CLEIDE APARECIDA BOMFETI

DESENVOLVIMENTO DE NÓDULOS EM *Sesbania virgata* E A IMPORTÂNCIA DOS EXOPOLISSACARÍDEOS EM SUA SIMBIOSE COM *Azorhizobium doebereinerae*

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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Microbiologia Agrícola, área de concentração em Microbiologia e Bioquímica do Solo, para a obtenção do título de Doutor.

Orientadora

Dra. Fatima Maria de Souza Moreira

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RESUMO

Sesbania virgata é uma leguminosa tropical pioneira que ocorre naturalmente no Brasil sendo usada para a recuperação de solos degradados e reflorestamento de matas ciliares. *S. virgata* desenvolve uma simbiose eficiente apenas quando inoculada com linhagens de *Azorhizobium doebereinerae*. A outra espécie do gênero *Azorhizobium*, *A. caulinodans*, apresenta características fenotípicas e genotípicas similares à *A. doebereinerae* e forma simbiose eficiente somente quando inoculada em *S. rostrata*, nativa da África. Apesar da similaridade observada, *A. caulinodans* induz a formação de pseudonódulos em *S. virgata* e *A. doebereinerae* não forma nódulos em *S. rostrata*. No presente trabalho, foi caracterizada a morfogênese dos nódulos radiculares de *S. virgata* através de microscopia de luz, fluorescência e eletrônica de transmissão. Além disso, para investigar os aspectos genéticos bacterianos do processo de nodulação, cerca de 2500 mutantes foram gerados através de inserções obtidas com transposos (Tn5). Como observado em *S. rostrata*, a nodulação das raízes de *S. virgata* em condições de alagamento ocorre através de fendas na base das raízes laterais. O desenvolvimento do tecido infectado envolve a formação de cordões de infecção e de um meristema apical, resultando em nódulos indeterminados. No entanto, em comparação com *S. rostrata*, a diferenciação completa da zona de fixação em *S. virgata* apresenta um desenvolvimento mais lento. Utilizando nossa biblioteca bacteriana, dois mutantes para a produção de exopolissacarídeos foram selecionados em meio YEB contendo 0,01% de Vermelho Congo. A caracterização destes mutantes foi visualizada pela formação de colônias róseas, com posterior escurecimento no centro, dando um aspecto as colônias mutantes de "olho de touro". Estes mutantes, quando inoculados em *S. virgata*, produziram apenas pequenos nódulos de coloração pálida somente 21 dias após a inoculação e a planta hospedeira apresentou características de deficiência de nitrogênio, evidenciando o papel imprescindível dos exopolissacarídeos no estabelecimento da simbiose.

Palavras-Chave: Leguminosa. Simbiose. Nodulação. Mutantes.

ABSTRACT

Sesbania virgata is a tropical pioneer legume which occurs naturally in Brazil and is used for the regeneration of degraded soils and reforestation of gallery forests. *S. virgata* only develops an efficient symbiosis when inoculated with strains of *Azorhizobium doebereinerae*. The other species of the genus *Azorhizobium*, *A. caulinodans*, has phenotypic and genotypic similarities with *A. doebereinerae* and establishes an efficient symbiosis when inoculated in *S. rostrata*, which is native to Africa. However, despite the similarity between *A. caulinodans* and *A. doebereinerae*, *A. caulinodans* forms pseudonodules in *S. virgata* and *A. doebereinerae* forms ineffective nodules in *S. rostrata*. In this work, we characterized the root nodule morphogenesis of *S. virgata* through light, fluorescence and transmission electronic microscopy. In addition, to investigate bacterial genetics aspects of this nodulation process, approximately 2000 mutants were generated by Tn5 insertions. As observed in *S. rostrata*, the root nodulation of *S. virgata* under flooded conditions occurs via cracks (crack entry). The development of the infected tissue involves the formation of infection threads and the establishment of an apical meristem resulting in indeterminate nodules. However, compared to *S. rostrata*, the complete fixation zone differentiation in *S. virgata* shows a slower development. Using our bacterial mutant library, two exopolysaccharides mutants were selected on YEB agar containing 0,01% Congo Red. The characterization of these mutants show the formation of rough pink colonies, with subsequently darkening at the center, giving a “bulls eye” appearance. Once inoculated in *S. virgata*, these mutants produced smaller and paler nodules only after 21 days of inoculation and the host plant showed features of nitrogen starvation demonstrating the importance of EPS in the symbiosis stabilisment.

Keywords: Legume. Symbiosis. Nodulation. Mutants.

SUMÁRIO

PRIMEIRA PARTE

1 INTRODUÇÃO

A fixação biológica de nitrogênio (N_2) atmosférico (FBN) é um processo mediado por uma parcela de bactérias encontradas no solo, que são capazes de invadir ou penetrar os tecidos radiculares e ou caulinares de plantas leguminosas, provocando uma hipertrofia (nódulos) nestes tecidos. Estas bactérias nomeadas de bactérias fixadoras de nitrogênio nodulíferas em leguminosas (BFNNL) ou rizóbios, se diferenciam nos nódulos em bacteróides reduzindo o N_2 a NH_3, disponibilizado-o para a planta. Esta, por sua vez, disponibiliza fontes de energia e carbono para as bactérias.

Na simbiose entre rizóbio e leguminosa, a invasão bacteriana pode seguir três rotas diferentes, na qual a mais conhecida é a invasão via pêlos radiculares. Neste caso, a bactéria induz o encurvamento dos pêlos em desenvolvimento e penetra na raiz através da hidrólise local da parede celular e invaginação da membrana plasmática. A penetração destas bactérias resulta na formação de um cordão de infecção intracelular que atinge o primórdio nodular liberando as bactérias no interior das células radiculares. Este tipo de formação de nódulos ocorre em ervilha, feijão, soja e alfafa além dos legumes *Medicago trunculata* e *Lotus japonicus* (VANCE et al., 1982; WOOD; NEWCOMB, 1989).

Outra rota de entrada de rizóbios em leguminosas ocorre via invasão intercelular na base das raízes laterais, e é observada em muitos legumes tropicais. A bactéria penetra na planta através de fendas formadas devido à protrusão das raízes laterais e coloniza grandes espaços vegetais intracelulares denominados de pacotes de infecção. A formação de nódulos via "crack entry" ocorre em espécies como *Sesbania rostrata*, *Neptunia* sp., *Aeschynomyces*, *Stylosanthes* e *Arachis* (CHANDLER et al.,1982; ALAZARD; DUHOUX,

1990; JAMES et al., 1992; LOUREIRO et al., 1995; BOOGERD; ROSSUM, 1997; GOORMACHTIG et al., 1997).

Um terceiro modo de infecção é encontrado na leguminosa *Mimosa scabrella*, e neste caso o rizóbio penetra diretamente entre células epidérmicas pela dissolução da lamela média e invade as células do hospedeiro através de estruturas semelhantes aos cordões de infecção (SPRENT, 1989).

Dois tipos principais de nódulos são descritos de acordo com a persistência da atividade meristemática envolvida nas divisões celulares durante a formação do tecido nodular, sendo denominados de nódulos determinados e indeterminados. Legumes de regiões temperadas (*Pisum*, *Medicago*, *Trifolium* ou *Vicia*) geralmente desenvolvem nódulos indeterminados de formatos cilíndricos, que são iniciados na parte interna do córtex da raiz e mantém um meristema apical persistente na qual todos os tecidos dos nódulos se originam. O desenvolvimento de zonas consecutivas pode ser observado no tecido infectado central de um nódulo maduro, correspondendo a um gradiente de diferenciação simbiótica do meristema apical para os tecidos basais aderidos a raiz (VASSE et al., 1990; TIMMERS et al., 2000). Nódulos determinados são característicos de alguns legumes tropicais como *Phaseolus*, *Glycine*, *Lotus* e *Vigna*. Estes nódulos são originados da divisão celular do córtex externo e não possuem um meristema persistente. A atividade meristemática cessa nos primeiros estágios do desenvolvimento nodular e o crescimento subseqüente é causado pela expansão da célula, resultando em nódulos de formato esférico (PATRIARCA et al., 1996).

Um dos passos iniciais da interação simbiótica envolve o reconhecimento de flavonóides específicos liberados pela planta, por uma proteína bacteriana regulatória (fatores Nod) que leva a expressão dos genes de nodulação (LEROUGE et al., 1990; SCHULTZE et al., 1991; SPAINK et al., 1991).

Os fatores Nod não são os únicos sinais bacterianos necessários para uma simbiose bem sucedida. Assim como em outras interações entre bactérias e plantas ou animais, polissacarídeos de superfície (SPS) especialmente exopolissacarídeos (EPS) e lipopolissacarídeos (LPS), atuam como importantes moléculas sinalizadoras no processo simbiótico (SPAINK, 2000; FRAYSSE et al., 2003; D'HAEZE et al., 2004).

Os EPSs são produtos extracelulares secretados no meio circundante e acumulados na superfície celular. São heteropolissacarídeos de unidades repetidas, compostos de hexoses e radicais piruvil, acetil, succinil e hidroxibutanoil (LEPEK; D'ANTUONO, 2005).

Eles parecem estar envolvidos nos processos de invasão ou infecção do tecido vegetal pela bactéria, no desenvolvimento do nódulo, na supressão da defesa da planta, no desenvolvimento do bacteróide e indução da expressão de alguns genes pela planta hospedeira (SPAINK et al., 2000; FRAYSSE et al., 2003).

Sesbania virgata é uma espécie arbórea nativa empregada em áreas de recuperação de solos degradados e em reflorestamento de matas ciliares. Todos os estudos de bactérias fixadoras de nitrogênio (FBN) envolvendo *S. virgata* mostram que esta espécie forma simbiose eficiente somente quando é inoculada com estirpes da espécie *Azorhizobium doebereinerae* (GONÇALVES; MOREIRA, 2004; MOREIRA et al., 2006; FLORENTINO; MOREIRA, 2009). A ocorrência deste microssimbionte, em solos do Sul de Minas Gerais, está relacionada com a presença do hospedeiro, *S. virgata* (FLORENTINO; MOREIRA, 2009). A outra espécie do gênero *Azorhizobium*, *A. caulinodans*, apresenta características culturais, fenotípicas e genotípicas similares à *A. doebereinerae* e forma simbiose eficiente somente quando inoculada em *S. rostrata*, nativa da África. (DREYFUS et al., 1988). Apesar da similaridade fenotípica e afinidade genética observada, *A. caulinodans* não forma nódulos em

S. virgata (FLORENTINO; MOREIRA, 2009) ou induz a formação de pseudonódulos (GONÇALVES; MOREIRA, 2004) e *A. doebereinerae* também não forma nódulos em *S. rostrata* (GONÇALVES; MOREIRA, 2004).

A importância dos EPSs na interação *Azorhizobium-Sesbania* tem sido verificada através do estudo das estirpes mutantes de *A. caulinodans* ORS571-X15 e ORS571-oac2. A mutação da estirpe ORS571-oac2 está localizada no gene *oac2*, que codifica a enzima dTDP-L-ramnose sintase e a mutação na estirpe ORS571-X15, está no gene *oac3*, que codifica a enzima dTDP-D-glicose sintase. Estes genes fazem parte de um cluster composto por quatro genes (*oac0*, *oac1*, *oac2*, *oac3*) envolvidos na síntese de hexoses, que fazem parte da estrutura do EPS (GOETHALS et al., 1994; GAO et al., 2001).

O mutante ORS571-oac2 produziu pequenas quantidades de EPS quando comparado com a estirpe selvagem, enquanto que ORS571-X15 foi completamente deficiente na produção de EPS (GOETHALS et al., 1994; D'HAEZE et al., 1998; GAO et al., 2001). Os mutantes ORS571-oac2 e ORS571-X15 quando inoculadas em raízes de *S. rostrata* não foram capazes de completar o processo de infecção, revelando a importância do EPS neste processo simbiótico (D'HAEZE et al., 2004).

Uma vez que investigações sobre os fatores fisiológicos, morfológicos e genéticos relacionados ao processo de FBN são de suma importância para o estabelecimento de uma relação simbiótica mais eficiente do ponto de vista agronômico, este trabalho teve como objetivo descrever o processo de formação dos nódulos e fixação biológica de nitrogênio na interação simbiótica entre *S. virgata* e *A. doebereinerae* e avaliar o papel dos exopolissacarídeos nesta simbiose.

2 REFERENCIAL TEÓRICO

2.1 Simbiose entre leguminosas e bactérias fixadoras de nitrogênio

A fixação biológica de nitrogênio (N_2) atmosférico (FBN) é um processo mediado por uma parcela de bactérias que possuem um complexo enzimático chamado de nitrogenase. Estas bactérias são encontradas no solo e podem invadir ou penetrar nos tecidos radiculares e ou caulinares de plantas leguminosas provocando uma hipertrofia (nódulos) nestes tecidos. Estas bactérias podem ser nomeadas de bactérias fixadoras de nitrogênio nodulíferas em leguminosas (BFNNL) ou rizóbios.

Nos nódulos, os rizóbios se diferenciam em bacteróides reduzindo o N_2, a NH_3, o qual é transformado em NH_4^+ e disponibilizado para a planta leguminosa. Esta, por sua vez, disponibiliza fontes de energia e carbono para as bactérias, formando a simbiose, onde os dois organismos se beneficiam.

A família Leguminosae compreende um número estimado de 20.000 espécies, dividida em três sub-famílias: *Papilionoideae, Mimosoideae e Caesalpinoideae* e calcula-se que em torno de 23% destas espécies tenham sido investigadas quanto à capacidade de nodular. A capacidade de nodular parece estar correlacionada ao grau de evolução da espécie dentro da família, de modo que nos grupos mais primitivos de *Caesalpinioideae*, a maioria das espécies são não nodulíferas; enquanto nas *Mimosoideae* e *Papilioniodeae*, o número de espécies não nodulíferas é bem menor (MOREIRA; SIQUEIRA, 2006).

Para o estabelecimento da simbiose é necessário à ocorrência de troca de sinais moleculares entre a planta e a bactéria. A planta libera flavonóides que são reconhecidos pela proteína NodD da bactéria, desencadeando a transcrição de genes da nodulação *nod*ABC e a síntese dos fatores Nod. A planta reconhece

estes fatores Nod e inicia-se o encurvamento do pelo radicular e posteriormente, a formação dos nódulos (REDMOND et al., 1986; SPAINK et al., 1991).

Enquanto a participação dos fatores Nod como moléculas atuantes nos primeiros estágios do processo simbiótico entre rizóbios e leguminosas é bem conhecida, o envolvimento de outras moléculas como polissacarídeos de superfície produzidos por estas bactérias ainda não está bem definido. Os polissacarídeos de superfície parecem estar relacionados com os processos de infecção pela bactéria, supressão da resposta de defesa pela planta hospedeira e desenvolvimento do bacteróide culminando no processo de nodulação com a consequente fixação biológica de nitrogênio (SPAINK et al., 2000; FRAYSSE et al., 2003).

Nas regiões tropicais, onde os solos são geralmente ácidos, pobres em nutrientes e com elevados teores de alumínio, o cultivo de espécies leguminosas capazes de formar simbiose com bactérias fixadoras de N_2 têm grande importância para manutenção do ecossistema. Isso porque estas espécies podem contribuir para a ciclagem de nutrientes de modo mais efetivo, uma vez que a qualidade do material aportado em termos de conteúdo de nitrogênio é geralmente superior àquele oriundo de espécies não leguminosas.

Segundo Franco e Faria (1997), as espécies leguminosas que são capazes de formar simbiose com rizóbios têm sido utilizadas para restaurar a fertilidade do solo em áreas degradadas, porque mantêm os níveis adequados de N no solo para o crescimento vegetal, dispensando o uso de fertilizantes nitrogenados que, além de caros, podem exercer impactos sobre o ecossistema.

A deposição das folhas e o crescimento das raízes estabilizam o solo, aumentam sua atividade biológica e criam condições propícias para o estabelecimento de outras espécies de plantas mais exigentes. O solo de áreas degradadas colonizadas por leguminosas capazes de se associarem com rizóbios

apresenta maior diversidade e biomassa em relação aqueles que não possuem espécies vegetais que formam associação com rizóbios (CAMPELO, 1998).

Dessa forma, a utilização de espécies leguminosas que formam simbiose com rizóbios e que apresentam rápido crescimento e capacidade de sobreviver às condições de solo de baixa fertilidade natural, torna-se uma alternativa viável, do ponto de vista econômico e ecológico.

2.2 Mecanismos de formação de nódulos

A interação entre rizóbio e legumes é caracterizada por vários estágios consecutivos e conservados que precedem o desenvolvimento de nódulos ativos na fixação de nitrogênio. Estes estágios incluem: 1) o reconhecimento específico e bidirecional entre planta hospedeira e microssimbionte, 2) aderência da bactéria a planta, 3) invasão bacteriana, 4) formação de um cordão de infecção e liberação das bactérias no interior das células da planta, 5) diferenciação do bacteróide e 6) maturação do nódulo. O desenvolvimento do meristema nodular ocorre concomitantemente com os estágios 2 a 5 e o periciclo e as células corticais da raiz são estimuladas a se dividir geralmente em sentido oposto ao pólo do xilema, próximo ao local de infecção para formar o primórdio nodular (NDOYE et al., 1994; CRESPI; GALVEZ, 2000; JONES et al., 2007).

Neste evento inicial, a troca de sinais envolve a produção de flavonóides pela planta e de fatores Nod pela bactéria. No processo seguinte, ocorre a aderência das bactérias às raízes das plantas, processo essencial para a invasão mediada pelo encurvamento dos pêlos radiculares, mas não para o processo de invasão que ocorre na base das raízes laterais (GOORMACHTIG et al. 2004). Este passo consiste de uma aderência inicial reversível, seguida de uma aderência irreversível, sendo ambas mediadas por diferentes moléculas de superfície provenientes de ambos os parceiros simbióticos. A primeira fase

consiste de uma ligação fraca e inespecífica em que as lectinas das leguminosas, as ricadesinas e polissacarídeos de superfície bacterianos (SPS) parecem estar envolvidos. A segunda fase requer a síntese de fibras de celulose bacterianas que causa uma ligação estreita e permanente entre as bactérias e as raízes (LAUS; KIJNE, 2004; D'HAEZE et al., 2004; BRENCIC; WINANS, 2005; RODRIGUEZ-NAVARRO et al., 2007; OLDROYD; DOWNIE, 2008).

O próximo passo inclui a invasão bacteriana e a infecção dos tecidos da planta na qual, dois diferentes mecanismos têm sido descritos: (I) o mecanismo de encurvamento do pêlo radicular que ocorre em leguminosas de regiões temperadas, como *Medicago sativa*, *Medicago truncatula, Pisum sativum*, *Vicia*, espécies de *Trifolium* (trevo), e em algumas espécies tropicais como *Lotus japonicus*, e (II) a infecção intercelular que ocorre na base das raízes laterais também conhecido como "crack entry", sendo observado para a maioria das leguminosas tropicais, tais como *Sesbania rostrata*, *Neptunia* spp., *Arachis hypogaea*, *Aeschynomene* e *Stylosanthes* (Figura 1) (GAGE, 2004; GOORMACHTIG et al., 2004).

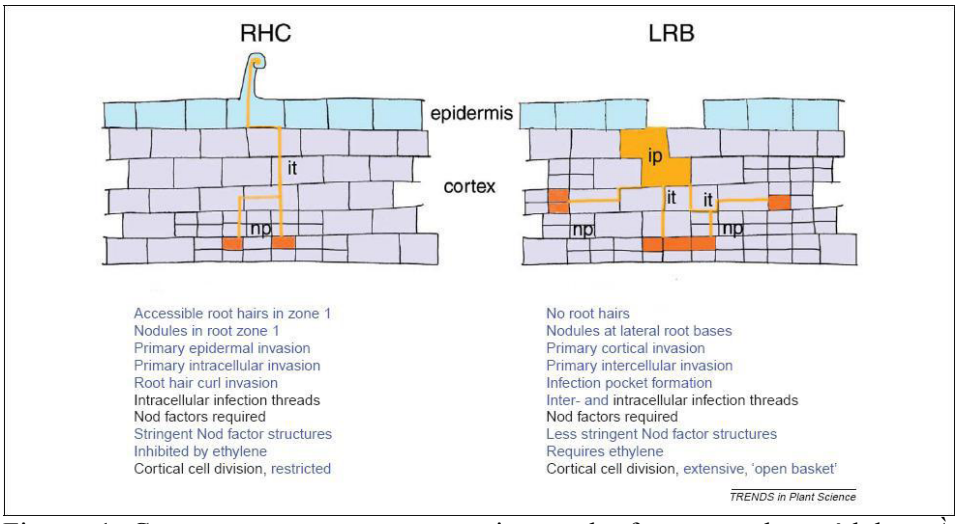

Figura 1 Comparação entre os mecanismos de formação dos nódulos. À esquerda, invasão via pêlo radicular. As bactérias são englobadas com o encurvamento de pêlo e induzem a formação de um cordão de infecção intracelular (it). O cordão de infecção prossegue através das células do primórdio nodular (np) onde as células vegetais são tomadas pelas bactérias. À direita, invasão via raiz lateral. As bactérias colonizam os espaços intercelulares entre as células corticais e induzem a formação de pacotes de infecção (ip), de onde cordões de infecções intra e intercelulares guiam as bactérias para o primórdio nodular

Fonte:(GOORMACHTIG et al., 2004)

A invasão que ocorre na base das raízes laterais é freqüentemente postulada como sendo mais primitiva, porém as interpretações atuais de árvores evolucionárias sugerem que o mecanismo de encurvamento de pelo radicular constitui-se no método ancestral de penetração das bactérias para formação dos nódulos. Investigação sobre *Sesbania rostrata*, que podem ser infectada pelos dois tipos de mecanismos, mostrou que a invasão intercelular é uma adaptação às condições submersas, e que o processo de encurvamento do pêlo radicular é o caminho ancestral percorrido pelos rizóbios para penetrar nas leguminosas (FERNANDEZ-LOPEZ et al., 1998; GOORMACHTIG et al., 2004).

(I) Penetração via pêlo radicular. No momento do reconhecimento dos rizóbios ocorre um aumento nos níveis de cálcio intracelular nos pêlos radiculares em desenvolvimento, seguido de um rearranjo do citoesqueleto. Em seguida, os pêlos radiculares começam a se encurvar, englobando assim uma colônia bacteriana na ponta deste pêlo (JONES et al., 2007; OLDROYD; DOWNIE, 2008). Ao mesmo tempo, as células do córtex radicular são estimuladas a reiniciar a mitose, preparando-se para receber os rizóbios invasores. Após a hidrólise local da parede celular, ocorre a invaginação da membrana celular vegetal por estas bactérias, formando assim uma estrutura intracelular tubular conhecida como cordão de infecção (GOORMACHTIG et al., 2004). O fenômeno da proliferação das células bacterianas localizadas no cordão de infecção, juntamente com a indução da síntese de membrana, são presumivelmente responsáveis para o crescimento interior do cordão de infecção contra o turgor das células do hospedeiro (JONES et al., 2007). Finalmente, os cordões de infecção se fundem com as paredes celulares e as bactérias são liberadas no espaço intercelular, onde um novo cordão é formado para invadir células da camada subjacente. Este processo repete-se sucessivamente em cada camada, levando a uma ramificação, que invade o interior dos tecidos vegetais (GAGE, 2004).

(II) Penetração via base de raízes laterais: Nas bases das raízes laterais ou adventícias, os rizóbios penetram nas células vegetais a partir de rachaduras ou fissuras na epiderme, formadas pela emergência das raízes durante o desenvolvimento do sistema radicular (NDOYE et al., 1994; OLDROYD; DOWNIE, 2008). As bactérias colonizam diretamente o córtex da raiz subepidérmica e, assim, contornam a etapa complexa de aderência. No córtex da raiz as bactérias induzem a morte celular local criando bolsas de infecção intercelular, preenchidas com rizóbio. A colonização e indução da morte celular são mediadas por espécies reativas de oxigênio e etileno (D'HAEZE et al.,

2003). Os próximos passos relacionados com a invasão celular são característicos de cada espécie vegetal. Em *Aeschynomene*, *Stylosanthes* e *Arachis* (Papilionoideae), as bactérias contidas nas bolsas de infecção têm acesso ao interior dos tecidos vegetais através do colapso das células. Em *Neptunia* spp. (Mimosoideae) e *S. rostrata* (Papilionoideae), cordões de infecções intercelulares e intracelulares surgem a partir das bolsas de infecção invadindo as camadas mais profundas de células e, finalmente, o primórdio nodular (GOORMACHTIG et al., 2004)

O passo final antes da maturação do nódulo é a liberação das bactérias e a internalização das mesmas pelas células corticais, o que acontece quando os rizóbios atingem a camada de tecido alvo, o primórdio nodular no córtex interno ou externo da planta (JONES et al., 2007). As bactérias são englobadas pela membrana plasmática da célula hospedeira em um processo parecido com uma endocitose. A estrutura recém-formada, que consiste nas bactérias diferenciadas rodeadas por uma membrana da célula vegetal (membrana peribacteroide), é denominada de simbiossoma (PRELL; POOLE, 2006). Depois que as bactérias concluem sua diferenciação em bacteróides e estando em um ambiente com baixo teor de oxigênio no simbiossomas funcionais, elas podem expressar as enzimas do complexo nitrogenase e iniciar a fixação do nitrogênio (OKE; LONG, 1999, JONES et al., 2007).

2.3 Tipos de nódulos

Os nódulos são separados em dois tipos principais, dependendo da duração da atividade meristemática no nódulo: o tipo indeterminado e o determinado (Figura 2) (CRESPI; GALVEZ, 2000).

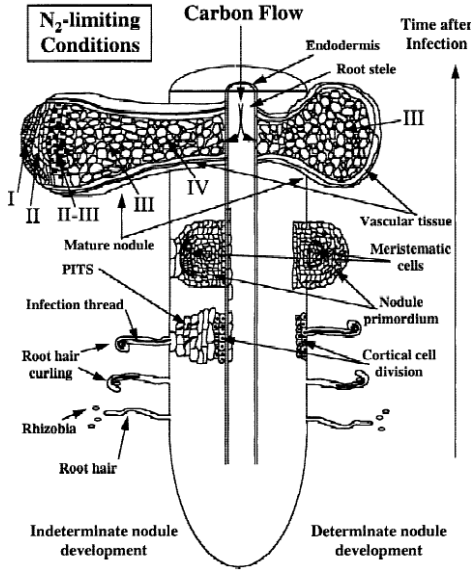

Figura 2 Desenvolvimento de nódulos indeterminados e determinados em que condições de escassez de nitrogênio são necessárias. I a IV= diferentes regiões presentes em nódulos maduros descritas no texto abaixo
Fonte: (CRESPI; GALVEZ, 2000)

O desenvolvimento de nódulos indeterminados começa com o desenvolvimento do primórdio nodular no córtex radicular interno. As células corticais que estão ativamente se dividindo para formar o primórdio acumulam grande quantidade de amiloplastos. Nódulos indeterminados maduros são caracterizados por um meristema apical persistente, localizado na extremidade externa do nódulo, que é responsável pelo crescimento do nódulo (zona I do nódulo) (CRESPI; GÁLVEZ, 2000). A zona I mantém uma população de células em divisão ativa e continua a crescer para fora durante o período ativo do nódulo. Os cordões de infecção crescem passando desta região para a zona de invasão ou zona II, onde as bactérias são liberadas. As células bacterianas são englobadas em uma membrana pré-bacteróide e ambos dividem-se sincronicamente, até que as bactérias se diferenciam em bacteróides fixadores de nitrogênio (OKE; LONG, 1999; JONES et al., 2007). A interzona II-III é

caracterizada por um acúmulo de amiloplastos e representa o ponto onde as bactérias são progressivamente transformadas em bacteróides. Os bacteróides são poliplóides devido a vários ciclos de endoreduplicação, são maiores em tamanho e mostram um aumento da permeabilidade da membrana (MERGAERT et al., 2006). O composto de armazenamento de carbono bacteriano poli-β-hidroxibutirato (PHB), com o progresso das bactérias, este composto é liberado, sendo utilizado como fonte de carbono e energia para o desenvolvimento do bacteróide. Aproximando-se do centro da raiz da planta, estabelece-se a zona de fixação de nitrogênio ou zona III. Esta área, onde o nitrogênio fixado é assimilado, é composta por células vegetais contendo bacteróides e pequenas células vegetais livre de bacteróides. Finalmente, a zona de senescência ou zona IV está localizada perto da endoderme, onde ambos bacteróides e as células da planta encontram-se em degeneração (CRESPI; GÁLVEZ, 2000; MERGAERT et al., 2006). Assim, em nódulos indeterminados, o crescimento e o funcionamento do nódulo ocorrem ao mesmo tempo, e todas as zonas de diferenciação podem ser observadas em uma única seção longitudinal de um nódulo (NDOYE et al., 1994).

O desenvolvimento de nódulos determinados começa com a formação do primórdio nodular no córtex radicular externo. A atividade de divisão celular, necessária para a formação do nódulo, cessa muito rapidamente depois da formação do nódulo Após sucessivas divisões, as células meristemáticas invadidas se diferenciam simultaneamente para formar o tecido central de fixação de nitrogênio, onde a fixação biológica ocorre. Após a liberação bacteriana nas células vegetais, simbiosomas individuais se fundem e os bacteróides continuam a se dividir, resultando em simbiosomas que contêm múltiplos bacteróides. Neste caso, os bacteróides são comparáveis às bactérias de vida livre em diversos aspectos, tais como seu conteúdo de DNA genômico, o tamanho das células e sua viabilidade. Além disso, os grânulos de PHB, por não

serem utilizados como fonte de energia continuam a acumular-se nos bacteróides maduros (CRESPI; GÁLVEZ, 2000; LODWIG; POOLE, 2003; MERGAERT et al., 2006; PRELL; POOLE, 2006). Portanto, os nódulos determinados não possuem um meristema persistente e o crescimento dos nódulos ocorre pela expansão celular (alargamento), em vez de divisão celular (alongamento) (NDOYE et al., 1994; MERGAERT et al., 2006). Assim, em nódulos determinados, o crescimento e funcionamento do nódulo não estão associados, apenas um padrão temporal de diferenciação pode ser observado, e todas as células no interior do nódulo proliferam, diferenciam e senescem sincronicamente (JONES et al., 2007).

2.4 Simbiose *sesbania-azorhizobium*

O gênero *Sesbania* pertence à família Leguminosae e compreende aproximadamente 70 espécies distribuídas nos continentes americano e africano, onde geralmente são encontradas em solos degradados, de baixa fertilidade e em áreas alagadas, apresentando grande potencial para utilização em programas de recuperação de solos degradados e em reflorestamento de matas ciliares (ALLEN; ALLEN, 1981). Esse grande potencial deve-se em parte, à capacidade das espécies desse gênero em formar simbiose com rizóbios presentes no solo, favorecendo o seu estabelecimento.

Dreyfus et al. (1988), isolaram nódulos radiculares e caulinares de *Sesbania rostrata* e descreveram o gênero *Azorhizobium*, com uma única espécie, *Azorhizobium caulinodans*. *Sesbania rostrata* é típica de solos alagados da região do Sahel na África Ocidental e forma nódulos fixadores de nitrogênio atmosférico nas raízes e em grande quantidade no caule, podendo fixar N_2 mesmo quando o conteúdo de nitrogênio no meio for alto, contribuindo para um alto potencial fixador de N_2 (DREYFUS et al., 1988). A presença de nódulos

caulinares em *S. rostrata* é verificada somente quando esta espécie é inoculada com a estirpe *A. caulinodans*, confirmando um alto grau de especificidade da espécie *A. caulinodans* com *S. rostrata*, apesar de nodular com outros gêneros nas raízes (LADHA et al., 1990; GONÇALVES; MOREIRA, 2004).

Moreira et al. (1998), estudando uma estirpe isolada de uma outra espécie do gênero *Sesbania*, *Sesbania virgata*, observaram que esta apresentava características culturais em meio de cultivo 79 (FRED; WAKSMAN, 1928), também denominado YMA (VINCENT, 1970), semelhantes à estirpe tipo de *A. caulinodans*, ORS 571^T; com taxa de crescimento rápido, produção de reação alcalina em meio de cultura e baixa produção de goma. Essas características distinguem o gênero *Azorhizobium* de outros gêneros de rizóbios (MOREIRA et al., 2006). Utilizando a técnica de sequenciamento parcial do gene 16S rDNA, estes autores verificaram que a estirpe isolada de *S. virgata* apresentou seqüências próximas à estirpe ORS 571^T, o que indicava uma nova espécie dentro do gênero *Azorhizobium* (MOREIRA et al., 1998).

Posteriormente, foram isoladas da mesma planta hospedeira, em diferentes regiões do sudeste brasileiro, outras estirpes com características semelhantes à BR 5401^T, que apresentava crescimento intermediário, alcalinização e pouca produção de goma (BARBERI et al., 1998; GONÇALVES; MOREIRA, 2004).

A partir das diferenças encontradas, a nova espécie foi apresentada e proposta com o nome *Azorhizobium johannense* no 12^{th} International Congress on Nitrogen Fixation, em Foz do Iguaçu-PR, 1999 (MOREIRA et al., 1999) e renomeada posteriormente como *Azorhizobium johannae* (GONÇALVES; MOREIRA, 2004). No entanto, o nome foi alterado para *A. doebereinerae* na publicação da nova espécie por MOREIRA et al. (2006).

S*esbania virgata* é uma espécie nativa que apresenta crescimento rápido, podendo atingir de 1 a 4 metros de altura, com diversos surtos de

florescimento por ano. A sua distribuição ao longo dos ecossistemas é desuniforme e está associada a áreas que sofreram algum tipo de impacto humano. Ela pode ser encontrada em campos alagáveis, solos arenosos ou argilosos e de baixa fertilidade natural. Por apresentar este aspecto rústico, esta espécie tem sido amplamente utilizada no reflorestamento da mata ciliar, na recuperação de solos degradados e no controle da erosão (ALLEN; ALLEN, 1981; FRANCO et al., 1996).

Segundo Pott e Pott (1994), esta planta pode ser utilizada na atividade apícola, como fornecedora de lenha, e para revegetação, uma vez que funciona como pioneira e local de pouso de aves que trazem sementes de trepadeiras e de outras plantas sucessoras. Na Argentina, a semente torrada substitui o café, sendo "café", um dos nomes populares sendo cultivada em jardins. Braggio et al. (2002) verificaram que as folhas de *S. virgata* apresentam atividades farmacológicas que reduzem o estímulo doloroso e o edema inflamatório.

Os estudos de FBN envolvendo *S. virgata* mostraram que esta espécie forma simbiose eficiente somente quando é inoculada com estirpes da espécie *Azorhizobium doebereinerae* (GONÇALVES; MOREIRA, 2004; MOREIRA et al., 2006; FLORENTINO; MOREIRA, 2009). De acordo com Gonçalves e Moreira (2004), não foi observada nodulação em *S. virgata,* quando inoculada com rizóbios dos gêneros *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium* e *Sinorhizobium*, corroborando a existência de especificidade simbiótica entre *S. virgata* e *A. doebereinerae*.

2.5 Exopolissacarídeos produzidos por rizóbios

Como todas as bactérias gram-negativas, os rizóbios produzem polissacarídeos constituintes da parede celular, que ficam aderidos à célula (polissacarídeos capsulares) ou podem ser excretados para o ambiente

(exopolissacarídeos). Nos rizóbios, os exopolissacarídeos (EPS) são na maioria das vezes, heteropolissacarídeos (compostos por diferentes tipos de monossacarídeos), consistindo de unidades repetitivas, que são espécies específicas. Uma grande diversidade nas estruturas químicas dos EPS pode ser encontrada entre os rizóbios de acordo com a composição dos açúcares e suas ligações em uma subunidade simples, repetindo o tamanho da unidade e o grau de polimerização (WORKUN et al., 1998; LAUS et al., 2005).

Os EPSs produzidos por rizóbios são essenciais para o estabelecimento da simbiose entre estas bactérias e as leguminosas. As propriedades reológicas e a localização periférica dos EPSs sugerem que eles são responsáveis pelo primeiro contato da bactéria com a planta. (LEPEK; D'ANTUONO, 2005). Estes compostos são essenciais para o efetivo estabelecimento da simbiose de estirpes de e de *Rhizobium* sp. em plantas de alfala, leucena e siratro, sendo que estas estirpes com fenótipo exo$^-$ (incapazes de produzir exopolíssacarideos) não foram capazes de infectar estas plantas (LEIGH et al. 1985; DJORDJEVIC et al.; 1987; BATTISTI et al., 1992).

Um mutante para produção de EPS de *Rhizobium leguminosarum* bv. *trifolii* formou nódulos ineficientes em plantas de trevo e o EPS de *Rhizobium leguminosarum* bv. *viciae* foi necessário para o processo de infecção e formação do encurvamento dos pêlos radiculares em *Vicia sativa* (SKORUPSKA et al., 1995; WORKUN et al., 1998).

Na simbiose *R. leguminosarum* bv. *trifolli* com *T. pratense*, mutantes deficientes em EPS induziram o acúmulo de compostos fenólicos e necrose nas células corticais da planta hospedeira indicando reações de defesa da planta em resposta ao processo de infecção (SHORUPSKA et al., 1995). Em mutantes que produziram pequenas quantidades de EPS, a reação de defesa da planta não foi tão severa, ocorrendo à formação do cordão de infecção, porém sem o conseqüente desenvolvimento dos bacteróides (BIALEK et al., 1995).

Um mutante de *S. meliloti* com modificações na estrutura do EPS induziu apenas nódulos inefectivos em *M. Sativa* (NIEHAUS et al., 1993). Ficou ainda demonstrado, que a conservação estrutural neste EPS de *S. meliloti* é necessária para o início da infecção e uma produção aumentada deste composto afeta a colonização dos pêlos radiculares (CHENG; WALKER, 1998), sugerindo que o EPS parece funcionar como uma molécula sinal que reconhece receptores complexos presentes nas plantas. Mutantes para EPS de *S. meliloti*, quando inoculados em *M. sativa* formaram pseudonódulos induzindo uma alteração na resposta de defesa da planta, com aumento da espessura da parede celular das células corticais, além de induzirem um acúmulo de compostos fenólicos nas células destes nódulos (NIEHAUS et al., 1993).

Estudos realizados por Parniske et al. (1993; 1994) indicaram que os EPSs são também, capazes de previnir a resposta de defesa da planta hospedeira. Analisando estirpes mutantes para EPS de *Bradyrhizobium japonicum* estes autores demonstraram que nos primeiros estágios da interação entre estes mutantes e *Glicine max* houve um acúmulo de fitoalexinas. Após 72 horas de incubação os níveis de fitoalexinas produzidas pelas plantas inoculadas com a estirpe mutante, foram 10 vezes maiores quando comparado com as plantas inoculadas com a estirpe selvagem.

Além disso, observou-se que os EPS exercem uma importante função nos processos envolvidos na nodulação e na adaptabilidade de estirpes de rizóbios quando submetidas às condições de estresse ambiental difrentes. Os EPS estão relacionados com a proteção da célula em situações de condições ambientais adversas, característica de grande importância para a sobrevivência destas em solos tropicais, que geralmente, apresentam problemas relacionados com a acidez e toxidez de Al^{3+} e Mn^{+2}. Solos com essas características, além de limitar o desenvolvimento das plantas (RITCHEY et al., 1980), podem também diminuir a população dos microrganismos no solo, principalmente dos

microrganismos benéficos ao desenvolvimento das plantas (GLENN; DILWORTH, 1994).

Estirpes de *Rhizobium* que produzem maior quantidade de EPS são mais tolerantes às condições de acidez quando comparadas com estirpes que produzem menor quantidade (CUNNIGAN; MUNNS, 1984). Além disso, uma maior produção de EPS em estirpes do gênero *Bradyrhizobium*, está relacionada com as condições de acidez presentes no meio de cultura, sendo que quanto maior o grau de acidez, maior a produção destes compostos (BARBERI et al., 2004; MIGUEL; MOREIRA, 2001). Por isso, existe um contínuo interesse em se elucidar a genética, a biologia molecular e a bioquímica destes polissacarídeos extracelulares de origem bacteriana.

2.6 Envolvimento dos EPS na simbiose *sesbania* x *azorhizobium*

Os processos de infecção, desenvolvimento do nódulo e fixação biológica de nitrogênio na interação simbiótica de *Azorhizobium caulinodans* e *Sesbania rostrata* têm sido atualmente, alvo de vários estudos. Como característica particular desta simbiose, a invasão bacteriana inicial pode ocorrer intercelularmente através de espaços no córtex externo na base das raízes adventíceas, ou intracelularmente via encurvamento de pêlos radiculares (D' HAEZE et al., 1998).

Para uma invasão bacteriana bem sucedida e conseqüente desenvolvimento dos nódulos nesta simbiose, é necessária a síntese e a percepção de moléculas sinais por ambos os parceiros. Para as bactérias, algumas moléculas, como os fatores Nod e os EPS recebem destaque, por estarem envolvidos diferencialmente nas diversas etapas do processo de formação dos nódulos e funcionalidade dos mesmos.

A participação dos EPS na interação simbiótica entre *A. caulinodans* e *S. rostrata*, tem sido verificada através do estudo das estirpes mutantes ORS571-X15 e ORS571-oac2. A mutação de ORS571-X15 está localizada em *oac3*, um gene ortólogo de *rfbA* em *Salmonella typhimurium* que codifica a enzima dTDP-D-glicose sintase envolvida na síntese de deoxihexoses. *Oac3* é parte de um cluster de quatro genes (*oac0*, *oac1*, *oac2*, *oac3*) que codificam enzimas para a síntese de dTDP-L- ramnose a partir de glicose 1-fosfato (GOETHALS et al.,1994; GAO et al., 2001). A enzima OAC3 catalisa o primeiro passo da via enquanto que a enzima dTDP-L-ramnose sintase, codificada pelo gene *oac2*, catalisa o último passo para a síntese de dTDP-L- raminose. A estirpe ORS571-oac2 carrega, portanto, uma mutação no gene da enzima codificada pela região *oac2* do cluster (GOETHALS et al.,1994; GAO et al., 2001).

A mutação no gene *oac3* pode ser visualizada através de modificações morfológicas nas colônias de ORS571^{T} que se tornaram enrugadas, e também através da precipitação celular em meio líquido, além da perda na capacidade de produção de EPS (D'HAEZE et al., 1998). A estirpe ORS571-oac2 também apresentou alterações, sendo capaz de produzir apenas pequenas quantidades de EPS quando comparada com a estirpe selvagem (GAO et al., 2001).

A estirpe mutante ORS571-oac2 quando inoculada em raízes de *S. rostrata* pode induzir a formação do cordão de infecção, mas foi afetada no momento de sua invasão nas células da planta hospedeira, que produziu sintomas de defesa como o desenvolvimento de paredes celulares espessas (GAO et al., 2001; MATHIS et al., 2004). O mutante ORS571-X15 teve sua ação bloqueada durante a formação do cordão de infecção, sem traços de infecção inter ou intracelular (D' HAEZE et al., 1998; D'HAEZE et al., 2004).

Assim como em outros gêneros de rizóbios, estirpes de *A. caulinodans* apresentando mutações em seu EPS não foram capazes de estabelecer uma simbiose bem sucedida com seu hospedeiro, confirmando mais uma vez, a

importância destes polissacarídeos no processo de FBN. No entanto, os mecanismos exatos do desenvolvimento da simbiose entre leguminosas e BFN ainda permanecem obscuros e estudos relacionados aos EPSs produzidos por estas bactérias são ainda, assunto de contínuo interesse.

3 CONSIDERAÇÕES FINAIS

A simbiose entre rizóbios e plantas leguminosas é de considerável importância para a manutenção da sustentabilidade dos ecossistemas porque fornece o nitrogênio (N) necessário para o crescimento das plantas e mantêm os níveis adequados de N no solo, favorecendo a ciclagem de nutrientes. Com isso, ocorre uma redução na utilização de fertilizantes nitrogenados, que além de caros, podem exercer impactos químicos sobre o meio ambiente. Para o estabelecimento dessa simbiose é necessário a atuação de moléculas químicas sinalizadoras, responsáveis pelo reconhecimento entre os dois organismos, como os flavonóides e as lectinas liberadas pela planta hospedeira e os fatores Nod e os exopolissacarídeos liberados pelos rizóbios. Porém, os mecanismos de desenvolvimento simbiótico e de infecção ainda não estão claros e estudos bioquímicos, genéticos e moleculares dos polissacarídeos extracelulares de origem bacteriana são de grande importância para elucidação destes mecanismos. Além disso, a simbiose específica e com alta efetividade entre *S. virgata* e estirpes de *A. doebereinerae* oferece um modelo de estudo que possibilitará um melhor entendimento da relação simbiótica entre plantas e bactérias, em um dos processos de maior importância na obtenção de nitrogênio pelos seres vivos.

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SEGUNDA PARTE

ARTIGO 1 EXOPOLYSACCHARIDES PRODUCED BY THE SYMBIOTIC NITROGEN-FIXING BACTERIA OF LEGUMINOSAE[1]

Normas da Revista Brasileira de Ciência do Solo (versão preliminar)

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SUMMARY

The exopolysaccharides (EPS) produced by the symbiotic nitrogen-fixing bacteria of Leguminosae, commonly known as α and β rhizobia, are mostly species-specific heteropolysaccharides consisting of units of common sugars linked to a variety of substituents. Such molecules are crucial for establishing a successful symbiosis between these bacteria and plants of the Leguminosae family, which are involved in the nodule development and the defence response of the plant. Data from the literature also show that the EPS produced by rhizobia are involved in adaptation to environmental stresses and play an important role in the aggregation of soil particles. Despite the interesting features of some EPS produced by these bacteria,

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such as thickening or stabilising properties, there are no studies showing the industrial application of rhizobia.

In this review we discuss the role of these compounds in the process of biological nitrogen fixation (BNF), in the adaptation of rhizobia to environmental stresses and in the process of soil aggregation. The possible applications of these biopolymers in industry are also discussed.

Index terms: nodulation, environmental stress, soil aggregation, rhizobia.

RESUMO: *EXOPOLISSACARÍDEOS PRODUZIDOS POR BACTÉRIAS FIXADORAS DE NITROGÊNIO SIMBIÓTICAS DE LEGUMINOSAE*

Os exopolissacarídeos (EPS) produzidos por bactérias fixadoras de nitrogênio simbióticas de Leguminosae, comumente denominados α e β rizóbios, são na maioria das vezes heteropolissacarídeos espécie-específicos formados por unidades de açucares comuns ligados a uma variedade de substituintes. Tais macromoléculas são cruciais para o estabelecimento de uma simbiose bem sucedida entre estas bactérias e plantas da família Leguminosae, estando envolvidos na formação dos nódulos e na resposta de defesa da planta. Dados da literatura também mostram que os EPS produzidos pelos rizóbios estão envolvidos na adaptação a estresses ambientais, além de possuirem grande relevância na agregação entre as partículas do solo. Apesar dos EPS produzidos por algumas espécies de bactérias possuirem, como por exemplo propriedades estabilizantes ou espessantes, não existem estudos mostrando a aplicação industrial de rizóbios.

Na presente revisão discutem-se o papel desses compostos no processo de fixação biológica de nitrogênio (FBN), na adaptação dos rizóbios a estresses

ambientais, bem como no processo de agregação do solo. As possíveis aplicações desses biopolímeros na indústria também são discutidas.

Termos de indexação: nodulação, estresse ambiental, agregação do solo, rizóbios.

INTRODUCTION

Nitrogen (N) is a constituent of various cellular components, such as amino acids, nucleic acids and chlorophyll. Thus, many fundamental biochemical reactions involve the presence of N, making it the fourth most consumed element in quantity by cultivated plants. In the case of some plants in the Leguminosae family, this nutrient can be fully or partially provided through the symbiosis of these plants with nodulating nitrogen-fixing bacteria, commonly known as rhizobia.

The rhizobia possess an enzyme complex called nitrogenase, which is responsible for the reduction of atmospheric nitrogen (N_2) to ammonia (NH_3). This process is known as Biological Nitrogen Fixation (BNF) and contributes to an important source of N in agricultural systems, consequently reducing the utilisation of this nutrient in the fertilisation of legume crops. In Brazil, the best example of the utilisation of the BNF process is the inoculation of soybean crops (*Glycine max*. (L.) Merril) with strains belonging to the genus *Bradyrhizobium*, which completely substitutes the nitrogen fertilisation and ensures greater competitiveness of the product in the external market.

With the stimulation of flavonoids exuded from the roots of legumes in the soil, the rhizobia synthesise signalling molecules that are responsible for the nodule development (Broughton et al., 2000; Shorupska et al., 2006). These signalling molecules called Nod factors are lipochitooligosaccharides (LCOs)

that posses various chemical substitutions. The Nod factors are responsible for initiating root hair curling, formation of the infection thread and activation of cellular division of the cortical cells, resulting in the formation of the nodules (Schulze et al., 1998). Within these nodules, the bacteria differentiate into bacteroids to complete the BNF process. In response, the plants provide carbohydrates as a source of carbon and energy to these bacteria.

The Nod factors are not the only bacterial signals necessary for the establishment of a successful symbiosis. As with other interactions between bacteria and plants or animals, surface polysaccharides (SPS) are also involved. These molecules act as important signals in symbiotic processes and are present in Gram-negative bacteria as cyclic glucans, lipopolysaccharides (LPS), capsular polysaccharides (CPS) and exopolysaccharides (EPS), (Spaink, 2000; Fraysse et al., 2003; D'Haeze et al., 2004).

The cyclic glucans are usually concentrated in the periplasmic space, where they are important regulatory compounds involved in the osmotic adaptation of bacteria (Breedeveld et al., 1993). The LPS are anchored in the bacterial outer membrane and consist of three parts: lipid A, the core olysaccharide and the O-antigen polysaccharide (Madigan et al., 2004). The CPS are surface polysaccharides that form a cohesive layer adherent to the bacterial cell surface while the term EPS is used for polysaccharides with little or no cell association (Fraysse et al., 2003; Shorupska et al., 2006).

This review is focused on the EPS and their function in the process of symbiotic BNF and the adaptation of rhizobia to environmental stresses. Brief considerations of the potential industrial applicability of these bacteria in the production of gums and the importance of these compounds in soil aggregation are also presented.

EXOPOLYSACCHARIDE PRODUCED BY α AND β-RHIZOBIA

The exopolysaccharides produced by rhizobia are mostly heteropolysaccharides species or strain-specific and are formed from repeating units of hexose residues such as glucose, galactose, mannose, rhamnose, galacturonic and glucuronic acids with pyruvate, acetyl, succinyl and hydroxybutanoic substitutions (Lepek & D'Antuono, 2005).

The chemical compositions of EPS produced by the rhizobia are highly diverse, varying in the composition of the sugars and their linkage in the single subunit, repeating unit size, and degree of polymerisation, as well a non-carbohydrate decoration (van Workun et al., 1998; Laus et al., 2005; Fraysse et al., 2003; Shorupska et al., 2006). Figure 1 shows the primary structure of EPS from different rhizobia species. Among the nine genera of known α-rhizobia, the composition of the EPS has only been characterised in *Rhizobium, Bradyrhizobium*, *Sinorhizobium* and *Azorhizobium*.

Strains of *Rhizobium leguminosarum*, despite belonging to different biovars (*trifolii*, *viciae* and *phaseoli*) and nodulating different host plants, have conserved EPS composed of glucose, glucuronic acid and galactose in a ratio of 5:2:1 (Robertsen et al., 1981; O'Neil et al., 1991) (Figure 1A). However, some strains secrete EPS with different sugar contents and chain lengths. In *R. leguminosarum* bv. *trifolii* 4S (Figure 1B), an EPS subunit is composed of seven sugars, and the galactose molecule is absent in this chain (Amemura et al., 1983). In *Rhizobium leguminosarum* bv. *viciae* 248 (Figure 1C) the EPS subunit has an additional glucuronic acid (Canter-Cremers et al., 1991). Similar to *S. Meliloti,* strains of *R. leguminosarum* can produce EPS that differ in molecular weight, meaning that they can produce both low as high molecular weight EPS (Djordevic et al., 1987; Mazur et al., 2003).

The chemical compositions of EPS produced by other species of the *Rhizobium* genus have also been described, such as the EPS from *R. tropici* CIAT899 (Figure 1D) that is composed of subunits made up of glucose and galactose sugars in a ratio of 6:2 (Gil-Serrano et al., 1990) and the EPS produced by *Rhizobium sullae* strain KYGT207, which is formed from monomers of glucose, galactose and mannuronic acid in a ratio of 2:1:1 (Kaci et al., 2005). *Rhizobium huakuii* isolated from nodules of *Astragalus sinicus* produces an EPS composed of glucose, galactose, ribose and glucuronic acid in a ratio of 5:1:1:1 (Hisamatsu et al., 1997).

In *Bradyrhizhobium japonicum*, differences in the composition of EPS (Table 1), DNA sequence, membrane lipid composition and antibiotic resistance led to the reclassification of this species into two groups (I and II), with one group remaining as *B. japonicum* (group I) while the other was renamed *B.elkanii* (group II) (Kuykendall et al., 1992). The EPS of *B. japonicum* (Figure 1E) is composed of mannose, galactose, glucose and galacturonic acid sugars in a ratio of 1:1:2:1 (Mort & Bauer 1980, 1982; Huber et al., 1984; Puvanesarajah et al., 1987; Louch & Miller, 2001), while *B. elkanii* (formerly referred to as *B. japonicum* group II) synthesises an EPS consisting solely of rhamnose and glucuronic acid in a 3:1 ratio (Huber et al., 1984; An et al., 1995) (Figure 1F)

Table 1 Composition of EPS from the different *Bradyrhizhobium japonicum* strains previously classified as group I and group II. (Modified and updated from Huber et al., 1984) (Note: Currently group II is classified as *B. elkanii*).

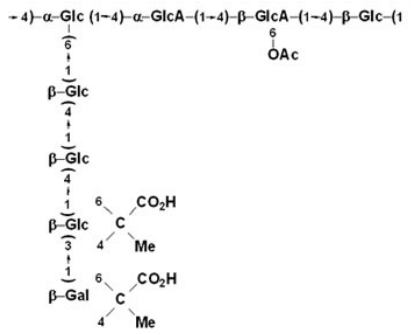
Group/Strain	Mannose	Glucose	Galactouronic acid	Galactose	4-*O*-Methyl galactose	Rhamnose	4-*O*-Methyl-galacturonic acid
I							
ATCC10324[a]*	1	1.54	0.87	0.57	0.25	-***	-
D193[a]	1	1.64	0.68	0.59	0.29	-	-
D209[a]	1	0.83	0.46	0.38	0.09	-	-
HS123[c]	1	2	-	0.6	0.4	-	-
M1E7[d]	1	2	0.81	0.81	-	-	-
THA6[a]	1	1.59	0.93	0.60	0.25	-	-
USDA 24[a]	1	1.59	0.91	0.60	0.25	-	-
USDA 38[a]	1	1.85	0.99	0.74	0.19	-	-
USDA 58[a]	1	2.03	0.52	0.73	0.15	-	-
USDA 62[a]	1	1.83	0.60	0.66	0.41	-	-
USDA 110[a]	1	2.20	1.10	0.52	0.42	-	-

USDA 115[a]	1	1.65	0.84	0.62	0.23	-	-
USDA 123[a]	1	2.17	0.71	0.35	0.70	-	-
USDA 140[a]	1	1.96	0.97	0.75	0.27	-	-
3I1b 138[b]	1	2.30	1.18	0.32	0.68	-	-
3I1b 110[b]	1	2.20	1.12	0.52	0.42	-	-
61A50[a]	1	1.75	0.75	0.50	0.32	-	-
5631[a]	1	1.74	0.88	0.76	0.27	-	-
5633[a]	1	1.58	0.79	0.45	0.39	-	-
II							
USDA 29[a]	-	-	-	-	-	3	-
USDA 46[a]	-	-	-	-	-	3	1
USDA 76[a]**	-	-	-	-	-	3	+****
USDA 86[a]	0.36	-	-	-	-	3	+
USDA 94[a]	0.25	0.10	-	-	-	3	+
USDA 117[a]	-	-	-	-	-	3	+
USDA 130[a]	0.19	0.34	-	-	-	3	+
61A76[a]	-	-	-	-	-	3	+

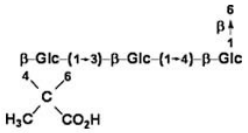
[a]Huber et al. (1984); [b]Mort & Bauer (1980), [c]Puvanesarajah et al. (1987); [d]Louch & Miller(2001)
Type strains of **B.japonicum* and ** *B.elkanii*[***] absence of the monosaccharide
[****] Presence of the monosaccharide but at an undetermined level. Adapted from Huber et al. (1984)

Among the best known EPS produced by the rhizobia is the succinoglycan (EPS I) produced by strains of *Sinorhizobium meliloti* (Figure 1G). This EPS is composed of an octasaccharide of repeating units containing one galactose and seven glucoses (Leigh et al., 1985; Reinhold et al., 1994). *S. meliloti* also has the ability to synthesise another exopolysaccharide, galactoglucan (EPS II), which is synthesised under low-phosphate conditions or when mutations in genes related to EPS I synthesis occur (Zhan et al., 1989; Zhan et al., 1991; Keller et al., 1995). EPS II is a disaccharide with repeating units composed of one glucose and one galactose (Zhan et al., 1989; Her et al., 1990) (Figure 1H). Both EPS I and II may be secreted in two different fractions, high or low molecular weight. The high molecular weight fractions of EPS I and II have hundreds to thousands of monomeric units (10^6-10^7 Da), while the low molecular weight fractions consist of monomers, dimers and trimers in the case of EPS I and oligomers (15 to 20 units) in the case of EPS II (Gonzalez et al., 1996; Gonzalez et al., 1998; Wang et al., 1999; Shorupska et al., 2006).

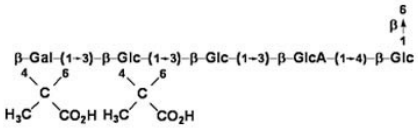
A) *R. leguminosarum* EPS



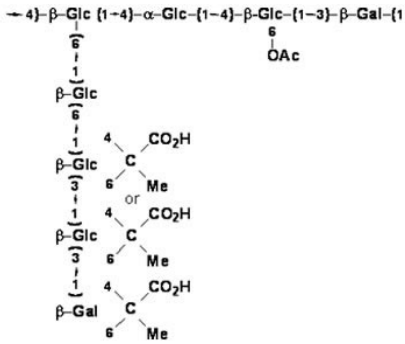
B) *R. leguminosarum* bv. *trifolii* 4S EPS



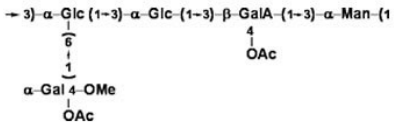
C) *R. leguminosarum* bv. *viciae* 248 EPS



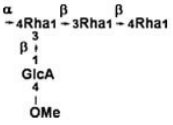
D) *R. tropici* CIAT899 EPS



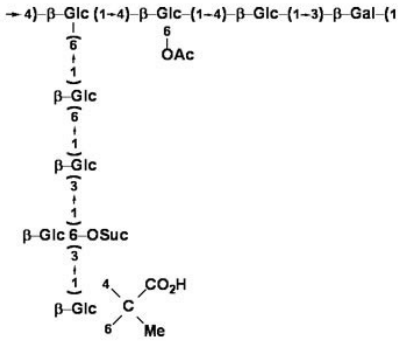
E) *B. japonicum* EPS



F) *B. elkani* EPS



G) *S. meliloti* EPS I



H) *S. melioti* EPS II

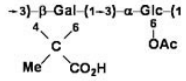


Figure 1. Primary structure of EPS of different rhizobia species A) *Rhizobium leguminosarum* (Robertsen et al., 1981; O'Neil et al., 1991), B) *R. leguminosarum* bv. *trifolii* 4S (Amemura et al., 1983), C) *Rhizobium leguminosarum* bv. *viciae* 248 (Canter-Cremers et al., 1991), D) *Rhizobium tropici* CIAT899 (Gil-Serrano et al., 1990), E) *Bradyrhizobium japonicum* (Mort & Bauer 1980, 1982; Huber et al., 1984; Puvanesarajah et al., 1987; Louch & Miller, 2001), F) *Bradyrhizobium elkani* (Huber et al., 1984; An et al., 1995). G) *Shinorhizobium meliloti* EPS I (Leigh et al., 1985; Reinhold et al., 1994), H) *Shinorhizobium meliloti* EPS II (Zhan et al., 1989; Her et al., 1990). Glc=glucose; Gal=galactose; GlcA=glucouronic acid; GalA=galactouronic acid; Mn=manose; Rha=rhamnose; Suc=succinate; Ac=acetate.

The EPS produced by *Azorhizobium caulinodans* strain ORS571^T, different from other EPS produced by species of rhizobia, is a linear homosaccharide composed only of 4,6-*0*-(1-carboxyetilideno)-D-galactosyl residues (D' Haeze et al., 2004).

The β-rhizobia genus *Burkholderia* contains both associative and symbiotic species. The EPS structure of the nitrogen-fixing strains of symbiotic species *B. caribensis* MWAP71 is composed of glucose and thalose in a 2:1 ratio (Vanhaverbeke et al., 2001). As an example of the EPS described for associative species of N_2–fixing *Burkholderia,* we can cite the *B. brasiliensis* strain M130, which produces two distinct EPS, EPS A and EPS B. EPS A is composed of rhamnose, glucose and glucuronic acid in a ratio of 2:2:1, whereas EPS B is composed of rhamnose, galactose, glucose and glucuronic acid in a ratio of 2:2:2:2 (Mattos et al., 2001). *B. tropica* Ppe8 is not actually a valid species, but a study of the composition of its EPS revealed that it is formed by subunits composed of rhamnose, glucose and glucuronic acid in a ratio of 2:2:1 (Serrato et al., 2008), . There is no available information about the EPS of the another genus of β-rhizobia: *Cupriavidus*.

A wide variety of the chemical structures have been described for the different species of rhizobia, however, some genera such as *Mesorhizobium* have not yet had the chemical composition of their EPS determined. The chemical characterisation of the EPS of other rhizobia species is still needed, as the characterisation of these compounds is highly relevant from both economic and agricultural perspective. We also emphasise that these studies were performed mainly with strains from temperate areas with weather and soil conditions different from tropical regions.

FUNCTION OF EXOPOLYSACCHARIDES IN THE PROCESS OF LEGUME NODULATION

The biological functions of EPS in Rhizobium-legume symbiosis are related to the different stages of plant infection by the bacterium. It has already been shown that these compounds are essential for the effective establishment of the symbiosis between *Rhizobium* sp. NGR234 and *Leucaena leucocephala* or *Macropitilium atropurpureum.* Mutant strains deficient in the production of EPS were unable to promote the formation of efficient nodules in these different hosts, and the ability to induce functional nodules with these mutants was restored by adding purified EPS from the parental strain (Djordjevic et al., 1987).

A mutation in *R. leguminosarum* bv. *trifolii* 24.1 for the production of EPS (*exo*$^-$ mutants) formed inefficient nodules in *Trifolium pratense* plants (Skorupska et al., 1995), and the EPS of *R. leguminosarum* bv. *viciae* ANU843 was necessary to induce root hair curling and the formation of the infection thread in *V. sativa* (van Workun et al., 1998). Inoculation with *exo*$^-$ mutants of *R. leguminosarum* RBL5523 in the roots of *V. sativa* subsp. *nigra* also blocked the formation of infection threads, which were aborted soon after the initiation of

the infection process (Laus et al., 2004, 2005). The co-inoculation of mutant *exo*$^-$ Nod$^+$ mutants with *exo*$^+$ Nod$^-$ mutants restored nodule development process in bacteria from the genus *Rhizobium*, demonstrating the importance of EPS in the process of nodulation (van Workun et al., 1998; Laus et al., 2005).

The influence of EPS in root hair curling and the invasion of bacteria into the nodule was assessed with different strains mutated for the production of EPS (*exo*$^-$), using *S. meliloti* Rm1021 symbiosis with *Medicago sativa* plants. Strains with the *exo*$^-$ phenotype were not capable of nodulating these plants efficiently (Leigh et al., 1985; Battisti et al., 1992; Urzainqui & Walker, 1992).

The addition of small quantities of EPS produced by wild strains during inoculation with *exo*$^-$ mutants allowed for the formation of functional nodules. The addition of low molecular weight EPS I (succinoglycan) at the moment of inoculation of *exo*$^-$ mutants of *S. meliloti* Rm1021 led to the formation of a nodule morphology similar to the wild type strain, with the presence of a large quantity of bacteroids (Battisti et al., 1992; Urzainqui & Walker, 1992).

A mutant of *S. meliloti* Rmm2011 unable to produce EPS I only induced the formation of pseudonodules that did not contain an infection thread or bacteroids in *M. sativa* (Niehaus et al., 1993). The intact structure of the EPS I of *S. meliloti* Rm1021 is required for the initial formation and elongation of the infection thread (Cheng & Walker, 1998), suggesting that the EPS functions as a signalling molecule that recognises complex receptors present in plants. A EPS I mutant of *S. meliloti* CXM1-118 inoculated into *M. sativa* and *M. trunculata* plants formed small, irregularly shaped and inefficient nodules that were formed 3 to 4 days after those induced by the parental strain (Zatovskaya et al., 2007). Electron microscopy analysis of these nodules revealed the presence of highly vacuolated cells that contained a reduced number of bacteroids.

In the case of *S. meliloti* Rm1021, EPS II is also involved in the formation of nodules that fix nitrogen efficiently, overcoming the symbiotic

defects of strains deficient in the production of EPS I, which are able to functionally replace succinoglycan (Glazebrook & Walker, 1989; Gonzalez et al., 1996). Moreover, the addition of low molecular weight EPS II to mutants deficient in the production of EPS I and II enabled the process of nitrogen fixation, producing nodules morphologically indistinguishable from those produced by the wild type strain (Gonzalez et al., 1996).

The EPS also play a fundamental role in the initial stages of the symbiotic interaction between *Bradyrhizobium japonicum* 110spc4 and *Glycine max* in preventing the defence response from the host plant. EPS mutant strains (*exo*$^-$) of *B. japonicum* stimulated the accumulation of phytoalexins (defence substances produced by plants) in the early stages of interaction with *Glycine max*. After 72 hours of incubation, the levels of phytoalexins produced by the plants were 10-fold higher than in plants inoculated with the wild type strain (Parniske et al., 1993, 1994).

Exo$^-$ mutants of *S. meliloti* Rm2011, when inoculated on *M. sativa*, formed pseudonodules that induced an alteration in the defence response of the plants, with increased cell wall thickness in the cortical cells and an accumulation of phenolic compounds in the cells of these nodules (Niehaus et al., 1993).

In the symbiosis of *R. leguminosarum* bv. *trifolli* 24.1 with *T. pratense*, EPS-deficient mutants induced the accumulation of phenolic compounds and necrosis in the cortical cells of the host plant, which indicates a plant defence reaction in response to the infection process (Shorupska et al., 1995). In mutants that produced small amounts of EPS, the defence reaction of the plant was not as strong, occurring the formation of the infection thread, but it resulted in the development of irregularly shaped bacteroids and an electron dense cytoplasm, both signs of degeneration (Bialek et al., 1995).

In the *Azorhizobium caulinodans* ORS571 - *Sesbania rostrata* symbiosis, mutants deficient in EPS production were unable to penetrate the tissues of the host plant due to the loss of protection exerted by the EPS upon exposure to the hydrogen peroxide (H_2O_2) produced by *Sesbania rostrata* as a defence mechanism (D' Haeze et al., 2004).

The function of EPS in the determination of rhizobia-host plant specificity is still very controversial (Shorupska et al., 2006). van Workun et al. (1998) demonstrated that *exo*$^-$ mutants of *R. leguminosarum* bv. *viciae* RBL5523 inoculated into *V. sativa* can overcome the lack of EPS production when homologous EPS (structurally similar), but not heterologous EPS (structurally different) are added, suggesting that there are some structural requirements for EPS to function as a signalling molecule in the process of symbiosis. A hybrid strain of *Rhizobium* sp NGR234 containing the genes of *S. meliloti* for the production of EPS I was capable of inducing the formation of nodules in *L. leucocephala* plants, but they were not able to fix nitrogen, indicating that the structure of the EPS is essential for the formation of efficient nodules (Gray et al., 1991).

In co-inoculation experiments using the RBL5833 *exo*$^-$ strain of *R. Leguminosarum* with other species of EPS producing bacteria (*Agrobacterium tumefaciens* LBA4301, *Rhizobium* NGR234, *R. leguminosarum* bv. *trifolii* ANU845 and LPR5045, *R. tropici* CIAT899, *S. meliloti* RCR2011) in *V. sativa* roots, nodules were formed only in roots inoculated with rhizobia producing EPS homologs (*R. leguminosarum* bv. *trifolii* ANU845 and LPR5045) and with rhizobia producing similar EPS (*R. tropici* CIAT899). However, the transformation of heterologous strains with the symbiotic pRL1J1 Sym plasmid of *R. leguminosarum* provided these strains with the ability to form efficient nodules in *V. sativa* plants, which indicates that the specificity of nitrogen-fixing bacteria is not determined only by the structure of EPS (Laus et al., 2005).

Considering that a limited number of rhizobia species deficient in the production of EPS were investigated for co-inoculation with non-homologous EPS-producing species, the hypothesis that EPS are involved in specificity with the host plant cannot be confirmed. Therefore, it is not possible to relate EPS with the specificity in nitrogen-fixing bacteria and legumes.

EXOPOLYSACCHARIDES IN THE PROCESS OF RHIZOBIA ADAPTATION IN LIMITED ENVIRONMENTAL CONDITIONS

For the establishment of the symbiosis between rhizobia and legumes, in addition to the requirements for recognition of specific chemical signals between the symbionts, the environmental conditions must be adequate for this interaction to develop. In tropical regions it is common to find highly acidic soils associated with toxic Al^{+3}, salinity, low levels of calcium and phosphorus, high temperatures, and other types of stress. Soils with these characteristics may limit not only plant growth, but also the survival of rhizobia in the soil, its infection of the plant and the process of BNF.

As described previously, the EPS produced by rhizobia are very diverse in composition and chemical structure. Under normal cultivation conditions, there is great variability in the production of EPS by rhizobia strains, both quantitatively and qualitatively. The strains with high levels of EPS production tend to be more tolerant to acidic conditions and salinity than strains that produce low levels of EPS (Cunningham & Munns, 1984; Eaglesham et al., 1987; Xavier et al., 1998; Freitas et al., 2007; Xavier et al., 2007). In the case of saline stress, the EPS surrounds the bacterial cells, decreasing the cell surface contact with the saline medium and providing greater cellular resistance to the osmotic effect (Elsheikh & Wood, 1990).

The BR 29 and SEMIA 587 strains of *Bradyrhizobium elkanii*, recommended as inoculants for soybean, produce greater amount of EPS when grown in acidic conditions than when cultivated at a neutral pH (Barberi et al. 2004; Miguel & Moreira, 2001). The same trend of increased production of EPS in acidic conditions and with limited Ca^{+2} was observed in the strain USDA 3187 of *Bradyrhizobium* sp. (Macció et al., 2002).

The limitation of some nutrients can also lead to increased production of EPS by certain rhizobia strains. In these cases, the increased production of EPS is considered an adaptation mechanism adopted by these bacteria (Barberi et al., 2004; Miguel & Moreira, 2001; Macció et al., 2002). Thus, the synthesis of EPS by the rhizobia can also be regulated by the culture conditions.

As previously mentioned, *Sinorhizobium meliloti* produces two types of EPS, and the concentration of phosphate in the medium regulates the production of one type of EPS at the expense of the other. In low phosphate conditions EPS II predominates, and the colonies of these bacteria have a more mucoid morphology. Under normal conditions, *S. meliloti* produces EPS I, with less mucoid colonies (Zhan et al., 1991; Mendrygal & Gonzalez, 2000).

However, abiotic stresses do not always induce greater production of EPS by rhizobia strains. This was observed for *Sinorhizobium meliloti* strains grown under acidic conditions in low concentrations of Ca^{+2}. Under these conditions, the limitation of Ca^{+2} in the culture medium drastically reduced the production of EPS by the strains (Dilworth et al., 1999; Delavechia et al., 2003).

The different genera of rhizobia have different behaviours with respect to EPS production when the strains are cultivated under different environmental conditions, which cause alterations in the production and the chemical composition of these compounds. The species or strain-specific EPS substances are essential for the establishment and effectiveness of the symbiosis between

rhizobia and legumes, and it is important to understand the influence of these characteristics on the process of nodulation.

Studies of EPS I and II produced by *S. meliloti* were performed in order to determine the function of these two compounds during nodulation of *M. sativa*. It was observed that EPS I is more efficient in mediating the invasion processes, although both EPS act in the process of *M. sativa* nodulation (Pellock et al., 2000). These authors report that the ability of *S. meliloti* to produce two types of EPS and their process of nodulation provides a competitive advantage to this strain, since even in limiting environmental conditions the process of nodulation and N_2 fixation is not affected.

Due to their predominantly anionic nature, the EPS have the capacity to strongly interact with metal cations and play an important role in the sequestration or immobilisation of these ions in the environment (De Philippis & Vincenzini, 1998). Despite the increase in EPS production in response to heavy metals that has been studied in other bacterial species, in rhizobia few studies have been performed (Santamaría et al., 2003). EPS produced by *Bradyrhizobium* (Chamaecytisus) BGA-1 (Corzo et al., 1994) and *Bradyrhizobium japonicum* USDA 110 (Santamaría et al., 2003) in the presence of solutions of Fe^{+3}, Al^{+3} and Th^{+4} form a gelatinous precipitate composed of EPS bound to these metals (Diaz-Marrero et al., 2004). The EPS of *Rhizobium etli* is also able to bind to metal ions, and are able to rapidly adhere to Mn^{+2} and Pb^{+2}, suggesting a potential application for this biopolymer in the field of bioremediation (Foster et al., 2000).

POSSIBLE INDUSTRIAL APPLICATIONS OF EPS PRODUCED BY RHIZOBIA

Exopolysaccharides produced by some species of microorganisms are widely used in various industries. These compounds, also called biopolymers, are hydrosoluble gums that have the ability to form gels and viscous solutions in an aqueous medium.

Microbial biopolymers vary greatly in their composition and consequently in their physical and chemical properties. Due to this wide diversity in both structure and physical properties, these compounds have various applications in the food, pharmaceutical, petroleum, cosmetic, textile, paint and agricultural product industries. They can be applied as emulsifiers, stabilisers, binders, gelling agents, coagulants, lubricants, film formers, thickeners and suspension agents.

Several biopolymers have been produced and used commercially because they have a wide range of applications, especially in the areas of food, biomedical, pharmaceutical and cosmetic applications. The vast majority are used as stabilisers of all kinds of food and thickeners of ice cream, yogurt, lotions, creams, and sunscreens, among others.

Dextran, xanthan and gellan produced by the bacteria *Leuconostoc* spp., *Xanthomonas* spp. and *Sphingmonas elodea* respectively, are still among the few microbial polysaccharides commercialised on a large scale, and these compounds are very important in the gums market. The structure of these polysaccharides is quite varied. Xanthan is composed of glucose, mannose and glucuronic acid in a ratio of 2:2:1. Dextran is a homopolysaccharide composed of glucose molecules, while gellan is a heteropolysaccharide consisting of glucuronic acid, glucose and rhamnose with glycerate and acetate groups in its structure. Economically, xanthan is the most important microbial

polysaccharide, with a worldwide production of about 40 to 50 thousand tons/year and a value of about 270 million U.S. dollars annually. The annual growth rate for demand is estimated to increase at a continuous rate of 5 to 10% (Pradella, 2006).

The search for new microorganisms that produce polysaccharides is economically attractive. Production of large quantities is a challenge being faced by several research groups, as microbial biopolymers can be produced in large quantities and fermentation offers the advantage of controlled production. This eliminates the problems found in the production of polymers from plants and algae, such as problems at harvest, climate conditions or marine pollution (Sutherland, 2001).

The atmospheric N_2 fixing bacteria (NFB) can be found as both free-living organisms and in association or symbiosis with leguminous plants (rhizobia). Studies of EPS that focus on commercial utilisations are more advanced for the free-living NFB, where we can cite the clairana produced by *Beijerinckia* sp. (Moreira et al., 2003) and the alginate produced by *Azotobacter vinelandii* (Garcia-Cruz et al., 2008).

Since there are no studies on the commercial production of gum by rhizobia, these can be considered unexplored sources of microbial polysaccharides, with great potential in industrial applications. These bacteria have elevated morphological, physiological, genetic and phylogenetic diversity, are not pathogenic and produce large amounts of EPS. In Figure 2 we illustrate the appearance of colonies characterised by high gum production on solid medium, and the precipitation of EPS from strains of *Mesorhizobium plurifarium* BR3804 and *R. tropici* CIAT899, recommended by MAPA (Ministério da Agricultura, Pecuária e Abastecimento) as inoculants for the species *Chamaecrista ensiformis* and *Phaseolus vulgaris* respectively,

demonstrating the high potential of these strains for the production of these biopolymers.

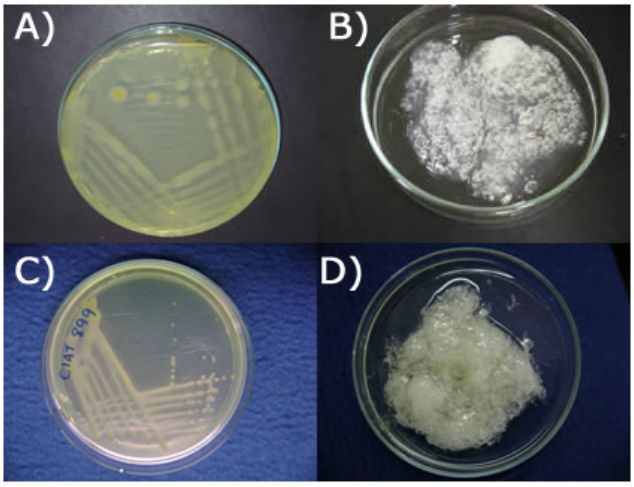


Figure 2. A) Colonies of the *M. plurifarium* BR3804 strain, grown in 79 medium with bromothymol blue; B) the biopolymer produced by *Mesorhizobium plurifarium* BR3804 after precipitation with ethanol (1.44 g dry weight EPS/L of medium) after 72 hours of culture; C) colonies of *R. tropici* CIAT899 strain, grown in 79 medium with bromothymol blue; D) biopolymer produced by *R. tropici* CIAT899 after precipitation with ethanol (3.0 g dry weight EPS/L of medium) after 72 hours of culture.

ROLE OF EPS IN SOIL AGGREGATION

Soil aggregation has a great importance for the growth and nutrition of cultivated plants. This process is dependent on several factors, such as the physical properties, climatic conditions and biological activities of the soil (Materechera et al., 1994; Bezzate et al., 2000).

The action of microorganisms, notably fungi and bacteria, in the process of soil particle aggregation is principally related to the production of EPS by these organisms (Alami et al., 2000). Microbial EPS can increase the adhesion

of soil particles to plant roots and the mechanical stability of rhizospherical soils, besides increasing the level of water retention in this environment (Chenu & Roberson, 1996; Amellal et al., 1998).

The inoculation of a field of wheat (*Triticum durum* L.) with the associative bacterium *Paenibacillus polymyxa*, selected by its ability to fix nitrogen, resulted in a 57% increase of the soil mass that adhered to the roots and an increased frequency of aggregates with sizes between 0.2 and 2 mm due to the production of EPS (levan), which contributed to the greater aggregation of this soil (Gouzou et al., 1993; Bezzate et al., 2000). The same effect on wheat was also observed after inoculation with other associative species. Inoculation of wheat seedlings in soil with 24% water content with *Pantoea aglomerans* provided an increase in the adhesion of soil particles to the roots, where 140 mg of soil adhered to 1 mg of roots in the inoculated treatment compared to 90 mg of soil that adhered in the control treatment. There was also a significant increase in the macroporosity of the inoculated soil (diameters of 10 to 30 μm) compared to the uninoculated control (Amellal et al., 1998).

The effect of inoculation of clay soils with the diazotrophic cyanobacterium *Nostoc* spp. resulted in an increased incidence of porosity in the inoculated soils (30%) compared with the control soil without inoculation (5%). Electron microscopy analysis allowed the visualisation of the primary soil aggregation that resulted from the interaction of the secreted EPS with particles of soil (Falchini et al., 1996). These cyanobacteria can establish symbiosis with several species of bryophytes and gymnosperms or live freely in the soil.

Some studies relating the production of EPS by strains of rhizobia and soil aggregation have also been described. Inoculation of a strain of *Rhizobium* sp. into *Triticum durum* L. plants significantly increased the percentage of aggregates with a diameter of 1.6 to 2 mm, and the water stability in this fraction was 42 ± 5% in the inoculated treatments compared with 30 ± 4% in the control

(Kaci et al., 2005). Another strain of *Rhizobium* sp., growth promoting of *Helianthus annuus* L. provided a significant increase in the volume of macro pores (from 12 to 60 μm in diameter) in soil inoculated with this bacterium. Under conditions of water stress, there was a change in the soil structure around the root system, avoiding the negative effects of water deficit on the growth of the inoculated plants (Alami et al., 2000).

Conformational studies of the structure of EPS from *Burkolderia caribensis* MWAP71 demonstrated that this strain produces an EPS that is responsible for the aggregation due to the adsorption capacity of the EPS to mineral surfaces and the adhesive properties of this biopolymer (Vanhaverbeke et al., 2003).

Although little attention has been given to the influence of microorganisms, particularly bacteria producing EPS, in the process of soil aggregation, microbial EPS are biological factors that influence the formation of soil structure. These compounds contribute to the stability and aggregation of particles and are potential agents for improving the structural quality of agricultural soil.

FINAL CONSIDERATIONS

The process of BNF achieved through the symbiosis between legumes and rhizobia provides high sustainability for agricultural systems. BNF increases the fertility and organic matter levels in the soil and reduces the need for nitrogen fertilisers, thus providing both economic and environmental benefits.

Despite all the data available in the literature about the possible roles of EPS in BNF, the exact function of these compounds in the symbiotic process is not yet completely understood, and further examination of the signalling mechanisms is required. Previous studies of the roles of EPS in the processes of

nodulation and BNF through the study of mutant strains for the production of these compounds are focused principally on the *Rhizobium* and *Sinorhizobium* genera, while the understanding of these processes in other species is insufficient. In depth research and comparisons between other genera are necessary to better understand the role of these compounds in the process of symbiosis between bacteria and legumes.

The production of these compounds by strains of rhizobia is regulated by environmental conditions, and in some cases the production of these compounds provides an increased ability to adapt to various stress conditions. Thus, the studies related to the importance of EPS in the process of adaptation of these bacteria to various edaphic and climate conditions are particularly important, as this affects the survival and functionality of these strains.

In recent years, the demand for industrial exopolysaccharides of bacterial origin (biopolymers) has grown significantly. These compounds have a broad applicability in various industries due to their diverse structural and physico-chemical properties. Bacterial EPS offer several advantages, such as the potential for controlled, high speed production with higher yield and greater purity and consistency than alternative sources.

These compounds also offer a potential application in agriculture due to their adhesive properties and their ability to form gels that promote the adhesion of soil particles into stable aggregates that contribute to better growth and development of plants. Although the role of bacterial EPS in soil aggregation is recognised, little information is available in the literature about the specific action of known bacteria and possible methods for their management.

Considering that none of the nodulating *Burkholderia* species or the α and β-rhizobia have been shown to be pathogenic, they can be generally characterised as an unexplored source of microbial polysaccharides with great potential in industrial applications and as stabilising agents in the soil. They

present high morphological, physiological, genetic and phylogenetic diversity, are not pathogenic and produce large amounts of these compounds. Furthermore, the role of these compounds in adaptation to stress may be important criteria for the selection of inoculant strains in different soil and climate conditions. The biodiversity of rhizobia in tropical soils offers a vast, unexplored field for research in this area.

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ARTIGO 2 Nodule development on the tropical legume *Sesbania virgata* under flooded and non flooded conditions

Normas da Revista Symbiosis (versão preliminar)

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Abstract

The tropical legume *Sesbania virgata* can be nodulated on its root system by the bacteria *Azorhizobium doebereinereae*. Here we investigate in detail the process of root nodulation showing that when cultivated in test tubes with nutritive solution (flooded condition) *Sesbania virgata* develops nodules exclusively at the base of secondary roots. Intercellular infections leads to the formation of infection pockets, which then give rise to infection threads that spread intra and intercellularly. Initialy the nodule differentiation is comparable to that of indeterminate nodules, with the meristematic cells located at the periphery and fixing cells at the nodule center. However the mature nodules develop uniformly around a central axis showing typical histology of determinate nodules, which are round in shape. Another peculiarity is that when cultivated in non flooded

conditions *Sesbania virgata* nodules are formed via root hair curling and mature nodules are of the indeteminate type.

Keywords nitrogen fixation, nodule formation, tropical legume

1 Introduction

The biological fixation of atmospheric nitrogen (N_2) is a process mediated by a number of bacteria that possess an enzyme complex called nitrogenase. These bacteria are found in soil and can invade or penetrate legume root tissues causing a hypertrophy in these tissues forming structures called nodules. In the nodules, the nitrogen fixing bacteria, commonly known as rhizobia, differentiate into bacteroids reducing N_2 to NH_3, which is transformed into NH_4 and available to the legume plant. The legume, provides energy and carbon sources to the bacteria, forming one symbiosis which both organisms are benefited.

Rhizobia have been reported to infect legume roots by three different mechanisms; the best known of which is via root hairs where rhizobia induce the curling of growing root hairs, are entrapped in the curl and enter the root hair by local hydrolysis of cell walls and invagination of plasma membrane. The inverted tip growth result in the formation of a tubular infection thread that guides the bacteria towards the inner cortical cells until reaching the nodule primordium were the bacteria are released. Within the host cell, bacteria are enclosed by a plant membrane and differentiate into nitrogen fixing bacteroids (Oldroyd and Downie, 2004).

Another mode of entry, via intercellular invasion at lateral root bases, has been observed in many tropical legumes. The bacteria enter via cracks formed by the protrusion of lateral roots and colonize larger intercellular spaces called infection pockets. In *Arachis hypogae* (Chandler, 1978), *Stylosanthes*

(Chandler et al., 1982) and *Aeschynomene* (Alazard and Duhoux, 1990), no infection threads are observed and the intercellular rhizobia invade the cortical cells through altered cell walls. In *Sesbania rostrata* (Ndoye et al., 1994) and *Neptunia* sp. (Subba-Rao et al., 1995) infection pockets narrow down to form intercellular infection threads, and subsequently intracellular infection threads intrude into nodule primordium.

A third mode of infection has been described in *Mimosa scrabella*, where rhizobia penetrate directly between undamaged epidermal cells by dissolution of the middle lamella of the radial cell walls and invade the host cells through infection threads (de Faria et al., 1988, Sprent 1989).

Nodules are separated into two main types depending on the duration of meristematic activity within the nodule: the indeterminate and determinate type. The determinate nodules originate from cell division in the outer cortex and do not have a persistent meristem. The development and growth of this type of nodules is caused by cell expansion resulting in spherical-shaped nodules (Patriarca et al., 1996). Determinate nodules are characteristic of legumes as *Phaseolus*, *Glycine*, *Lotus* and *Vigna*.

Indeterminate nodules are usually cylindrical-shape, and are initiated in the inner root cortex. They maintain a persistent apical meristem from which all nodule tissue are formed and in this way, a consecutive developmental zone can be observed in the infected central tissue of a mature nodule (Timmers et al., 2000). Indeterminate nodules can be found in legumes as *Pisum*, *Medicago*, *Trifollium* and *Vicia*.

The genus *Sesbania* belongs to the Leguminosae family, subfamily Papilionoideae. It comprises about 70 species distributed mainly in the African and American continents. Many plants of this genus can form a symbiosis with rizobia, which favors development in depleted and low natural fertility soils (Allen and Allen, 1981). *Sesbania virgata*, native to South America, is found in

the South, Southeast, and Midwest regions of Brazil, as well as in Paraguay, Argentina, and Uruguay (Pott and Pott, 1994). It is a fast-growing pioneer species and produce flowers and fruits several times a year and it is also adapted to flooding conditions. These properties allows it to be used in recovery programs of degraded areas and gallery forest reforestation. This species has pharmacological properties and the treatment of mice with the lyophilized juice of the leaves reduced the reaction to painful stimulation and inflammatory edema (Braggio et al., 2002). *S. virgata* only develops an efficient symbiosis when inoculated with strains of *Azorhizobium doebereinerae* (Goncalves and Moreira, 2004; Moreira et al., 2006), demonstrating that this symbiosis is a highly specific process between this bacterial species and its host plant. *S. virgata* plants also present a highly branched root system, which protects the soil against the erosive action of water waves (Zanadrea et al., 2009).

Although this species has a recognized ability for recovery of degraded areas and also the specific and highly effective symbiosis with strains of *A. doebereinerae,* its nodulation process remained unknown. The objective of this study was to describe the root nodule organogenesis of *S. virgata* and compare this nodulation process with that occurring in the other nodulating species *S. rostrata*. Differences in the nodulation under flooded and non flooded conditions were also analyzed.

2 Materials and Methods

2.1 Bacterial strains, growth and transformation

Azorhizobium doebereinerae 5401 was grown at 28 ^{o}C in YEB medium (Geremia et al., 1994) and *Escherichia coli* DH5α was grown at 37 ^{o}C in Luria Bertani medium (Sambrock et al., 1989). To construct a strain that is detectable

by GFP analysis, the plasmid pBBR5-hem-gfp5-S65T (Van den Eede et al., 1992) was mobilized into *A. doebereinerae* 5401 via triparental conjugation using pRK2013 (Ditta et al., 1980) as a helper. Gentamicin-resistant derivatives of 5401 were obtained by selection on YEB medium supplied with 25 μg/ml gentamicin.

2.2 Plant culture and inoculation

In order to facilitate germination, *Sesbania virgata* seeds were scarified with concentrated sulphuric acid for 50 minutes, and then surface desinfected for 3 minutes in 13 % (v/v) sodium hypochlorite. Surface desinfected seeds were rinsed with 1 liter of sterile distilled water, and germinated on 0.8 % (w/v) agar plates in darkness at 28^{o} C for 5 days. The five-day-old seedlings are transferred aseptically to test tubes with Norris nitrogen free solution. The shoots were allowed to grow out of the tube, which was closed with aluminium foil. The tubes were kept in a greenhouse with following conditions: 28-32 °C, 16hlight/8hdark and high humidity. After one week the plants were inoculated with 70 μl of BR5401 or 5401-GFP cultures. Inocula were prepared by growing bacterial cultures to late exponential phase in shaken flasks containing YEB at 28 ^{o}C. Uninoculated controls were used in all experiments.

2.3 GFP analysis and light microscopy

The infection process was monitored daily (1-15 days after infection) and the GFP bacteria were visualized using a MZFLII (Leica) stereomicroscope equipped with a blue-light source and a Leica GFP plus filter set. Nodules samples were collected after 4, 6, 8, 10 and 15 day post-inoculation. The root nodules were fixed by a mixture of 2.5 % formaldehyde and 2.5 %

glutaraldehyde in 0.1M of sodium cacodylate buffer, dehydrated, and embedded in Tecnovit 7100 (Heraeus Kulzer, Wehrheim, Germany). Semi thin (2 μm) sections were cut, using a microtome RJ2040 (Reichert-Jung, Germany), and placed on SuperFrost® Plus glass slides (Menzel-Glaser, Germany). The sections were stained with 0.05 % toluidine blue and after drying the slides were mounted with DPX (BDH Chemicals, U.K.). A Diaplan microscope equipped with bright- and dark-field optics was used to examine the sections.

2.4 Transmission electron microscopy

Nodules collected 4 and 10 days after inoculation were harvested, cut in half with a sterile scalpel, and immersed into freshly prepared fixative buffer (3 % gluteraldehyde and 3-4 % paraformaldehyde in 0.1 Na-cacodylate buffer; pH 7.2). Thereafter, the samples were postfixated with 1 % OsO_4 (osmium tetroxide) and 1.5 % $K_3Fe(CN)_6$ in 0.1 M Na-cacodylate buffer (pH 7.2) dehydrated, and embedded in SPUUR's resin. Ultrathin sections of fixed and embedded nodules were cut using an ultramicrotome (Leica) EMUC6, followed by a post-staining with uranyl acetate and lead citrate and collected on formvar-coated copper (Cu) slot grids. The photographs were taken using a transmission electron microscope 1010 (JEOL, Japan).

2.5 Nodulation under non flooded conditions

To compare the nodule formation process of *S. virgata* under flooded and non flooded conditions, seedlings of *S. virgata* were grown in test tubes and in Leonard jars The comparison of root morphology and nodulation traits between hydroponically and vermiculite grown *S. virgata* plants was done 7 days postinoculation and roots and roots hair were stained with methylene blue

(D'Haeze et al., 2000). After 30 days of inoculation, the nodules of plants under flooded and non flooded conditions were counted considering the distribution of the nodules along the roots or at the lateral root base.

3 Results

3.1 Colonization of *Sesbania virgata* roots

The typical morphology of inoculated *Sesbania virgata* plants after 1 month grown in test tubes and a closer view of its nodulated roots are show in Figure 1.

Fig. 1 *Sesbania virgata* plants grown in test tubes with Norris nutrient solution without mineral nitrogen 30 days after inoculation. **a** the entire plant; **b** root system showing the nodules distributed along the roots. Scale bar= 1cm.

In the root system of *S. virgata*, the nodules were first observed on the upper portion of the root system (main root/ lateral root) with 4-5 days after inoculation. Young nodules were most spherical and white and after 8-10 days of inoculation they became distinctly pink inside. After 30 days, we could still observe nodules with round shape resembling the determinate nodule type. Using GFP-labelled *A. doebereinerae* it was possible to monitor infections and early nodules development (Fig 2). Four days following inoculation, the base of lateral roots was seen to swell and the rhizobial colonization of the root surface was evident (Fig 2a). Six days after inoculation, nodulation sites were exclusively restricted to the base of the lateral roots, with the formation of a globular nodule primordia, confirming that the swellings corresponded to centers of meristematic activity (Fig 2b). Also, the fluorescent bacteria could be detected in the nodule primordia. At 8, 10 and 15 days after inoculation, the root nodule of *S. virgata* was completely occupied by the strain 5401 GFP-labeled (Fig. 2 c,d,e). Despite a detailed observation, we could detect neither root hair curling nor the development of infection threads at the apex of root hairs induced in the nodulation region. Also, for older inoculated plants (30 days postinoculation), nodulation sites was exclusively restricted to the base of lateral roots.

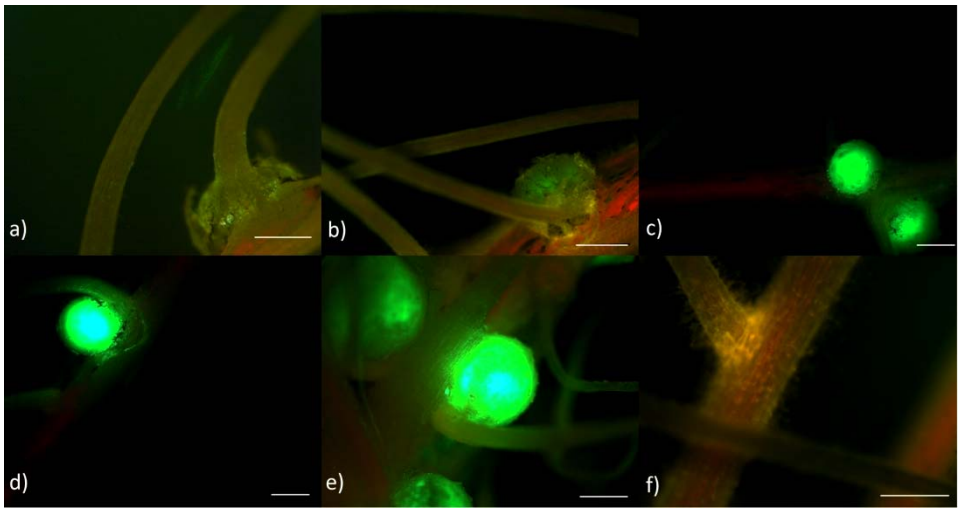

Fig. 2 *S. virgata* nodules infected with *Azorhizobium doebereinerae* strain 5401-GFP viewed under epifluorescent light. **a** 4 days; **b** 6 days; **c** 8 days; **d** 10 days; **e** 15 days after inoculation; **f** control without inoculation. Scale bar= 1mm.

3.2 Infection process and nodule initiation

Light microscopy of developing nodules confirms that the nodule meristem originates within the root cell layers before bacterial infection had progressed and consists of small cells with a dense cytoplasm (Fig. 3a). The mitotic activity of these cortical cells gave rise to nodule primordia that could be detected 4 days after inoculation. The bacteria invade the cortical cells and colonize the intercellular spaces (Fig 3b), forming infections pockets (Fig 3c). From those infection pockets, narrowed extensions protrude inwards, becoming smaller and thus forming intercellular infection threads which subsequently pass the cortical cells intracellularly (Fig 3d) guiding the bacteria towards the primordium cells. At 4 days after inoculation, the meristem appeared as a globular nodule primordia structure. Ultrastructurally, the infection pockets appeared to be filled

with proliferating rhizobia embedded in an electron dense matrix (Fig 6a) and the invasion by intra and intercellular infection treads were confirmed (Fig 6b).

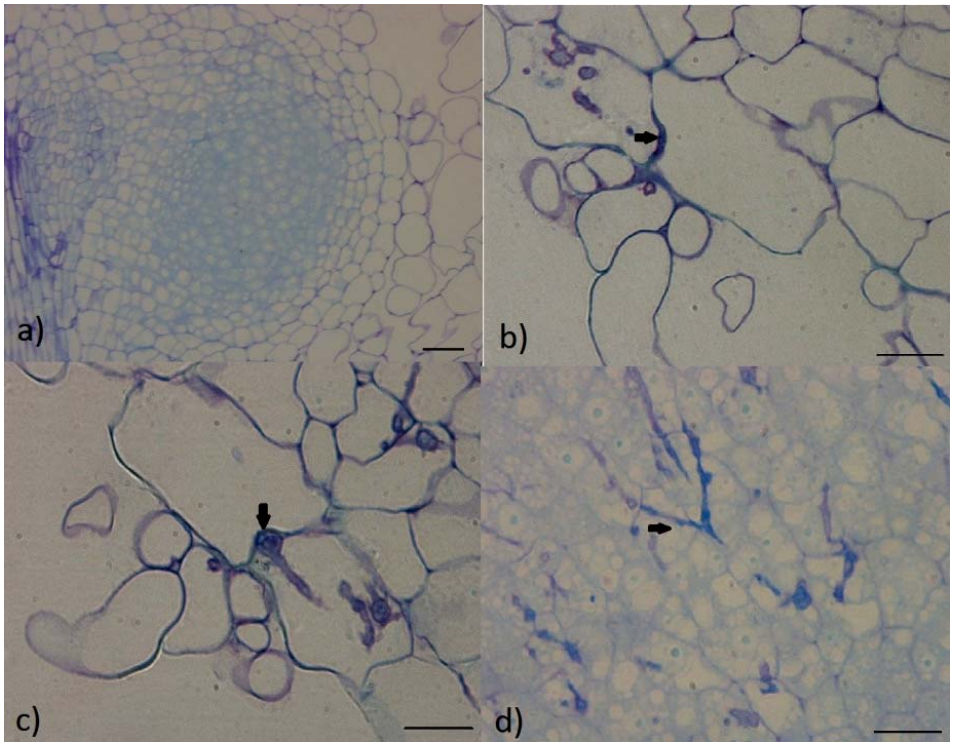

Fig. 3 Light microscopy analysis of *S. virgata* nodule development 4 days after inoculation. **a** meristematic tissue showing small cells with a dense cytoplasm; **b** a clear intercellular infection tread occurring between 2 cells (arrow); **c** infection pockets (arrow); **d** and intracellular infection threads (arrow). Scale bar = 100 µm.

At 6 days after inoculation, the meristem appeared as a condensed spherical structure (Fig 4a) that consists of dividing meristematic cells (Fig 4b) surrounding the infection centre (Fig 4c). The infection centre is defined as the cells which contain intracellular infection threads wherein the bacteria are guided towards the target cells for invasion (Fig 4d). In this way, a street of cortical cells containing infection treads is formed which guide the bacteria from

the infection pockets inside the nodule primordium. At this stage, also the nodule vascular elements appear (Fig 4a).

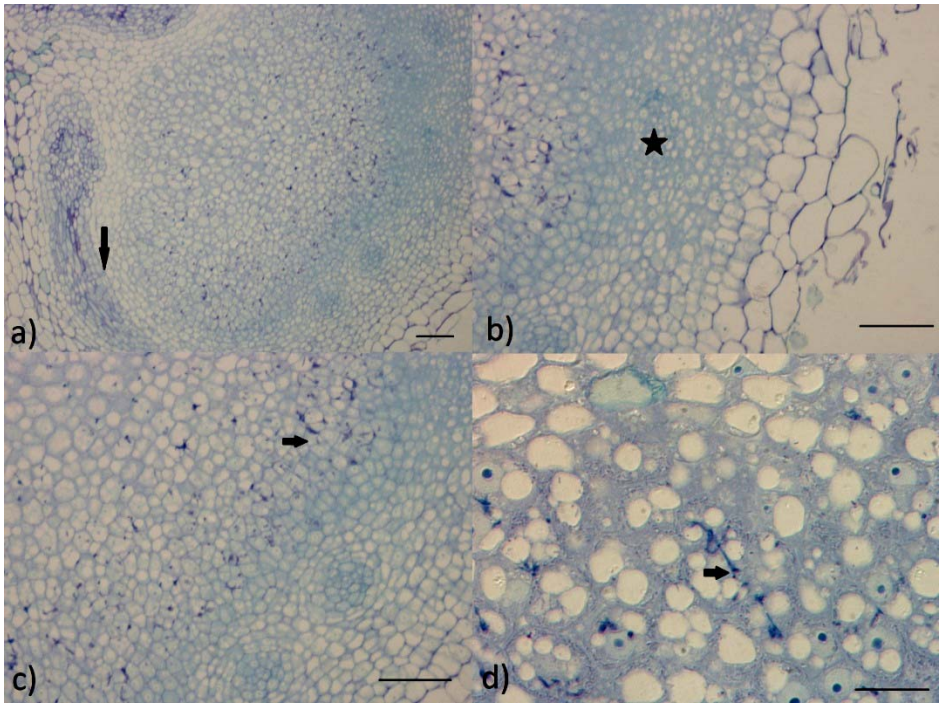

Fig. 4 Histology of *Sesbania virgata* 6 days old nodule **a** the structure of developing nodule showing the meristem (asterisk) and vascular elements (arrow); **b** aspect of the nodule meristem surrounding the infection zone (star); **c** and aspect of the infection zone showing the spread of infection treads; **d** magnification of panel c showing the central nodule zone where the bacteria start to invade the cells (arrow). Scale bar = 100 µm.

3.3 Nodule development

In the period of 4 days to 10 days after inoculation, the differentiation of the nodule occurred. In further stages of development (8 days after inoculation), bacterial invasion and differentiation progrees and the fixation zone is expanded.

In contrast to the young infected cells, the cytoplasms of mature infected cells are completely filled with bacteria (Fig 5a). Between the bacteria filled cells, some smaller uninfected cells are observed and they are highly vacuolated (Fig 5b). Nodules at 10 days after inoculation were already mature and presented a central fixation region and a reduced pheripheral zone (Fig 5c). The central tissue is surrounded by the nodule parenchyma that is surrounded by an outer cortical cell region which consists of slightly larger cells which are not so highly packed together (Fig 5d). At the border of the nodule parenchyma, the nodule vascular tissues are located.

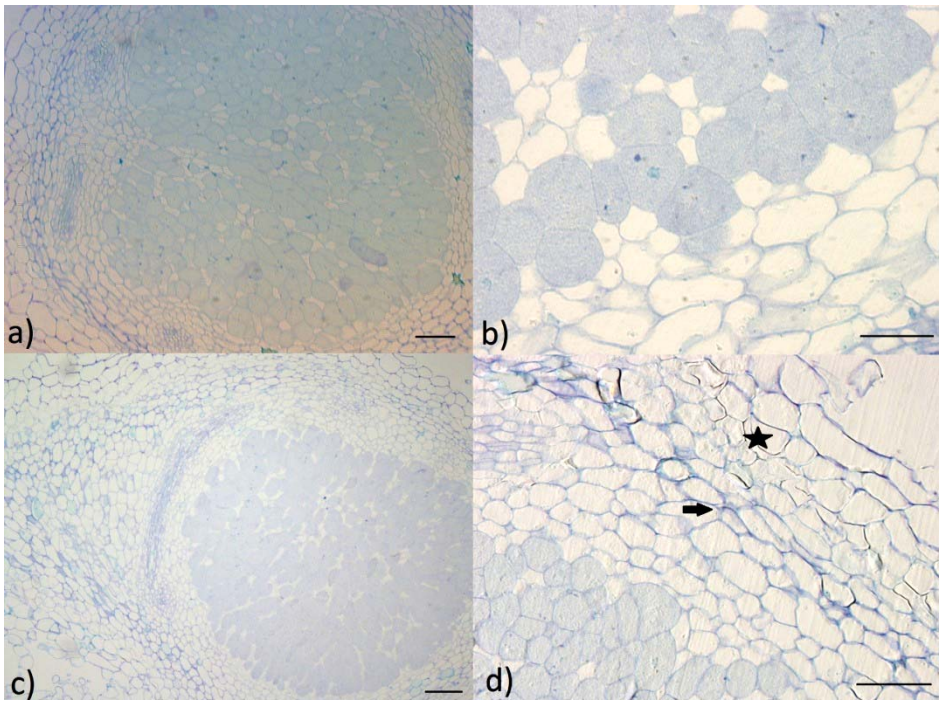

Fig. 5 *Sesbania virgata* nodules structure of 8 days (a-b) and 10 days (c-d) after inoculation. **a** clear central fixation tissue showing the cells completely filled with bacteria; **b** magnification of the fixation zone that comprise both invaded and noninvaded cells; **c** nodule aspect at 10 days after inoculation showing the central fixation zone with cells occupied by bacteria; **d** magnification showing the reduced peripheral zone with the parenchyma (arrow) and outer cortical layer (star). Scale bar = 100 µm.

Ultrastructurally the cytoplasm of the mature infected cells is completely filled with symbiosomes (Fig. 6b). Mature symbiosomes can enclose multiples bacteroids (Fig. 6c). The bacteroid appeared electron dense, and the peribacteroid membrane displayed an irregular shape.

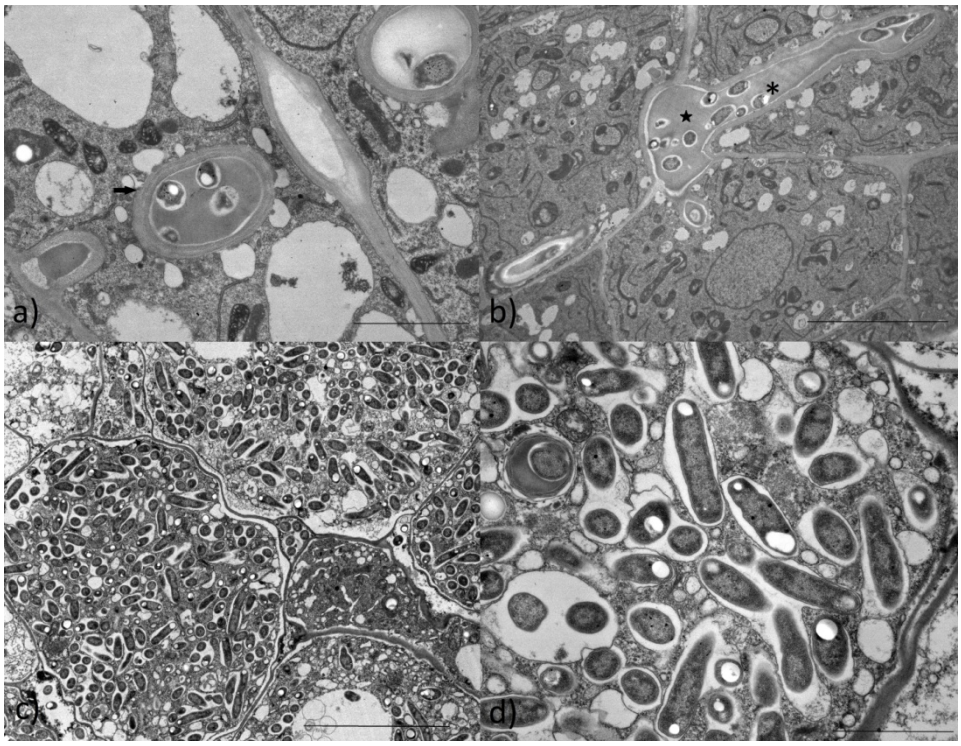

Fig. 6 Transmission electron microscopy of *Sesbania virgata* nodules 4 days (a, b) and 10 days (c,d) after inoculation. **a** Infection threads (arrow) penetrating into the plant cell cytoplasm embedded in a dense matrix; **b** infection thread penetrating inter (star) and intracellularly (asterisk); **c** mature infected cell completely filled with the symbiossomes **d** symbiossomes containing multiples bacteroids,. Scale bar: a= 2µm; b= 6µm; c=10µm; d= 2µm.

Histologically it was seen that *S.virgata* nodules at 6 days after inoculation presented an actively dividing nodule meristem, an intermediated zone enriched with infection treads and one central region where the bacteria start to invade the cells (Fig. 7a). This histological organization is similar to that of indeterminate nodules however at 10 days after inoculation the presence of a meristematic region and the gradient of cell differentiation is not clear anymore, with the nodules growing acquiring a round shape, which is a characteristic of

determinate nodules (Fig 7b). Mature nodules display the histological organization of determinate nodules with one clear central region and peripheral tissues (Fig 7c). A central gap surrounded by the fixation tissue was persistent in these nodules, reflecting the position of the original site of infection (Fig 7c).

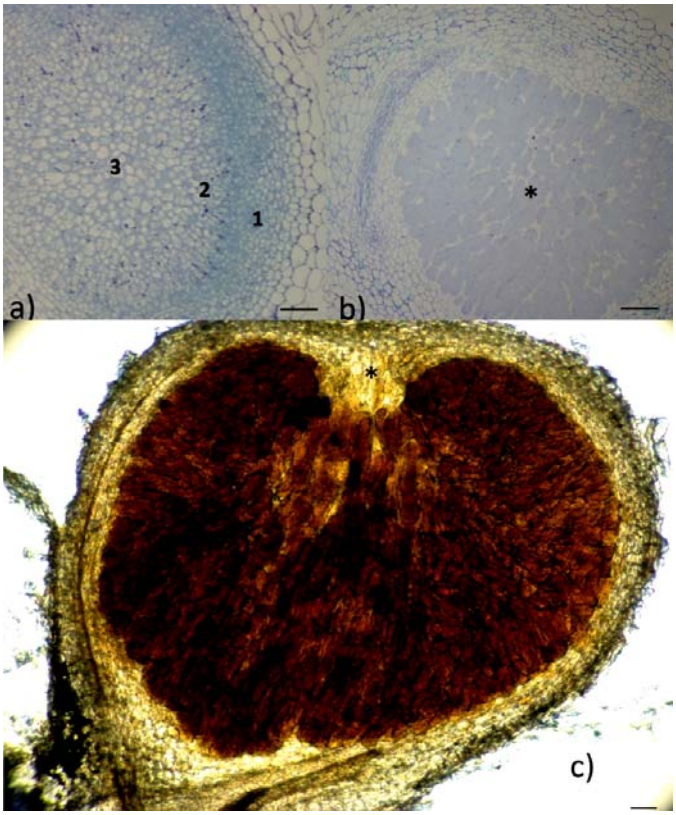

Fig. 7 a Light microscopy of Sesbania virgata nodule 6 days after inoculation, with the distinguish zones 1: meristematic zone; 2: intermediated zone enriched with infection threads; 3: central region where the bacteria starts to invade the cells; **b** nodule 10 days after inoculation with a clear fixation zone and peripheral tissues; **c** a tick longitudinal section of one nodule, 3 weeks after inoculation where the nitrogen fixing tissue (star) surrounds the original site of infection (asterisk). Scale bar = 100 µm.

3.4 Nodulation under flooded and non flooded conditions

On hydroponic roots of *S. virgata*, nodules arose at the bases of lateral roots, (Fig 8a) and the bacteria invaded the nodule primordia between cortical cells. However, when plants were grown on vermiculite in Leonard jars, nodules were distributed all over the vermiculite grown roots and not preferentially at the lateral root base (Fig 8b). Upon inoculation with *A. doebereinerae* 5401 (pBBR5-hem-gfp5-S65T) which can be visualized by fluorescence microscopy, curled root hair with infection treads could be detected (Fig 8e, f).

On hydroponic roots the bacteria entered the plant solely by invasion at lateral root base, but on vermiculite-grown roots, both types of invasion occurred with a clear preference for root hair invasion. The tips of hydroponic roots hardly had root hairs and were always naked (Fig 8c) and root hairs present only at the upper, oldest part of the root did not curl upon inoculation. On the contrary, when grown in vermiculite, roots were completely covered with root hairs, starting from the zone just above of the tip (Fig 8d) and inoculation resulted in deformation and curling of the root hairs.

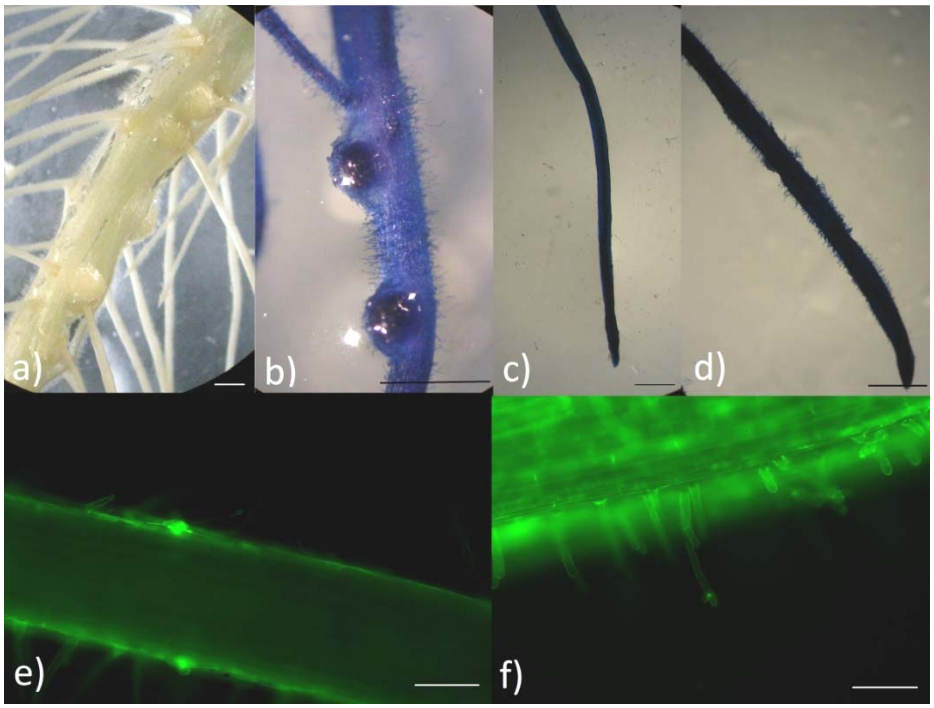

Fig. 8 a *Sesbania virgata* roots under flooded condition showing the distribution of the nodules at lateral root base; **b** nonflooded conditions where the nodules are formed all over the roots; **c and d** methylene blue stained root tip of hydroponically and vermiculite grown *S. virgata* roots, respectively; **e, f** curling and penetration of GFP labeled *A. doebereinerae* in roots growing in Leonard jars. Scale bar: (a-d) = 1cm; (e-f) = 100µm.

Other difference between the growth of *S. virgata* hydroponically or in Leonard jars is related to the nodule types formed in both conditions. When cultured in test tubes *S. virgata* developed nodules with round shape and as discussed before, when mature they reassemble of determinate type. However when cultivated in vermiculite, the plants developed round and also long shape nodules (Fig 9a), the latter being typical of indeterminate nodules. Microscopic analysis confirmed the presence of an apical meristem in these mature nodules (Fig 9b) that could not be detected for nodules developed in plants growth under

flooded conditions. In this way, under non flooded conditions, *S. virgata* can develop both determinate and indeterminate nodules and under flooded conditions only the determinate type was observed.

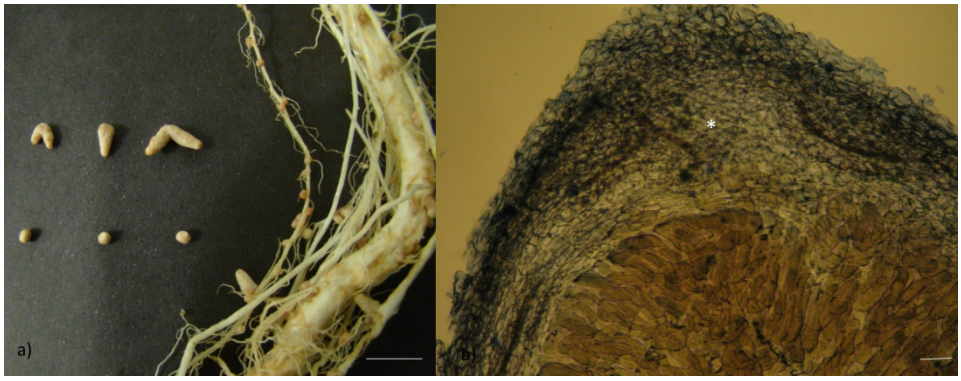

Fig. 9 a Determinate and indeterminate nodules formed in the same *Sesbania virgata* plants grown in Leonard jars; **b** section of a long shape nodule confirming the presence of an apical meristem (asterisk) typical of indeterminate nodules.Scale bar: a= 1cm, b= 100µm.

4 Discussion

This investigation has shown that *Sesbania virgata* has many of the morphological and ultrastructural features that are characteristic of the nodules in the other semiaquatic legume *Sesbania rostrata* (Ndoye et al., 1994). Like the *S. rostrata* mycrosymbiont *A. caulinodans*, the bacteria *A. doebereinerae* enter the plant via cracks at lateral or adventitious root base in a process that is also common to various tropical legumes as *Arachis* (Chandler, 1978), *Stylosanthes* (Chandler et al., 1982), *Aeschynomene* (Napoli et al., 1975) and *Neptnuia* (James et al., 1992). Similar to *S. rostrata*, the process of nodule ontogenesis includes an intercellular infection, followed by the formation of intercellular infection pockets and finally the development of infection treads which spread intra and

intercellularly into the root cortex. The intercellular invasion is a common characteristic not only for *S. rostrata* root and stem nodules (Duhoux, 1984; Ndoye et al., 1994) but also for *A. hypogaea* (Clandler, 1978) *Stylosanthes* spp. (Chandler et al., 1982), *A. americana* (Napoli et al., 1975) and *Neptunia plena* (James et al., 1992). However, the presence of intracellular infection treads described in this study has only been observed for the tropical semi aquatic legume *S. rostrata* (Duhoux, 1984; Ndoye et al., 1994) and also for the tropical aquatic legumes *Neptunia plena* (James et al., 1992) and *Neptunia natans* (Suba-Rao et al., 1995). For the species *A. hypogea* (Clandler, 1978) neither intercellular infections pockets nor infections treads have been described.

One interesting aspect is the delay for the formation of infection pockets in the nodule ontogenesis in *S. virgata* compared with *S. rostrata*. For *S. virgata*, the formation of the infection pockets is visible only 6 days after inoculation, while for *S. rostrata* its appear as early as 12 hours after inoculation (Ndoye at al., 1994). The nodule meristem formation is induced in *S. rostrata* simultaneously with infection in the middle and cortical cell layers during the first hours following inoculation (Ndoye et al., 1994) but in *S. virgata* the meristematic activity in the lateral roots base could be detected only 4 days after inoculation. This delay for the infection pockets formation and nodule development should be related to the seed nutrients accumulation that is higher in *S. virgata* once the seeds of this species are much bigger than the ones from *S. rostrata*. In this way, *S. rostrata* needs a fast nodule developing and further nitrogen fixation, to supply the plants nutritional nitrogen requirements.

Once more similarly to *S. rostrata*, the meristem appears first as a mass of cells but after some days this meristem suffers a uniform expansion that reassembles as an open basket shape structure, typical for *S. rostrata* nodulation, surrounding the infection site (Ndoye et al., 1994). The release of the bacteria in *S. virgata* root nodules probably occurs via a similar mechanism as described for

S. rostrata where the infection droplets, each carrying one or two bacteria are budded off in the plant cell cytoplasm from the wall-lacking tip of the infection treads. Next, symbiossomes are formed in which the bacteria differentiate into bacteroids and start to fix nitrogen. The symbiosomes of both species can enclose a large number of bacteroids, and this could result either from division of the bacteroids within the symbiosomes or from fusion of several symbiosomes (Ndoye et al., 1994).

In young *S. virgata* root nodules, the meristematic zone, infection region and infected zone could be observed at the same time and the appearance of these developmental zones is very similar to what is seen during the development of indeterminate nodules as pea and alfafa (Vasse et al., 1990). However after 8-10 days after inoculation a uniform enlargement of the nodules was observed, with the infected tissue surrounding the initial infection site what resemble the developmental pathway typically described for determinate nodules (Sprent 1989; Pawlowsky and Bisseling, 1996). Indeed, the pattern of differentiation of central tissue is very similar for both nodules type; however at the end, the nodules display a round shape and the overall histological organization of determinate nodules typical of *S. rostrata* (Ndoye et al., 1994).

During the symbiosis between the semi aquatic legume *S. virgata* and its microsymbiont *A. doebereinerae*, when cultivated under flooded conditions, the bacteria enter the plant via cracks at lateral or adventitious root bases, in a manner very similar to that of *S. rostrata* (Goormachtig et al., 2004). However, on well aerated roots growing in Leonard jars in vermiculite, nodules do not arise at the lateral root bases, but are distributed mostly over the root system. In contrast to hydroponically grown roots, which are bare, the well aerated roots are covered with root hairs. After inoculation with *A. doebereinerae* we could see that the root hairs were curled and contain infection threads guiding the bacteria. Sometimes the development of the nodule at the lateral root base could

be detected and as suggest by Goormachtig et al., (2004), the bacteria can also colonize cracks that appear once the nodule primordium expands from the cortex. Also, on hydroponically grown *Neptunia plena* roots, nodules arose at lateral root bases, and infection took place intercellurlarly as in *S. rostrata* (Goormachtig et al., 2004). On the other hand, in Leonard jars the nodules are distributed all over the lateral roots, and infection treads were detected with curled root hairs (Goormachtig et al., 2004).

The observation that *S. virgata* displays versatility in entry mode is the second observation in the literature that semiaquatic legumes can switch the mode of invasion according to the ambient growing conditions. Some other authors suggest that the intercellular invasion was recruited to allow infection where root hair invasion is inhibited, but these articles never focus on the infection process (Loureiro et al., 1995; James and Sprent, 1999).

The nodule type also differs according to the cultivation conditions. It was observed that mature nodules of *S. virgata* can be either determinate or indeterminate depending on conditions of root growth. Interestingly for *S .rostrata* there is a switch from determinate to indeterminate reflecting conditions of water logging versus aerated soil in nature (Fernandez-Lopez et al., 1998). The infection process and nodule formation in *S. virgata* is very similar to the process that occurs in *S. rostrata* (Ndoye et al., 1994; Fernandez-Lopez et al., 1998, Goormachtig et al., 2004). To adapt to situations of flooding or partial flooding these two species developed versatile mechanisms.

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ARTIGO 3 Mutants of *Azorhizobium doebereinerae* deficient in Exopolysaccharide Production form ineffective nodules on *Sesbania virgata*

Normas da Revista Soil Biology and Biochemistry (versão preliminar)

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Abstract

Azorhizobium doebereinerae 5401 is able to nodulate roots of the tropical legume *Sesbania virgata*. Two 5401 Tn5 insertion mutants, strains 5401-IV1A and 5401-XVI6A, had a rough colony morphology and are impaired in the exopolysaccharide production. We have examinated the nature of symbiotic deficiency of these mutants and our observations indicated that the inoculation of *Sesbania virgata* with these exopolysaccharide deficient mutant results in the formation of nodule-like structure and the reduction of nitrogen content and shoot dry weight in the plants inoculated with the mutants. Microscopic analysis of pseudonodules induced by these non-mucoid mutants revealed that the

nodules contain neither infection threads nor bacteroids. These data contribute to our understanding that exopolysaccharides are required for nodule development in *S. virgata*, probably acting at the early stages of nodule formation.

Keywords: Bacteria; EPS; Mutation; Legume; Nitrogen Fixation

1. Introduction

The biological nitrogen fixation process is mediated by some bacterial species found in soil that are able to penetrate the root tissues of leguminous plants, causing a hypertrophy called nodule. In the nodules, the infecting bacteria differentiate into N_2-fixing bacteroides that provide the plant with a source of nitrogen by reducing N_2 to NH_3.

One of the initial steps in this symbiotic interaction involves the recognition of specific plant-exuded flavonoids by a regulatory bacterial protein (the Nod D protein). This recognition leads to the production of a bacterial return signal, the Nod factors, that induces root hair curling and initiate nodule development (Lerouge et al., 1990; Schultze et al., 1991). However, the Nod factors are not the only bacterial signals needed for a successful symbiosis. Like in other interactions between bacteria and plants or animals, surface polysaccharides (SPS), especially exopolysaccharides (EPS) and lipopolysaccharides (LPS), act as important signaling molecules in the symbiotic process (Spaink, 2000; Fraysse et al., 2003; D'Haeze et al., 2004). EPS are extracellular molecules secreted into the surrounding media and can accumulate on the cell surface. They are heteropolysaccharides formed by a repeated unit made of hexose residues and with pyruvyl, acetyl, succinyl and hydroxybutanol substitutions (Lepek and D'Antuono, 2005). EPS seems to be involved in the invasion of plant tissue by the bacteria, the development of the nodule, the suppression of the

plant defense, the development of bacteroids and the expression of some genes by the host plant (Spaink et al., 2000; Fraysse et al, 2003).

Sesbania virgata is a tropical legume used for the regeneration of degraded soils and the reforestation of gallery forests. Previous studies involving different nitrogen-fixing bacteria and *S. virgata* have demonstrated that *S. virgata* only develops an efficient symbiosis when inoculated with strains of *Azorhizobium doebereinerae* (Gonçalves and Moreira, 2004; Moreira et al., 2006). The occurrence of this bacterium in soils of the Brazilian state of Minas Gerais is related to the presence of the host, *S. virgata* (Florentino and Moreira, 2009). The other species of the genus *Azorhizobium*, *A. caulinodans*, has cultural, phenotypic and genotypic similarities with *A. doebereinerae* and establishes an efficient symbiosis when inoculated in *S. rostrata*, which is native to Africa (Dreyfus et al., 1988). However, despite the similarity between *A. caulinodans* and *A. doebereinerae*, *A. caulinodans* form pseudonodules in *S. virgata* and *A. doebereinerae* form ineffective nodules in *S. rostrata* (Gonçalves and Moreira, 2004).

The role of EPS in the *Azorhizobium-Sesbania* interaction has been verified through studies of the mutant strains of *A. caulinodans*, ORS571-X15 and ORS571-oac2. The mutation in the ORS571-oac2 strain is located in the *oac2* gene, which encodes the dTDP-L-rhamnose synthase enzyme and the mutation in the strain ORS571-X15 is in the *oac3* gene, which encodes the dTDP-D-glucose synthase enzyme. These genes are part of a cluster composed of four genes (*oac0*, *oac1*, *oac2* and *oac3*) that are involved in the synthesis of hexoses (Goethals et al., 1994; Gao et al., 2001). While the ORS571-oac2 strain produced small amounts of EPS when compared to the wild type strain, the ORS571-X15 strain was completely deficient in the production of EPS (Goethals et al., 1994; D' Haeze et al., 1998; Gao et al., 2001). Additionally, both the ORS571-oac2 and ORS571-X15 strains were unable to complete the

infection process when inoculated in *S. rostrata* roots. This demonstrates the importance of EPS in this symbiotic process (D'Haeze et al., 2004).

Although the role of EPS in the process of nodulation in *S. rostrata* has been examinated, no study examining the involvement of EPS in the symbiosis between *A. doebereinerae* and *S. virgata* has been performed. The studies of the molecular aspects of EPS in the highly specific symbiosis between this bacterial species and its host plant as well as its relationship with *A. caulinodans* and *S. rostrata* will enable us to have a better understanding of the process of nodulation and biological nitrogen fixation.

Therefore, in order to investigate the role of EPS in the symbiotic relationship between *S. virgata* and *A. doebereinerae*, the effect of mutation in EPS production on nodule development and nitrogen fixation in this symbiosis were analyzed.

2. Materials and Methods

2.1. Bacterial Strains and construct used in this tudy

Azorhizobium doebereinerae wild type strain 5401 was grown at 28 ^{o}C in YEB (Geremia et al., 1994) or MMO (Goethals et al., 1989). Preformed transposase-transposon complexes (transposome) EZ-Tn5 <Kan-2>Tnp was obtained from Epicentre technologies (Madison, WI, U.S.A).

2.2. Preparation of electrocompetent *A. doebereinerae* cells

One loop of *A. doebereinerae* 5401 fresh culture from YEB agar plates was inoculated in 100 mL of liquid medium. These liquid cultures were allowed to grown to 2-3 days at 28 ^{o}C; then the cells was adjusted to optical density at 600 nm of 0.5-0.8 with fresh YEB medium. The cells were harvest by centrifugation (10000 rpm) at 4 ^{o}C for 15 minutes and washed twice in 50 mL of cold sterile

water, concentrated by centrifugation, and suspended in 2 mL of cold 10 % glycerol. The suspension was centrifuged at 6000 rpm for 15 minutes at 4 ^{o}C, resuspended in 200 µl of 10 % glycerol and held on ice until electroporated with the transposon constructions.

2.3. *A. doebereinerae* electroporation protocol

Fifty microliters of concentrated, electrocompetent cells were transferred to a cold, 1.5-mL microcentrifuge tube containing 20 ng of transposome DNA. The cell-DNA mixture was placed between the chilled electrodes of one electroporation chamber (BIO RAD) and subjected to a single, high voltage pulse. Pulses (2.5 KV, 200Ω, 25µF) were generated with Gene Pulse Electroporation System (BIO RAD). After the pulse delivery, the cells were immediately removed from the electroporation chamber and inoculated into 1 mL of YEB liquid medium. The cells were incubated for 4 hours at 28 ^{o}C with constant shaking (100 rpm). After incubation, the electroporated sample was plated entirely on 1 plate of YEB medium containing 50 µg/mL of Kanamycin. The control consisted of 50 µl of concentrated cells, which was electroporated with water instead of transposon DNA.

2.4. Isolation of Genomic DNA from *A. doebereinerae* Tn5 mutants

Eight Tn5 mutants were aleatory selected and grown in 10 mL of YEB liquid medium containing Kanamycin (50µg/mL) at 28 ^{o}C for 3 days. After the incubation period, the cultures were centrifugated (10 minutes, 14000 rpm) and the genomic DNA was extracted as described in the ZR Fungal/Bacterial DNA kit (Zymo Research Corp.).

2.5. Southern Blot analyses of Tn5 mutants

The ^{32}P-labeled Tn5 DNA was used as a probe in Southern Hybridization analysis of restriction digested DNA from Tn5 mutants. Genomic DNA from 8 randomly selected transposed *A. doebereinerae* colonies were digested overnight with *PvuI* restriction enzyme (cuts the transposon in 2 parts) according to the manufacture's condition. *PvuI* digested genomic DNAs were electrophoresed, alakali-denaturated and transferred to a nitrocellulose membrane (Sambrook et al., 1989). Prehybridization and hybridization were carried out in Dig-Easy Hybridization Buffer (Roche). Post-hybridization wash conditions were performed as 3xSSC, O.1 % SDS and 1xSSC, 0.1 % SDS at 65 ^{o}C. The same process was repeated for the EPS mutants detected, but in this case, the enzyme *EcoRI* that does not cut inside of the transposon was used.

2.6. Selection of mutants altered in EPS production

From the mutagenesis experiments, approximately 2500 Tn5 mutants of *A. doebereinerae* were randomly picked up and stored at -80 ^{o}C as glycerol stocks in 96 well plates. To visualize cell surface differences, the strains were simultaneously replica plate on MMO medium (determination of auxotrophic mutants), YEB and YEB agar containing 0.01 % of Congo Red (YEB+ CR), and incubated for 5 days at 28 ^{o}C. EPS mutant strains were indicated by colonies grown on YEB+ CR with a dry consistency and darkening at the center of the colonies, giving sometimes a bull eyes appearance (Freeman et al., 1989). Defects in EPS biosynthesis were confirmed following the Sudan Black B screening procedure (Liu et al, 1998) that is based on the differential dye uptake by exopolysaccharide producing and mutant strain. Each Tn5 generated mutant with altered mucoid phenotype was spread as single colony onto YEB+CR agar plates to confirm the EPS deficient production. EPS mutants detected, are

referred as 5401-I8B, 5401-IV1A, 5401-XI4C, 5401-XIII11B, 5401-XVI6A, and 5401-XIX4F in this manuscript.

2.7. Plant Nodulation Tests

Sesbania virgata seeds were submerged in sulphuric acid for 50 minutes and afterwards thoroughly washed in 1L of sterile water. Then, they are treated with concentrated NaOCl (13 %) for 2 minutes and once again rinsed in 1L of sterile water. The sterile, swollen seeds were germinated on sterile plates with 0,8 % agar, and placed in a 28 °C incubator in total darkness for 5 days. The 5 days old seedlings are transferred in a sterile environment to glass tubes, containing 70 ml of N-free Norris solution and sealed from the outside with aluminium-foil, which was disinfected with 70 % ethanol. The tubes were kept in a greenhouse with 28-32 °C, 16h light/8h dark and high humidity. After one week the plants were inoculated with 70 µl of 3 days culture of *A. doebereinerae* 5401, and the EPS mutants.

After 4 weeks of inoculation, the nodules were counted, and nitrogen accumulation of the plants was obtained. For the nitrogen accumulation, the plants were harvested and oven dried for 48 hours at 80^{o}C and weighted. The dry material was grounded and mixed, and the total nitrogen content was determined by a Kjeldahl digestion (Bremmer 1960). The weight of shoot dry matter was also determined to compare the fixation capacity between the wild type and the mutants. All the nitrogen fixation assays were performed in triplicate and uninoculated plants were used as control.

2.8. Microscopy Studies

Nodules harvested 4 weeks after inoculation were treated for light and electronic microscopy. Nodules were fixed by a mixture of 3% formaldehyde and 3-4 % glutaraldehyde in 0.1 M of sodium cacodylate buffer, dehydrated, and embedded

in SPUUR's resin. Sections of 2 µm were stained with 0.5 % toluidine blue and examined by light microscopy. Thin sections (60 nm) were stained with 2 % uranyl acetate for 12 minutes followed by 1 % lead citrate for 5 minutes and then examined by transmission electron microscopy 1010 (JEOL, Japan).

3. Results

3.1. Isolation of EPS defective mutants

Random Tn5 mutagenesis were carried out by electroporating EZ-Tn5 <Kan-2>Tnp in *A. doebereinerae* 5401 strain and Southern Blot analysis of 8 Kam^r colonies confirmed the transposon insertion. From these eight mutants; which the DNA was treated with the enzyme *PvuI* and that cuts the transposon in 2 parts; three mutants (5401-X10C, 5401-III8B, 5401-XX1A) presented a single insertion and four strains (5401-XII7B, 5401-III4B, 5401-XII3B, 5401-XI5B) presented 2 transposon insertions. For one sample (5401-IV2C) DNA was not well digested and the appearance of the insert was not clear (Fig 1).

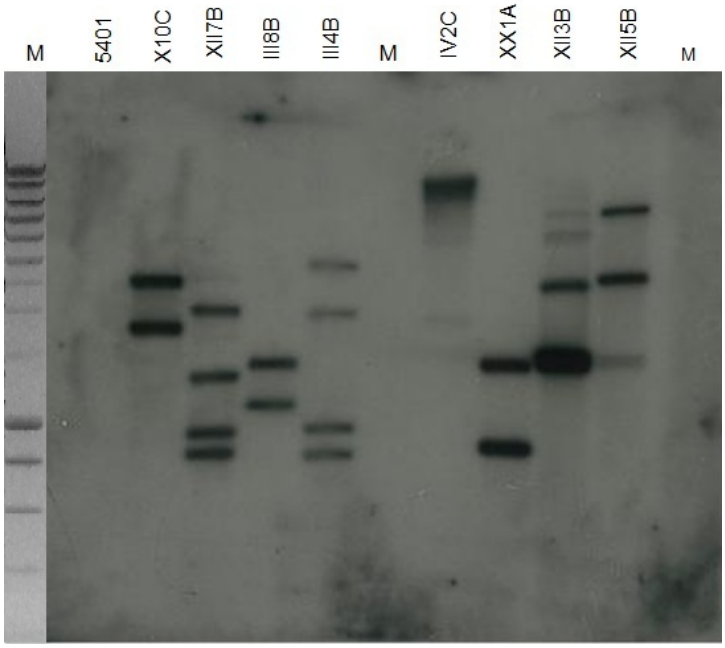

Fig. 1. Southern analysis of transposition clones of *Azorhizobium doebereinerae*. Approximately 500 ng of genomic DNA were digested by *PvuI* restriction enzyme that cleaves the transposon in the middle. The wild type strain 5401 was used as a control. M= DNA marker.

Amongst the Km^r transconjugants, we detected several non-mucoid, rough colonies that appeared on YEB and MMO. To confirm the non-mucoid phenotype, the strains were also growing in YEB+ Congo Red or grown on YEB and stained with Sudan black. The colonies with dry crystalline consistency on YEB+ CR and those that stained with Sudan Black were considered non slime producers. From the 2500 Tn5 mutagenized *A. doebereinerae* 5401 strains, which were screened for a loss in their ability to produce EPS, 92 colonies were yielded. Each of these colonies was purified by single colony isolation. Six of these mutants, 5401-I8B, 5401-IV1A, 5401-XI4C, 5401-XIII11B, 5401-XVI6A,

and 5401-XIX4F, presented colonies with pink dry appearance, and occasionally darkening at the centre of the colonies occurred (Fig 2).

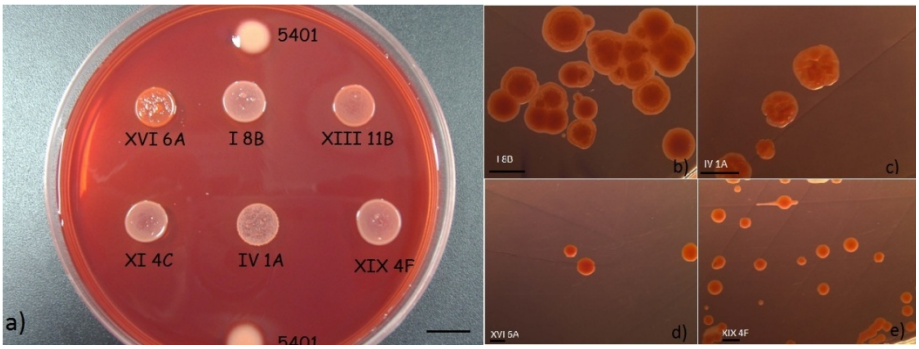

Fig. 2. (a) *Azorhizobium doebereinerae* wild type 5401 and the EPS mutant strains grown on YEB+ Congo Red, showing the dry appearance of the mutants; (b-d) Colonies aspects of four EPS mutants that presented pink dry colonies. Scale bar: a= 1cm; b-e= 1mm.

To confirm the presence of Tn5 sequence in each EPS mutant, a Southern Blot analysis of EcoRI-digested genomic DNA was carried. This enzyme does not cleave the transposon sequence, and for all mutants a single hybridizing band was observed (Fig 3).

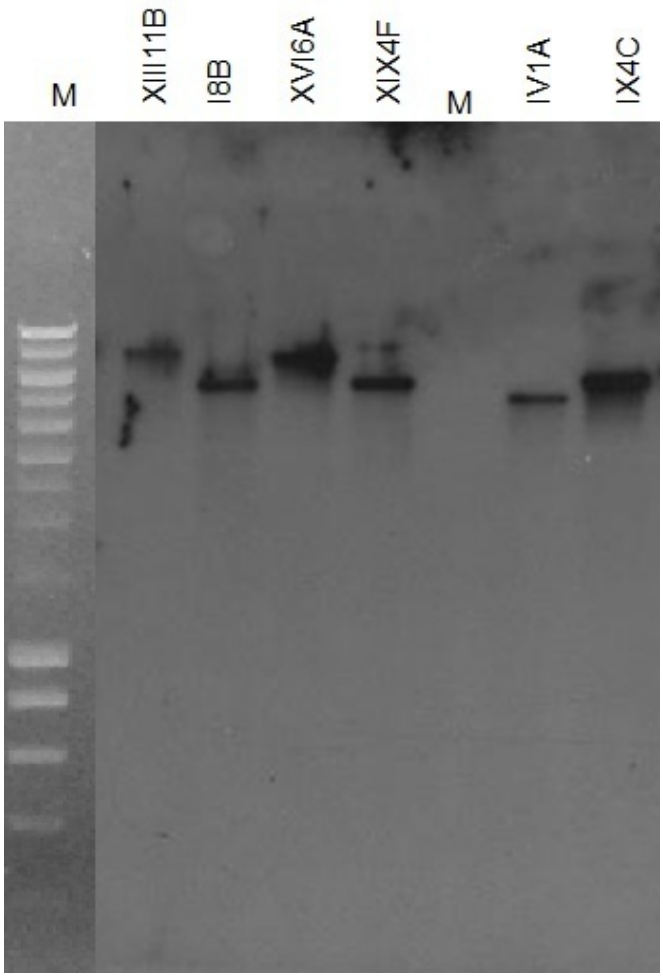

Fig. 3. Southern blot analysis of six *Azorhizobium doebereinere* EPS mutants. Approximately 500 ng of genomic DNA were digested by *EcoRI* restriction enzyme that does not cleave the transposon insertion. M= DNA ladder

3.2. Symbiotic phenotypes of the mutants

Nodulation assays in glass tubes containing nitrogen-free Norris solution showed that the mutants 5401-I8B, 5401-IX4C, 5401-I XII11B and 5401-XIX4F when inoculated in roots of *S. virgata* produced normal nitrogen fixing nodules

able to support growth of the inoculated plants in the same way as observed for the wild type. The nodules shape were round, the formation of nodules started at 4-5 days after inoculation and the plants presented similar aspects of growth and development when inoculated with the mutants or with the wild type 5401 (Fig 4).

However, plants inoculated with the mutants 5401-IV1A and 5401-XVI6A starts to develop nodules only after 21 days from inoculation. In three inoculation experiments done with these mutants strains, only 3 plants inoculated with the strain 5401-IV1A presented few pseudonodules smaller and paler than the wild type nodules. For the strain 5401-XVI6A after 3 weeks of inoculation the plants start to develop some nodules, but with 1 month postinoculation only 2 to 3 nodules per plant were present compared with 23±3 formed by the wild type. The nodules formed by this strain were also paler and smaller when compared with the ones formed by the wild type.

As the mutant strains 5401-IV1A and 5401-XVI6A presented interesting symbiotic phenotypes these two strains were further analysed. After 30 days, the host plants inoculated with the mutants were yellowish showing features of nitrogen starvation (Fig 4). The nitrogen starvation, were checked by the dry weight and nitrogen content in *S. virgata* shoot and these parameters differed markedly between plants inoculated with mutants and wild-type strains, confirming a disturbed nodulation process following the inoculation with these EPS mutants (Fig 5).

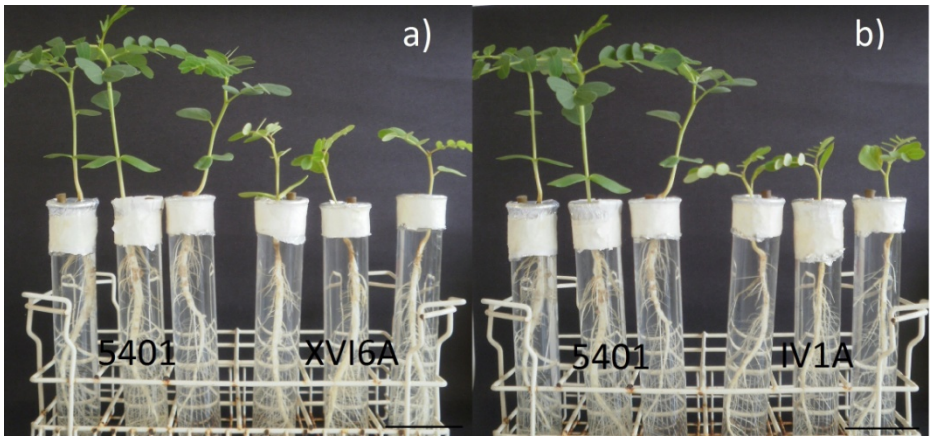

Fig. 4. *Sesbania virgata* plants inoculated with the wild type strain 5401 and the mutants (a) 5401-XVI6A and (b) 5401-IV1A, showing the features of nitrogen starvation, 30 days after inoculation. Scale bar = 4 cm

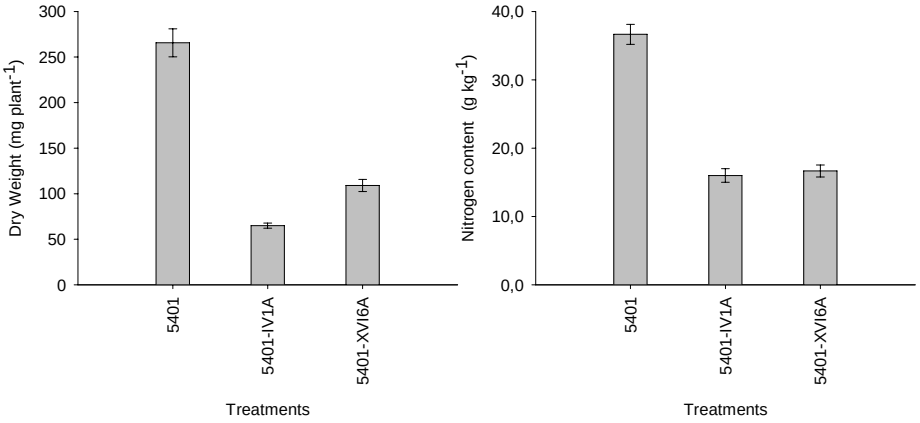

Fig. 5. (a) Dry weight and (b) nitrogen content of *S. virgata* plants inoculated with wild type 5401 and the mutant strains 5401-IV1A and 5401-XVI6A.

Microscopic studies on four-week-old-nodules elicited by the EPS mutants 5401-IV1A and 5401-XVI6A showed only non-infected or empty nodules. The central tissues of these nodules contain abundant empty cells with large vacuoles and no infection threads or infected cells were observed. In nodule cells infected

by the wild type strain, many bacteroids with electron-translucent deposit of poly-β-hydroxybutyrate could be seen (Fig 6).

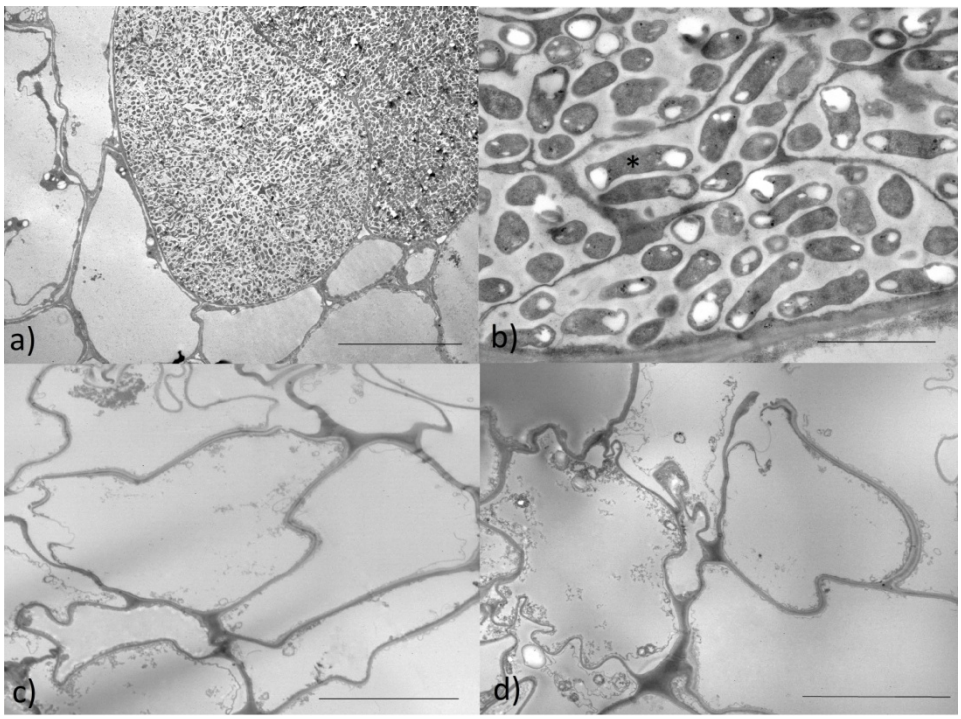

Fig. 6. Transmission electron micrographs of *Sesbania virgata* nodules induced by 5401 wt and EPS mutants (a) 5401 wt (b) a closer view of 5401 wt nodules showing the bacteroids (asterisk) (c) 5401-XVI6A (d) 5401-IV1A. Scale bars: a= 20µm, b= 2µm, c= 10µm, d= 10 µm.

4. Discussion

Rhizobial plant symbionts produce EPS that play an essential role in the interaction with the host. EPS appear to be essential for the early infection stages, as the invasion of plant tissue by the bacteria, formation of the infection threads and development of bacteroides (Fraysse et al., 2003). Another

symbiotic role proposed for rhizobial EPS is that these molecules could function non-specifically in avoiding a plant defense response (Shorupska et al., 2006).

Toward better understanding this process, six EPS–impaired mutants were obtained (5401-I8B, 5401-IV1A, 5401-XI4C, 5401-XIII11B, 5401-XVI6A, and 5401-XIX4F) from the wild type *S. virgata* symbiont strain. In YEB or MMO media, the mutants did not produce detectable levels of ethanol precipitated EPS and formed small rough colonies in both media. Although, all EPS mutants displayed a non-mucoid phenotype, only 2 strains (5401-IV1A and 5401-XVI6A), that qualitatively produced less EPS induced the formation of empty nodules defective in nitrogen fixation in *S. virgata* roots. A lower percentage of *S. virgata* plants were nodulated when inoculated with these mutants, and the few nodules that could be detected, starts to develop only 21 days after inoculation. Also, the plants inoculated with this two mutant strains presented shoot dry weight and nitrogen content diminished by around 50% when compared with the plants inoculated with the wild type strain.

These mutants are probably mutated in genes that control the early stages of *S. virgata* infection, and induced non-infected nodules devoided infection treads and bacteroids. The empty nodules induced by these mutants essentially resembled the nodules induced on alfafa, by non-infecting EPS mutants of *Rhizobium meliloti* (Leight et al., 1985; Finnan et al., 1986). The strains ORS571-oac2 and ORS571-X15 of *A. caulinodans* induced ineffective nodule-like structures on *S. rostrata* (Gao et al., 2001; D´Haeze et al., 2004). No formation of nodules was seen on peas infected with *R. leguminosarum* EPS mutant or when *Leucaena* was inoculated with *Rhizobium* sp. NGR234 EPS mutant (Chen et al., 1988; Diebold and Noel, 1989).

The severe disturbation of nodule development in *S. virgata* roots inoculated with the strains 5401-IV1A and 5401-XVI6A was also detected at the microscopic level. Electron microscopic observation of *S. virgata* nodules

infected with these cultures confirmed the non nodule occupancy. No infection treads or bacteroids were observed, confirming that these two selected Tn5 mutants impaired in the EPS production induces only empty non- nitrogen-fixing nodules on this legume. Inoculation with EPS mutants of *R. leguminosarum* RBL5523 in the roots of *V. sativa* subsp. *nigra* also blocked the formation of infection threads, which were aborted soon after the initiation of the infection process (Laus et al., 2004, 2005). A mutant of *S. meliloti* Rmm2011 unable to produce EPS induced the formation of pseudonodules that did not contain an infection thread or bacteroids in *M. sativa* (Niehaus et al., 1993).

The defect in nodule development reported for EPS mutants varies with the plant-rhizobia partners. In this way, our data provide evidences that EPS are involved in the interaction between *S. virgata* and *A. doebereinerae* in the early stages of the nodulation process. In this context and as infection treads were not formed when these mutants were inoculated, it is tempting to speculate if EPS are involved in the reduction of plant defense response. Further experiments with the 5401-IV1A and 5401-XVI6A mutants might contribute to answer many question that still remain about the role of EPS in the process of nitrogen fixation in legumes. Detailed genetical analysis of EPS mutants is also needed to explain the differences in the morphology and structure of *S. virgata* nodules.

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