 in three northeastern regions: PB, PE and AL. Roots and stems were submitted to surface disinfestation and the semi-selective media NFb, JMV, LGI-P and JNFb were used for counting and isolation, and then the phenotypic and physiological characterization of the bacterial isolates was carried out. Fifty-two native endophytic bacteria were isolated and characterized, which were evaluated for their ability to fix nitrogen, nitrogenase activity (ARA), inorganic phosphate solubilization and indole acetic acid (IAA) production. The results obtained showed that the highest

natural occurrence of endophytic diazotrophic bacteria in sugarcane occurred in the region of Alagoas. Concerning the biotechnological potential of the bacterial community, 57% of the total isolates presented *in vitro* N_2-fixation ability, 69% presented solubilization halo, with solubilization index (SI) ranging from 1.4 to 3.3 in culture medium with inorganic phosphate, and practically all isolates were able to produce IAA (concentrations of 1.97 μg/mL to 150.68 μg/mL of the culture medium) and showed positive results of *in vitro* nitrogenase activity, with values ranging from 0.98 to 4.60 nmol $C_2H_4.h^{-1}$. Regarding the molecular identification, most of the isolates belong to the families Enterobacteriaceae and Pseudomonadaceae.

Keywords: bioprospection, growth promotion, biological nitrogen fixation, phosphate solubilization, IAA.

1 Introduction

Sugarcane is one of the main crops in the Brazilian agricultural landscape and considered one of the great alternatives for the biofuels sector due to the great potential in the production of ethanol and its by-products (CONAB, 2016).

Bacteria can promote plant growth through several mechanisms, such as nitrogen fixation (Vermaet al., 2013), phosphate solubilization (Krey et al., 2013), production of hormones such as auxins, gibberellins and zeatin (Cassan et al., 2009), including through biological control of pathogens (Wang et al., 2009).

Biological nitrogen fixation (BNF) is the significant process by which N_2 is reduced to inorganic NH_3, making N available to plants by a specialized group of prokaryotic organisms, so-called diazotrophs, through the enzyme nitrogenase (Spatzal, 2015). These microorganisms are able to perform BNF due to the presence of the enzyme nitrogenase, capable of promoting the breaking reaction of nitrogen atoms at room temperature and normal pressure, using energy from photo- and chemosynthetic processes, or obtained from carbohydrates (from fermentation or respiration) and stored in the form of ATP (Figueiredoet al., 2008).

Endophytic diazotrophic bacteria are those that fix atmospheric N_2 and colonize the interior of plant tissues without causing disease symptoms and are genetically diverse. Several genera of alpha-, beta- and gamma-proteobacteria, including *Azospirillum,Azorhizobium,Azoarcus,Burkholderia, Citrobacter, Enterobacter, Gluconacetobacter diazotrophicus, Herbaspirillum, Klebsiella, Serratia, Pseudomonas* and *Rhizobium*, have been identified (Magnaniet al., 2010; Santi et al., 2013).

Considering the great vegetal biodiversity of Latin American countries with tropical climate, it is estimated that each plant species has yet unclassified endophytic microorganisms and, therefore, presenting genetic diversities capable of providing structurally diversified compounds and with little known bioactivity. Endophytic bacteria can offer various benefits to the host plant, particularly growth promotion and protection against pathogens; in diverse environmental conditions, these bacteria are able to communicate and interact with the plant more efficiently than rhizospheric bacteria (Ali et al., 2012; Coutinho et al., 2015).

For a more efficient selection of isolates in the process of colonization and growth promotion, the isolates need to be initially identified through *in vitro* biochemical analysis (Bernardeset al., 2010). It is also important to perform the taxonomy of selected microorganisms with biotechnological potential in agriculture and, for this reason, 16S rRNA sequencing has been successfully used to identify genera such as *Bacillus* (Deepa et al., 2010), *Rhizobium*, *Burkholderia* (Ferreira et al., 2012), *Enterobacter*, *Pantoea*, *Serratia*, among others (Tian et al., 2009).

In this context, based on the knowledge that Plant Growth-Promoting Bacteria (PGPB) are a biotechnological tool that is of primary importance in the survival and development of cultivated plants, the hypothesis to be tested is that the use of homologous bacteria native to the northeast region may have a positive effect on the plant, since these bacteria are adapted to the environmental conditions of the region under study. This hypothesis was tested to investigate the isolation and to characterize the biochemical and physiological properties in the identification of endophytic diazotrophic bacteria and their biotechnological potential associated with the sugarcane variety RB867515 for use in agriculture.

2 Materials and methods

2.1 Study area

The collection of the plant material (stem and roots) of the sugarcane variety RB867515 was carried out in the cultivation field of the São José Plant (Igarassu-PE-Brazil), having as geographical coordinates: 07º 43' 16.2" S latitude and 34º 55' 06.1" W longitude; in the cultivation field of the Miriri Plant (Mamanguape-PB): 06° 57' 25.6" S latitude and 35º 07' 06.1" W longitude; and in the cultivation field of the Coruripe Plant (Coruripe-AL): 10º 08' 48.4" S latitude and 36º 17' 25.1” W longitude (Table 1).

2.2 Collection of samples

Plant samples were collected between April and May 2014. Samples of shoots and roots of the sugarcane variety RB867515 were packed in plastic bags inside an ice cube box and promptly transported to the Laboratory of Soil Biology of the Agronomic Institute of Pernambuco (IPA) -

PE/Brazil. At the time, soil was also collected from the sample collection areas for physical and chemical analysis (Table 1).

2.3 Counting and isolation of endophytic bacteria

The counting and isolation of endophytic bacteria were carried out using the method of Döbereiner et al. (1995), from the plant samples (stem and roots).

2.4 Physiological characterization

2.4.1 Inorganic phosphate solubilization

The inorganic phosphate solubilization was tested by the bacterial isolates in insoluble phosphate medium following the method of Verma et al., 2001 and Rodriguez et al., 2000. The diameter of the solubilization halo was observed as a translucent area around the colony after seven days of incubation, indicating a positive response of the isolate to solubilization of calcium phosphate. The data were used to calculate the Solubilization Index (SI), determined by the ratio between halo diameter and colony diameter, proposed by Berraquero et al. (1976).

2.4.2 *In vitro* N_2-fixation

Bacterial isolates were evaluated for their ability to fix N2 *in vitro* by the growth in culture medium without nitrogen source. For this purpose, the isolates were inoculated in test tubes containing 10 mL of NFb culture medium. The formation of a surface film indicates the presence of nitrogen-fixing microorganisms (Dobereineret al., 1995).

2.4.3 Indole acetic acid (IAA) production

IAA quantification was performed by the method of Kuss et al. (2007). The isolates that formed a red color in the period of 30 min were evaluated in a spectrophotometer with a wavelength of 520 nm. The concentration of indole compounds was estimated with a standard curve, previously prepared with uninoculated sterile culture medium, and known amounts of IAA: 0.5, 10, 30, 50, 70, 90 and 100 μg.mL, according to the equation $Y = 0.0121X - 0.0075$ ($R^2 = 0.9995$).

2.4.4 Acetylene reduction assay (ARA)

The quantification of the nitrogen-reducing activity performed by the nitrogenase complex was measured using the method of Boddey et al. (1990).

The quantification of the ethylene produced in the samples was carried out in a gas chromatograph with flame ionization detector. The acetylene reduction activity is given by nmol of ethylene produced per hour of incubation.

2.5 PCR amplification and sequencing of the 16S rRNA gene

DNA bacterial extraction was conducted according to Sambrook et al. (2001), using 5mL of fresh cultures of the endophytic isolates cultured in LB medium for 24 h at 28°C. After quantification, DNA of each isolate was submitted to the PCR reaction.

The amplification was performed by PCR (Polymerase Chain Reaction) with primers fD1 (5′AGA GTTTGAT CCTGGCTCAG 3′) and rD1 (5′AAGGAGGTGATC CAGCC 3′) (Weisburg et al. 1991). The cycle included an initial denaturation at 95°C for 2 min, 30 cycles of 15 s at 94°C, 45 s at 93°C, 45 s at 55°C, 2 min at 72°C, followed by a final extension of 5 min at 72°C. Purification with 7.5 M ammonium acetate was performed according to Menna et al. (2006). Ultrapure water was used to re-suspend the DNA. The amount and purity were assessed on agarose gel 1.5% (w/v), adjusting the final concentration at 16 ng/μL. The amplified PCR products were sequenced and analyzed using GenBank database, using CLUSTALW software (Yushmanov and Chumakov 1988). Phylogenetic trees were constructed with the MEGA program (Tamura et al., 2013) version 6 integrated with the ClustralW program.

2.6 Statistical analysis

Statistical analyses were performed using the program SASM-agri. Data were submitted to analysis of variance (ANOVA) using the Scott-Knott test at 5% probability.

3 Results and discussion

3.1 Quantification of endophytic diazotrophic bacteria

The results indicate the occurrence of endophytic diazotrophic bacteria associated with the sugarcane variety RB867515 in three states of the northeast.

The highest most probable number (MPN) of bacteria was obtained in roots, compared to plant parts such as the stem (Table 1). This is due to the fact that this region is where the compounds exuded by the roots of the plants become more concentrated, being loaded with energy sources and nutrients necessary for the cycles of bacterial cells (Silva et al., 2012). Often, a greater number of diazotrophic bacteria is found associated with *Poaceae* roots (Prakamhang et al., 2009), which represent the initial point of infection of the plants by the bacteria, suggesting that the main entry of endophytes is the roots and basal regions of the host, where from this point, they can spread within the plant. Similar results were found in sugarcane, soybean and rice crops in other research studies (Oliveira et al., 2009; Jha et al., 2009; Silva et al., 2012).

The highest bacterial densities were obtained in roots of the Alagoas area (Coruripe Plant) in the LGI-P medium ($1.1x10^5$ cells/g fresh root), followed by JNFb and JMV ($4.5x10^4$ cells/g fresh root) and NFb ($1.4x10^4$ cells/g fresh root). In Pernambuco, the roots also had the highest densities in the JMV medium ($1.5x10^3$ cells/g fresh root), followed by LGI-P ($9.5x10^2$ cells/g fresh root), NFb ($9.5x10^2$ cells/g fresh root) and JNFb ($4.5x10^2$ cells/g fresh root). The area of Paraíba (Miriri Plant) had the lowest density of bacteria regarding roots, the JMV medium showing ($9.5x10^2$ cells/g fresh root), followed by NFb ($0.9x10^2$ cells/g fresh root) and LGI-P ($0.4x10^2$ cells/g fresh root).

The population of plant endophytes also depends on many variables, such as the stage of plant growth; plant tissue analyzed; plant health; the nutritional status of the plant; type of soil and its condition (including pH and moisture content); altitude; temperature, etc. (Hardoim et al., 2008). Some of these factors may influence the populations of these bacteria.

There are also some factors that interfere qualitatively and quantitatively in the biodiversity of the endophytic microbiota. The following are highlighted: plant growth and development stage, the rhizosphere effect, the advancement of the species cycle and the increase in the production of mucilages excreted by root cells in the rhizosphere (Walker et al., 2003), which can also be a consequence of both the desquamation of cells due to soil friction as they grow and the senescence thereof (Monteiro et al., 2012). Genotypes of cultivated plants are also considered as important factors for determining the composition of plant-associated bacterial communities.

In general, the maximum values of density of diazotrophic bacteria obtained in this study ranged between 10^2 and 10^5. The endophytic bacteria population obtained varied in relation to the collection sites, perhaps due to soil properties, such as the presence of organic matter or water issues in each region.

3.2 Physiological characterization

A total of 52 endophytic isolates were evaluated for their nitrogen-fixing ability *in vitro*, IAA production and inorganic phosphate solubilization in the culture medium, considering these characteristics as important mechanisms for plant growth promotion (Zaid et al., 2009), in addition to the acetylene reduction assay.

In this study, virtually all the isolates were able to produce the phytohormone IAA in the presence of the amino acid L-tryptophan. The isolates produced IAA at concentrations of 1.97 μg/mL to 150.68 μg/mL of the culture medium (Table 2). Szilagyi-Zecchin et al. (2014) also observed a similar response, all strains were positive for IAA production. According to Naveed etal.

(2015), L-tryptophan may promote an increase in the productivity of IAA by bacteria, since its presence in the culture medium causes a stimulatory effect on the pathways of physiological activation, responsible for the assimilation of this amino acid used as a precursor for bacterial biosynthesis of IAA.

A large variation was found in the amounts of IAA produced by the isolates from the different study areas. Some speculation can be made about the role of the IAA-producing bacterial community on the variety of a particular region, since the area that obtained the highest percentage of isolates with good IAA rates was Alagoas (Coruripe Plant), with 25% of the isolates, followed by Pernambuco, with 20%; Paraíba did not present isolates with good rates for this test (Table 2).

The IAA is able to provide both rapid responses to plant development, as in the case of cell elongation, and slow ones, as in the case of cell differentiation and division, interfering with the increase of roots and the amount of root hairs, and can also act on the tropism and apical dominance of plants (Sukumar et al., 2013), suggesting an important role in the bacterium-plant interaction. It is one of the main properties of the microbial contribution to plants, helping in promoting plant growth.

In addition to the production of phytohormones, the ability of bacteria to solubilize inorganic phosphate is an interesting contribution. After nitrogen, phosphorus is the nutrient that limits plant growth, because despite having large reserves in the soil, it is in a form unavailable to plants, and solubilization can improve this condition (Postma et al., 2010), being this a possible mechanism for promoting plant growth under field conditions. Endophytes are known to promote plant growth through phosphate solubilization (Collavino et al., 2010). In this aspect, 69% of the total isolates presented solubilization halo, with solubilization index (SI) ranging from 1.4 to 3.3 in culture medium with inorganic phosphate (Table 2), suggesting that this group of bacteria could have a more important role during this stage of development of the host plant, depending on the plant genotype.

Santos et al. (2012) found similar results when evaluating 14 root endophytic diazotrophic bacteria from two sugarcane varieties, where SI values ranged from 1.00 to 2.33. The area that obtained the highest percentage of isolates with good inorganic phosphate solubilization index was Pernambuco, with 87% of the isolates, followed by Alagoas, with 39% of the isolates, and Paraíba, with 33% of the isolates. However, Alagoas was the region that presented the isolates with the highest IAA and phosphate solubilization rates, obtained from the stem, differing statistically at 5% probability level by the Scott-Knott test.

BNF has been one of the most studied mechanisms involved in the microorganism-plant interaction and contributes significantly to the nitrogen nutrition of sugarcane plants, increasing productivity in a sustainable manner, even in an area of low N availability to plants (Schultz et al.,

2014). Thus, BNF was evaluated, being observed a high percentage of these bacteria with N_2-fixing ability *in vitro*. It was observed that 57% of the total isolates presented this ability (Table 2).

Of the isolates from the area of Paraíba (PB), 90% were able to fix N_2*in vitro*, followed by Pernambuco, with 66%, and Alagoas, with 42%. In addition, 4 of these isolates (IPA 44, IPA 59, IPA 166 and IPA 167), besides the ability to fix N_2*in vitro*, showed good IAA production and inorganic phosphate solubilization (Table 2). This corroborates postulated information that many diazotrophic bacteria are ubiquitous, performing, in addition to BNF, solubilization of insoluble phosphates and IAA production (Zaid et al., 2009). BNF in sugarcane plants is a complex process involving a range of factors related to the genotype of plants and the bacteria associated with them. It should also be noted that photosynthetic efficiency, nutritional requirements and resistance to unfavorable conditions, among others, are characteristics related to the genotype of plants that may influence the efficiency of nitrogen fixation by the associated bacteria. The genetic diversity of diazotrophic bacteria of the same species related to the genotype of the plant should also be considered as an important factor when analyzing the differences regarding BNF in the sugarcane crop (Reis Junior et al., 2000). The root was the plant part that originated most of the isolates (67%) (Table 2).

The ability of the nitrogenase complex to reduce nitrogen can be measured indirectly by the acetylene reduction activity (ARA), a competitive inhibitor of the enzyme nitrogenase reductase. Thus, the confirmation of the diazotrophic character was performed through this technique, being only tested in the isolates that were positive for the *in vitro* N_2-fixation test. All isolates that were submitted to the acetylene reduction technique showed positive results for *in vitro* nitrogenase activity, cultivated in pure cultures, ranging from 0.98 to 4.60 nmol $C_2H_4.h^{-1}$ (Table 2). The statistical analysis of the nitrogenase activity result indicated a significant difference between the isolates.

Regarding the acetylene reduction in the culture medium, the biological fixation ability of bacteria is varied; even though it is positive for the ARA, it is not known which of them fix nitrogen actively due to several factors (Fischer et al., 2012). It is believed that the low nitrogenase activity and the quantity and quality of the carbon source in the culture medium may interfere with the enzymatic activity (Pedrinho, 2009).

Table 1 - Location of areas, soil analysis, plant parts, culture media, colony-forming units and number of bacterial isolates, obtained from thesugarcane variety RB867515 in different regions of the northeast.

Area	Density g/cm^3		pH	P	Na	K	Ca	Mg	H	Al	CEC	V	M	Soil texture				Plant part	Culture medium	No. cells/mL	No. of bacterial isolates
	Ad	Rd	H_2O	mg.dm^3			Cmolc/dm^3					(%)		Clay	Silt	GS	FS				
														%							
1	1.50	2.62	6	68	0.1	0.15	4	1.1	2.72	0	8.1	66	0	9	7	57	27	Root	JNFb	$4.5x10^4$	5
																			JMV	$4.5x10^4$	5
																			NFb	$1.4x10^4$	6
																			LGI-P	$1.1x10^5$	3
																		Stem	JNFb	$9.5x10^2$	3
																			JMV	$9.5x10^2$	4
																			NFb	$1.4x10^4$	3
																			LGI-P	$0.4x10^2$	-
2	1.53	2.59	5.5	33	0.04	0.69	3.45	1.15	5.31	0.05	10.7	50	1	12	5	56	27	Root	JNFb	-	-
																			JMV	$9.5x10^2$	8
																			NFb	$0.9x10^2$	1
																			LGI-P	$0.4x10^2$	-
																		Stem	JNFb	-	-
																			JMV	$2.5x10^2$	-
																			NFb	-	-
																			LGI-P	-	-
3	1.47	2,34	6.9	60	0.06	0.11	3.65	0.95	3.00	0	7.7	60	0	9	6	57	27	Root	JNFb	$4.5x10^2$	3
																			JMV	$1.5x10^3$	-
																			NFb	$9.5x10^2$	3
																			LGI-P	$9.5x10^2$	2
																		Stem	JNFb	$1.5x10^2$	1
																			JMV	$0.9x10^2$	2
																			NFb	$0.9x10^2$	3
																			LGI-P	$4.5x10^2$	1

Area 1: Alagoas (Coruripe Plant): Area 2: Paraíba (Miriri Plant); Area 3: Pernambuco (São José Plant). Ad = apparently density; Rd = real density; GS = Gross Sand; FS = Fine Sand; V = base saturation; m = saturation of aluminum.

These results show that there is not only a greater genotypic diversity of bacteria in the sugarcane variety RB867515, but also a greater metabolic diversity among these bacteria, which shows that in these areas there must be potential microorganisms for the promotion of plant growth.

Table 2 - Result of the acetylene reduction activity (ARA), N_2-fixation*in vitro*, indole acetic acid (IAA) production and inorganic phosphate solubilization by endophytic bacterial isolates from three sugarcane varieties, collected in three regions of northeastern Brazil.

Isol	Area	ARA (nmol $C_2H_4.h^{-1}$)	N2-fixation *in vitro*	IAA* production (µg.mL)	Phosph. Solub.*(SI)	Isol	Area	ARA (nmol $C_2H_4.h^{-1}$)	N2-fixation *in vitro*	IAA* production(µg.mL)	Phosph.Solub.* (SI)
						IPA 94	PB	1.33d	+	11.89l	1.73k
IPA 09	PE	ND	+	65.61g	2.33g	IPA 95	PB	1.10d	+	37.51i	0.00m
IPA 10	PE	*	-	145.88a	1.80k	IPA 96	PB	4.60a	+	46.65h	0.00m
IPA 11	PE	*	-	90.89f	2.26h	IPA 101	PE	1.77c	+	31.91j	2.55f
IPA 17	AL	2.08c	+	4.78l	0.00m	IPA 102	PE	0.98d	+	31.80j	2.22h
IPA 18	AL	0.98d	+	34.84i	2.40g	IPA 112	AL	*	-	25.61j	2.25h
IPA 36	PB	2.45c	+	12.05l	2.75e	IPA 113	AL	2.09c	+	1.97l	0.00m
IPA 40	PE	3.08b	+	12.27l	2.44g	IPA 114	AL	1.88c	+	3.21l	1.87j
IPA 41	PE	*	-	9.30l	0.00m	IPA 115	AL	*	-	32.58j	1.40l
IPA 42	PE	2.60b	+	5.19l	2.96d	IPA 116	AL	*	-	39.16i	1.90j
IPA 43	PE	*	-	16.02k	2.62f	IPA 117	AL	*	-	13.24k	0.00m
IPA 44	PE	4.37a	+	117.75d	3.18b	IPA 118	AL	*	-	10.70l	0.00m
IPA 45	PE	*	-	11.45l	2.66e	IPA 119	AL	*	-	102.60e	0.00m
IPA 51	AL	*	-	13.13k	0.00m	IPA 120	AL	*	-	16.74k	1.70k
IPA 52	AL	*	-	15.19k	0.00m	IPA 151	PE	3.35b	+	17.78k	3.21b
IPA 53	AL	*	-	17.06k	0.00m	IPA 152	PE	1.20d	+	13.62k	2.20h
IPA 54	AL	*	-	11.00l	0.00m	IPA 153	PE	2.09c	+	11.03l	2.32g
IPA 55	AL	*	-	26.74j	0.00m	IPA 154	PE	1.77c	+	10.70l	3.00d
IPA 56	AL	*	-	16.93k	0.00m	IPA 161	AL	2.97b	+	36.74i	2.57f
IPA 57	AL	*	-	147.73a	1.83k	IPA 162	AL	2.81b	+	20.29k	2.74e
IPA 58	AL	*	-	140.54b	2.40g	IPA 163	AL	1.37d	+	21.01k	3.37a
IPA 59	AL	2.15c	+	148.69a	2.73e	IPA 164	AL	1.65c	+	14.26k	0.00m
IPA 89	PB	2.03c	+	8.08l	1.96j	IPA 165	AL	1.29d	+	10.12l	3.12c
IPA 90	PB	4.27a	+	10.60l	2.37g	IPA 166	AL	1.33d	+	150.68a	3.09c
IPA 91	PB	3.32b	+	15.47k	2.11i	IPA 167	AL	2.67b	+	138.89b	2.50f
IPA 92	PB	*	-	24.06k	0.00m	IPA 168	AL	*	-	127.40c	2.37g
IPA 93	PB	3.75a	+	9.60l	1.80k	**C.V.**		**8.84%**		**3.56%**	**4.07%**

*Evaluation by the Scott-Knott test at 5%. Means followed by distinct letters differ from eachother by the Scott-Knott test (P<0.01). ND = not determined; (SI) = Solubilization Index; (*) = absent; (+) = positive test; (-) = negative test. (AL) = Coruripe Plant; (PB) = Miriri Plant; (PE) = São José Plant.

3.3 Molecular characterization of the isolates

The total DNA from 15 bacterial isolates was used to amplify their 16S rRNA genes. The product of the amplification reaction with the oligonucleotides rD1-fD1 generated a single fragment of approximately 1,500 bp. However, it was not possible to amplify the 16S rDNA of all strains with this pair of oligonucleotides. Phylogenetic analysis was performed in order to help the correct identification of strains, as well as to determine the relationships with related species. The consensus sequences of each isolate were compared to sequences from the GenBank public database through the BLAST program (NCBI - www.ncbi.nih.gov). The 16S rRNA phylogenetic tree was dominated by *Enterobacteriaceae* and *Pseudomonadaceae*, including *Klebsiella oxytoca*, *Burkholderia tropica*, *Candidimonas* sp., *Bordetella petrii*, *Enterobacter oryzae*, *Sphingomonas oligophenolica*, *Kosakonia oryzae* and *Stenotrophomonas maltophilia*, *Pseudomonas entomophila*, *Uncultured Rhizobiaceae bacterium*. This analysis showed identity rates ranging from 77% to 99% (Figure 1).

Studies on the diversity of nitrogen-fixing bacteria in symbiosis with sugarcane have been increasingly explored with the use of molecular techniques. The primer pair termed fD1 and rD1 is capable of amplifying a wide variety of bacterial types. It is possible to extend species diversity by substituting rD1 for rP1, notwithstanding, from the perspective of amplifying the maximum number of 16S rDNA nucleotides, rD1 is preferable. It is close to termination 3'.

The identification obtained by the partial sequencing of the 16S rRNA gene showed that the sequences of strains isolated from sugarcane are very similar. In the family *Enterobacteriaceae*, the identity ranged between 77% - 98%. Magnani et al. (2010) also isolated endophytic microorganisms belonging to the family *Enterobacteriaceae* in sugarcane.

The sequences related to the family *Pseudomonaceae* ranged between 97 - 99% of similarity with the bacterial genus/species identified. Of the 15 isolates analyzed by 16S rRNA, 8 had high similarity with *Pseudomonas* sp. This result corroborates the studies of several authors that isolated this genus from several crops of rice (*Oryza sativa*), canola (*Brassica napus* L.), maize (*Zea mays* L.) and sugarcane (*Saccharum* L.), revealing the ability of *Pseudomonas* sp. in colonizing different plant regions and promoting growth (Araújo et al., 2013; Sivasakthi et al., 2013; Sulochana et al., 2014).

The genera *Enterobacter*, *Klebsiella* and *Burkholderia* have been found as sugarcane endophytes in several studies, related to growth promotion (Magnani et al., 2010; Luvizotto et al., 2010; Wei et al., 2014).

*Klebsiella*sp. is also known to have an endophytic association as diazotrophs with plants such as wheat, corn and rice (Jha and Kumar, 2007).

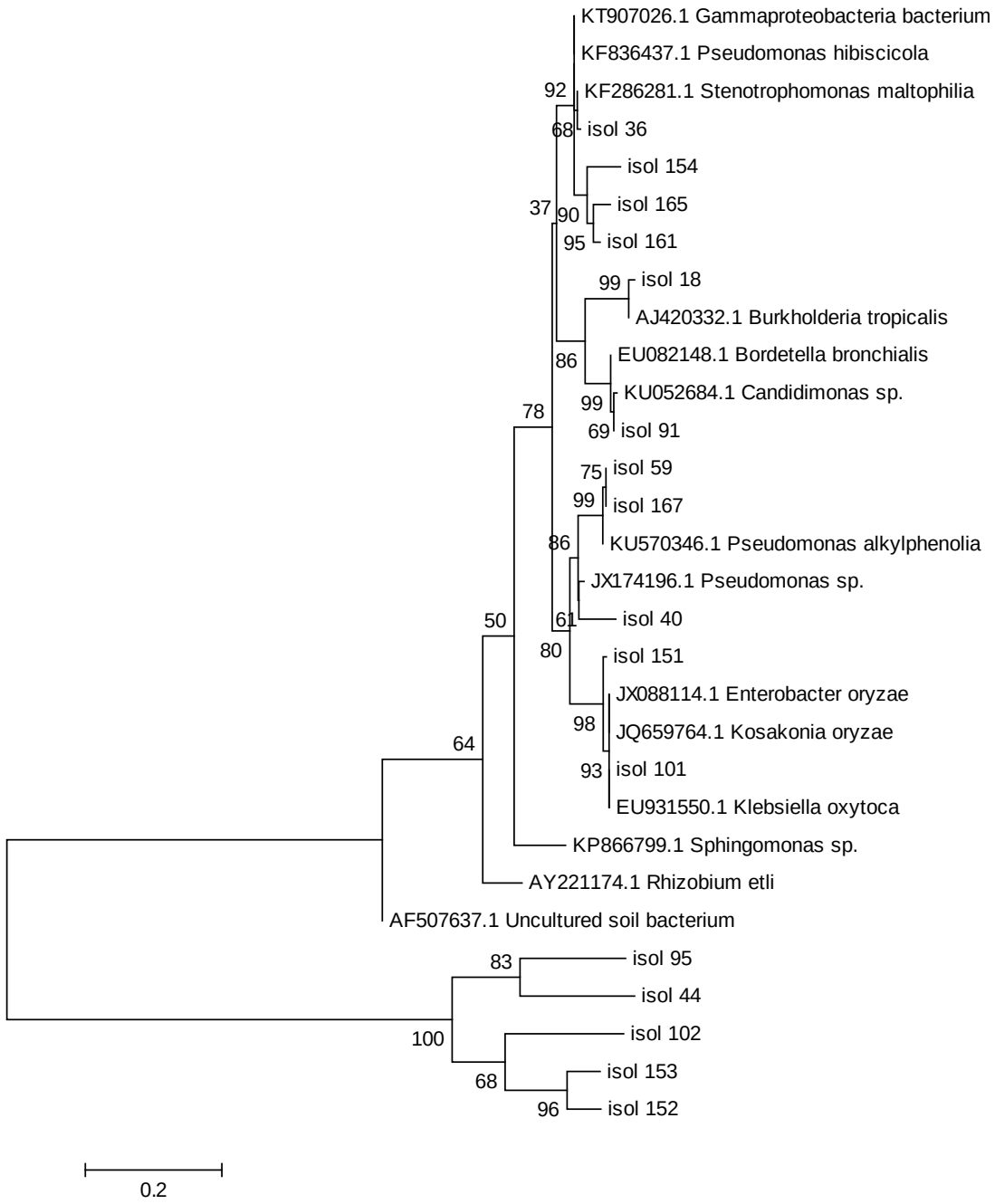

Figure 1 - Phylogenetic tree constructed with MEGA 6 program and neighbor-joining algorithm based on 16S rRNA gene sequences of the endophytic bacteria isolated from sugarcane. GenBank accession numbers of the sequences are given along with the names of the species. Bootstrap values based on 1,000 reolications are shown at branch nodes.

The isolate IPA 44 showed 82% similarity with soil bacteria.

Thaweenut et al. (2011) observed the presence of *nif*H sequences similar to those of *Bradyrhyzobium* sp. and*Azorhizobium caulinodans* in the roots and stem of plants of the sugarcane variety NiF8. Researchers from Embrapa-CNPAB also found a diversity of *nif*H sequences belonging to the genera *Bradyrhizobium* sp. and*Rhizobium* sp. in roots of the sugarcane variety RB867515 grown in the field of the station located in the city of Seropédica-RJ (Fischer et al., 2012).

Isolates of the genus *Burkholderia* are mainly associated with sugarcane plants and are responsible for a range of physiological activities. Bacteria of this genus, in addition to being able to multi-replicate, have genomes with 2 to 3 chromosomes, sized 6 to 9 Mb, characteristics that confer to these bacteria genomic plasticity and metabolic adaptability (Luvizotto et al., 2010).

Due to the genomic plasticity of the bacteria, great differences between isolates living in similar environments, such as the interior of the plant root and rhizosphere, are very difficult to find. This fact may explain the high degree of relationship between the isolates observed by 16S rRNA.

4 Conclusions

In all the environments studied, there was a great diversity of endophytic bacteria, with a higher percentage of these bacteria in the roots compared to the stem of sugarcane plants. The sugarcane endophytic bacteria presented potential to promote plant growth, since they have the ability to produce IAA, to solubilize inorganic phosphate and to fix N_2, in addition to showing good rates of nitrogenase activity. Both the percentage and the total number of endophytic diazotrophic bacteria reflected an interaction between the sugarcane variety RB867515 and the environments, especially in the area of Alagoas (Coruripe Plant), where the highest densities of endophytic diazotrophic bacteria were observed, showing that there is interaction between the sugarcane genotype and the environment. Most of the isolates belong to the families *Enterobacteriaceae* and *Pseudomonadaceae*. The bacterial isolates IPA 44 (*Rhizobium*sp.), from the state of Pernambuco, IPA 59 (*Pseudomonas* sp.), from the state of Alagoas, and IPA 167 (*Pseudomonas* sp.), from the state of Alagoas, showed potential for application as inoculant in the sugarcane variety RB867515. The isolated bacteria should be investigated in future research as alternatives in the biotechnological processes applied to agriculture.

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6 CAPÍTULO III

INOCULATION OF ENDOPHYTIC DIAZOTROPHIC BACTERIA IN MICROPROPAGATED SEEDLINGS OF SUGARCANE (*Saccharum officinarum* sp.) VARIETY RB867515

(Artigo a ser submetido ao periódico Soil Biology and Biochemistry)

Inoculation of endophytic diazotrophic bacteria in micropropagated seedlings of sugarcane (*Saccharum officinarum* sp.) variety RB867515

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ABSTRACT

The objective of this study was to evaluate the effects of inoculation (alone and in mixture) of different strains of endophytic bacteria from the sugarcane variety RB867515, collected in the northeast of Brazil, on its growth at the initial growth stage (45 DAI and 120 DAI). With this purpose, two experiments were carried out in a greenhouse at the Agronomic Institute of Pernambuco (IPA), located in the city of Goiana - PE, Brazil, in a completely randomized design. The first experiment, at 45 days after inoculation (DAI) in tubes, was composed of: uninoculated plants; plants inoculated *in vitro* with three individual endophytic bacterial isolates; and a mixture with micropropagated seedlings. The second experiment, at 150 DAI, consisted of: inoculated plants transplanted to pots with non-sterile soil without nitrogen fertilization; and uninoculated plants with nitrogen fertilization equivalent to 80 kg of N ha^{-1}. The variables analyzed were: dry mass of shoots and roots, tillering and N content accumulated in the plant. At 45 days after inoculation, there was no significant difference between the inoculated plants and the uninoculated

control. The inoculation of nitrogen-fixing bacteria native to the northeast region in micropropagated seedlings of sugarcane variety RB867515 grown in pots, at 150 DAI, promoted plant development and also presented similar performance to the nitrogen treatment.

Keywords: BNF, *Pseudomonas* sp., mixture of strains, *in vitro* cultivation of plants.

1 Introduction

Currently, Brazil plays an important global role in the production and trade of agricultural commodities, which grows each year through the adoption of new technologies and increased consumption of chemical inputs. Among the main Brazilian agricultural crops, sugarcane (*Saccharum* spp. L.) is one of the most important, with Brazil being the largest producer in the world. According to the National Supply Company (CONAB), the area planted in the 2015/2016 season was around 8.7 million hectares, with an estimated production for the 2016/17 season of 684.77 million tons (CONAB, 2016). In order to obtain the current levels of production, it is estimated that in the year 2016, 31.4 million tons of fertilizers were used, of which 22.3 million tons were imported (National Association for the Diffusion of Fertilizers and Correctives - ANDA, 2016).

In the 2016/17 season, the sugarcane production will increase by 2.9% in relation to the previous season (CONAB, 2016). To achieve such production, increasing quantities of fertilizers, mainly nitrogen fertilizers, are required. N is one of the nutrients with the lowest rate of utilization by sugarcane, and about 50% of all fertilizer applied is lost by leaching in the form of nitrate (NO^{3-}), contaminating the water table, or by denitrification in the form of nitrous oxide (N_2O), contributing to the worsening of global warming (Spatzal, 2015).

Some alternatives have been sought to reduce the environmental impact of nitrogen fertilizer, and one of them is the Biological Nitrogen Fixation (BNF), which is one of the most important natural processes on the planet. This process is mediated by microorganisms called diazotrophs. These microorganisms are able to grow in medium free of combined nitrogen, using as nitrogen source the gaseous form (N_2), which is reduced to ammonia (NH_3) through the enzymatic complex nitrogenase, and assimilated as amino acids by plants (Franche et al., 2009).

Some research companies have conducted several studies with the purpose of developing inoculants for grasses with the ability to stimulate increased productivity and/or dry matter and N accumulation by the plant. There are reports in the literature that inoculants with diazotrophic bacteria promote an increase in plant development, including productivity, showing similarity to the addition of 120 Kg ha^{-1} N, as in sugarcane varieties RB867515 and RB72454 (Schultz et al., 2012).

The stimulus to plant development by some species of bacteria can be realized through the availability of nutrients such as phosphorus, by its solubilization, and nitrogen, by the biological fixation of atmospheric nitrogen (Hardoim et al., 2008; Berg et al., 2009; Compant et al., 2010).

The technique of regeneration of plants from meristem culture facilitates the inoculation of N_2-fixing bacteria and makes it possible to select strains or even to inoculate bacteria, aiming to increase the contribution of BNF (Reis et al., 2004).

Thus, the hypothesis of this work was tested to verify whether the inoculation of endophytic diazotrophic bacteria isolated from the northeast of Brazil, originated from the same variety, RB867515 (homologous), shows high potential for biological nitrogen fixation. This hypothesis was tested using different bacteria that were inoculated alone and mixed in the interaction with sugarcane in order to supply the N necessary to the development of the crop as well as to promote growth. Several biological parameters were analyzed in this study.

2 Materials and Methods

2.1 Location of the experiment

The experiments were conducted at the Plant Tissue Culture Laboratory (LCTV) and in a greenhouse at the Itapirema Experimental Station (7º 38' 33.33" S and 34º 56' 50.80" W), located in the city of Goiana, Zona da Mata Norte of Pernambuco (Brazil), both belonging to the Agronomic Institute of Pernambuco (IPA).

2.2 Plant Material

The plant material used is the commercial variety RB867515, the most cultivated in Brazil. This variety has fast growth, better performance in soils of light texture and medium fertility, in addition to medium tillering ability, good sprouting in cane plant and first ratoon, high sucrose content, good productivity and erect growth (RIDESA, 2010), being widespread in northeastern Brazil.

2.3 *In vitro* micropropagation of sugarcane

The micropropagated sugarcane seedlings were given by the Santa Tereza Plant at the initial stage of propagation. Sugarcane seedlings were micropropagated according to a method described by Hendre et al. (1983), using the apical meristem. This methodology uses the MS medium (Murashige and Skoog, 1962) modified in relation to the hormonal concentration to promote callus

multiplication (phase I), shoot multiplication (phase II) and root multiplication (phase III) for 80 to 90 days.

The plants micropropagated in the rooting phase were individualized and transferred to flasks with 20 mL of MS medium, modified by Reis et al. (1999). After 48 hours of transfer of the plants, the flasks that did not show contamination were selected to receive the bacterial inoculum.

2.4 Preparation of the inoculum

The endophytic bacterial isolates were cultured in 10 mL of liquid Dyg's culture medium (Rodrigues Neto et al., 1986) for 24 h under shaking at 30 °C. The absorbance of the samples was measured at 560 nm and the bacterial density adjusted to 0.8 O.D. mL^{-1}. The endophytic bacteria used individually and in mixture to inoculate the sugarcane plants are listed in Table 1.

Table 1 - Bacteria used in this study and sources ofisolation.

Bacteria	Symbol	Sugarcane variety/Place of origin
Rhizobium etli	Rhz	RB867515 (PE)
Pseudomonas sp.	Ps1	RB867515 (AL)
Pseudomonas sp.	Ps2	RB867515 (AL)

The inoculation of the *in vitro* micropropagated seedlings was performed according to the method described by Reis et al. (2004). Each vial containing 5 seedlings was inoculated with 0.1 mL of a bacterial suspension in the final rooting phase and maintained for up to 7 days at 25 °C under artificial light with photoperiod of 12 hours of light.

2.5 Evaluation of micropropagated sugarcane seedlings at 45 Days After Inoculation (DAI)

The first experiment was conducted in a greenhouse at the Agronomic Institute of Pernambuco (IPA), in tubes filled with commercial substrate Basaplant® sterilized. In this trial, individual and mixed inoculation were evaluated with the following treatments:

- Four types of inoculation: individual inoculation (Rhz), individual inoculation (Ps1), individual inoculation (Ps2) and mixed inoculation (Ps1, Ps2 and Rhz) without nitrogen fertilization.

- Control treatment without inoculation and without nitrogen fertilization.

The experimental design was a randomized complete block design with 15 replicates. The plants were kept in tubes (acclimatized) for 45 days.

The root system and the shoots were separated, dried in an oven at 65 ºC until constant mass, and weighed in a semi-analytical balance to determine the dry mass (g) of roots (DMR) and shoots (DMS) for evaluation of the effect of inoculation.

Samples of the dry mass of shoots were passed through a Wiley mill (2 mm) for determinations of the total nitrogen accumulated in the plant tissues by the method of Kjeldahl (Alves et al., 1994).

2.6 Evaluation of sugarcane seedlings at 150 Days After Inoculation (DAI)

The second experiment was carried out in a greenhouse with the transplanting of micropropagated sugarcane seedlings, at 45 DAI, in pots containing 8 kg of soil without sterilization, collected in the 0-20 cm layer. The site chosen for soil collection was an area of sugarcane cultivation at the Itapirema Experimental Station, IPA (Goiânia/PE- Brazil). The attributes of the soil used in the experiment can be seen in Table 2. The soil received NK fertilization according to the recommendation of the crop and based on the chemical analysis thereof. Fertilization was carried out with the equivalent of 80 kg of N ha^{-1} as urea for nitrogen treatment, in addition to 1 mL of Hoagland's micronutrient solution (Sarruge, 1975) per pot.

Three bacterial isolates were selected based on the performance presented in a previous work under controlled conditions and for having positive characteristics presented in *in vitro* evaluation, such as capacity to grow in medium free of nitrogen source, to produce indole acetic acid (AIA) and to solubilize inorganic phosphate.

Table 2 - Chemical analysis of the soil and substrate (Basaplant®) used in experiments.

	mg/dm^3					pH	$cmol_c/dm^3$							
	Fe	**Cu**	**Zn**	**Mn**	**P**		**K**	**Na**	**Al**	**Ca**	**Mg**	**H**	**S.B**	**CTC**
Substrate	-	-	-	-	82.1	5.76	11.0	-	0.3	10.0	6.8	9.2	16.90	20.99
Soil	44.20	0.60	8.30	7.10	154	6.3	0.9	0.06	0.0	2.00	0.60	2.60	5.55	8.15

The experiment was conducted in a factorial scheme (5X1), composed of Ps1, Ps2 and Rhz, alone and in a mixture (Ps1, Ps2 and Rhz) (inoculated in the *in vitro* phase of the plant) without nitrogen fertilization, and a control treatment with 10 replicates per treatment. The control treatment received nitrogen fertilization without inoculation of the diazotrophic bacteria.

The inoculation efficiency was evaluated at 150 days after inoculation. The dry mass of roots and shoots and the number of tillers were evaluated, being also carried out the determination of the total nitrogen accumulated in the plant tissues by the Kjeldahl method (Alves et al., 1994).

2.7 Statistical analysis

The design was completely randomized. Data were submitted to analysis of variance (ANOVA) by Tukey's test ($P<0.05$), through the SASM-agri program.

3 Results and Discussion

3.1 Evaluation of micropropagated sugarcane seedlings at 45 Days After Inoculation (DAI)

The results observed in this first evaluation showed no significant difference between the treatments inoculated with the three strains of diazotrophic bacteria (Ps1, Ps2 and Rhz), alone and mixed, and the uninoculated control by Tukey's test ($p<0.05$).

Table 3 presents a summary of the different growth parameters evaluated in the sugarcane plants submitted to the five different treatments. Regarding the accumulation of dry mass of shoots of sugarcane, treatments with *in vitro* inoculation of bacteria did not present averages statistically superior to the control, presenting mean weights of 0.76 g in this phase (Table 3).

Regarding the dry mass of roots, the treatments inoculated with *Peudomonas* (Ps1 and Ps2) were statistically inferior (0.23 g and 0.22 g, respectively) in relation to the uninoculated control and the inoculation with *Rhizobium*sp. (Rhz) (0.30 g and 0.29 g, respectively) (Table 3).

Table 3 - Mean values of the different treatments for dry mass of shoots and roots, number of tillers and N content, evaluated in plants of sugarcane variety RB867515 at 45 DAI.

	Control	Ps1	Ps2	Rhz	Ps1,Ps2andRhz	C.V. (%)
DMS (g)	0.72a	0.76a	0.77a	0.77a	0.80a	17.35
DMR (g)	0.30a	0.23b	0.22b	0.29a	0.27ab	24.39
No.Tillers	0.13a	0.13a	0.06a	0.06a	0.00a	12.25
N content (mg/g)	10.06a	12.20a	11.50a	11.16a	10.96a	9.11

DMS= Dry mass of shoots. DMR = Dry mass of roots. Means followed by the same letter do not differ by Tukey test ($p<0.05$). Ps1 and Ps2 = *Pseudomonas*sp.; Rhz = *Rhizobium* sp.

According to Malavolta (1980), the evaluation of the dry matter allows a better measurement of the ability of a plant to accumulate mass, because after the dehydration of the material, only the weight of the biomass remains, that is, the accumulation of compounds and metabolites by the plant.

As for the number of tillers, there was no significant difference between the treatments by Tukey test ($p<0.05$), although the mixed inoculation did not present tillering (Table 3). The tillering of sugarcane begins around 40 days after planting, which may justify the result.

Regarding the N content in the shoots of plants, the results show that the inoculation of endophytic bacteria did not present a significant difference between the inoculated treatments and the uninoculated control in this phase, by Tukey test ($p<0.05$), with a slight increase in the inoculation with *Pseudomonas*sp. (Ps1) (12.20g) (Table 3). These results suggest that in some sugarcane-bacterial interactions, the assimilation of the fixed N occurs later.

Canuto et al. (2003) also observed, in their experiment with micropropagated seedlings of sugarcane variety SP 70-1143 (evaluated at 65 days), that between treatments with inoculation of diazotrophic bacteria and the uninoculated control, there was no significant statistical differences.

The difficulty in selecting seedlings of the same size among the micropropagated seedlings obtained for the study, also evidenced by Canuto et al. (2003), is an aspect that hinders the comparison of the tested treatments and their possible effects in a short period of time, especially for long-cycle plants such as sugarcane.

3.2 Evaluation of sugarcane seedlings at 150 Days After Inoculation (DAI)

In the second experiment, conducted in pots with non-sterilized soil, the N treatment (nitrogen control) was significantly better than the remaining treatments in the four parameters evaluated: dry mass of shoots and roots, tillering and N content. Notwithstanding, the plants inoculated with Ps1, Ps2, Rhz and (Ps1, Ps2 and Rhz) (9.98g, 9.00g, 11.25g and 8.65g respectively) did not differ statistically from the plants with nitrogen fertilization (16.04g) in the dry biomass of shoots (Table 4). This shows that the inoculation had a positive effect on the development of plants.

Table 4 - Effect of the different treatments on the growth parameters evaluated in plantsof sugarcane variety RB867515at 150 DAI.

	Nitrogen control	Ps1	Ps2	Rhz	Ps1,Ps2 and Rhz	C. V. %
DMS (g)	16.04a	9.98a	9.00a	11.25a	8.65a	22.61
DMR (g)	4.98a	3.66b	3.30bc	3.55bc	2.43c	26.17
No.Tillers	0.10a	0.80a	0.90a	0.00b	0.00b	19.97
N content (mg/g)	12.94a	8.82bc	8.8bc	10.44ab	7.78c	14.00

DMS= Dry mass of shoots. DMR = Dry mass of roots. Means followed by the same letter do not differ by Tukey test ($p<0.05$). Ps1 and Ps2 = *Pseudomonas*sp.; Rhz = *Rhizobium* sp.

Since N is directly related to the growth of plants (Lainé et al., 1995), the biomass gain evaluates, besides the efficiency in the process of assimilation of this element, a possible contribution of the biological fixation in some sugarcane varieties (Donato et al., 2003), which would explain the fact that the higher accumulation of dry mass of shoots in the inoculated treatments did not differ statistically from the nitrogen control.

Positive results were also observed by Garcia et al. (2013) and Gírio et al. (2015), in the initial development of the sugarcane variety RB867515 with the inoculation of diazotrophic bacteria. The variety RB867515 is more demanding on soil fertility (RIDESA, 2010), which justifies its response to nitrogen fertilizer and inoculation. Schultz et al. (2012) and Gírio et al. (2015) have observed that the genotype of this variety is more responsive to the inoculation of growth-promoting bacterial strains.

A lower accumulation of dry mass of roots was observed with the inoculation, especially in the (Ps1, Ps2 and Rhz) (2.43 g) mixtures, which promoted a negative effect on root biomass (Table 4). Similar results were obtained by Lima et al. (2011), who observed lower root development in plants that received inoculation with two strains in sugarcane plants of the variety SP813250 under greenhouse conditions, and Gírio et al. (2015), when using a bacterial inoculant in pre-sprouted seedlings in the same variety (RB867515), observing that the dry mass of roots did not increase, but there was an increase in root length.

Although the mass of the root system was not favored by the inoculation, it is possible to infer that the growth-promoting bacteria modified the root system architecture (Hari and Srinivasan,

2005; Gosal et al., 2012). Inoculation provided a thinner root system, which allows a greater surface contact with the soil and increased uptake of water and nutrients (Bhattacharjee et al., 2008).

As for the number of tillers, the plants treated with Rhz inoculation and the Ps1 + Ps2 + Rhz mixture did not show tillering (Table 4), however, treatments Ps1 and Ps2 (0.80 and 0.90, respectively) favored the tillering, not differing significantly in relation to the nitrogen control (0.10), which may have been caused by ethylene. According to Mishra et al. (2013), ethylene favors the tillering of sugarcane.

Regarding the nitrogen content in the shoots of plants, the results showed that the inoculation with the strain Rhz (10.44 mg/g N) did not present a significant difference in relation to the nitrogen treatment (12.94 mg/g N). Notwithstandin, the inoculation with the mixture of strains Ps1, Ps2 and Rhz (7.78 mg/g N) showed a significant difference by Tukey test ($p<0.05$), presenting a slight increase in N accumulation in the shoots compared to the nitrogen treatment (Table 4).

The results of nitrogen content in shoots can show the photosynthetic capacity obtained by the crop through the treatments. The photosynthetic capacity is strongly influenced by the amount of nitrogen in the leaf (Chapin et al., 1987). Knowing that *Rhizobium*sp. (Rhz) has the *nif*H gene, we can assume that there were adequate conditions inside the plant for the nitrogenase to be active and that the nitrogen was supplied to the plant by biological fixation. Similar results were obtained in rice plants when the strain RI-530 of *R. leguminosarum* significantly increased the percentage of nitrogen in the leaves (Chi et al., 2009).

These results show a different behavior for BNF in the inoculation of the different strains in the variety RB867515; the inoculation with the strain Rhz was equivalent to the nitrogen control, favoring a greater accumulation of dry mass of shoots and a greater accumulation of N in the plants (Table 4).

Increases in shoot biomass attributed to the effect of rhizobia on non-leguminous plants were reported by several authors, citing increases in shoot and leaf biomass, height and/or photosynthetic activity (Chi et al., 2009; Kaci et al., 2005; Singh et al., 2005).

On the other hand, due to factors intrinsic to the bacterium, we observed that the tillering was satisfactory in the strains Ps1 and Ps2. These results are probably related to the complexity of the biological N_2 fixation process in sugarcane plants, which involves a range of factors, such as the genetic characteristics of the cultivars and the bacteria associated with them. The beneficial effect of the inoculation of diazotrophic bacteria on plants in early stages has already been observed in

tomato and red pepper inoculated with bacteria of the genera *Pseudomonas*sp. and*Serratia* sp., which mainly promoted increased plant vigor (Islam et al., 2013).

The interaction of plants with beneficial microorganisms at the beginning of the development of plants is of great importance, being reported by several authors (Aziz et al., 2012; Canellas et al., 2013; Garcia et al., 2013; Gírio et al., 2015; Vargas et al., 2012). For plants that go through the nursery stage, for example, the association with growth-promoting bacteria is of great importance, since it anticipates the transplanting time for the field, stimulating the early growth of the seedling and, consequently, reducing its time of acclimatization, which increases productivity, the turnover in the occupation of infrastructure and the efficiency of the use of specialized labor (Silveira et al., 2003).

All tested isolates have potential mechanisms for promoting plant growth and one or more of the mechanisms may be responsible for the higher growth of sugarcane under the conditions tested.

4 Conclusion

The inoculation of nitrogen-fixing bacteria in micropropagated seedlings of sugarcane variety RB867515 promoted plant development and also presented similar performance to the nitrogen treatment. When used in the individual inoculation, the bacterial genera *Pseudomonas* (Ps1 and Ps2) promoted a better tillering, while the genus *Rhizobium* (Rhz) presented higher dry biomass of shoots and N content in relation to the treatment with nitrogen fertilization, promoting the growth of sugarcane plants. The isolated bacteria Ps1, Ps2 and Rhz are able to and efficient in fixing atmospheric nitrogen, while the mixture of the strains (Ps1, Ps2 and Rhz) did not show a good synergism.

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7 CONCLUSÕES GERAIS

1. Este estudo mostrou que é possível a inoculação pelo uso da técnica de biorreator de imersão temporária (BIT), sendo o primeiro relato de inoculação em mudas micropropagadas por este sistema, sugerindo um grande potencial de resposta à inoculação e otimização do processo em escala comercial;
2. O uso de estirpes homólogas também pode ter contribuído no beneficiamento da interação com a cana-de-açúcar na variedade RB92579;
3. Em todos os ambientes do Nordeste estudados houve grande diversidade de bactérias endofíticas, tendo ocorrido maior percentual dessas bactérias nas raízes comparativamente aos colmos das plantas de cana-de-açúcar;
4. As bactérias endofíticas da cana-de-açúcar apresentaram potencial para promoção de crescimento vegetal, pois possuem a capacidade de produzir AIA, solubilizar fosfato inorgânico, fixar N_2 e possuem bons índices da atividade da nitrogenase;
5. Tanto o percentual quanto o número total de bactérias diazotróficas endofíticas refletiram uma interação entre a variedade RB867515 de cana-de-açúcar e os ambientes, especialmente na área de Alagoas (Usina Coruripe), onde as maiores densidades de bactérias diazotróficas endofíticas foram observadas, mostrando haver interação entre o genótipo da cana e o ambiente;
6. Através da identificação molecular foi possível observar que a maioria dos isolados obtidos da cana-de-açúcar da variedade RB92579 pertence às famílias *Enterobacteriaceae* e *Pseudomonadaceae*;
7. Os isolados bacterianos IPA 44 (*Rhizobium*sp.), IPA 59 (*Pseudomonas* sp.) e IPA167 (*Pseudomonas* sp.) apresentaram potencial para aplicação como inoculante na variedade RB867515 de cana-de-açúcar;
8. A inoculação de bactérias fixadoras de nitrogênio em mudas micropropagadas de cana-de-açúcar na variedade RB867515 promoveu o desenvolvimento e também apresentouperformance semelhante ao tratamento nitrogenado;
9. As estirpes dos gêneros Ps1, Ps2(*Pseudomonas* sp.) e Rhz (*Rhizobium* sp.) apresentaram capacidade e eficiência de fixar o nitrogênio atmosférico, onde as estirpes de *Pseudomonas* sp.(Ps1 e Ps2) promoveram um melhor perfilhamento, enquanto a estirpe de *Rhizobium*sp. (Rhz) apresentou maior biomassa seca da parte aérea e teor de N comparado à adubação nitrogenada, utilizados na inoculação individualmente, promovendo o crescimento vegetal da cana-de-açúcar (RB867515).

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ANEXOS

Maria do Carmo Barreto <mcsbarreto@gmail.com>

Your co-authored submission

1 mensagem

Microbiological Research <EviseSupport@elsevier.com> 8 de abril de 2017 20:32

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Dear Dr. Barreto,

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Journal: Microbiological Research

Title: Biotechnological potential of endophytic bacteria to improve the seedling micropropagated of variety RB92579 sugarcane (Saccharum officinarum L.)

Corresponding Author: Vera Lima

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Soil Biology & Biochemistry publishes original, scientifically challenging research articles of international significance that describe and explain biological processes occurring in soil. These include the possible applications of such knowledge to issues of soil and environmental quality insofar as such studies inform our understanding of the role of soil biology and biochemistry in mediating soil functions, agricultural sustainability and ecosystem services. The ecology and biochemical processes of soil organisms, their effects on the environment and their interactions with plants are major topics. The applications of new molecular, microscopic and analytical techniques to understanding and explaining population and community dynamics is of great interest. The journal also publishes state-of-the-art reviews of contemporary research that present significant and novel hypotheses, as well as comments and arguments about specific and often controversial aspects of life in the soil.

SCOPE

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AUDIENCE

Soil biologists, biochemists, plant scientists, agricultural scientists, environmental scientists, earth scientists, botanists, ecologists and entomologists.

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GUIDE FOR AUTHORS

INTRODUCTION

This journal is a forum for research on soil organisms, their biochemical activities and their influence on the soil environment and plant growth. It publishes original work on quantitative, analytical and experimental aspects of such research. Soil biology and soil biochemistry cover many scientific disciplines but a single journal brings together the results and views of research workers working in a wide variety of research areas. The scope of this journal is wide and embraces accounts of original research on the biology, ecology and biochemical activities of all forms of life that exist in the soil environment. Some of the subjects which have proved to be prominent are the biological transformations of plant nutrients in soil, nitrogen fixation and denitrification, soil-borne phases of plant parasites, the ecological control of soil-borne pathogens, the influence of pesticides on soil organisms, the biochemistry of pesticide and pollution decomposition in soil, microbial aspects of soil pollution, the composition of soil populations, modelling of biological processes in soil systems, the biochemical activities of soil organisms, soil enzymes and the interactions of soil organisms with plants and the effects of tillage on soil organisms and soil biochemistry. Sequence data Papers dealing with amino acid sequences of proteins or with nucleotide sequences must carry a statement that the data have been deposited with an appropriate data bank, e.g., the European Molecular Biology Laboratory (EMBL) or GenBank Data Libraries. The data base accession number must be given at the end of the Materials and Methods section of the manuscript under the separate heading 'Accession numbers'. For example: Coordinates and structure factors have been deposited in the Protein Data Bank with accession number 2XYZ. Lengthy nucleotide sequences will be published only if, in the judgement of the Editorial Board, these results are of general interest and importance.

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