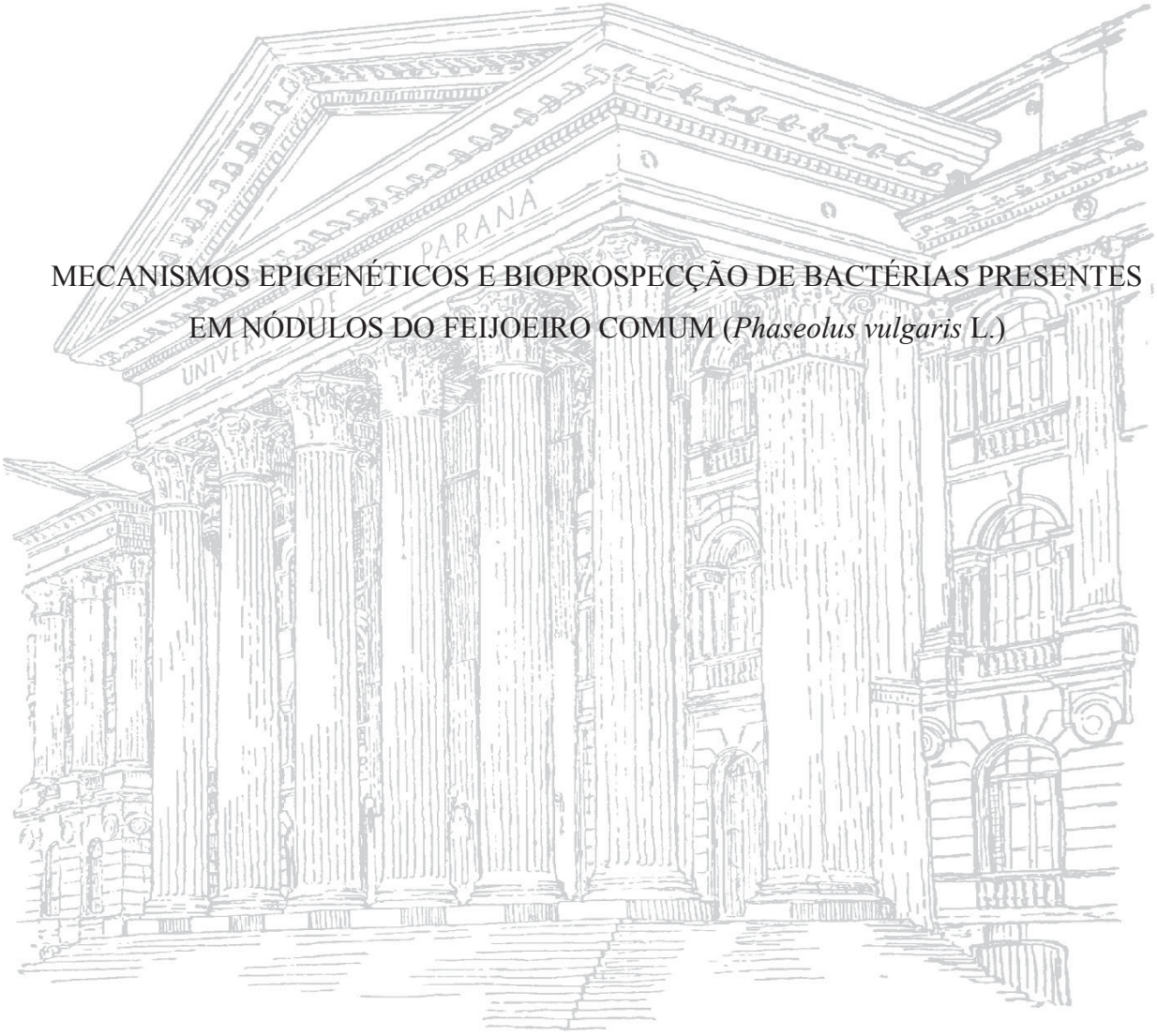
UNIVERSIDADE FEDERAL DO PARANÁ

FRANCIÉLE DE LIMA



MECANISMOS EPIGENÉTICOS E BIOPROSPECÇÃO DE BACTÉRIAS PRESENTES EM NÓDULOS DO FEIJOEIRO COMUM (*Phaseolus vulgaris* L.)

CURITIBA

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MECANISMOS EPIGENÉTICOS E BIOPROSPECÇÃO DE BACTÉRIAS PRESENTES EM NÓDULOS DO FEIJOEIRO COMUM (*Phaseolus vulgaris* L.)

Dissertação apresentada ao Programa de Pós-Graduação em Genética, Setor de Ciências Biológicas, Universidade Federal do Paraná como requisito à obtenção do título de Mestre em Genética.

Orientadora: Profª. Drª. Lygia Vitória Galli Terasawa

Co-orientadora: Profª. Drª. Daiani Cristina Savi

Co-orientador: Prof. Dr. Douglas Adamoski Meira

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“Without passion, there is no genius”

Theodor Mommsen

RESUMO

O nitrogênio gasoso (N_2) é a forma mais estável de nitrogênio no planeta Terra, no entanto, precisa ser convertido em formas químicas assimiláveis pelas plantas, através da Fixação Biológica do Nitrogênio (FBN). Após a fotossíntese, esse é o processo biológico mais importante para a manutenção dos ecossistemas naturais. Bactérias fixadoras de nitrogênio denominadas "rizóbios" possuem a capacidade de formar nódulos, estruturas específicas nas raízes das leguminosas para a FBN. A bioprospecção de bactérias eficientes para a realização da FBN leva em consideração duas linhas diferentes de pesquisa, uma que busca dentro de uma diversidade de bactérias e outra que explora algo mais especifico, como por exemplo um padrão de regulação da expressão gênica em uma bactéria alvo. Portanto, o objetivo geral deste trabalho foi contribuir para a compreensão sobre os mecanismos epigenéticos que regulam a nodulação no feijoeiro comum e na soja através de uma revisão de literatura, isolar, identificar e verificar o potencial para a fixação biológica do nitrogênio de bactérias presentes em nódulos de duas cultivares, BRS Esteio e IPR Sabiá, do feijoeiro comum (*Phaseolus vulgaris* L.). A revisão de literatura foi realizada entre os meses de agosto e dezembro de 2020, sendo os mecanismos epigenéticos encontrados a metilação do DNA, modificação de histonas e pequenos RNAs, ocorrendo concomitantemente na bactéria e na planta. Para a bioprospecção de novas linhagens, 176 bactérias foram isoladas a partir de nódulos de feijoeiro utilizados como iscas, as quais foram agrupadas pela metodologia BOX-PCR livre de extração e separadas em 33 grupos considerando dissimilaridade de 80%. Segundo o índice de diversidade de Shannon, a cv. BRS Esteio coletada em solo com pH 5.84 apresentou a maior diversidade genética para os isolados bacterianos Um representante de cada grupo foi escolhido aleatoriamente para a identificação por análise filogenética do gene 16S rDNA. 57% dos isolados pertencem ao gênero *Rhizobium*, os demais foram identificados como *Bacillus*, *Pseudomonas*, *Herbaspirillum* e *Enterobacter*. Os 33 isolados foram inoculados em sementes da cv. IPR Sábia em ensaio em casa de vegetação a fim de estimar seu potencial na FBN, um isolado do gênero *Rhizobium* e um isolado do gênero *Herbaspirillum* apresentaram resultados significativos com relação a massa seca da parte aérea e massa seca da raiz. Este trabalho visou contribuir para o conhecimento atual sobre regulação epigenética durante o processo de nodulação, assim como investigar *in situ* se bactérias indígenas fixadoras de nitrogênio isoladamente podem proporcionar benefícios à planta hospedeira. Esta pode ser uma perspectiva na obtenção de novos inoculantes, que são fundamentais para o estabelecimento de uma agricultura com mais qualidade, viabilidade e ganho econômico que beneficiem tanto o agricultor quanto o meio ambiente.

Palavras-chave: Fixação Biológica do Nitrogênio. Feijoeiro comum. *Rhizobium*. Simbiose.

ABSTRACT

Gaseous nitrogen (N_2) is the most stable form of nitrogen on Planet Earth, however it needs to be converted into chemical forms assimilable by plants, through Biological Nitrogen Fixation (BNF). After photosynthesis, this is the most important biological process for the maintenance of natural ecosystems. Nitrogen-fixing bacteria, called "rhizobia" has the ability to form nodules, specific structures in the legume roots, for BNF. The bioprospecting of efficient bacteria for the BNF process takes into account two different lines of research, one that search within a diversity of bacteria and another that explores something more specific, such as a pattern of gene expression regulation in a target bacterium. Therefore, the general objective of this work was to contribute to the understanding of the epigenetic mechanisms that regulate nodulation in common bean and soybean through a literature review, to isolate, to identify and to verify the potential for biological nitrogen fixation of bacteria present in nodules of two cultivars, BRS Esteio and IPR Sábia, of the common bean (*Phaseolus vulgaris* L.). The literature review was carried out between August and December 2020, with the epigenetic mechanisms found being DNA methylation, histone modification and small RNAs, occurring concomitantly in the bacteria and the plant. For the bioprospecting of new strains, 176 bacteria were isolated from common bean nodules used as baits, which were grouped by the extraction-free BOX-PCR methodology and separated into 33 groups considering 80% dissimilarity. According to Shannon's diversity index, cv. BRS Esteio collected in soil with pH 5.84 showed the greatest genetic diversity for bacterial isolates. One representative from each group was chosen at random for identification by phylogenetic analysis of the 16S rDNA gene. 57% of the isolates belong to the genus *Rhizobium*, the others were identified as *Bacillus*, *Pseudomonas*, *Herbaspirillum* and *Enterobacter*. The 33 isolates were inoculated in seeds of cv. IPR Sábia in a greenhouse test in order to estimate its potential in BNF, one isolate of genus *Rhizobium* and one isolate of the genus *Herbaspirillum* showed significant results in relation to the shoot dry mass and root dry mass. This work aimed to contribute to the current knowledge on epigenetic regulation during the nodulation process, as well as to investigate *in situ* whether indigenous nitrogen-fixing bacteria alone can provide benefits to the host plant. This can be a perspective in obtaining new inoculants, which are fundamental for the establishment of an agriculture with more quality, viability and economical gains that benefits both the farmer and the environment.

Keywords: Biological Nitrogen Fixation. Common beans. *Rhizobium*. Symbiosis.

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1 INTRODUÇÃO

A população mundial passou de 2.5 bilhões em 1950 a 7.5 bilhões em 2017 (UNITED NATIONS, 2019). Uma vez que o número de pessoas no mundo está aumentando cada dia mais a produção agrícola precisa se adaptar a esse novo cenário. Alguns estudos demostraram que a produção atual precisa ser duplicada até 2050 para que possa suprir as demandas da população (RAY *et al*., 2013; PASTOR *et al*., 2019). Sendo assim, a agricultura deve ser repensada para que seja não só mais produtiva, mas sim sustentável e que preze pelo uso consciente dos recursos naturais. Um dos pontos chave para essa forma de agricultura é a Fixação Biológica do Nitrogênio (FBN) (HUNGRIA; VARGAS, 2000). A FBN é realizada por microrganismos que são capazes de captar o nitrogênio atmosférico e converter em amônia (VIEIRA, 2017).

Produtos comerciais derivados da amônia obtida pela transformação química do gás natural (EMPRESA DE PESQUISA ENERGÉTICA, 2019) são aplicados nas lavouras com a finalidade de reverter a deficiência de nitrogênio. Durante a síntese química da amônia são geradas grandes quantidades de CO_2, que, por sua vez, é um gás de efeito estufa. Consequentemente, caracteriza uma forma de produção prejudicial ao meio ambiente (VIEIRA, 2017). Por outro lado, a FBN é um processo natural que, nas espécies da família Leguminosae como por exemplo o feijoeiro comum (*Phaseolus vulgaris* L.), ocorre em estruturas especificas presentes nas raízes dessas plantas. A interação simbiótica entre bactérias fixadoras de nitrogênio e o feijoeiro comum é de extrema importância, uma vez que, essa leguminosa é fonte de proteína em muitos países da América do Sul e Central, Ásia e África (ASSEFA *et al*., 2019; TORRES *et al*., 2020).

Os microrganismos responsáveis pela FBN habitam a rizosfera. Nessa região, ocorre a interação entre plantas e partículas do solo sendo este um sistema dinâmico que abriga um conjunto complexo de comunidades microbianas interagindo entre si. Por essa razão, ela é importante para a dinâmica dos ecossistemas pois é onde ocorre a ciclagem dos nutrientes através da decomposição da matéria orgânica e parte dos processos relacionados aos ciclos biogeoquímicos, como o do carbono, da água e do nitrogênio (AHKAMI *et al*., 2017). A FBN é um processo bioquímico realizado por bactérias e arqueas (MASSON-BOVIN; SACHS, 2018), que ocorre devido a ação de um complexo enzimático responsável pela redução do nitrogênio atmosférico (N_2) a amônia (NH_3) (VIEIRA, 2017). Após a fotossíntese, a FBN é o processo biológico mais importante do

planeta porque contribui com grande parte do nitrogênio introduzido nos ecossistemas naturais (JUNIOR; MENDES; HUNGRIA, 2018).

A compreensão dessa interação possibilita que novas tecnologias agrícolas sejam desenvolvidas. Uma dessas tecnologias é a aplicação de inoculantes comerciais, segundo o Decreto nº 4.954 de 14 de janeiro de 2004 (atualização: Decreto nº 8.384 de 29 de dezembro de 2014), são produtos que contém microrganismos vivos com atuação favorável ao crescimento de plantas. Bactérias fixadoras de nitrogênio pertencentes ao grupo dos rizóbios foram os primeiros microrganismos utilizados na formulação de inoculantes para a FBN (SANTOS; NOGUEIRA; HUNGRIA, 2019). No contexto atual, o uso de inoculantes microbianos não possui somente a função de aumentar a produtividade nas lavouras, mas também de iniciar uma "microrrevolução verde" no setor agropecuário (FUKAMI; CEREZINI; HUNGRIA, 2018).

Desse modo, este trabalho tem como objetivo contribuir com a área da fixação biológica de nitrogênio em três eixos: a) realizar uma revisão de literatura sobre os principais mecanismos epigenéticos, tanto na planta quanto na bactéria, que regulam a nodulação no feijoeiro comum e na soja; b) aprimorar uma metodologia para rápida discriminação de isolados bacterianos quanto a diversidade genética visando a triagem de bactérias com potencial para a composição de inoculantes e c) isolar, identificar a nível de gênero e verificar o potencial para a fixação biológica do nitrogênio de bactérias selvagens presentes em nódulos de duas cultivares do feijoeiro, sendo elas cv. BRS Esteio e cv. IPR Sábia cultivados em solos com distintas características químicas.

2 REFERENCIAL TEÓRICO

2.1 RHIZOBIA – BACTÉRIAS FIXADORAS DE NITROGÊNIO

"Rhizobia" é um termo usado para denominar bactérias, que pertencem ao gênero *Rhizobium* ou que são filogeneticamente relacionados a ele, capazes de nodular e fixar nitrogênio em associação com leguminosas (WILLEMS, 2006). Essa interação entre plantas da família Leguminosae e bactérias capazes de realizar a FBN contribui para estudos envolvendo simbiose (MASSON-BOIVIN *et al*., 2009; LINDSTRÖM; ASERSE; MOUSAVI, 2015) e objeto de muitos outros que buscam uma forma mais

sustentável de prover nitrogênio às plantas. Seu grande benefício é ser uma alternativa ao uso de fertilizantes químicos, especialmente nos solos tropicais, diminuído a emissão de gases de efeito estufa e a poluição das águas por nitrato (HUNGRIA; MENNA; DELAMUTA, 2015).

As leguminosas possuem a habilidade de aumentar a fertilidade dos solos, e esta habilidade está relacionada com o potencial de prover nitrogênio atmosférico fixado, via ácidos orgânicos, aminoácidos entre outros produtos do metabolismo dessas plantas, durante o seu crescimento e durante a decomposição das mesmas (SÁNCHEZ-NAVARRO *et al*., 2019; HASANUZZAMAN *et al*., 2019). Dois mil anos atrás, já era descrito na literatura Chinesa que leguminosas tinham efeitos benéficos na fertilidade dos solos, mas somente em 1675 os nódulos nas raízes dessas plantas foram descritos por Marcello Malpighi (WANG *et al*., 2019). No século XIX, Hellriegel e Wilfarth estabeleceram que os microrganismos presentes nos nódulos das raízes eram os responsáveis pela obtenção do nitrogênio atmosférico. Esses microrganismos, que inicialmente eram considerados pertencentes a uma única espécie, foram nomeados de *Bacillus radiciola* por Beijerinck em 1888. Um ano depois Frank sugeriu o termo *Rhizobium* para nomear as bactérias que induziam a formação de nódulos, com a espécie original *R. leguminosarum* (WILLEMS, 2006; VIEIRA, 2017; WANG *et al*., 2019).

Em 1921 L*ö*hnis e Hansen propuseram uma divisão das bactérias conhecidas como rizóbios em dois grandes grupos: um de crescimento rápido que estabelece relações simbióticas com espécies de trevo (*Trifolium sp*.), alfafa (*Medicago sp.*), ervilha (*Pisum sp.*) e feijão (*Phaseolus sp*.), e um de crescimento lento que se associa à soja (*Glycine sp*.) e ao caupi (*Vigna unguiculata***)**. Em 1932 seis espécies do gênero *Rhizobium* já haviam sido reconhecidas devido as características morfofisiológicas dessas bactéria, sendo elas *R. leguminosarum*, *R. trifolli*, *R. phaseoli*, *R. meliloti*, *R. japonicum* e *R. lupini* (VIEIRA, 2017).

Atualmente, os rizóbios compreendem um grupo parafilético com mais de 200 espécies, as quais estão distribuídas em 18 gêneros de Alphaproteobacteria e Betaproteobacteria (DE LAJUDIE *et al*., 2019; TAYLOR; SIMMS; KOMATSU, 2020). Os gêneros mais conhecidos são de alphaproteobacteria, sendo eles: *Rhizobium*, *Ensifer* (anteriormente *Sinorhizobium*), *Mesorhizobium*, *Azorhizobium* e *Bradyrhizobium* (VIEIRA, 2017). *Bradyrhizobium* é amplamente distribuído, predominante nos solos

tropicais e é provavelmente o gênero ancestral de todos os rizóbios descritos até hoje (HUNGRIA; MENNA; DELAMUTA, 2015; HELENE *et al*., 2020). Por outro lado, *Rhizobium* é um gênero de bactérias pertencentes a classe α-Proteobacteria, ordem Rhizobiales e família Rhizobiaceae. Atualmente, o gênero já possui mais de 90 espécies descritas, sendo todas Gram-negativas, não-esporulantes e aeróbicas (TONG *et al*., 2018). O ciclo de vida dessas espécies inclui uma fase saprófita quando a bactéria habita o solo, uma fase simbionte quando a bactéria habita o interior do nódulo e uma fase endofítica quando se associa a plantas não nodulantes (WANG *et al*., 2019).

2.2 FIXAÇÃO BIOLÓGICA DO NITROGÊNIO E A RELAÇÃO SIMBIÓTICA ENTRE LEGUMINOSAS E RHIZOBIA

A Fixação Biológica do Nitrogênio é catalisada por um complexo enzimático altamente conservado ao longo da evolução - denominado Nitrogenase - sendo que este é o único sistema biológico capaz de fixar nitrogênio atmosférico (MASSON-BOVIN; SACHS, 2018). A nitrogenase é formada por duas proteínas denominadas dinitrogenase e dinitrogenase redutase. A dinitrogenase redutase é um dímero constituído por duas subunidades idênticas e, já a dinitrogenase é um tetrâmero $\alpha_2\beta_2$. As duas proteínas contêm ferro (Fe) e a dinitrogenase contém também molibdênio (Mo) (RUBIO; LUDDEN, 2005 e 2008).

Os genes envolvidos na síntese da nitrogenase (genes *nif*) foram inicialmente estudados em *Klebsiella oxytoca* estirpe M5a1 (antigamente identificada como *K. pneumoniae*), nessa estirpe os 20 genes *nif* estão organizados em várias unidades transcricionais clusterizadas em uma região de 23 kb (RUBIO; LUDDEN, 2008). De acordo com Santos e colaboradores (2012) aproximadamente todas as bactérias diazotróficas contém um conjunto mínimo de 6 genes que codificam os componentes estruturais (genes *nifHDK*) e biossintéticos (genes *nifENB*) da nitrogenase e este conjunto deve ser utilizado para a predição computacional de potenciais fixadores de nitrogênio.

Bactérias anaeróbicas não apresentam dificuldades com relação a atividade da nitrogenase, entretanto, aeróbios obrigatórios apresentam certa dificuldade, uma vez que a dinitrogenase redutase é irreversivelmente inativada pelo oxigênio (O_2), portanto a FBN pode ocorrer somente sob microaerobiose. Este é um paradoxo para rizóbios com aerobiose restrita que pode ser resolvido quando em simbiose com leguminosas já que

dentro do nódulo existe um microambiente propício para a realização da FBN (MASSON-BOVIN; SACHS, 2018). O segundo problema envolvendo a nitrogenase está relacionado com o fato de a fixação biológica do nitrogênio ser um processo com alto gasto de energia na forma de ATP (adenosina trifostato). Devido a tripla ligação entre os átomos de nitrogênio, 16 ATPs são necessários para a redução de N_2 a 2 NH_3, este problema também pode ser resolvido quando em associação simbiótica com leguminosas (VIEIRA, 2017).

Para estabelecer essa relação simbiótica leguminosas precisam reconhecer os rizóbios da comunidade microbiana presente na rizosfera e regular os investimentos energéticos depositados na biogênese do nódulo. Com isso, as plantas mantêm o custo-benefício com relação à FBN ao seu favor (SACHS; QUIDES; WENDLANDT, 2018). Esse reconhecimento é denominado de especificidade simbiótica e ocorre por meio da troca de sinais químicos entre o hospedeiro e os microrganismos durante os estágios iniciais da interação (WANG; LIU; ZHU, 2018).

Quando os rizóbios invadem os tecidos das leguminosas, como *Phaseolus vulgaris* L., estas bactérias mudam a sua forma celular e não se dividem mais, sendo então chamadas de bacteroides. Esses são envoltos por uma membrana derivada da planta hospedeira denominada de membrana peribacterioide ou membrana simbionte (SM). O bacteroide envolto pela SM é denominado de simbiossoma, que pode então atuar como uma organela temporária para a fixação do nitrogênio (LA PENA *et al*., 2018).

2.2.1 O PROCESSO DE NODULAÇÃO EM LEGUMINOSAS

A nodulação é uma interação complexa que se inicia com a exsudação de compostos fenólicos, principalmente flavonoides e isoflavonoides pelas raízes da planta hospedeira. As bactérias presentes na rizosfera respondem a esses flavonoides ativando as proteínas de nodulação (*nodD*), essas proteínas atuam como reguladores transcricionais. nodD é responsável por iniciar a expressão coordenada dos genes de nodulação (*nod*, *nol* e *noe*) através da ligação em uma região conservada upstream do operon *nod*. Os genes *nod* são responsáveis pela síntese e secreção de lipoquitooligossacarídeos, denominados de fatores *Nod*. Os fatores *Nod* se ligam a receptores presentes na membrana plasmática das células na raiz da planta induzindo uma variedade de respostas moleculares, fisiológicas e celulares a fim de dar continuidade ao processo de infecção e nodulação. As bactérias

entram nas células da raiz e se deslocam, através do cordão de infecção, até o meristema do nódulo onde se diferenciam em bacteroides e fixam nitrogênio atmosférico (VIEIRA, 2017; SANCHS; QUIDES; WENDLANDT, 2018; CLÚA *et al*., 2018; WANG; LIU; ZHU, 2018).

Diferentes flavonoides secretados por diferentes espécies de leguminosas são reconhecidos por diferentes reguladores transcricionais *nodD*, este é o primeiro passo da interação onde deve haver compatibilidade (LIU; MURRAY, 2016). Espécies de rizóbios tem de um a cinco genes regulatórios *nodD*. *Rhizobium tropici* CIAT 899 possui a capacidade de nodular uma variedade de legumes, e é o simbionte de maior sucesso na nodulação de *Phaseolus vulgaris* L em solos ácidos. O sequenciamento do genoma dessa estirpe permitiu a identificação de cinco genes *nodD* diferentes, sendo que *nodD1* é essencial para a nodulação em *Leucaena leucocephala*, *Lotus japonicus* e *Macroptiluim atropurpureum*, enquanto que *nodD2* é essencial para a simbiose com *P. vulgaris* L. Essa variação em qual *nodD* é importante durante a nodulação, também pode ser caracterizada como uma estratégia de nodular sob stress abiótico, por exemplo em altas concentrações salinas e altas concentrações de manitol, *nodD2* é o principal ativador transcricional ao invés de *nodD1* (DEL CERRO *et al*., 2015, 2015, 2017 e 2019).

Johnston e colaboradores (1978) demostraram que a habilidade de nodular plantas de ervilha pode ser transferida de uma estirpe de *R. leguminosarum* noduladora para um estirpe da mesma espécie mas não noduladora, concluindo assim que os genes necessários para o processo de nodulação estariam localizados em plasmídeos. Hoje em dia já é reconhecido que os clusters de genes necessários para a nodulação e fixação do nitrogênio estão localizados em um plasmídeo simbiótico e que além desse, outros plasmídeos podem ser encontrados quando o genoma desses microssimbiontes é caracterizado. *Rhizobium tropici* CIAT 899 possui três plasmídeos, um megaplasmídeo (pRtrCIAT899c), um plasmídeo simbiótico (*pSym*) e um plasmídeo menor (pRtrCIAT899a). Enquanto que, *Rhizobium* estirpe PRF 81 possui 4 plasmídeos, um megaplasmídeo (pPRF81d), um plasmídeo simbiótico *pSym* (pPRF81C) e dois plasmídeos pequenos pPRF81a e pRF81b. Além desses, cada uma das espécies possui um cromossomo circular onde se encontram genes envolvidos com o metabolismo celular (ORMEÑO-ORRILLO *et al*., 2012).

O segundo passo na interação, onde deve haver compatibilidade, é o reconhecimento dos fatores de nodulação (fatores *nod*) pela planta. Após a interação dos reguladores transcricionais *nodD* com a região promotora (*nod box*) do operon *nodABC*, os genes de nodulação são transcritos e traduzidos em produtos proteicos necessários para a biosíntese dos fatores de nodulação. Fatores de nodulação são lipoquitooligosacarídeos estirpe-específicos (WANG *et al*., 2019) que podem ser inativados por enzimas (hidrolases) produzidas pelas plantas, entretanto quando há compatibilidade os fatores de nodulação são reconhecidos por receptores de alta afinidade presentes na membrana plasmática das células da epiderme da raiz. A ligação dos fatores de nodulação aos receptores gera um padrão de sinalização intracelular que se inicia com a percepção dos sinais, a transdução desses sinais e a transcrição de genes importantes para a formação do cordão de infecção e organogênese do nódulo (MBENGUE; HERVÉ; DEBELLÉ, 2020).

Quando o nódulo é estabelecido, planta e bactérias se envolvem em uma relação simbiótica. A planta se beneficia dessa relação pelo fato de ter uma fonte de nitrogênio disponível. Já as bactérias têm acesso a carboidratos e a intermediários de Krebs (principalmente malato e succinato) disponibilizados pelas plantas que podem ser usados como fonte de energia, permitindo à bactéria fixar nitrogênio em grandes quantidades e, resolvendo assim o problema com relação ao alto gasto energético necessário para o funcionamento da nitrogenase. O segundo problema, com relação a toxicidade do O_2, é resolvido justamente pela relação com a planta. As plantas produzem uma hemeproteína ligadora de oxigênio, a leg-hemoglobina, sua função é se ligar a grande parte do oxigênio presente no nódulo, fazendo assim com que ele não interfira no processo de fixação do nitrogênio (MASSON-BOVIN; SACHS, 2018). Esta hemeproteína confere uma coloração rosada aos nódulos que estão ativos, diferenciando-os assim de nódulos ineficientes na fixação que apresentam coloração branca ou esverdeada (VIEIRA, 2017).

2.2.1.1 EPIGENÉTICA NA FIXAÇÃO BIOLÓGICA DO NITROGÊNIO: UMA INTRODUÇÃO

A compreensão dos diferentes níveis de complexidade da interação planta-microrganismo é um instrumento para o desenvolvimento de novas tecnologias aplicadas ao aumento da produtividade aliada a sustentabilidade ambiental (FIORILLI et al., 2020). Um desses níveis é a regulação da expressão gênica através de mecanismos epigenéticos

que podem ser descritos em 3 pontos distintos: (1) na planta que participa da relação simbiótica, (2) no microrganismos que estabelece a relação simbiótica com a planta e (3) no momento da interação planta-microganismo.

Nas plantas (1), o silenciamento gênico via pequenos RNAs, modificação de histonas e metilação do DNA, são mecanismos essenciais durante a interação com microrganismos patogênicos ou simbiontes (ROBERTS E SÁNCHEZ, 2019). Os pequenos RNAs, principalmente microRNAs, regulam a expressão de genes em um nível pós-transcricional e são amplamente estudados em plantas. Alguns miRNAs já foram descritos como potenciais reguladores do desenvolvimento dos nódulos, homeostase hormonal e autoregulação da nodulação (HOANG, TÓTH e STACEY, 2020). Em *Medicago truncatula*, miR169 e o miR166 regulam a nodulação (NAIR E MANICKAVELU, 2020).

Em bactérias (2) a metilação do DNA está envolvida com a regulação da replicação do cromossomo, reparo do DNA, controle da atividade de transposons, regulação do ciclo celular e transcrição. Desoxiadenosina metiltransferase (Dam) e Metiltransferase do ciclo celular (CcrM) são duas metiltransferases codificadas pelo genoma procarioto (ADHIKARI e CURTIS, 2016; SÁNCHEZ-ROMERO e CASADEÚS, 2020). O status de metilação de adeninas muda durante a interação planta-microrganismo, por exemplo em *Mesorhizobium loti* MAFF303099 regiões não metiladas no genoma (SUMs) se tornam metiladas durante a simbiose com leguminosas. SUMs estão presentes no genoma de uma grande variedade de bactérias e estas regiões podem ter um papel significativo como um mecanismo regulatório. Estirpes de *M. loti* superexpressando CcrM apresentam hipermetilação do genoma, e quando em simbiose com *Lotus japonicus* é observado um atraso no desenvolvimento dos nódulos, indicando assim um papel dessa metiltransferase nas fases iniciais da organogênese dos nódulos (ICHIDA et al., 2007; 2009).

Pequenos fragmentos de RNA podem atuar como um mecanismo de "comunicação entre reinos" durante a interação entre leguminosas e rhizobia (3), estes fragmentos são derivados de RNAs transportadores codificados pelo genoma da bactéria e são denominados de tRNA de Rhizobia-derivado de pequenos fragmentos de RNA (tRFs) (REN *et al*., 2019; SU *et al*., 2020).

A metilação do DNA é o mecanismo epigenético mais bem estudado em bactérias, mas muitas questões sobre a metilação no genoma desses microrganismos permanecem sem respostas, tais como: “As regiões não metiladas apresentam uma função específica? ” “Quais outros processos biológicos são dependentes da regulação por metilação? ” (ADHIKARI e CURTIS, 2016).

Devido à falta de informações especificas e organização dos dados referentes as plantas de interesse, foram formuladas algumas questões para serem respondidas em uma revisão de literatura: além da metilação do DNA, em procariotos, existem outros mecanismos epigenéticos já descritos na literatura que regulam o processo de nodulação, no feijoeiro comum e na soja? E com relação às plantas, quais miRNAs regulam esse processo? Além de pequenos RNAs existem outros mecanismos?

2.2.2 DENTRO DO NÓDULO: UM GÊNERO ESPECÍFICO OU UMA COMUNIDADE MICROBIANA COMPLEXA?

Nos nódulos formados quando em associação com leguminosas os gêneros de rizóbios são em sua maioria os microssimbiontes dominantes, mas outros gêneros podem ser isolados, entretanto ainda não está claro como a competitividade entre estes microssimbiontes ocorre em diferentes condições ambientais e nem qual é a ordem de nodulação competitiva entre as espécies. (JI *et al*., 2017). Bactérias endofíticas isoladas de nódulos podem ser consideradas uma nova fonte biotecnológica, quando isoladas dos mesmos, exploradas quanto aos meios pelos quais promovem o crescimento vegetal, e avaliadas quando inoculadas nas sementes das plantas hospedeiras (ZHAO; XU; LAI, 2018).

Chinnaswamy e colaboradores (2018) isolaram de nódulo de raízes de *Medicago polymorpha* a espécie *Bacillus megaterium*. Essa estirpe endofítica apresentou alta tolerância a salinidade e metais pesados, e atividade promotora de crescimento em plantas *in vivo*. Genes de fixação e nodulação foram identificados no genoma de *B. megaterium* NMp082, e na ausência de rizóbios, foi verificado que essa estirpe é capaz de induzir o desenvolvimento de estruturas que lembram nódulos nas raízes da planta hospedeira. Segundo os autores é provável que uma transferência lateral dos genes *nifH* e *nodD* tenha ocorrido entre bactérias simbióticas e endofíticas. Gêneros endofíticos como *Enterobacter*, *Acinetobacter*, *Pseudomonas* e *Bacillus* já foram isolados de nódulos da

soja (*Glycine max* L.), e estes apresentam a capacidade de produção de sideróforos, ácido indol acético (AIA) e fixação do nitrogênio (ZHAO; XU; LAI, 2018).

P. vulgaris L. é uma espécie de leguminosa capaz de estabelecer uma relação simbiótica e ser nodulada por vários simbiovars, essa característica é denominada de promiscuidade do hospedeiro (SHAMSELDIN; VELÁZQUEZ, 2020). O termo simbiovar é usado para agrupar estirpes dentro de uma espécie que são capazes de estabelecer simbiose com uma leguminosa específica (PEIX et al., 2015). Muitos trabalhos já confirmaram a promiscuidade de *Phaseolus vulgaris* L., como por exemplo, Grange e Hungria (2004) ao isolarem pelos menos três gêneros diferentes dos nódulos dessa leguminosa, sendo eles *Rhizobium*, *Sinorhizobium* e *Mesorhizobium*. Outros gêneros também já foram isolados de *P. vulgaris* L., como *Pseudomonas*, *Stenotrophomonas*, *Arthrobacter*, *Chryseobacterium*, *Enterobacter* e *Agrobacterium* (SÁNCHEZ, *et al*., 2014; DELAMUTA, *et al*., 2020), segundo dados da literatura *Herbaspirillum seropedicae* é também capaz de se aderir e colonizar internamente as raízes de *P. vulgaris* (SCHMIDT et al., 2011) e a nova espécie *Herbaspirillum lusitanun* foi isolada de nódulos de *P. vulgaris* coletados em solo de Serra Peneda em Portugal (VALVERDE et al., 2003).

2.3 O FEIJOEIRO COMUM (*Phaseolus vulgaris* L.)

Phaseolus vulgaris L. originou-se na mesoamérica, provavelmente na região do atual estado do México e se espalhou para o sul em direção aos Andes. Assim, ao menos dois eventos independentes de domesticação dessa leguminosa ocorreram e determinaram a formação de dois *pools* genéticos distintos, o mesoamericano e o andino. A principal consequência desta domesticação ao nível de genoma é a redução da diversidade genética (GEPTS *et al*., 1986; GEPTS; BLISS, 1986; GEPTS, 1990; VITTORI, *et al*., 2017). Atualmente essa leguminosa é considerada a mais importante fonte de proteína vegetal, principalmente para as populações de baixa renda dos países em desenvolvimento. O feijão também se constitui uma fonte de ferro, cálcio, magnésio, zinco, vitaminas (principalmente do complexo B) carboidratos e fibras (MESQUITA *et al*., 2007; ZILIO, 2017; FIDELIS, 2019; NTATSI, 2020).

Esta leguminosa figura entre os três mais importantes grãos cultivados no mundo, juntamente com a soja (*Glycine max* L.) e o amendoim (*Arachis hypogaea* L.)

(SHAMSELDIN; VELÁZQUEZ, 2020). É uma espécie anual, cultivada em praticamente todos os estados do país, considerada atípica por ser capaz de proporcionar três safras ao ano (safra das águas, safra da seca e safra de inverno) (CONAB, 2019). Essa cultura é considerada exigente em condições climáticas, pois pode sofrer danos severos com umidade do ar, temperaturas e índices pluviométricos extremos. Pouca disponibilidade de água também afeta o seu desenvolvimento pois não é resistente a períodos de estiagem e possui escassa capacidade de recuperação após estresse hídrico, especialmente por apresentar sistema radicular pouco desenvolvido (FRANCISCO *et al*., 2016).

Seu ciclo de desenvolvimento é dividido em dois estádios principais: o vegetativo (V) e o reprodutivo (R). O período vegetativo do feijoeiro engloba os estádios da germinação, emergência e desenvolvimento das folhas primárias e compostas. O reprodutivo vai desde o período de pré-floração, floração, formação das vagens, enchimento das vagens e maturação. Esses estádios são importantes para indicar quais manejos e decisões devem ser tomadas durante o desenvolvimento da cultura. Os estádios variam de acordo com os cultivares, podendo ser superprecoces (ciclo com cerca de 60 dias) e tardio (ciclo de 115 dias) (OLIVEIRA, 2018). Os cultivares BRS Esteio e IPR Sábia apresentam um ciclo médio da emergência das plântulas até a maturação fisiológica (colheita) de 85 a 90 dias e de 87 dias, respectivamente (Embrapa Arroz e Feijão, 2015; IAPAR, 2018).

O estado do Paraná é o maior produtor nacional da leguminosa, contribuindo com cerca de 22% da produção do país (MORAES, 2017; CASTRO, 2019). Na safra de 2020/2021(CONAB, 2021) a produção nacional foi de 3,250 mil toneladas. Para um maior rendimento da cultura do feijoeiro é indicado a aplicação de fertilizantes nitrogenados (nitratos e formulações de NPK – Nitrogênio, Fósforo e Potássio), pois estes disponibilizam o nitrogênio necessário para a produção de clorofila, ácidos nucléicos, proteínas entre outros componentes importantes para o funcionamento da célula vegetal. Esse nutriente é o mais absorvido pela cultura, e aumenta o número de vagens e peso dos grãos pois 50% do N absorvido é destinado a produção dessas estruturas (OLIVEIRA, 2018).

Apesar de ser recomendada a aplicação de fertilizantes nitrogenados não é a alternativa mais sustentável. A FBN é a alternativa biológica para o suprimento deste elemento. Um fornecimento adequado de nitrogênio durante o desenvolvimento das plantas garante maior taxa e eficiência da nodulação (KOUKI, 2015). Assim, o uso associado de fertilizantes nitrogenados e biofertilizantes ainda é visto como uma forma do agricultor obter melhores resultados. Porém, devido ao aumento da demanda por

alimento ao mesmo tempo em que questões ambientas estão sendo discutidas em um panorama global, juntamente com casos relatados de resistência a patógenos e pesticidas, o interesse em inoculantes microbianos cresce a cada dia, tornando-se assim um campo de estudo em expansão (SANTOS; NOGUEIRA; HUNGRIA, 2019).

2.4 ESTIRPES UTILIZADAS EM INOCULANTES COMERCIAIS PARA CULTURA DO FEIJOEIRO

Rhizobium tropici CIAT 899 foi isolado de nódulos do feijão comum coletados na Colômbia, e foi descrita como uma bactéria aeróbica, gram-negativa, não formadora de esporos, com colônia circular, convexa, semitranslúcida, de 4 a 5 mm de diâmetro, sendo capaz de nodular e fixar nitrogênio em associação simbiótica com *P. vulgaris*, *Leucaena esculenta* e *Leucaena leucocephala* (MARTÍNEZ-ROMERO *et al*., 1991).

Rhizobium freirei PRF 81 foi isolado de nódulos do feijão comum coletados no estado do Paraná entre os anos de 1992/93, de regiões com solo ácido (pHs 3.5 – 5.4), sendo capaz de nodular plantas de *Phaseolus sp.* e *Leucaena sp*. Tanto *R. tropici* CIAT 899 quanto *R. freirei* PRF 81 apresentaram, em condições de casa de vegetação, a capacidade de nodulação em diversas espécies de leguminosas (HUNGRIA *et al*., 2000).

Morón e colaboradores (2005) demostram que a capacidade de CIAT 899 de nodular uma diversidade de espécies, mesmo em condições de baixo pH, é devido a um aumento na biossíntese dos fatores de nodulação e alteração na estrutura dos mesmos em condições acidas. Sugerindo assim, que a atividade dos produtos de alguns genes de nodulação em CIAT 899 é dependente de acidez e que as modificações que ocorrem nos fatores de nodulação provavelmente contribuem para o aumento da estabilidade dessas moléculas contra a degradação.

Atualmente, *R. tropici* CIAT 899 é usado como inoculante para as cultivares do feijoeiro comum em vários países da América do Sul e da África (HUNGRIA *et al*. 2000). Essa estirpe apresenta alta tolerância a estresses abióticos, eficiência na fixação biológica do nitrogênio e produção de compostos indólicos que auxiliam no crescimento da planta hospedeira (TULLIO *et al*. 2018). *R. freirei* PRF 81^{T} também é usada comercialmente, uma vez que, assim como CIAT 899, apresenta resistência as condições ambientais características de regiões tropicais como solo ácido e temperatura elevada.

Tullio e colaboradores (2019) utilizaram bactérias identificadas como *R. freirei* PRF 81^T crescidas em duas condições: controle (pH 6.8) e estresse ácido (pH 4.8) e realizaram procedimentos de extração total das proteínas, eletroforese em gel de agarose, espectrometria de massa, e identificação *in silico* das proteínas extraídas. Nesta estirpe os resultados obtidos foram a mudança no perfil de proteínas locais. Os padrões moleculares modificados podem representar o meio pelo qual *R. freirei* PRF 81^T se adapta as condições ambientais presentes nos solos brasileiros.

2.5 MÉTODOS DE *SCREENING* E IDENTIFICAÇÃO DOS ISOLADOS BACTERIANOS

A identificação da comunidade bacteriana envolvida na FBN por muito tempo foi a nível morfológico, fisiológico, bioquímico e simbiótico (CHUEIRE *et al*. 2003). Atualmente, o uso de ferramentas moleculares tem sido amplamente disseminado entre os pesquisadores, que buscam a identificação dos microrganismos envolvidos no processo simbiótico. As ferramentas mais utilizadas são a formação de agrupamentos por BOX-PCR (Box Element), ERIC-PCR (*Enterobacterial Repetitive Intergenic Consensus*) e rep-PCR (*Repetitive Extragenic Palindromic*), o sequenciamento parcial do gene ribossomal 16S para a identificação a nível de gênero e análise Multilocus para a identificação a nível de espécie.

2.5.1 BOX-PCR (Box Element)

Em uma das técnicas mais utilizadas, a BOX-PCR o primer BOX A1R é utilizado para a amplificação de sequências de DNA repetitivas e interespaçadas presentes ao longo do genoma bacteriano. O primer BOX A1R foi desenhado por Koeuth, Versalovic e Lupski (1995), juntamente com mais sete primers, baseados na sequência consenso de DNA das subunidades *boxA*, *boxB* e *boxC* do genoma de *Streptococcus pneumoniae*. Os elementos Box são sequências repetidas em mosaico compostas por várias combinações dessas subunidades, que possuem 59, 45 e 50 pares de base, respectivamente. As sequências de DNA desses elementos *box* são inteiramente diferentes das sequências de DNA de ERIC e REP, entretanto são similares com relação ao tamanho, número de cópias e capacidade para formar estruturas estáveis em forma de alça. O resultado da amplificação dos elementos Box constitui-se de marcadores polimórficos de DNA de

diferentes tamanhos produzindo assim “impressões digitais” que são únicas para cada uma das estirpes dentro de uma espécie.

A fim de, otimizar o tempo de preparo das amostras antes da reação de PCR com os primers específicos para a região *boxA* inúmeros trabalhos vem utilizando a técnica de PCR livre de extração e esta tem apresentado resultados positivos durante a amplificação, mesmo com variações entre os reagentes ou passos usados durante a aplicação de protocolos (LEE; WONG, 2009; ZHU, 2010; DAMNJANOVIC; HARVEY; BRIDGE, 2019). As técnicas de ERIC e REP também resultam na amplificação de sequências repetitivas presentes no genoma bacteriano. Estes elementos podem ser utilizados como sítios eficientes para a ligação de primers durante a PCR com o objetivo de produzir, assim como elementos Box, “impressões digitais” nos diferentes genomas bacterianos (VERSALOV; KOEUTH; LUPSKI, 1991). Esses padrões de amplificação ou podem ser usados para a separação das estirpes em grupos que podem ser posteriormente identificados através do sequenciamento parcial do gene *16S rDNA*.

2.5.2 16S rDNA

A sequência do gene ribossomal (16S em procariotos e 18S em eucariotos) é altamente conservada ao longo da evolução em todos os organismos, pois a funcionalidade do ribossomo é primordial para a manutenção das atividades metabólicas de uma célula, assim a comparação das sequências de *rRNA* é uma ferramenta poderosa para a elucidação das relações evolutivas e filogenéticas entre bactérias, arqueas e organismos eucariotos distantes. A amplificação do gene *16S rDNA* resulta em um fragmento de aproximadamente 1500 pares de base (WEISBURG *et al*., 1991). O gene *16S rDNA* contém nove regiões menos conservadas ou variáveis (VI-V9) que se localizam entre regiões conservadas. As regiões variáveis são uteis para estudos de taxonomia e filogenia, já as essas regiões conservadas são utilizadas para o desenho de primers universais (BAKER; SMITH; COWAN, 2003).

A mensuração do número de diferenças presentes nas regiões variáveis do gene *16S rDNA* pode ser usada afim de estimar o tempo de divergência entre as espécies, tornando assim, possível a visualização de relações evolutivas por meio de uma árvore filogenética. Os atributos mais importantes da variação na sequência de *16S rDNA* para este propósito são: a existência de padrões reconhecíveis de variação, ou seja, a tendência de que uma

mesma variação ocorra dentro de grupos filogenéticos e a variação existente na frequência com que essas mudanças ocorrem de posição para posição dentro da sequência. Assim assinaturas na sequência nucleotpidica podem ser estabelecidas e a amplificação dessas sequências permite acessar a diversidade genética e para a compreensão da estrutura das comunidades, especialmente de organismos que habitam ambientes extremos (WOESE, 1987; BAKER; SMITH; COWAN, 2003).

3 OBJETIVOS

3.1 OBJETIVO GERAL:

Contribuir para a compreensão da regulação epigenética durante a nodulação e isolar, identificar e determinar o potencial para a fixação biológica do nitrogênio de bactérias presentes em nódulos de duas cultivares do feijoeiro comum (*Phaseolus vulgaris* L.).

3.2 OBJETIVOS ESPECÍFICOS:

- Realizar uma revisão de literatura sobre os mecanismos epigenéticos que regulam a nodulação no feijoeiro comum (*Phaseolus vulgaris* L.) e na soja (*Glycine max* (L.) Merr.).
- Isolar as bactérias presentes nos nódulos de duas variedades do feijoeiro comum (*Phaseolus vulgaris* L.): BRS Esteio (Grupo Preto) e IPR Sábia (Grupo Carioca) cultivadas em solos com diferentes valores de pH.
- Caracterizar morfofisiologicamente os isolados obtidos através de análise das características morfológicas, uso de meios de cultura seletivos e teste de Gram.
- Otimizar um protocolo para a realização de BOX-PCR a partir de um método livre de extração de DNA.
- Utilizar os padrões de bandas obtidos através da BOX-PCR para a separação dos isolados em grupos.
- Identificar um isolado representante de cada grupo formado por análise filogenética baseada na sequência do gene *16S rDNA*.

- Verificar a eficiência dos isolados representantes de cada um dos grupos formados no processo de fixação biológica de nitrogênio (FBN) quando inoculados em sementes da cv. IPR Sábia em casa de vegetação.

4 Capítulo 1. Epigenetics' role in the common bean (*Phaseolus vulgaris* L.) and soybean (*Glycine max* (L.) Merr.) nodulation: a review

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Key message

Key message is not needed for reviews

Abstract

Nitrogen-fixing bacteria establish symbiosis with legumes for the biological nitrogen fixation process in specific root structures called nodules. Among these legumes, common bean and soybean are two of the most economically relevant crops relying on the process for efficient nitrogen acquisition. This review proposes to answer two problem questions: What are the already known epigenetic mechanisms involved in the regulation in common bean and soybean nodulation, and how they act during the nodulation process. DNA methylation, histone modification, and small RNAs were the epigenetic mechanisms found. Adenine methylation pattern of bacterial genome changes during the symbiosis and rhizobial tRNA-derived small RNA fragments are transferred to plant cells to cleave specific mRNA targets, increasing nodule number. On the other side of the interaction, plant DNA methylation is essential during the nodule's life cycle,

and histone lysine methyltransferases can also influence both bacterial colonization and nodule number. The microRNAs are the best-studied epigenetic modifiers that regulate legume nodulation. These epigenetic mechanisms can also be used for bacterial or plant autoregulation of nodulation since biological nitrogen fixation is a mandatory but energy-consuming process. This review summarizes the state of the art of the epigenetic mechanisms involved in the regulatory network during common bean and soybean nodulation. Understanding of its mechanisms is essential to modulate and enhance nodulation in legume crops.

Keywords: epigenetic, nodulation, nitrogen-fixing bacteria, common bean, soybean

1 Introduction

The epigenetics unfolding occurred in studies of evolution and development. Nowadays, it is crucial to understand the molecular mechanisms that regulate gene expression in eukaryotes (Felsenfeld 2015). Epigenetics can be defined as the set of reversible and potentially inherited genome changes that occur without altering the DNA sequence. In bacteria, the most studied epigenetic mechanism is DNA methylation, which has a role in chromosome replication, mismatch repair, control of transposon activity, regulation of the cell cycle, and transcription (Adhikari and Curtis 2016; Sánchez-Romero and Casadeús 2020). Compared with bacteria, more epigenetic mechanisms are already described in plants: DNA methylation, chromatin remodelling and histone post-translational modifications (PTMs), and modifications due to non-coding RNAs as RNAi, long non-coding RNA, or microRNAs (Vaschetto 2015; Perrone and Martinelli 2020).

Plants are sessile organisms; therefore, they need a *plant immune system* that depends on a wide variety of strategies, many of which have some epigenetic regulation to adapt to environmental changes or biotic interactions. Some biotic interactions are detrimental, while others are beneficial or essential for plant growth and development, as the interaction between plants and microorganisms such as rhizobia (Alonso et al. 2019; Salgotra and Gupta 2019; Fiorilli et al. 2020; Perrone and Martinelli 2020). Rhizobia is a group of bacteria capable of, through a biochemical process, reduce the atmospheric nitrogen (N_2) to plant- assimilable ammonia (NH_3) (Vieira 2017), in a process called Biological Nitrogen Fixation (BNF). After photosynthesis, the BNF is the most important biological process for the maintenance of natural ecosystems (Junior et al. 2018) and is

catalyzed by a highly conserved enzyme complex called nitrogenase (Masson-Bovin and Sachs 2018).

Currently, rhizobia comprise a paraphyletic group with more than 200 species, distributed in 18 genera of α-proteobacteria and γ-proteobacteria (De Lajudie et al. 2019; Taylor, Simms and Komatsu 2020). Nitrogen-fixing bacteria, such as those of the genus *Rhizobium*, *Bradyrhizobium*, *Mesorhizobium*, and *Sinorhizobium*, can develop specific structures in the host legumes roots for BNF's process, the nodules . Nodulation is a complex interaction that begins with the exudation of phenolic compounds, mainly flavonoids and isoflavonoids, through the host plant's roots. The bacteria present in the rhizosphere respond to these flavonoids by activating transcriptional regulators responsible for initiating the coordinated expression of nodulation genes (*nod, nol and noe*). The *nod* genes are responsible for the synthesis and secretion of lipoquitooligosaccharides, called Nod factors. Nod factors bind to receptors on the plasma membrane of cells at the root of the plant, inducing various physiological, cellular, and molecular responses to continue the process of infection and nodulation. Bacteria enter the cells from the legume root to the nodule's meristem, where they differentiate into bacteroids and fix atmospheric nitrogen (Vieira 2017; Sanchs, Quides and Wendlandt 2018; Clúa et al. 2018; Wang, Liu and Zhu 2018; La Pena et al. 2018).

The Leguminosae family has importance for agriculture, and economy, since some species are consumed by millions of people worldwide. Common bean (*Phaseolus vulgaris* L.) is a protein source in many South and Central America, Asia, and Africa countries (Schwember et al. 2019; Torres et al. 2020). Other important legume is the soybean (*Glycine max* (L.) Merr.), which is one of the most important source of vegetable oil. Soybean and the common bean provide critical components of healthy human diets, and are more promising to diversity cropping systems, enhancing soil fertility management (Franke, Brand and Giller 2014).

Therefore, we searched the literature for epigenetic mechanisms involved in the regulation of nodulation in common bean and soybean and understand how they act during the nodulation process. This will serve as a point of reference for building new hypotheses for investigation since epigenetics is a field of study in expansion. To our knowledge, there are no reviews on epigenetic mechanisms explicitly involved in the

regulation of nodulation in these two legumes of extreme importance for the world economy and food security.

2 DNA methylation in microbial genome and nodule's life cicle

Cell-cycle regulated methyltransferase (*CcrM*) and deoxyadenosine methyltransferase (*Dam*) are two DNA methyltransferases encoded by the microbial genome. *CcrM* is essential in α-proteobacteria, where it catalyzes the transfer of a methyl group to the adenine nucleotides present in the 5'-GANTC-3` sequence. On the other hand, *Dam* is fundamental for DNA methylation in γ-proteobacteria and catalyzes the transfer of a methyl group to adenine in 5`-GATC-3` motifs (Adhikari and Curtis 2016; Blow et al 2016; Sánchez-Romero and Casadeús 2020).

Most of the 5'-GANTC-3' sites in the *Mesorhizobium loti* MAFF303099 genome are strictly methylated by *CcrM*, but this genome also has the presence of unmethylated regions (SUMs). These regions are stably maintained if environmental conditions do not change, however, during the establishment of plant-bacteria symbiosis, SUMs become strictly methylated (Ichida et al. 2007) (Fig. 1a). Mutant strains designated *M. loti* MlccrM-OX that overexpress *CcrM* consequently present hypermethylation of the genome. The genome hypermethylation, in this strain, induces a delay in nodule development, thus indicating that CcrM contributes to the regulation of early stages of nodule development by altering the DNA adenine methylation pattern (Ichida et al 2009).

During the free lining bacteria-to-bacteroid transition of *Bradyrhizobium diazoefficiens*, a key event in the symbiotic relationship settlement with soybean (*Glycine max* (L.) Merr.), five motifs are differentially methylated ($CAY(N)_6CTC$, $GAGA(N)_6RTG$, GANTC, CRAGGAT, CCTTGAC, this later one methylated only during symbiosis). This differential methylation alters the expression of 89 genes, of which nine are involved in symbiosis and nodule development, such as sulfurylation of the Nod factor (*noeE*), efficiency of nodule development (*nfeC*), number of infection events (*nolY*), maturation of the nitrogenase complex (*nifB*, *nifE*, *nifX*, and *nifZ*), and the development of symbiotic nitrogen fixation (*fixA* and *fixC*). The expression of genes involved in metabolism, environmental signal processing, cellular process, and others

with unknown function are also altered during the bacteria morphological transition (Davis-Richardson et al 2016).

More recently, DNA methylation role in the nodule's lifecycle was analyzed by whole-genome bisulfite sequencing (BS-seq). This strategy revealed dynamic changes of cytosine methylation in protein coding genes during three developmental stages of soybean nodules (12, 22, and 36 days after inoculation with *B. diazoefficiens* USDA110). These stages represent the formation, development, and nodule senescence, respectively. Identification of differentially methylated regions (DMRs) that are hypo and hypermethylated according to the nodule's lifecycle, suggested that there is a DNA methylation increase with nodule age (Fig. 1b). Genes differentially expressed and differentially methylated are mostly involved in the jasmonic acid biosynthetic process, defense responses, signal transductions, DNA metabolic process, and beta-glucan biosynthetic process (Niyikiza et al. 2020).

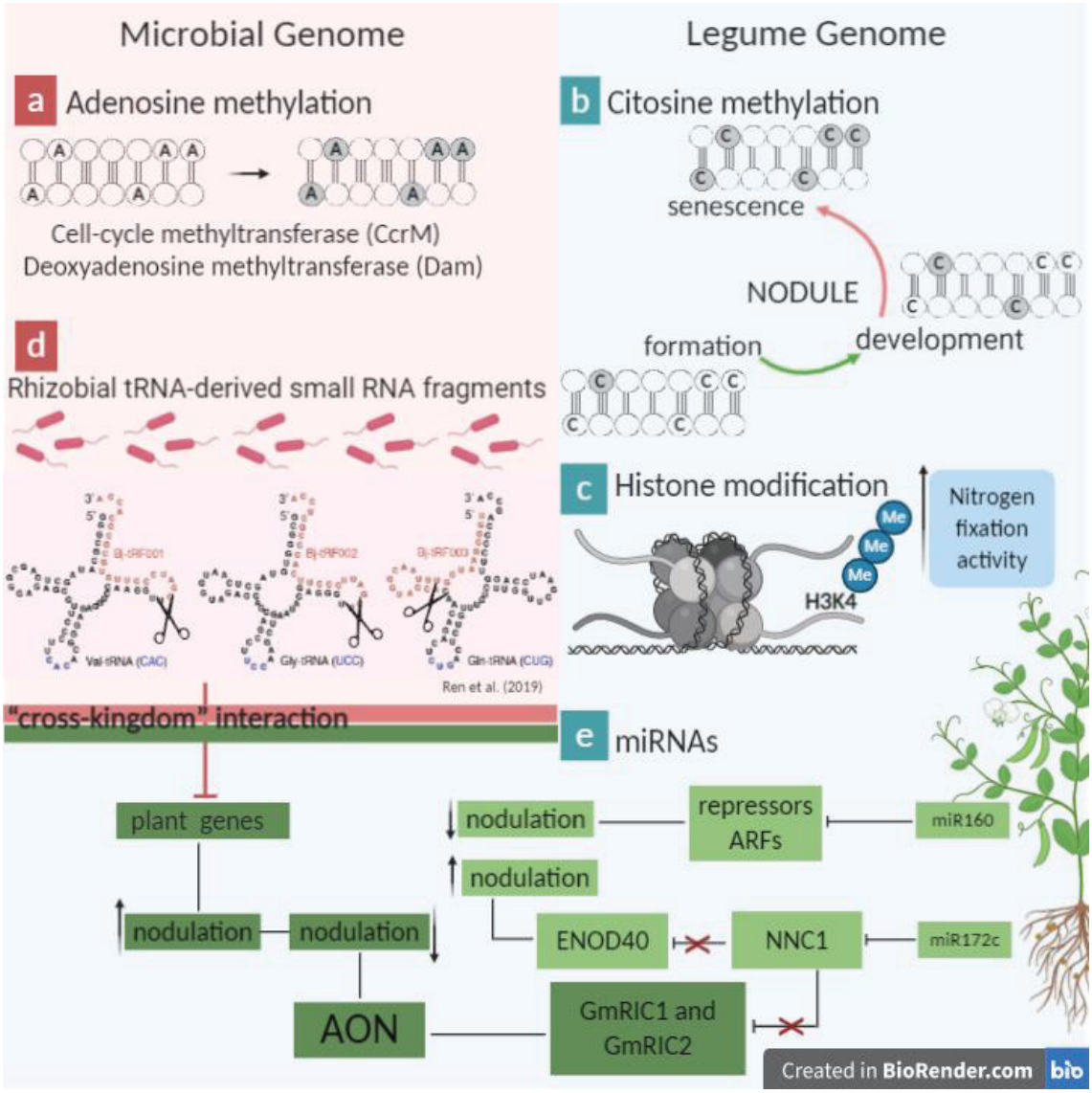

Figure 1. Summary of the epigenetic mechanism found in this systematic review. (**a**)The microbial genome is regulated by DNA adenine methylation, while the legume genome presented a nodule methylome (**b**). Histone lysine methyltransferases are responsible for (c) histone modifications **(d)** Rhizobial tRNA-derived small RNA fragments (the black scissors represent that only the red sequence of the tRNA will give rise to the small regulatory RNA) (**e)** miR160 acts in repressors of auxin response factors (ARFs) for decrease the nodule number. The miR172 target the Nodule Number Control 1 (NNC1) repressing its transcription. The NNC1 is a negative regulator of noduline genes such ENOD40. NNC1 is also a transcriptional repressor of Rhizobia-Induced CLE1 (GmRIC1 and GmRIC2) genes. When NNC1 is inactivated by miR172c, another pattern avoid excessive nodulation, this pattern is termed autoregulation of nodulation (AON) and this involve the *GmRIC1* and *GmRIC2* genes. tRFs are bacterial autoregulator of nodulation that acts as a positive regulator (dark green boxes) while GmRIC1 and GmRIC2 genes are part of the plant autoregulation of nodulation that acts to avoid excessive nodulation (dark green boxes). The pink and green bars between (**d)** and (**e)** represent the "cross-kindong" interaction.

3 Histone methyltransferases encoded by plant genome

Histone methylation is able to increase or decrease gene transcription, depending on the exact lysine/arginine position in the histone that is methylated and how many methyl groups are added to these amino acids. Methylation on lysine (K) residues of histone H3 and H4 are the best-studied post-translational modifications. They are catalyzed by histone lysine methyltransferases (HKMTs). Most HKMTs contain a conserved SET domain with methyl transferring activity, and all of these use S-adenosylmethionine (*SAM*) as a methyl donor. In *Arabidopsis*, histone methylation has roles on seed development, dormancy, and germination; organ morphogenesis (as roots, shoots, and leaves), flowering transition (through transcriptional modulation of flowering-regulatory genes), reproductive development, and callus formation (Cheng et al 2020; Zhou et al 2020).

In *Phaseolus vulgaris* L. genome, some genes already described encode HKMTs: *PvuASH1h, PvASHH2h, PvATXR3h*, and *PvuTRX1h*. *PvuTRX1h* had a stronger expression in the early stages of nodulation, whereas *PvuASH1h* transcript levels increased throughout the development of an efficient nitrogen-fixing nodule. Analysis by quantitative RT-PCR observed differential expression of these genes in nodules of BAT477 and DOR364 common bean cultivars inoculated with *Rhizobium etli* strain CE3. After silencing by RNA interference (RNAi), *PvASHH2h* downregulation was directly associated with the non-hairy-root phenotype and, consequently, with no nodule formation. On the other hand, *PvATXR3h*-RNAi and *PvTRX1h*-RNAi transgenic hairy roots formed nodule primordia that developed in determinate nodules (Quiceno-Rico, Camas-Reyes and Alvarez-Venegas 2012; Barraza et al 2015).

More recent research aimed to understand the role of *PvTRX1h,* specifically in nodule development and auxin biosynthesis, also using knockdown *PvTRX1h* expression by RNAi. *PvTRX1h* was downregulated by approximately 50%, resulting in a global decrease of H3K4me3 marks, increased auxin production and nodule number per plant, reduced bacterial survival inside of nodules, and consequently, a reduced nitrogen fixation activity compared to control nodules (Fig 1c.) Thus, according to the authors, *PvTRX1h* enzyme acts through histone modifications and could alter the auxin signaling network to determine bacterial colonization, nodule number, starch accumulation, hormone levels, and cell proliferation (Barraza et al 2018). Therefore, transcriptional

downregulation of *PvuTRX1h* and *PvATXR3h* and upregulation of *PvuASH1h* and *PvASHH2h* could be involved in nodule development.

The global H3K4me3 profile was first described for plant-bacterial interation pair in soybean cv. C08 inoculated with *Sinorhizobium fredii* CCBAU45436. The genes encoding the leghemoglobin (Lba, Lbc1, Lbc2 and Lbc3) and nodulin genes including *nodulin -16, -20, -22, -24, -44* and *ENOD93* are highly marked with H3K4me3 in soybean nodules. The increase of this histone mark is positively associated with the expression of genes encoding metabolic enzymes within the biosynthesis pathway of intermediate molecules for the assimilation of nitrogen by plant cells. Furthermore, the expression of some genes encoding for the carbon metabolism pathway is modulated by H3K4me3 (most of these genes are up-regulated with higher H3K4me3 levels) (Fig. 1c). A decrease of this histone mark in several transcription factors (TF) genes, mainly WRKYs TF, can coordinate the repression of host plant defense responses, thus enabling the nodule maintenance. Therefore, as well as in *P. vulgaris,* in soybean H3K4me3 marks are involved in regulation of BNF, in the nodule development and efficiency by upregulation of genes involved in important biosynthesis pathways (Wang et al 2020).

4 Small RNA involved in nodulation

Small RNAs are short non-coding molecules involved in posttranscriptional gene regulation. Most of the small RNAs required a longer double-stranded RNA (dsRNA) precursor. These dsRNAs are processed inside the cell by the ribonuclease III-like enzyme Dicer or Dicer-like proteins (DCLs) to generate fragments of 21-24 nt length, are then bound by Argonaute (AGO) proteins and incorporated into RNA-induced silencing complex (RISC) to act in some target sequence in the most cases causing gene silencing by cleavage or transcriptional inhibition. Plant small RNAs can be divided into two types: microRNAs (miRNAs) or small-interfering RNAs (siRNAs), both are involved in plant abiotic and biotic interactions (Alvarez-Venegas, De la Peña and Casas-Mollano 2014; Huang et al 2019; Zeng et al., 2019).

Ebeling, Kuding and Hennecke (1991) reported for the first time the role of RNA in nodule development, when analyzed the mutation in *B. japonicum* Tn5 strain 259. Soybean inoculated with this mutant strain presented numerous small, pseudonodules without nitrogen fixation activity in the first 20 days after inoculation. They discovered

that this mutation was located in a gene, called *sra* (symbiotic ribonucleic acid), and that this gene coded for a short RNA molecule instead of a protein. The *sra* gene could be found in *Rhizobium meliloti* and *Rhizobium leguminosarum* biovars viciae, trifolli and phaseoli but not in *Pseudomonas aeruginosa*, *Azotobacter vinelandii*, *Klebsiella pneumoniae* and *Escherichia coli*, wherefore, probably *sra* gene plays a general metabolic role that become more crucial under symbiotic conditions.

In common bean plants, Argonaute5 (AGO5) protein is preferentially expressed in nodules and roots. This protein accumulation is detected after one hour of rhizobia inoculation. Plants with AGO5 silencing by RNAi presented a reduced root hair deformation and reduced expression of the symbiotic genes as Early Nodulin40 (*ENOD40*) in response to rhizobia inoculation, compared to controls. Down-regulation of AGO5 by RNAi resulted in a 60% reduction in nodule number, and the nodules that reached maturity in these plants were irregular, smaller, and white. Similarly to common bean, AGO5 accumulated in nodules and roots of soybean, and its transcripts increased one hour post *B. diazoefficiens* inoculation. AGO5 silencing in soybean roots resulted in 50% fewer nodules than the control plants. The mature nodules were smaller and white, indicating a lack of leghemoglobin. Thereby, not only miRNAs but also Argonaute proteins are involved in establishing nodules and in their efficiency in nitrogen-fixing (Reyero-Saavedra et al. 2017).

New evidence demonstrated that some sRNAs can travel between the host (plant or mammalian) and interacting microbes/parasites to silence target genes of the partner *in trans*. These mechanisms are called "cross-kingdom sRNAs" (Huang et al 2019; Zeng et al., 2019). One of these RNAs that travel between two kingdoms is a recently discovered new class of small non-coding RNAs derived from Transfer RNA (tRNA) named Rhizobial tRNA-derived small RNA fragments (tRFs). These tRFs can regulate protein translation, silence genes by base-pairing with target mRNAs, sequester RNA-binding proteins and regulate transposable elements and non-coding RNA activity (Su et al 2020).

Recently, tRFs were described as a mechanism by which bacteria regulate nodules' number (Fig. 1d). These rhizobial tRFs hijacked the soybean Argonaute 1 (AGO1) to catalyze tRF-guided cleavage of target mRNAs in the plant cells, so like a cross-kingdom mechanism of action and/or interaction. A total of 52 genes in the soybean genome were predicted to be targets of rhizobial tRFs, of these Root Hair Defective 3

(*GmRHD3a/GmRHD3b*), Hairy Meristem 4 (*GmHAM4a/GmHAM4b*), and Leucine-Rich Repeat Extensin-Like 5 (*GmLRX5*), all orthologs of *Arabidopsis* genes, are essential for root hair and plant development. The overexpression of *GmRHD3b, GmHAM4a,* or *GmLRX5* genes reduced nodule number while silencing of three soybean tRFs (Bj-tRF001, Bj-tRF002, and Bj-tRF003 derived from Val-1-tRNA(CAC), Gly-1-tRNA(UCC), and Gln-1-tRNA(CUG), respectively) resulted in decreased nodule number, suggesting that these tRFs are positive regulators of nodulation. The presence of these tRFs was also analyzed in common bean-*Rhizobium etli* symbiosis. *R. etli* tRFs were primarily derived from the 3' ends of the tRNAs, and these are part of a different set of tRNAs targeting a different set of genes, compared with soybean-*B. japonicum* symbiosis (Ren et al 2019).

According to Baldrich and Mayers (2019), there are still many questions about these tRFs, as "Could this tRFs regulation be critical to distinguish symbiotic and pathogenic bacterias?", "Are these tRFSs produced under normal growing conditions or the specific tRFs necessary to trigger nodulation are only produced when needed?" and "Are these tRFs transported in vesicles from bacterial cell to the host and if they are, how the vesicles guide the tRFs to the appropriate AGO protein?". More studies are needed to answer these questions and the next ones that probably willemerge in the following years.

5 Specific microRNAs regulates soybean and common bean nodulation

MicroRNAs (miRNAs) are another class of small non-coding RNAs (20-24 nucleotides in size) that predominantly repress target genes post-trancriptionally in most eukaryotes. In plants, miRNAs are produced from specific stem regions of single-stranded hairpin precursors, and their location in the genome can be "intergenic" or "intronic". These regions are processed in pre-miRNAs, then recognized by Dicer-like RNase III endonucleases resulting in a mature miRNA, the mature miRNA bind to Argonaute proteins to form miRNA-induced silencing complex (miRISC). These non-coding RNAs are key regulators in plant development, being involved in the regulation of phenotypic plasticity, abiotic and biotic stress response and in the regulation of symbiotic processes (Song et al 2019; Wang, Mei and Ren 2019).

Nowadays several small RNAs, mainly microRNAs, have already been described as potential regulators of the nodulation initiation and formation, hormone homeostasis, and autoregulation of nodulation (Hoang, Tóth and Stacey 2020). For example soybean miRNAs: miR482, miR1507, miR1512, miR1515 miR1514, gma-miR2606b (legume-specific), gma-miR4416, and TAG_2383310 (soybean-specific). Ten days post *B. japonicum,* inoculation of miR482, simulating its overexpression, resulted in a dramatic increase in the number of mature nodules compared with the control roots. Overexpression of miR1515 and miR1512 was also associated with a higher nodule count but smaller than miR482 results. The nodule number also increased in hairy soybean roots ectopically expressing gma-miR2606b and decreased in roots ectopically expressing gma-miR4416. Therefore, miR482 and gma-miR2606b expression (and perhaps its target genes) can be crucial for the proper regulation of nodule numbers in soybean-*B. japonicum* symbiosis (Li et al 2010; Yan et al 2016).

Since there are recent reviews aiming specifically the miRNA mediated regulation of legume symbiosis (Hoang, Tóth and Stacey 2020; Nair and Manickavelu 2020; Tiwari et al 2020) and RNA-induced silencing complex (RISC) (Valdés-López et al 2019), our objective with this particular section is to point out most relevant proposed mechanisms involved in the regulation of common bean and soybean nodulation.

5.1 miRNAs that act via auxin response

The phytohormone cytokinin promotes nodule formation, and the phytohormone auxin inhibits nodule formation; therefore, a low auxin activity during nodule initiation is essential to normal nodule development (Suzaki, Ito and Kawaguchi 2013). The miR160 is a key signaling element determining the auxin/cytokinin balance during nodule development in soybean (Fig. 1e). Overexpression of this miRNA to silence a set of Auxin Response Factors (ARF) repressors belonging to the ARF10/ARF16/ARF17 family was used to obtain auxin-hypersensitive soybean plants. After 14 days of inoculation with *B. japonicum,* these plants had an average of 20.2 ± 1.8 nodules per plant, while miR160 overexpression had an average of 9.6 ± 1.7. Exogenous cytokinin restores nodule formation in miR160 overexpressed roots. GFP fluorescence data suggest that the primary mode of action of miR160 in nodules is transcript cleavage, whereas translational repression seems unlikely. miR160 can regulate infection during nodule

development and possibly rhizobial colonization of the nodule primordia (Turner et al. 2013; Nizampatnam et al. 2015).

Other miRNAs that act via auxin response are miR167 family members. Auxin response factors GmARF8a, GmARF8b, and GmARF6b function as direct target genes of miR167, and of them, GmARF8a may be the primary target of this miRNA. Knockdown of miR167 in soybean roots resulted in significantly fewer nodules than the controls, 28 days post *B. japonicum* strain USDA110 inoculation. Overexpressing of miR167c was also used to evaluate nodule numbers. The average number of nodules per root overexpressing miR167c exceeded 30; in contrast, the control roots had an average of 12 nodules per root; thus, miR167c is a positive regulate of soybean nodulation. When Rhizobia are absent, miR167 members are maintained at a low level and their targets, such as GmARF8, are able to activate the transcription of auxin-response genes to inhibit root nodulation. In the presence of rhizobia, miR167 was induced and inhibited GmARF8 activity, thus inhibiting the transcription of auxin-responsive genes from promoting nodulation (Wang et al 2015). The miR393d also regulates nodulation by directly repressing the auxin receptor *GmTIR1C* gene, but this miRNA will be discussed in the next topic.

5.2 miRNAs as negative regulators of the nodule number

The miR319d and miR393j-3p/miR393d are negative regulators of common bean and soybean nodulation, respectively. Overexpression of miR319d in *P. vulgaris* cv. BAT 93 roots resulted in early infection thread formation (2 days post *Rhizobium tropici* CIAT 899 inoculation), but a lower nodule number and nitrogenase activity (24 days post-inoculation). The TEOSINTE BRANCHED/CYCLOIDEA/PCF (TCP) is a class of transcriptional factors targeted by miR319d. These transcriptional factors bind to the Jasmonic acid (JA) biosynthetic gene (*LOX2*) promoter and directly regulate its transcription. JA has a negative effect on rhizobia infection but a positive effect on the nodule development of common beans. In soybean cv. Williams 82 roots inoculated with *B. japonicum* strain USDA110 ectopically expressing miR393j-3p, presented a decrease in nodule number. The miR93j-3p target the 3' UTR (untranslated region) of Early Nodulin 93 (*ENOD93*) mRNA. The miR393d overexpressed in soybean roots also

resulted in significantly reduced curled root hair number and nodule number at 6 days post-inoculation (Yan et al. 2015; Cai et al. 2017; Martín-Rodríguez et al. 2018).

The miR171 is another miRNA already studied in soybean nodulation. In nodules 3 weeks post inoculated with *B. japonicum*, the expression of *gma-miR171o* was suppressed, while of *gma-miR171q* was induced. These miRNAs are also negative regulators of nodule formation, once ectopic expression resulted in a reduction in nodule formation 4 weeks post inoculation. Two GRAS transcription factor superfamily called Scarecrow like-6 (*GmSCL-6*) and Nodulation-signaling pathway 2 (*GmNSP2*) are targets of gma- miR171o and gma-miR171q, respectively. *GmSCL-6* and *GmNSP2* expression silencing in roots resulted in a reduction in nodule formation. Soybean roots expressing mutated versions of *gma-miR171o* or *gma-miR171q*, lacking the ability to cleave mRNA targets, showed normal nodulation. Therefore, these two miRNAs regulate *GmSCL-6* and *GmNSP2*, which in turn, influence expression of the Nodule Inception (*NIN*), Early Nodulin 40 (*ENOD40*), and Ethylene Response Factor Required for Nodulation (*ERN*), in order to suppress soybean nodulation (Hossain et al. 2019).

5.3 miRNAs as positive regulators of the nodule number

Considering whole literature evidence miR172 is the best-known miRNA positively regulating the soybean and common bean nodulation (Fig. 1e). Overexpression or ectopic expression of miR172 in soybean resulted in increased root hair deformation, number of infection foci, nodule numbers, nitrogenase activity, and mRNA levels of some leghemoglobin (Lb) genes, compared with the control. miR172 targets the plant-specific transcription factor gene APETALA2 (AP2), which regulates juvenile to adult-phase transition, floral organ identity, and flowering time and development. miR172 promotes flowering by repressing AP2 in another plants. With a reduction in the transcript abundance of miR172c, the number of nodules formed 28 days post *B. japonicum* inoculation was reduced significantly, thus activation of miR172c during nodule initiation is required for soybean nodulation. Nodule Number Control 1 (*NNC1*) was a direct miR172c target during nodulation. *NNC1* encodes a transcription factor that negatively regulates nodule number (as a transcriptional repressor). Both miR172c overexpression and *NNC1* knockdown increased nodule number. NNC1 directly targets the Early Nodulin (*ENOD40*) gene promoter and represses their transcription activity. NNC1 is

also a transcriptional repressor of Rhizobia-Induced CLE1 (*GmRIC1* and *GmRIC2*), which are root-derived nodulation-specific peptides, while the Nodule Inception (NIN) in soybean (*GmNINa*) is a transcriptional activator of *GmRIC1* and *GmRIC2* genes. When miR172c inhibits NNC1 translation, the autoregulation of nodulation (AON) blocks excessive nodulation (the super nodulation phenotype) via the *GmRIC1* and *GmRIC2* genes. GmRIC1 and GmRIC2 activate a leucine-rich repeat receptor (NARK) encoded by *NODULE AUTOREGULATION RECEPTOR KINASE*. NARK is found on the plasma membrane of leaves and is responsible for inducing shoot-derived cytokinins that negatively regulate the miR172c activity. (Yan et al. 2013; Wang et al. 2014; Wang et al. 2019).

In common bean cv. Negro Jamapa 81-*Rhizobium etli* CE3 plants miR172 overexpression (OE172) resulted in improved root growth, increased rhizobia infection, increased expression of early nodulation, and autoregulation of nodulation genes, and improved nodulation and nitrogen fixation. miR172 acts silencing translation of the APETALA2 gene (*AP2-1*), which is a transcriptional repressor of *ENOD40.* Recover of *AP2-1* expression is associated with nodule senescence. The AP2 is a superfamily of transcriptional factors that may act as repressors or activators of transcription. The best candidates for regulation by these TFs are genes repressed in mature nodules and induced in senescent nodules. Some of these are genes encoding enzymes from the ethylene biosynthetic pathway since ethylene plays a positive role in nodule senescence. Also, miR172 could be related to decreased sensitivity to external nitrate inhibition of *R. etli* nodulation. It was observed that a higher nitrate concentration (3mM) totally blocked the nodulation in control common bean plants, while OE172 plants were able to form active nodules at 21 days post-inoculation (Nova-Franco et al. 2015).

6 Main Conclusions

In this review, we summarized epigenetic mechanisms acting in bacterial and legume genomes during symbiosis. The DNA cytosine methylation is crucial for normal nodule development and probably for the nodule's life cycle. Histone modifications also were found. Four Histone lysine methyltransferases (HKMTases) were already described (*PvuTRX1h*, *PvuASH1h, PvATXR3h*, and *PvASHH2h*) as potential regulators involved in nodule number, bacteroids survival, and nitrogen fixation activity. *PvTRX1h* is

responsible for H3K4me3 marks, present in soybean and common bean epigenome and involved with nitrogen fixation activity.

The miRNAs are the best known epigenetic regulators in plants. They act via auxin response and/or increase or decrease nodulation by other pathways. They also have specific targets, most of them involved in biogenesis, development, or nodule senescence. The miR172 has the most complex regulation pattern described, targeting transcriptional repressors of genes expressed in the first phase of plant-bacterial interaction (Early Nodulin), essential for the formation of the nodule, and genes involved in plant autoregulation of nodulation.

In the bacterial genome the DNA adenine methylation changes are probably partially responsible for activating or repressing genes during the transition from the free-living phase to the bacteroid phase. Among the class of small RNAs encoded by the bacterial genome, Rhizobial tRNA-derived small RNA fragments (tRFs) act like miRNA-AGO1 for cleavage or transcriptional repression of target genes in the plant in order to increase nodulation. Therefore, the discovery of this tRFs showed that there is also a bacterial autoregulation of nodulation, and these tRFs can be a new form of "cross-kingdom" interaction (Bars between Fig. 1d and Fig. 1e).

Several epigenetic mechanisms can be used by the bacteria and the plant for establishing the symbiosis and for precise regulation of nodulation, since BNF is an important biological process but with high energy expenditure. The present review article summarizes the state of the art in this investigation field and is intended as a reference for epigenetic mechanisms involved in the nodulation regulatory network. Understanding these mechanisms could lead to nodulation enhancement in these legumes crops and could be mimetized in cereals and other non-leguminous crops.

Declarations

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Conflicts of interest

The authors declare that there is no conflict of interest.

Availability of data and material

Not applicable

Code availability

Not applicable

Ethics approval

Not applicable

Consent to participate

Not applicable

Consent for publication

All authors read and approved the final manuscript.

Author contributions

FL and ABWB idealized the article, FL performed the literature revision, FL and DA drafted, FL, ABWB, VMK, LVGT, and DA critically read the text.

7 References

Adhikari S & Curtis PD (2016). DNA methyltransferases and epigenetic regulation in bacteria. FEMS Microbiology Reviews, 40(5), 575–591. https://doi.org/10.1093/femsre/fuw023

Alonso C, Ramos-Cruz D, Becker C. (2019) The role of plant epigenetics in biotic interactions. New Phytologist 221:731-737. https://doi.org/10.1111/nph.15408

Alvarez-Venegas R, De la Peña C, & Casas-Mollano JA (2014) Epigenetics in Plants of Agronomic Importance: Fundamentals and Applications. https://doi.org/10.1007/978-3-319-07971-4

Baldrich P & Meyers BC (2019) Bacteria send messages to colonize plant roots. Science, 365(6456), 868–869. https://doi.org/10.1126/science.aay7101

Barraza A et al (2018) Downregulation of PvTRX1h increases nodule number and affects auxin, starch, and metabolic fingerprints in the common bean (*Phaseolus vulgaris* L.). Plant Science, 274(May), 45–58. https://doi.org/10.1016/j.plantsci.2018.05.006

Barraza A, Luna-Martínez F, Chávez Fuentes JC, & Álvarez-Venegas R (2015) Expression profiling and down-regulation of three histone lysine methyltransferase genes

(PvATXR3h, PvASHH2h, and PvTRX1h) in the common bean. Plant OMICS, 8(5), 429-440.

Blow et al (2016). The Epigenomic Landscape of Prokaryotes. PLoS Genetics, 12(2), 1–28. https://doi.org/10.1371/journal.pgen.1005854

Cai Z et al (2017). GmTIR1/GmAFB3 based auxin perception regulated by miR393 modulates soybean nodulation. New Phytologist, 215(2), 672–686. https://doi.org/10.1111/nph.14632

Cheng et al (2020). Histone tales: Lysine methylation, a protagonist in Arabidopsis development. Journal of Experimental Botany, 71(3), 793–807. https://doi.org/10.1093/jxb/erz435

Clúa J, Roda C, Zanetti ME, Blanco FA (2018) Compatibility between Legumes and Rhizobia for the Establishment of a Successful Nitrogen-Fixing Symbiosis. Genes. v.9, 1-21.

Davis-Richardson AG et al (2016) Integrating DNA methylation and gene expression data in the development of the soybean-*Bradyrhizobium* N2-fixing symbiosis. Frontiers in Microbiology, 7(APR), 1–10. https://doi.org/10.3389/fmicb.2016.00518

De Lajudie PM et al (2019) Minimal standards for the description of new genera and species of rhizobia and agrobacteria. International Journal of Systematic and Evolutionary Microbiology, *69*(7), 1852–1863.

Ebeling S, Kundig C, & Hennecke H (1991) Discovery of a rhizobial RNA that is essential for symbiotic root nodule development. Journal of Bacteriology, 173(20), 6373–6382. https://doi.org/10.1128/jb.173.20.6373-6382.1991

Felsenfeld, G (2015) A Brief History of Epigenetics. In: Allis CD, Caparros ML, Jenuwein T, Reinberg D (ed) Epigenetics, 2nd edn. Colds Spring Harb Perspect Biology, pp 1-10.

Fiorilli V, Catoni M, Lanfranco L, Zabet NR (2020) Editorial: Interactions of plants with bacteria and fungi: Molecular and Epigenetic Plasticity of the Host. Frontiers in Plant Scince 11:274. https://doi.org/10.3389/fpls.2020.00274

Franke AC, Van den Brand GJ, & Giller KE (2014). Which farmers benefit most from sustainable intensification? An ex-ante impact assessment of expanding grain legume production in Malawi. European Journal of Agronomy, 58, 28–38. https://doi.org/10.1016/j.eja.2014.04.002

Hoang NT, Tóth K & Stacey G (2020) The role od microRNAs in the legume-*Rhizobium* nitrogen-fixing symbiosis. Journal of Experimental Botany, 71(5), 1668-1689. http://doi.org/10.1093/jxb/eraa018

Hossain MS, Hoang NT, Yan Z, Tóth K, Meyers BC, & Stacey G (2019) Characterization of the spatial and temporal expression of two soybean miRNAs identifies SCL6 as a novel regulator of soybean nodulation. Frontiers in Plant Science, 10(April), 1–14. https://doi.org/10.3389/fpls.2019.00475

Huang CY, Wang H, Hu P, Hamby R & Jin H (2019) Small RNAs – Big Players in Plant-Microbe Interactions. Cell Host and Microbe, 26(2), 173–182. https://doi.org/10.1016/j.chom.2019.07.021

Ichida H, Matsuyama T, Abe T, & Koba T (2007) DNA adenine methylation changes dramatically during establishment of symbiosis. FEBS Journal, 274(4), 951–962. https://doi.org/10.1111/j.17424658.2007.0563.x

Ichida H, Yoneyama K, Koba T, & Abe T (2009) Epigenetic modification of rhizobial genome is essential for efficient nodulation. Biochemical and Biophysical Research Communications, 389(2), 301–304. https://doi.org/10.1016/j.bbrc.2009.08.137

Junior FBR, Mendes LC, Hungria M (2018) Fixação biológica de nitrogênio: fundamentos e aplicações. In: Azevedo JÁ, Pamphile JÁ, Quecine-Verdi MC, Lacava PT (Org). Biotecnologia: microbiana ambiental. Maringá: Eduem, pp 125-152.

La Pena TC, Fedorova E, Pueyo JJ, Lucas MM (2018) The Symbiosome: Legume and Rhizobia Co-evolution toward a Nitrogen-Fixing Organelle? Frontiers in Plant Science. v.8, 1-26.

Li H, Deng Y, Wu T, Subramanian S, & Yu O (2010) Misexpression of miR482, miR1512, and miR1515 increases soybean nodulation. Plant Physiology, 153(4), 1759-1770. https://doi.org/10.1104/pp.110.156950

Martín-Rodríguez J, Leija A, Formey D, & Hernández G (2018) The microRNA319d/TCP10 node regulates the common bean – Rhizobia nitrogen-fixing symbiosis. Frontiers in Plant Science, 9(August), 1–13. https://doi.org/10.3389/fpls.2018.01175

Masson-Bovin C, Sachs JL (2018) Symbiotic nitrogen fixation by rhizobia – the roots of a success story. Current Opinion in Plant Biology. v. 44, 7-15.

Nair MN, Manickavelu A (2020) MicroRNAs as fine-turners of gene regulation in plant-microbe interactions. Current Science, v. 119, n. 8, 1282-1290.

Niyikiza D et al (2020) Interactions of gene expression, alternative splicing, and DNA methylation in determining nodule identity. The Plant Journal. https://doi.org/10.1111/tpj.14861

Nizampatnam NR, Schreier SJ, Damodaran S, Adhikari S, & Subramanian S (2015) MicroRNA160 dictates stage specific auxin and cytokinin sensitivities and directs soybean nodule development. Plant Journal, 84(1), 140–153. https://doi.org/10.1111/tpj.12965

Nova-Franco B et al (2015) The micro-RNA172c-APETALA2-1 node as a key regulator of the common bean-*Rhizobium etli* nitrogen fixation symbiosis. Plant Physiology, 168(1), 273–291. https://doi.org/10.1104/pp.114.255547

Perrone A, Marinelli F (2020) Plant stress biology in epigenomic era. Plant Science, pp 1-15. https://doi.org/10.1016/j.plantsci.2019.110376

Quiceno-Rico JM, Camas-Reyes JA, & Alvarez-Venegas R (2012) Molecular cloning and characterization of two Trithorax Group genes from *Phaseolus vulgaris* roots and symbiotic nodules. Plant OMICS, 5(2),103–114.

Ren, B., Wang, X., Duan, J., & Ma, J. (2019) Rhizobial tRNA-derived small RNAs are signal molecules regulating plant nodulation. Science, 365(6456), 919–922. https://doi.org/10.1126/science.aav8907

Reyero-Saavedra MdelR et al (2017) Gene silencing of argonaute5 negatively affects the establishment of the legume-rhizobia symbiosis. Genes, 8(12), 1–19. https://doi.org/10.3390/genes8120352

Salgotra K and Gupta M (2019) Exploring the Role of Epigenetic in Cereal and Leguminous Crops Exposed to Abiotic Stress. In: Alvarez-Venegas R, De-la-Pena C, Casas-Mollano JA (ed) Epigenetics in Plants of Agronomic Importance: Fundamentals and Applications, 2nd edn. Spring, 149-170.

Sánchez-Romero, M. A., & Casadesús, J. (2020). The bacterial epigenome. Nature Reviews Microbiology, 18(1), 7–20. https://doi.org/10.1038/s41579-019-0286-2

Sanchs JL, Quides KW, Wendlandt CE (2018) Legumes versus rhizobia: a model for ongoing conflict in symbiosis. New Phytologist 219:1199-1206.

Schwember AR, Schulze J, del Pozo A, Cabeza RA (2019) Regulation of Symbiotic Nitrogen Fixation in Legume Root Nodules. Plants, 1-18. https://doi.org/ 10.3390/plants8090333

Song, X., Li, Y., Cao, X., & Qi, Y. (2019). MicroRNAs and Their Regulatory Roles in Plant-Environment Interactions. Annual Review of Plant Biology, 70, 489–525. https://doi.org/10.1146/annurev-arplant-050718-100334

Su Z, Wilson B, Kumar P & Dutta A (2020). Noncanonical Roles of tRNAs: tRNA Fragments and Beyond. Annual Review of Genetics, 54(1), 1–23. https://doi.org/10.1146/annurev-genet-022620-101840

Suzaki T, Ito M & Kawaguchi M (2013) Genetic basis of cytokinin and auxin functions during root nodule development. Frontiers in Plant Science, 4(MAR), 1–6. https://doi.org/10.3389/fpls.2013.00042

Taylor BN, Simms EL, Komatsu KJ (2020) More Than a Functional Group: Diversity within the Legume-Rhizobia Mutualism and Its Relationship with Ecosystem Function. Diversity, 12(2).

Tiwari M et al (2020) Dynamics of miRNA mediated regulation of legume symbiosis. Plant Cell and Environment, 1-13. https://doi.org/10.1111/pce.13983

Torres AR, Brito B, Imperial J, Palacios MJ, Ciampitti IA, Ruiz-Argueso T, Hungria M (2020) Hydrogen-uptake genes improve symbiotic efficiency in common beans (*Phaseolus vulgaris* L.) Antonie van Leeuwenhoek 687-696. https://doi.org/ 10.1007/s10482-019-01381-6

Turner M et al (2013) Ectopic expression of miR160 results in auxin hypersensitivity, cytokinin hyposensitivity, and inhibition of symbiotic nodule development in soybean. Plant Physiology, 162(4), 2042–2055. https://doi.org/10.1104/pp.113.220699

Valdés-López et al (2019) Argonaute Proteins : Why Are They So Important for the Legume-Rhizobia Symbiosis? Frontiers in Plant Science, 1-10. https://doi.org/10.3389/fpls.2019.01177

Vaschetto LM (2015) Exploring an emerging issue: crop epigenetics. Plant Molecular Biology 33:751-755. https://doi.org/10.1007/s11105-014-0796-z

Vieira RF (2017) Ciclo do Nitrogênio em Sistemas Agrícolas. Brasília, DF: Empresa Brasileira de Pesquisa Agropecuária – Embrapa, pp 165.

Wang L et al (2019) A GmNINa miR172c-NNC1 Regulatory Network Coordinates the Nodulation and Autoregulation of Nodulation Pathways in Soybean. Molecular Plant, 12(9), 1211–1226. https://doi.org/10.1016/j.molp.2019.06.002

Wang Q, Liu J, Zhu H (2018) Genetic and Molecular Mechanisms Underlying Symbiotic Specificity in Legume-Rhizobium Interactions. Frontiers in Plant Science. v.9, 1-18.

Wang Q, Yung WS, Wang Z & Lam HM (2020) The histone modification H3K4me3 marks functional genes in soybean nodules. Genomics, (September), 1–13. https://doi.org/10.1016/j.ygeno.2020.09.052

Wang Y et al (2014) Soybean miR172c targets the repressive AP2 transcription factor NNC1 to activate ENOD40 expression and regulate nodule initiation. Plant Cell, 26(12), 4782–4801. https://doi.org/10.1105/tpc.114.131607

Wang Y et al (2015) microRNA167 directed regulation of the auxin response factors GmARF8a and GmARF8b is required for soybean nodulation and lateral root development. Plant Physiology, 168(3), 101-116. https://doi.org/10.1104/pp.15.00265

Wang J, Mei J & Ren G (2019) Plant microRNAs: Biogenesis, homeostasis, and degradation. Frontiers in Plant Science, 10(March), 1–12. https://doi.org/10.3389/fpls.2019.00360

Yan Z et al (2013) miR172 regulates soybean nodulation. Molecular Plant-Microbe Interactions, 26(12), 1371–1377. https://doi.org/10.1094/MPMI-04-13-0111-R

Yan Z et al (2016) Identification and functional characterization of soybean root hair microRNAs expressed in response to *Bradyrhizobium japonicum* infection. Plant Biotechnology Journal, 14(1), 332–341. https://doi.org/10.1111/pbi.12387

Yan, Z et al (2015) Identification of microRNAs and their mRNA targets during soybean nodule development: Functional analysis of the role of miR393j-3p in soybean nodulation. New Phytologist, 207(3), 748–759. https://doi.org/10.1111/nph.13365

Zeng J, Gupta VK, Jiang Y, Yang B, Gong L & Zhu H (2019) Cross-Kingdom Small RNAs Among Animals, Plants and Microbes. Cells, 8(4), 371. https://doi.org/10.3390/cells8040371

Zhou et al (2020). The function of histone lysine methylation related SET domain group proteins in plants. Protein Science, 29(5), 1120–1137. https://doi.org/10.1002/pro.3849

5 Capítulo 2. The use of extraction-free BOX-PCR coupled with R analyses to cluster bacteria isolated from environmental samples

This article will be submitted to the BioTechniques Journal (A4)

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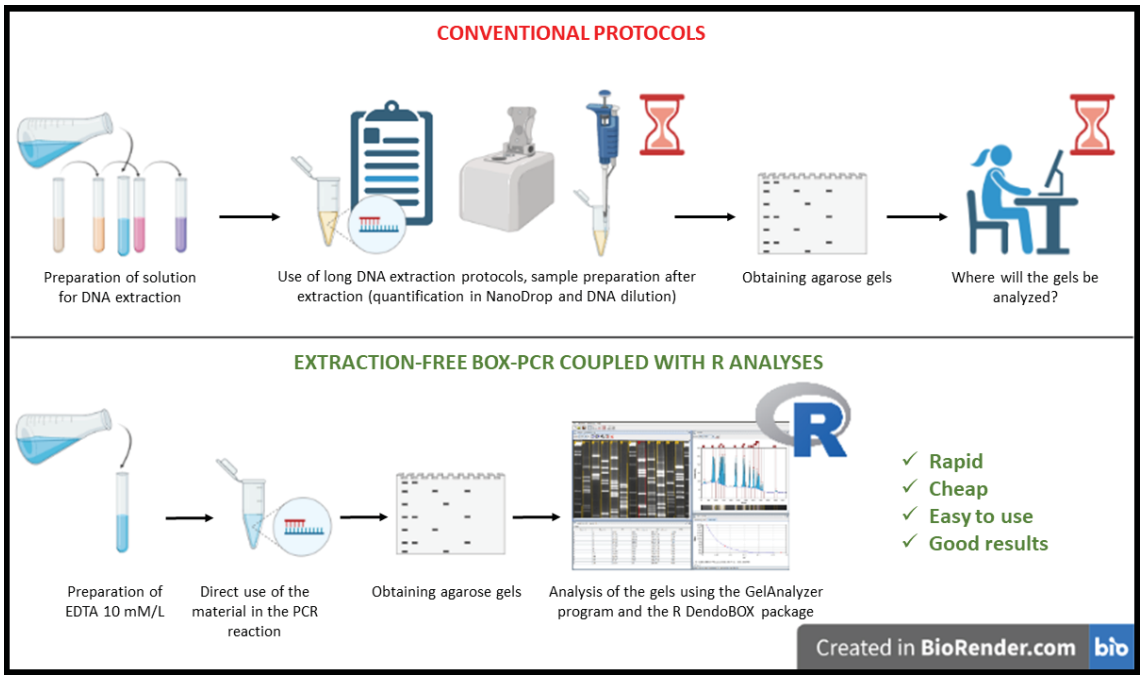
Abstract: Extraction-free PCR is a method that does not require DNA purification; therefore, this method makes the analysis cheaper, faster, and less work-intensive. It is commonly used to screening and differentiate isolates in a molecular assay. PCR-based fingerprinting methods take advantage of the presence of repetitive sequences, and BOX-PCR is one of these methods. It is a cheap and straightforward procedure, has good reproducibility and discrimination power. However, it is a method with difficulties in interpretation. The use of bioinformatic tools, such as GelAnalyzer is a way to maintain a reliable analysis. In this study, a method for screening and differentiating bacteria by BOX-PCR was optimized. The bacteria were grown in a liquid medium, removed by centrifugation, and the cell was treated with EDTA, vortex, and boiling. The BOX-PCR (conducted with the primer BOX-A1R) obtained patterns were analyzed in GelAnalyzer by comparing them to molecular weights standards. To evaluate those patterns, we developed an R pipeline to construct a binary matrix necessary for the calculation of the Jaccard similarity coefficient (J) by the Unweighted Pair Group Method Using Arithmetic Averages (UPGMA) and evaluating the branch support using the bootstrap approach. We provide a reliable and free-of-charge pipeline to evaluate rep-PCR band patterns with reproducibility and scalable to thousands of isolates.

- **Graphical abstract:**



- **Keywords:** Extraction-free PCR; BOX-PCR; Bioinformatic tools; Ambiental screening; Bacteria.

- **Main body of the text:**

1 Introduction

Extraction-free PCR or colony PCR is a method that does not require DNA purification. DNA extraction and purification are labor-intensive, time-consuming and costly method. The extraction-free PCR has the advantage of being more practical and cheaper (Borba, 2020). This method is commonly used to screen *Escherichia coli* transformants and can be used to most bacterial species for fragment amplification (Woodman, 2008).

Repetitive sequence-based PCR is a DNA amplification technique for bacterial genomic fingerprinting using repetitive DNA elements present within the bacterial genome. There are four main types of repetitive sequences used for molecular typing, which include enterobacterial repetitive intergenic consensus (ERIC) sequence, repetitive extragenic palindromic sequences (REP elements), polytrinucleotide (GTG)5, and BOX-PCR (Box Elements) (Borba, 2020; Bilung, 2018). BOX elements are highly conserved

DNA sequences discovered to be located within the intergenic region of the chromosome of gram-positive *Streptococcus pneumoniae*. The Box Elements are composed of three subunits, boxA, boxB and boxC, with 59, 45, and 50 nucleotides, respectively. The BOX consensus element is formed by only one unit of each subunits and forms a 154 bp sequence. The element BOX's function may be related to the regulation of the transcription of genes that are regulated by a single promoter (Borba, 2020).

The primer sequences designed for the *boxA* region (BOXA1, BOXA1R, and BOXA2R) amplify these fragments in many bacterial species (Koeuth, Versalovic, and Lupski, 1995). The BOX-A1R primer is often a primer of choice due to the high degree of conservation and diversity of the produced amplicons. Inversion and clustering of the *boxA* region permit a single primer to generate a range of product sizes in PCR with high diversity across and within species. Based on the diversity of interspersed short tandem repeats sequences in the genome, the BOX-PCR has been widely used for molecular typing of strains due to its good reproducibility and discrimination power (used to differentiate bacterial strains), simple procedure and low cost, advantages over other methods of Rep-PCR (Zhu, 2014).

The BOX-PCR results are complex and subjective to interpret; therefore, bioinformatics tools are fundamental for a reliable analysis of the information obtained by the BOX-PCR; some of them are listed in Table 1. After the gel-image acquisition, some free software can be used for this analysis, amongst them GelAnalyzer 2010a (gelanalyzer.com). This software requires less user intervention and is the most cited tool (Heras *et al*. 2016). GelAnalyzer was efficiently used to analyze the results of amplified polymorphic DNA band patterns in several researches (Hassan and Youssef, 2011; They *et al*. 2015; Youssef, Mansour and Solliman, 2010; Rajeev *et al*. 2019; Faria, 2015; Moura et al. 2015). Despite being a widely used tool, it is still little used for analysis of BOX-PCR fragments, and it does not have the capacity to produce clustering, for that it is necessary to use another tool such as the programming language R (R Development Core Team v.3.3.3).

Table 1. Other bioinformatic tools that analyze repetitive sequence-based PCR.

Software	Free	Open-source	Clustering	Sites
Bionumerics (AppliedMaths)	No	No	Yes	applied-maths.com/bionumerics
PyElph (Phyton)	Yes	Yes	Yes	sourceforge.net/projects/pyelph/
ImageJ	Yes	Yes	No	imagej.nih.gov/ij/
QuantityOne (Bio-Rad)	No	No	Yes	bio-rad.com
GelQuest (SequentiX)	No	No	No	sequentix.de/gelquest/index.php
GelAnalyzer (Java)	Yes	No	No	gelanalyzer.com

Following band pattern definition and the construction of a binary matrix, a possible approach to classify the isolates is to calculate the Jaccard similarity coefficient (J) (Jaccard, 1992) using the unweighted arithmetic means (UPGMA - Unweighted Pair Group Method Using Arithmetic Averages) method. UPGMA always produces an ultrametric tree, that is, a dendrogram, in which the distances from the root to each branch tip are equal (Makarenkov, Kevororkov and Legendre, 2006). Thereby, the aim of this study was to optimize a protocol for performing extraction-free BOX-PCR on many bacteria isolated from environmental samples and subsequently construction of an R script, to be further released as a package, for cluster analysis based on the data obtained by GelAnalyzer (19.1).

6 Materials & Methods

Methods for performing extraction-free PCR

Before performing extraction-free PCR was necessary to check whether the amount of DNA influences BOX-PCR's band pattern or not, for this was performed an amplification test independent of DNA concentration. Three methods were evaluated, the

first described by Zhu J. (2010), the second described by Packeiser *et al*. (2013), and the third is a combination of the two methods already mentioned (Supplementary Methods).

Bacterial Growth

A hundred and seventy six individual bacterial colonies isolated from *Phaseolus vulgaris* L. nodules by Lima *et al*. (2021) (unpublished data) were used for the extraction-free BOX PCR reaction. The bacteria were grown in 6 mL of YM liquid medium (Yeast-Mannitol) for 16 hours in the shaker (C24 Incubator Shaker, New Brunswick Scientific) at 180 revolutions per minute (rpm) at 28°C. As a positive control of each reaction, DNA extracted from one of the isolates at a concentration of 25 ng was used. The total DNA extraction reaction was carried out according to Raeder and Broda (1985).

Extraction-free PCR

1 mL from each culture was centrifuged at 10000 xg for 5 min and the supernatant was removed. The cells were resuspended in 40 µL 10mM EDTA, vortexed for 10 seconds, and heated to 100°C for 5 minutes. 360 µL of ultrapure water was added to the material and centrifuged at 10000 xg for 5 min. After this, 2 µL from the resulted solution was used in the BOX-PCR reaction.

BOX-PCR reaction

In the PCR reaction, the specific primer BOX A1R (5'-CTACGGCAAGGCGACGCTGACG-3', Invitrogen™) (Koeuth, Versalovic, and Lupski, 1995) was used. The reaction conditions were performed according to Kaschuck *et al.* (2006) with some modifications. For the extraction-free BOX-PCR reaction the following reagents and concentrations were used, 1X Buffer, 0.2 mM dNTPs, 3 mM $MgCl_2$, 0.6 mM Primer A1R, and 1.5 U/Taq DNA Polymerase Reaction.

The amplification followed an initial denaturation at 95 °C for 7 minutes; 35 cycles of 1 min at 94 °C, 1 minute at 53 °C, and 3 minutes at 72 °C, and final 4-minute extension at 72 °C (Veriti 96 Well Thermal Cycler, Applied biosystems). The amplified fragments were separated by electrophoresis (Electrophoresis Power Supply PS3002, Life Technologies, GIBCO) in 1.5% agarose gel diluted in TAE buffer with a running time of 4 hours at 100 volts (V) (approximately 2.8 V / cm). The molecular weight marker

Invitrogen 1 Kb Plus DNA Ladder and the Loading Dye for DNA 6X were used in all gels. The gels were stained with ethidium bromide 10 mg / mL viewed in a transilluminator with UV lamp (Transilluminator 4000, Stratagene) and photographed with a Nikon Coolpix L310 camera.

Cluster Analysis

The banding patterns generated by BOX-PCR were analyzed using GelAnalyzer version 19.1. As a result of this analysis, a table was obtained with each of the bands' molecular weights for each of the samples. The molecular weights were obtained through comparing the bands' location from each lane in the gel with the band of the marker Invitrogen 1 Kb Plus DNA Ladder in the same position. The molecular weights obtained in GelAnalyzer (19.1) were used as input data from an R script (R Development Core Team v.3.3.3) (Supplementary Methods) for the construction of a binary matrix necessary for the calculation of the Jaccard similarity coefficient (J) by the Unweighted Pair Group Method Using Arithmetic Averages (UPGMA). The support value of the branches of the dendrogram was obtained by *bootstrap* calculation.

7 Results and Discussion

Extraction-free BOX-PCR

According to the results obtained (Figure 1 Supplementary Methods) the amount of DNA did not influence the band pattern of the BOX PCR. Therefore, it is possible to perform the amplification by BOX PCR without standardizing the amount of DNA applied in the reaction, which is inviable in extraction-free PCR methodology. Also, is possible to verify a variation of the DNA amount used in BOX-PCR reactions in the literature, ranging from 50 ng (Wasserman, 2020 and Kaschuk et al. (2006) to 100 µg (Borba and collaborators (2020) in a single PCR reaction.

Of the tested methods, only the methodology of Zhu, J. (2010) did not present satisfactory results, since many bands were lost compared to the two other methods (Figure 2 Supplementary Methods). The other two methods tested showed similar performance, however we choose Packeiser et al. (2013) methodology since it is faster and more straightforward due to omitting one step. Further was removed the cleared

lysate transfer step and applied the protocol to liquid cultures, yielding satisfactory results (Figure S3, protocol M2B).

Zhu, J. (2010), tested four methods of preparing *Escherichia coli* cells to perform BOX A1R PCR. These methods included cell lysis by heat, washing the entire cell suspension in NaCl and direct use in PCR, alkaline lysis of the cell suspension, and purified genomic DNA. The method of washing the cell suspension in NaCl and direct use in PCR was the method we used here since, according to the author's results, this was the best in reproducibility. The Packeiser and collaborators (2013) tested four different lysis buffers, 10 mM Tris / 1 mM EDTA, 0.2% SDS, 10 mM EDTA and Y-PER, for Colony PCR reactions in Gram positive and Gram negative bacteria species, fungi and microalgae. According to the results found, the 10 mM EDTA lysis buffer performed well in amplifying the 16S gene in *Bacillus* sp., but an effective dilution must be performed; otherwise the EDTA may remain in the material and impair the PCR reaction, since this compound is a chelates divalent cations such as magnesium (Mg^{+2}) which is a cofactor of the DNA polymerase enzyme.

In all tests all isolates showed amplification of boxA fragments (Figures 2 and Figure 3 Supplementary Methods). Zhu J. (2010) used extraction-free PCR to amplify boxA fragments and obtained satisfactory results for *E. coli*, whereas Packeiser and collaborators (2013) used the method for amplifying the 16S gene and obtained good results.

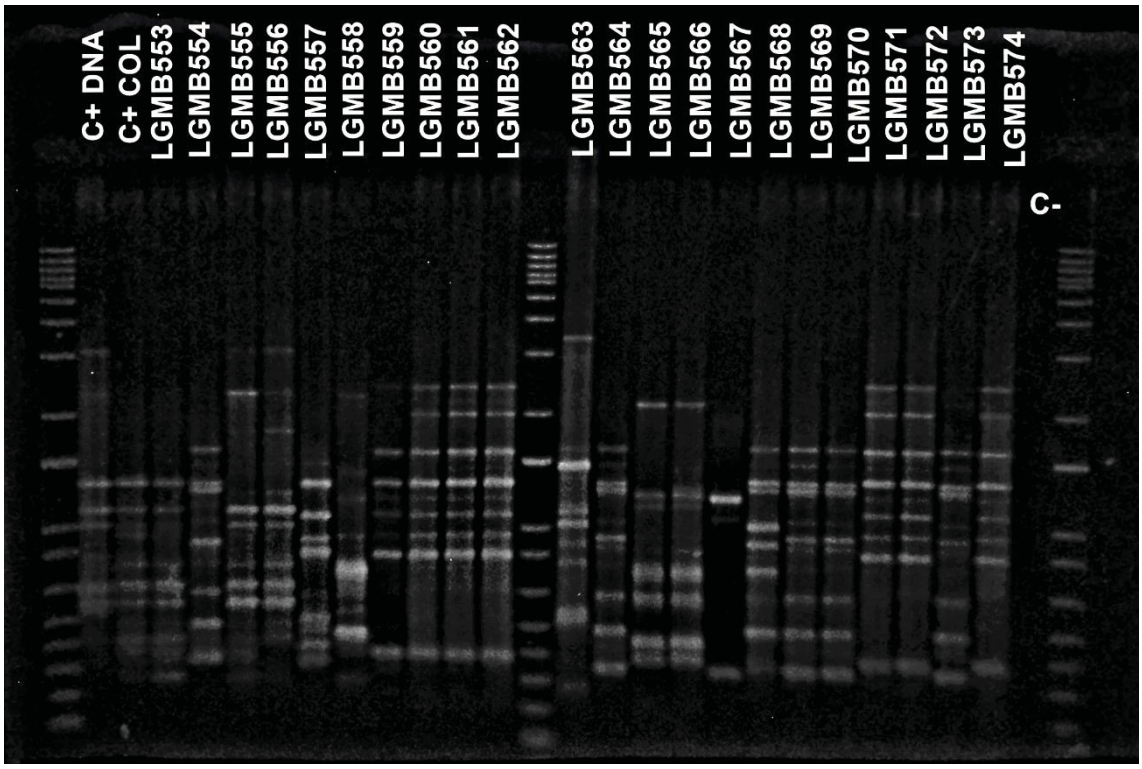


Figure 1. Agarose gels resulting from amplification of box fragments by extraction free PCR methodology. 3 lanes of DNA ladder molecular weight marker (1 Kb Plus DNA ladder) are present in each of the gels, the first lane, the middle lane, and the last lane. Each of the other lanes has the bands pattern of a specific bacteria sample named by a code followed by the number of the bacterium belonging to the collection of the Laboratory of Genetics of Microorganisms of the Federal University of Paraná. C+ DNA = genomic DNA, C+COL = positive control of extraction-free PCR.

BOX-PCR

A hundred and seventy six bacteria had their band patter amplified through the methodology optimized for extraction-free BOX PCR (Figure 1). The bacteria were named here with the LGMB code followed by the number of the bacterium belonging to the collection of the Laboratory of Genetics of Microorganisms of the Federal University of Paraná. The results obtained in this work with the use of the optimized protocol were satisfactory since all the isolates presented a band pattern detectable by the GelAnalyzer program (19.1) thus, all bands received a molecular weight necessary for the dendogram construction by the R script.

To optimize the sample preparation time before the BOX-PCR reaction, numerous studies used the extraction-free PCR technique and this has shown positive results during amplification in small samples or species known as *E. coli*, even with variations between the reagents or protocols steps. Lee and Wong (2009) used the extraction-free PCR

method to amplify boxA fragments for *E. coli* samples. In the reaction, the authors applied one washing step in distilled water followed by cell lysis by heat. In more recent studies Damnjanovi, Harvey and Bridge (2019) used the methodology for BOX A2R PCR reactions to differentiate *Lactococcus lactis*, *Leuconostoc mesenteroides* and *Streptococcus thermophilus*. To perform extraction-free PCR, the authors only collected the colonies from the plates using a sterile inoculation loop and added to the tube containing the final 25 μL volume of the PCR mix (which contained in addition to the commonly used reagents Tris-HCl 10 mM (pH 9) and 50 mM potassium chloride). The amplification resulted in distinguishable band patterns, indicating that the method employed can be a useful tool for the differentiation of these species. Puentes-Téllez and Salles, 2018 also screened environmental samples able to contribute to lignocellulose degradation using colony BOX-PCR.

The results obtained by using extraction-free PCR may vary according to the different methods applied for sample preparation. However, even so, it is a fast, reproducible, and low-cost method. Therefore, based on the researchers' results mentioned above and mainly by this research, we indicate the use of the extraction-free BOX PCR methodology in situations where many bacterial isolates need to be clustered and prioritized for further experiments and evaluations.

Parametrization of Cluster Analysis

The data obtained by GelAnalyzer (19.1) were used as input data in an R script to build the dendrogram. To describe a dendrogram that best represents the relationships between the bacteria, limit values were tested in base pairs referring to the definition of minimum distance between one band and another so that they are considered different bands (Figure 2). The tested values were established based on the results of GelAnalyzer (19.1), since the bacteria showed patterns with some very close bands, such as the lower molecular weight band of LGMB474 and LGMB475 in the Figure 2 differs by only 3 bp, low values were tested, these being 10, 20, 30, and 40 base pairs (bp). The 50 pb limit was not used because the Figure 2 showed that this limit is probably above the ideal limit because it is considering different bands as shared bands.

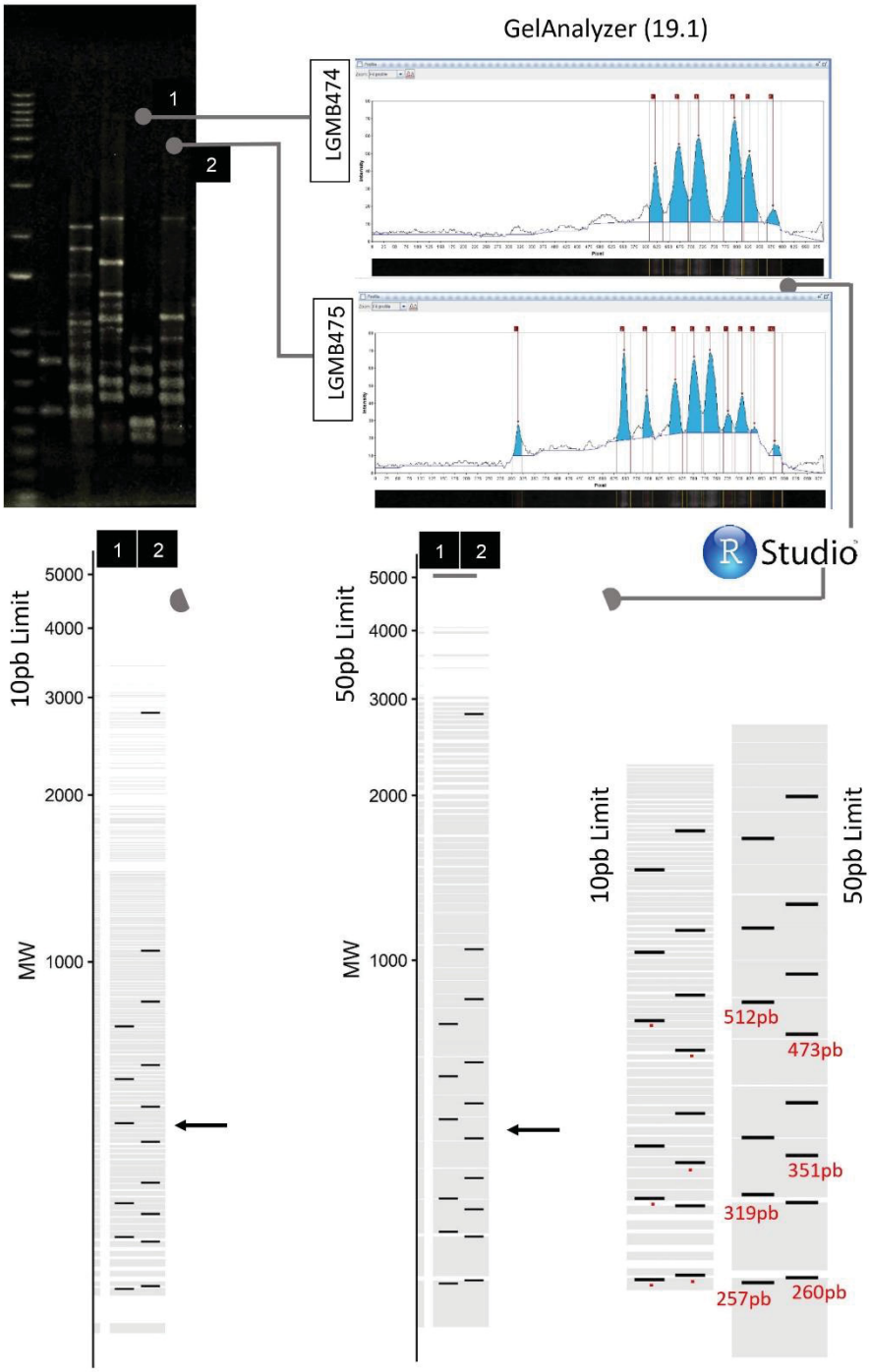


Figure 2. Explanatory scheme on how the band patterns were analyzed and the difference between using the 10 bp and 50 bp limit. After photographing the gels were analyzed in the program GelAnalyzer (19.1), the data resulting from this analysis were used as input data for an R script, this script generated the dendrogram used in this work. In this scheme, LGMB474 and LGMB475 were used to represent the difference between using the 10 bp and 50 bp limit. Each gray rectangle represents the limit used, so the bands that are within a given rectangle are considered the same band in each of these bacteria. For example, the third band of the LGMB474 has 512 bp, and the sixth band of the LGMB475 has 473 bp (indicated by the black arrow / indicated in the figures in the lower right corner). At a

limit of 10 bp these two bands are considered different bands, so these bands add up as a difference between these two bacteria, while at a limit of 50 bp these two bands are considered as the same band, so at this limit they count as one band shared between these two bacteria. The molecular size of some bands is indicated in red just below it at the 50 bp limit, and each corresponding band at the 10 bp limit is indicated with a red square just below it.

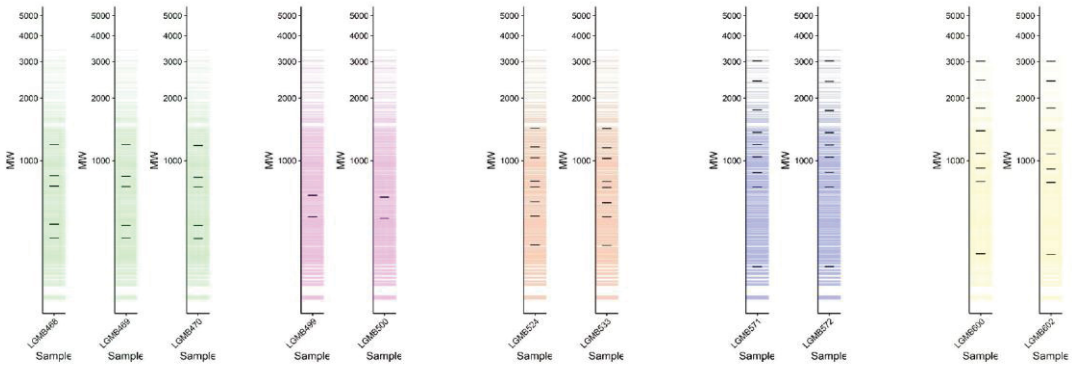
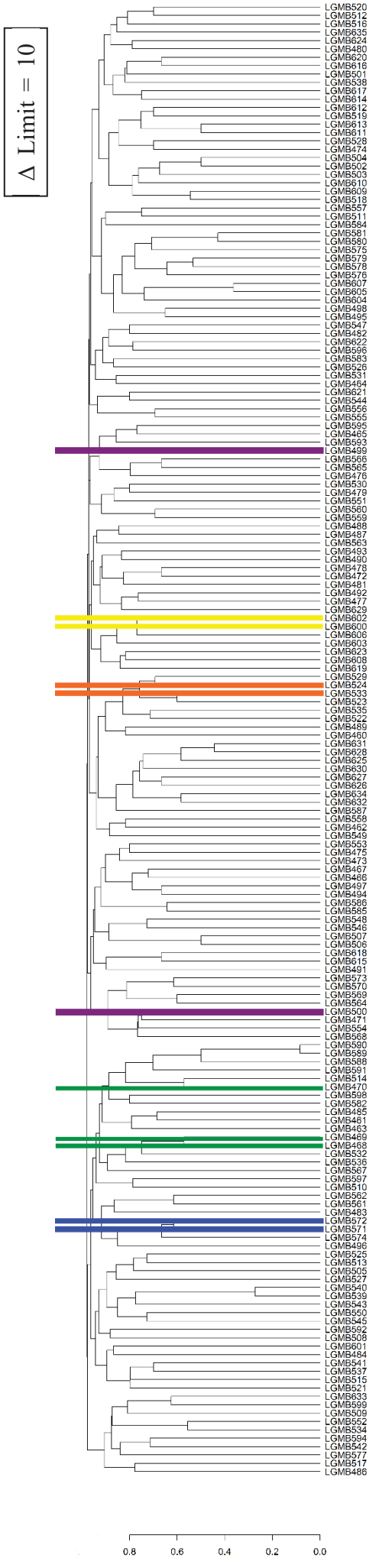


Figure 3. Guide relations: LGMB469, LGMB468 and LGMB470 indicated by green, LGMB500 and LGMB499 indicated in violet, LGMB533 and LGMB524 indicated in orange, LGMB572 and LGMB571 indicated in blue, LGMB602 and LGMB600 indicated in yellow.

To test these values, some "guide" relationships were established. These relationships served as a method of comparison between the dendrograms resulting from the different limits. They were selected based on the similarity of the band pattern visually defined by the images of the gels and the results obtained in GelAnalyzer (19.1). In one of these relationships (LGMB499 and LGMB500) morphophysiological similarity was also used as a criterion. The “guide” relationships chosen were LGMB469, 468 and 470, LGMB500 and 499, LGMB533 and 524, LGMB572 and 571, LGMB602 and 600 (Figure 3).


Δ Limit = 10
LGMB520
LGMB512
LGMB516
LGMB635
LGMB624
LGMB480
LGMB620
LGMB616
LGMB501
LGMB538
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LGMB612
LGMB519
LGMB613
LGMB611
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LGMB595
LGMB485
LGMB593
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LGMB530
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LGMB492
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LGMB515
LGMB521
LGMB633
LGMB599
LGMB509
LGMB552
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LGMB594
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LGMB577
LGMB517
LGMB486
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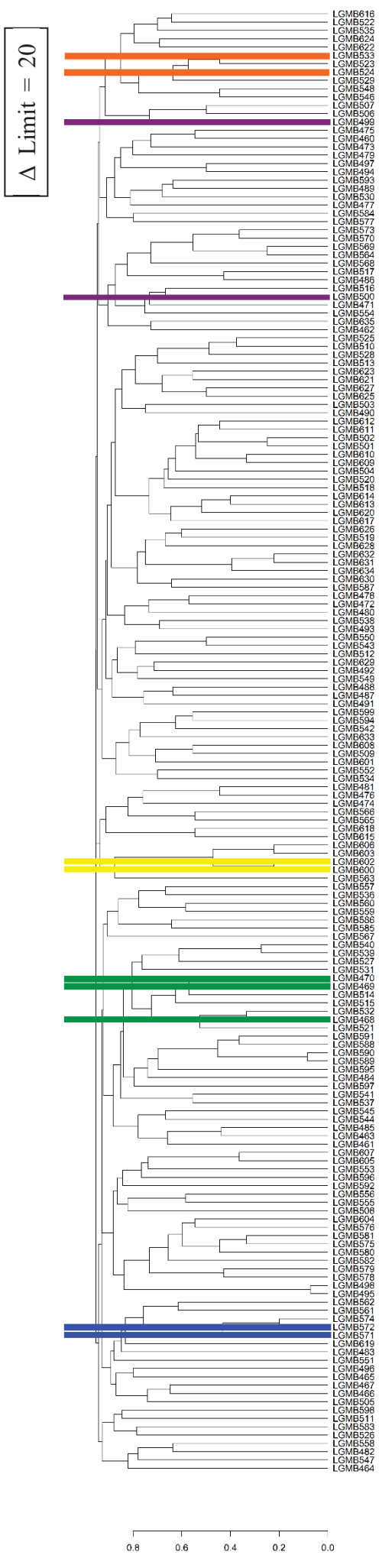
0.6
0.4
0.2
0.0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0.2
0.0

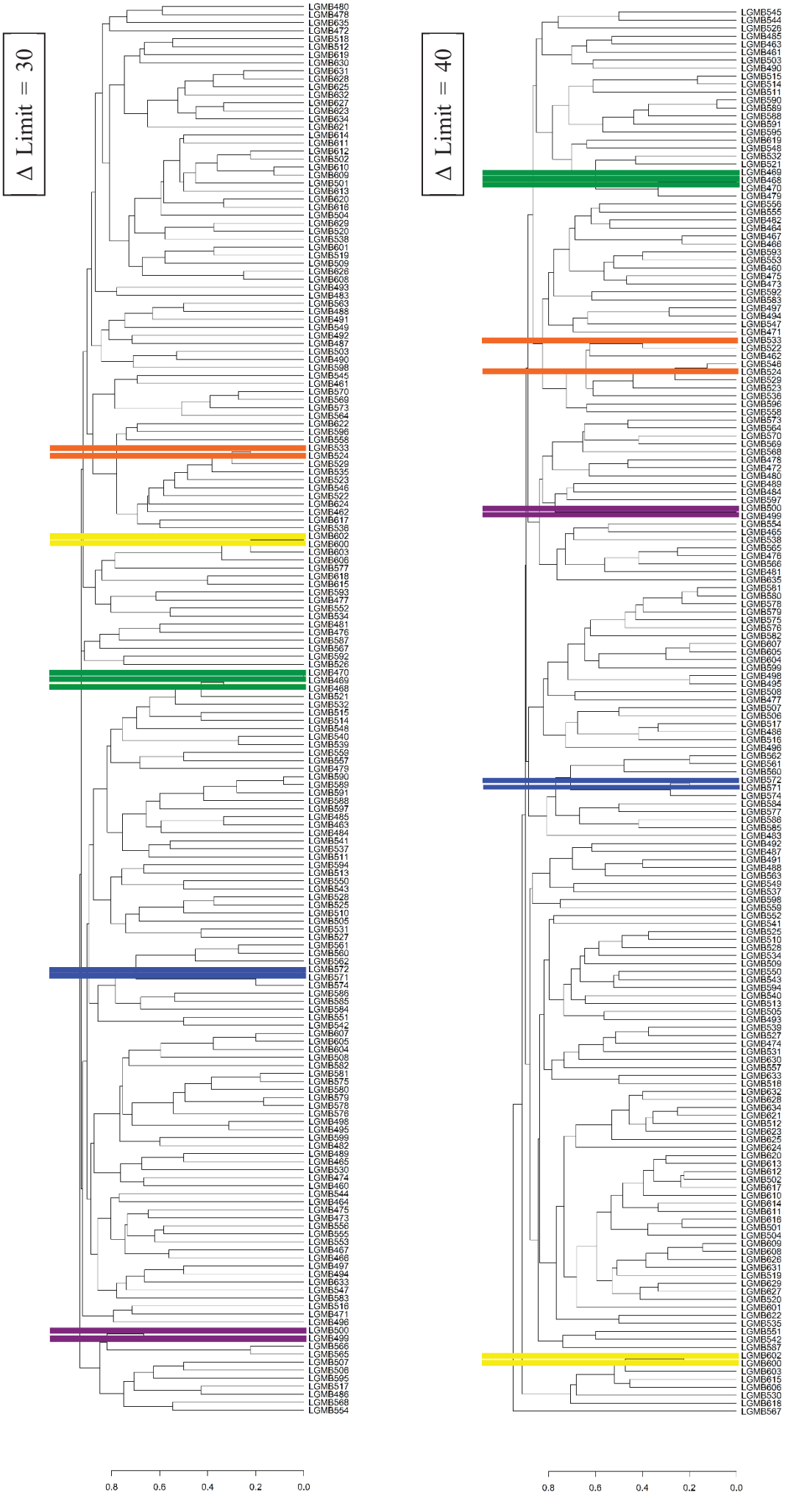
Δ Limit = 30
Δ Limit = 40
0.8
0.6
0.4
0.2
0.0

Figure 4. Distribution of the guide relations in the tested dendrograms. LGMB469, LGMB468 and LGMB470 isolates indicated by green, LGMB500 and LGMB499 indicated in violet, LGMB533 and LGMB524 indicated in orange, LGMB572 and LGMB571 indicated in blue, LGMB602 and LGMB600 indicated in yellow. In the rectangles on the left the limits used in each of the dendrograms are indicated.

Initially, the analysis was carried out with the limit value of 10 bp and it was verified how these relations were dispersed throughout the dendogram. As it is possible to see in the first dendrogram of Figure 4 that the relations LGMB500 and LGMB499, LGMB469, LGMB468 and LGMB470 are distant. The second dendrogram is the result of a limit of 20 bp and in this it is possible to verify the change in the position of some isolates, while the some isolates are still distant. In the dendrogram with a limit of 30 bp, all "guide" relations are established, whereas, in the dendrogram resulting from a 40 bp limit, LGMB533 and LGMB524 indicated in orange are become distant (Figure 4). In order to make the analysis as accurate as possible, the smaller limit capable of describing the guide relationships must be chosen. As a limit of 30 bp better describes the relationships between our isolates, used for comparison, we chose the dendrogram resulting from this limit (the complete dendogram resulting from this limit is shown in Figure 2 of the next article).

8 Conclusion and Future Perspectives

Here we presented a modificated methodology for BOX elements amplification present in the bacterial genome. Increasingly, scientists demand using such techniques that are cheaper, faster and require less labor from all areas around the world. The development of new methods opens new possibilities for success from different researches.

Author Contributions: FM and GMD: Perfomed the experiments, analysed the data and wrote the manuscript. DMA, DCS, VMK and LVGT: designed and supervised the experimental work. All authors read, revised and approved the manuscript.

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9 References

Bilung LM, Pui CF, Su'ut L, Apun K. Evaluation of BOX-PCR and ERIC-PCR as molecular tying tool for pathogenic *Leptospira. Disease Markers.* (2018).

Borba MP, Ballarini, AE, Witusk JPD, Lavin P, Sand SVD. Evaluation of BOX-PCR and REP-PCR as molecular typing tools for Antarctic *Streptomyces. Current Microbiology.* v.77, p.3573-3581 (2020).

Damnjanovic D, Harvey M, Bridge WJ. Applications of colony BOXA2R-PCR for the differentiation and identification of lactic acid COCCI. *Food Microbiology* 82: 277-286 (2019).

Faria G, Guimaraes RM, Carvalho ER, Sousa, DP. Método para a avaliação da atividade de isoenzimas em sementes de girassol. *Journal of Seed Science.* v.37, n.3, p211-217 (2015).

Hassan AA & Youssef MAH. Isolation and characterization of *Bacillus thuringiensis* bacteriophages using electron microscope and RAPD-PCR. *Journal of Agricultural Chemistry and Biotechnology*, *2*(12), 343–359 (2011).

Heras J, Domínguez C, Mata E *et al*. A survey of tools for analysing DNA fingerprints. *Briefings in bioinformatics* 17: 903-911 (2016).
Jaccard P. The distribution of the flora in the alpine zone. *New Phytologist*. v. 11, n. 2 (1912).

Kaschuk G, Hungria M, Santos JCP & Berton-Junior JF. Genetic diversity of rhizobia associated with common bean (*Phaseolus vulgaris* L.) grown under no-tillage and conventional systems in Southern Brazil. *Applied Soil Ecology.* v.32, n. 2, p. 210-220 (2006).

Koeuth T, Versalovic J, Lupski JR. Differential Subsequence Conservation of Interspersed Repetitive Streptococcus pneumoniae BOX Elements in Diverse Bacteria. *Genome Research*. 408-418 (1995).

Lee A & Wong E. Optimization and the Robustness of BOX A1R PCR for DNA Fingerprinting Using Trout Lake *E. coli* Isolates. *Journal of Experimental Microbiology and Immunology* 13: 104-113 (2009).

Makarenkov V,; Kevorkov D, Legendre P. Phylogenetic network construction approaches. *Applied Mycology and Biotechnology*. v. 6, p. 61-97 (2006).

Moura L, Pereira G, Costa S *et al*. Cancro do castanheiro na região do Minho: Estrutura populacional e variabilidade genética de populações de Cryphonectria parasitica. *Revista de Ciências Agrárias*, *38*(2), 238–247 (2015).

Packeiser H, Lim C, Balagurunathan B *et al.* An extremely simple and effective colony PCR procedure for bacteria, yeasts, and microalgae. *Applied Biochemistry and Biotechnology*, 169(2), p. 695–700 (2013).

Puentes-Téllez PE, Salles JF. Construction of effective minimal active microbial consortia for lignocellulose degradation. *Microbial Ecology*. v.76, p.419-429 (2018).
Raeder U and Broda P. Rapid preparation of DNA from filamentous fungi**.** *Letters in Applied Microbiology*., n. 1, p.17-20 (1985).

Rajeev M, Sushmitha TJ, Toleti SR & Pandian SK. Culture dependent and independent analysis and appraisal of earlystage biofilm-forming bacterial community composition in the Southern coastal seawater of India. *Science of the Total Environment*, *666*, 308–320 (2019).

They NH, Ferreira LMH, Marins LF & Abreu PC. Bacterial Community Composition and Physiological Shifts associated with the El Niño Southern Oscillation (ENSO) in the Patos Lagoon Estuary. *Microbial Ecology*, *69*(3), 525–534 (2015).

Wassermann, E., Montecchia, M. S. Garaventa, V. S., Correa, O. S., Romero, A. M. Virulence and pCM1 plasmid carriage are related to BOX-PCR fingerprint type in strains of *Clavibacter michiganensis* subsp. *michiganensis* that cause bacterial wilt and canker of tomato in Argentina. *Plant Pathology*. 69: 723-732 (2020).

Woodman, M. E. Direct PCR of intact bacteria (colony PCR). *Current Protocols in Microbiology*, (SUPPL. 9), 1–6 (2008).

Youssef MA, Mansour A and Solliman SS. Molecular Markers for New Promising Drought Tolerance Lines of Rice under Drought Stress via RAPD-PCR and ISSR Markers. *Journal of American Science*, 6(12) 355-363 (2010).

Zhu J. Comparison of Four Template Preparation Methods and Optimization of BOX A1R PCR for DNA Fingerprinting of Escherichia coli Isolates. *Journal of Experimental Microbiology and Immunology*. p. 153–160 (2010).

Zhu L, Xu H, Zhang Y, Fu G, Wu PQ, Li Y. BOX-PCR and PCR-DGE analysis for bacterial diversity of a naturally fermented functional food (Enzyme®). *Food Bioscience*. v.5, p115-122 (2014).

10 Supplementary Methods

Reagents

- Sodium chloride (NaCl) 1mol/L (Raeder & Broda, 1985)
- Autoclavaed ultrapure water
- Dissodium ethylenediamine tetraacetic acid (EDTA) 10 mM/L (Raeder & Broda, 1985)

Methods

Optimization of protocol for the extraction-free BOX-PCR

MET1. Zhu J. (2010) methodology

Several colonies were collected after growth in the YMA medium, to obtain a large amount of material, approximately 3 µL, which was diluted and homogenized in 500 µL of Sodium Chloride (NaCl) 1 mol/L. The preparation of the NaCl stock solution was carried out according to Raeder and Broda (1985). The samples were then centrifuged at 10000 xg for 5 minutes. The supernatant was discarded, and the pellet was resuspended and homogenized in 500 µL of 1 mol/L NaCl. The material was centrifuged at 10000 x*g* for 5 minutes. Again, the supernatant was discarded, and the resulting pellet was resuspended in 500 µL of ultrapure autoclaved water, continuing a new centrifugation at 10000 xg for 5 minutes. The supernatant was discarded, and the pellet was resuspended and homogenized in 20 µL of autoclaved ultrapure water. Of this sample, 1 µL of the supernatant was used in a 25 µL PCR reaction.

MET 2. Packeiser and collaborators (2013) methodology

From the growth of the bacteria in the YMA medium, a colony, approximately 1 µL was collected and resuspended in 20 µL of 10 mM EDTA. The samples were homogenized in vortex for 10 seconds and boiled for 5 minutes in a heating block. The material was centrifuged at 10000 xg for 5 minutes and 10 µL were transferred to a new tube, where they were diluted in 90 µL (1:10) of autoclaved ultrapure water. Of this sample, 1 µL of the supernatant was used in a 25 µL PCR.

MET 3. Combination of Zhu J. (2010) methodology and Packeiser *et al*. (2013) methodologies

One colony, approximately 1 µL was resuspended and homogenized in 500 µL of autoclaved ultrapure water, centrifuged at 10000 xg for 5 minutes and the supernatant was discarded, the material was resuspended in 20 µL of 10 mM EDTA, homogenized in vortex for 10 seconds and boiled for 5 minutes in a heating block, centrifuged at 10000 xg for 5 minutes and 10 µL were transferred to a new tube, where they were diluted in 90 µL (1:10) of autoclaved ultrapure water, 1 µL of the supernatant was used in a 25 µL PCR.

M2B. Packeiser and collaborators (2013) methodology with modification. The modification was the remove of a step of transferring 10 µL of the material to a new tube according to the methodology.

Amplification test independent of DNA concentration

Before performing extraction-free PCR was necessary to check whether the amount of DNA influences the band pattern of BOX-PCR or not. For this, a test was performed with the dilution of the DNA of an isolate of the genus *Bacillus* sp. belonging to the collection of the Laboratory of Microorganisms and Genetics located at Federal University of Paraná. After dilution, concentrations of 200 ng, 150 ng, 100 ng, 50 ng, 40 ng, 30 ng, 20 ng, and 1 ng of DNA were obtained. All concentrations of DNA were used in the BOX PCR reaction.

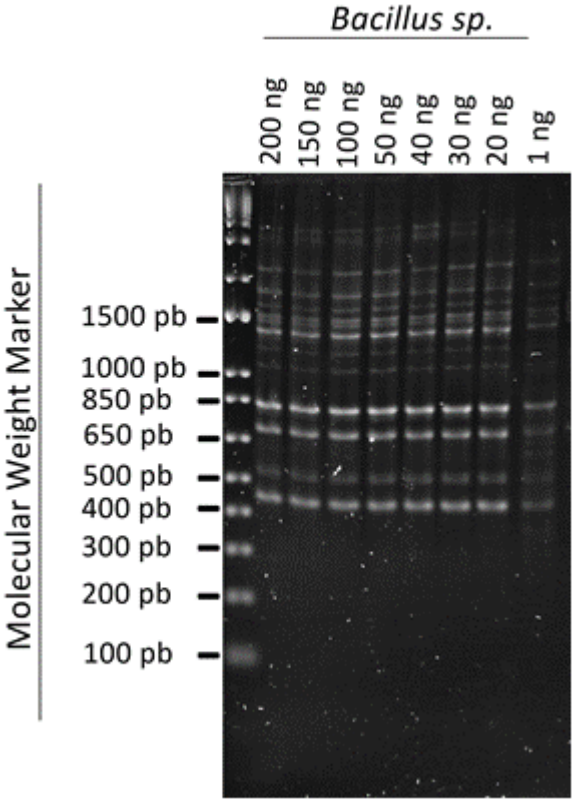

Figure 1. BOX PCR using DNA dilution from one *Bacillus* sp. isolated. The horizontal line represents the nanogram concentrations of DNA from the isolate and the vertical line represents the size of the bands of the 1 Kb Plus DNA ladder molecular weight marker (Manufacturer's Catalog: 10787018).

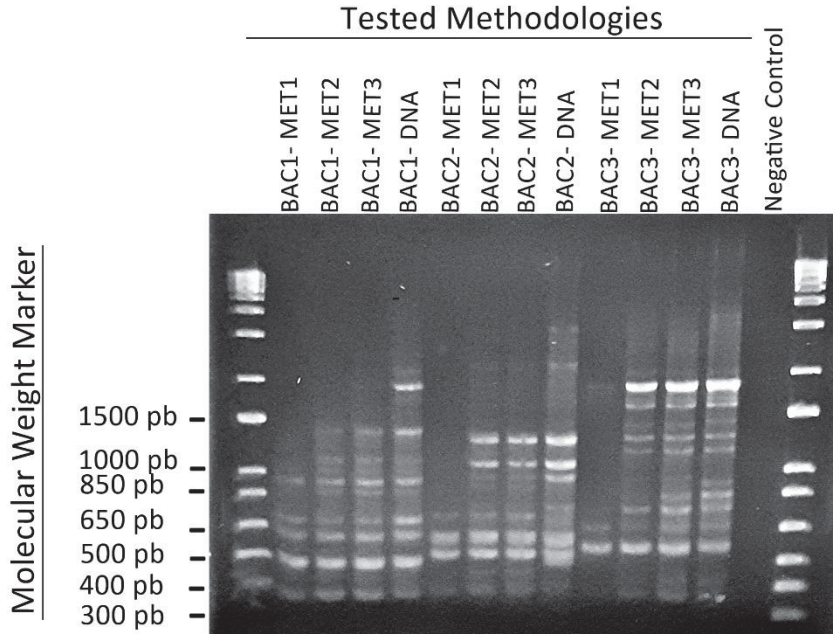

Figure 2. The three extraction-free PCR methodologies tested: MET1 (Zhu J. (2010) methodology), MET2 (Packeiser and collaborators (2013) methodology) and MET3 (Combination of Zhu J. (2010) methodology and Packeiser *et al*. (2013) methodologies). The horizontal line represents the amplification resulting from each methodology and the

vertical line represents the size of the bands of the 1 Kb Plus DNA ladder molecular weight marker (Manufacturer's Catalog: 10787018).

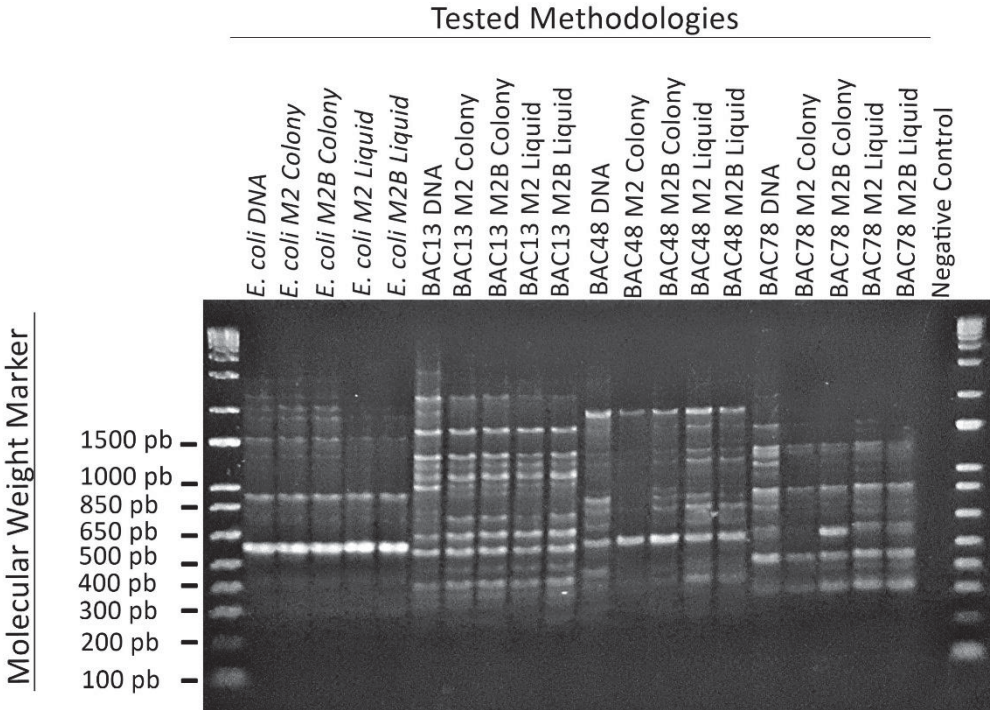

Figure 3. Amplification resulting from methodology of Packeiser et al. (2013) (M2) and methodology of Packeiser et al. (2013) modified (M2B). Liquid = Bacterial culture in liquid medium was used according to Zhu, J. (2010). Colony = Colonies from solid culture medium was used according to Packeiser et al. (2013). The horizontal line represents the amplification results from each methodology using the material in liquid media or solid media and the vertical line represents the size of the bands of the 1 Kb Plus DNA ladder molecular weight marker (Manufacturer's Catalog: 10787018).

11 Capítulo 3. Isolated bacteria from root nodules of *Phaseolus vulgaris* L.: identification and symbiotic efficiency

This paper will be submitted to Research in Microbiology (B1)

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Keywords: fixing biological nitrogen; rhizobia; common bean

1 Introduction

Dinitrogen (N_2) is the principal constituent of the atmosphere; this gas can be converted into ammonia (NH_3) by the industrial Haber-Bosch process or via Biological Nitrogen Fixation (BNF) [1]. The BNF is after photosynthesis, the most important biological process for the maintenance of natural ecosystems [2]. Some bacteria can reduce atmospheric nitrogen (N_2) to ammonia (NH_3) [3, 4], due to the presence of a minimum set of genes in their genome that encode the components of an enzyme complex called nitrogenase [5].

"Rhizobia" is a term used to describe bacteria, which belong to the genus Rhizobium or are phylogenetically related to it, capable of inducing the formation of nodules for carrying out the BNF in association with legumes [6]. Currently, rhizobia comprise a paraphyletic group with more than 200 species distributed in 18 genera of Alphaproteobacteria and Betaproteobacteria [7, 8]. Some of these genera are *Rhizobium*, *Ensifer* (previously *Sinorhizobium*)*, Mesorhizobium, Azorhizobium,* and *Bradyrhizobium* [3]. *Bradyrhizobium* is widely distributed, prevalent in tropical soils, and is probably the ancestral genus of all rhizobia described to date [9, 10]. The genus *Rhizobium* now has more than 90 described species, Gram-negative, non-sporulating, and aerobic [11].

In common bean (*Phaseolus vulgaris* L.) *Rhizobium* is the most frequently found genus in symbiosis with this legume, but other genera have already been isolated, such as Pseudomonas, Stenotrophomonas, Arthrobacter *Chryseobacterium*, *Enterobacter,* and

Agrobacterium [12, 13]. The symbiotic interaction between nitrogen-fixing bacteria and the common bean is essential since this legume is a primary protein source in many developing countries in South and Central America, Asia, and Africa [14, 15]. The common bean is a significant source of vitamins, minerals, carbohydrates, and fibers. This legume also has great importance for the economy, one of the three most cultivated legumes in the world [16].

Microbial inoculants are justified by their sustainability and an efficient alternative to providing nitrogen to plants, increasing food production without damaging the environment [17]. In this study we isolate, identified, and evaluated the potential for biological nitrogen fixation of bacteria present in nodules of two common bean cultivars.

12 Materials and Methods

Local characterization and soil analysis

The biological material was isolated from root nodules of common bean cultivars BRS Esteio and IPR Sabiá. The plants were 30-40 days old and were collected randomly from two private properties located in the city of Campo Largo, state of Paraná, Brazil (sampling site 1- 25º24’38’’S 49º29’38’’W and sampling site 2- 25º20’54’’S 49º30’33’’W). Soil samples were collected from the two collecting spots from the topsoil layer (0-20 cm depth). The samples were analyzed by Laborsolo (Curitiba, PR) for the parameters of pH, the content of phosphorus, potassium, calcium, magnesium, sulphur, manganese, copper, iron, aluminium, and organic matter.

Isolation of strains and morphophysiological features

All the nodules were extracted for each plant collected, washed with distilled water, and surface sterilized with 99% ethanol for 1 min, 3% chlorine hypochlorite for 3 min, followed by subsequent washing (eight times) with distilled water [18]. Surface-sterilized nodules were individually crushed, and the nodule juice was streaked onto Yeast, Manitol Agar- rose bengal (YMA) medium plate. Plates were incubated at 28º C for 4-5 days. The bacteria that grew in these plates were purified by repeated subculturing of single colonies. Each purified colony was cultured in YMA- congo red and YMA-bromothymol blue (0.5%) at 28ºC for 5 days. Morphological features of colony size,

pigmentation, consistency, and mucosity were evaluated. Gram analysis was also performed.

Extraction-free BOX-PCR and Cluster analyses

The extraction-free BOX-PCR and de the cluster analyses were conducted according to Lima et al. (2021) (unpublished data). Diversity was calculated by using the Shannon index ($H' = -\sum[\left(\frac{n1}{N}\right) ln \left(\frac{n1}{N}\right)]$) [19]. Grouping for BOX-PCR was obtained by considering a level of 80% dissimilarity.

Partial sequencing of 16S rDNA for genera identification

One representant of each group identified in the BOX-PCR had the DNA extracted as described by Raeder and Broda [20]. A fragment of about 1.5 kb of the 16S rDNA gene was amplified by PCR with universal primers fD1 and rD1 as described by Weisburg et al. [21], and the PCR conditions were described by Menna et al. [22]. The amplified fragments were observed in agarose gel 1% stained with ethidium bromide, visualized under UV radiation. The PCR products were sent to GoGenetic (Curitiba, Brazil) and sequenced by the Sanger methodology.

Phylogenetic analysis

The 16S rDNA sequences obtained in this study were edited in the BioEdit software version 7.2.5 and blasted in NCBI [23]. The 16S rDNA sequences of the reference species were downloaded from the LPSN database [24]. The sequences were globally aligned in the MAFFT online version 7 [25]. The global alignments were viewed in MEGA X 10 [26]. Bayesian phylogenetic inference trees were generated using the MrBayes software (3.2.7a) in the CIPRES Science Gateway (www.phylo.org/). Four tree was generated, *Herbaspirillum* species tree (Betaproteobacteria), *Rhizobium* species tree (Alphaproteobacteria), *Bacillus* species tree (Terrabacteria group), and *Pseudomonas* and *Enterobacter* species tree (Gammaproteobacteria). The external group for each of the phylogenetic trees was chosen after an analysis using the Seqmatch of Ribosomal Database Project (rdp.cme.msu.edu/). The trees were edited in the FigTree version 1.4.3.

Greenhouse experiment

The study of symbiotic properties (nodulation and nitrogen uptake) was carried out in sterilized Leonard jar assemblies containing 1:2 sterilized sand and vermiculite. The jars were arranged in a complete randomized design with four replicates *P. vulgaris* seed (cv. IPR Sabiá) were surface sterilized for 3 min in 98% ethanol, 1 min in 3% chlorine hypochlorite, and rinsed three times in distilled sterile water [18]. The seed was previously pre-germinated in germitest paper, for three days under 28ºC and a photoperiod of 14 hours.

Two healthy germinated seeds were planted in each jar. Each seed was inoculated with 1 mL of a log phase strain culture (10^6 CFU/mL). The treatments comprised one strain, selected randomly, from each group identified in the BOX-PCR analysis, and four controls, composed of two commercial *Rhizobium* strains (*R. freirei* PRF81^T and *R. tropici* CIAT899) and one without inoculation and nitrogen application (using urea-46%N) and the last treatment was composed without inoculation and without nitrogen application. Routine management practices were carried out by adding a nutritive solution [27] in the Leonard jars until the termination of the experiment.

The plants were harvest for 35 days, and the roots were carefully washed to remove sand and vermiculite particles, after which nodules from each jar were removed and counted. The plants were separated into the roots and the aerial parts using a clean scissor. Nodules, roots, and aerial parts from each jar were put in a pre-labeled separate paper envelope and dried at 60ºC for 48 h in oven dry and weighted on sensitive balance. The aerial part was then sent to ITL Laboratories (Londrina, Brazil) to quantify nitrogen uptake.

The data (number of nodules, dry mass of roots, aerial part, and nitrogen content) were subjected to Analysis of Variance (ANOVA). Post-hoc Tukey test was applied when adequated with $p \leq 0.05$ probability level.

13 Results and Discussion

Local characterization and soil analysis

The collection of cv. BRS Esteio was carried out on November 14, 2019, while the collection of cv. IPR Sábia was carried out on February 10, 2020. Collection's areas are located on the map in Figure 1. The total number of plants collected by collecting area and cultivar is present in Table 1, and the results of the chemical analysis of the soil samples are listed in Table 2. Of the analyzed chemical parameters, the soil pH is the most important since this parameter was used to compare and discuss the groups formed by the extraction-free BOX PCR.

Table 1. Collecting area, soil sample, number of plants and cultivar collected in Campo Largo – PR

Collecting area	Soil Sample	Plants collected	Cultivar
1	1	5	BRS Esteio
1	2	8	BRS Esteio
2	1	7	IPR Sábia
1	2	6	IPR Sábia

Table 2. Chemical analysis results of each soil sample taken from regions of P. vulgaris L. cultivars collecting.

Chemical analysis	Collection area 1 Soil sample 1	Collection area 1 Soil sample 2	Collection area 2 Soil sample 1
pH in H_2O	5.24	5.84	5.77
Phosphorus	18.77 mg/dm³	34.59 mg/dm³	6.87 mg/dm³
Potassium	0.66 cmolc/dm³	0.54 cmolc/dm³	0.69 cmolc/dm³
Calcium	4.62 cmolc/dm³	8.09 cmolc/dm³	6.81 cmolc/dm³
Magnesium	2.88 cmolc/dm³	2.91 cmolc/dm³	3.81 cmolc/dm³
Sulfur	1.48%	716%	210%
Manganese	11.49 mg/dm³	29.72 mg/dm³	21.35 mg/dm³
Copper	1.12 mg/dm³	4.01 mg/dm³	1.05 mg/dm³
Iron	162.94 mg/dm³	157.05 mg/dm³	203.30 mg/dm³

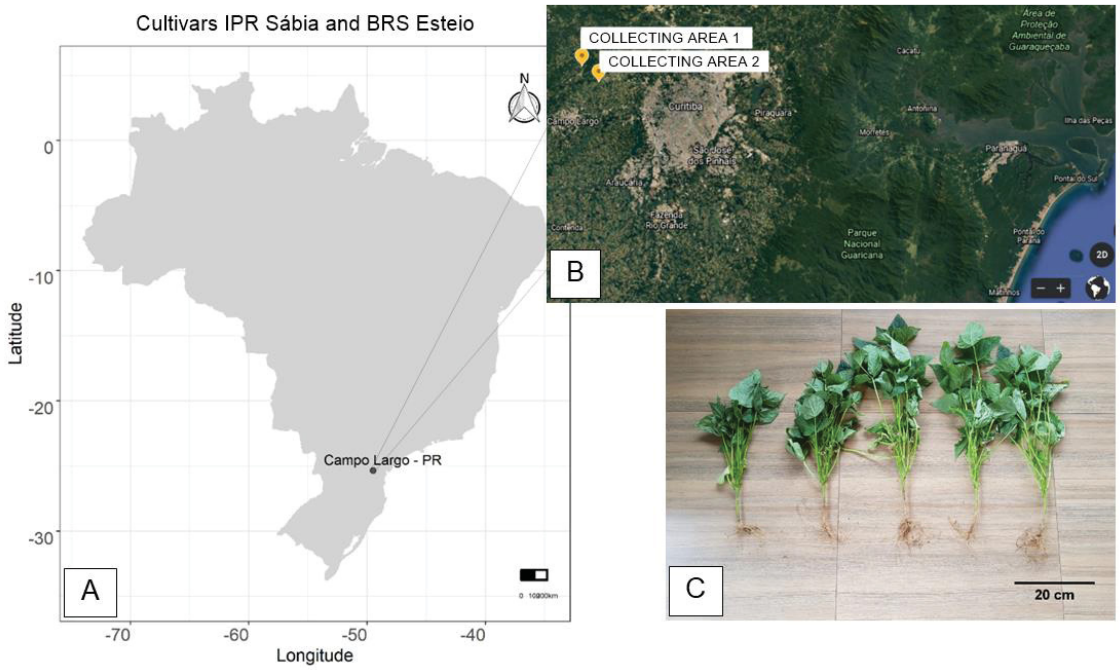


Figure 1. Collecting areas for the IPR Sábia and BRS Esteio cultivars A) Map showing the location of the Municipality of Campo Largo – PR B) Google Earth image with the location of private rural properties (in yellow), COLLECTING AREA 1: 25º24'38"S 49º29'38"W and COLLECTING AREA 2: 25º20'54"S 49º30'33"W C) *Phaseolus vulgaris* L. samples collected.

All plants were grown at acidic pH. An increase in soil acidity can result in a reduction of the intrinsic activity of the microbial community by several routes, including a reduction in the production of exudates by the plants; inhibition of certain microorganisms such as nitrifiers, thus decreasing the diversity of the microbial community; and increased aluminium (Al) in the soil, which increases results in reduced substrate bioavailability and toxicity [28].

Several studies have already reported that isolates of the Rhizobium genus have greater growth in culture media (YMA) with neutral pH and that in extreme variations (pH 4.0 and pH 9.0) there is a decrease in the number of cells or even no bacterial growth [29, 30]. Despite this pH tolerance range of the culture medium, the isolation of acid pH tolerant strains is already described in the literature, such as the strains currently used in Brazil as commercial inoculants, namely *Rhizobium tropici* CIAT 899 [31] and *Rhizobium freirei* PRF 81, the latter was isolated from acidic soil (pH 3.5-5.4) and can grow in a culture medium with pH 4.0 [32].

Isolation of strains and morphophysiological features

A hundred and seventy-six bacteria were isolated from the nodules of the cultivars BRS Esteio and IPR Sábia. Each of the isolates received a code from the collection belonging to the Genetics of Microorganisms Laboratory located in the Genetics Department of the Federal University of Paraná. This code consists of the acronym LGMB followed by a number between 460 and 635.

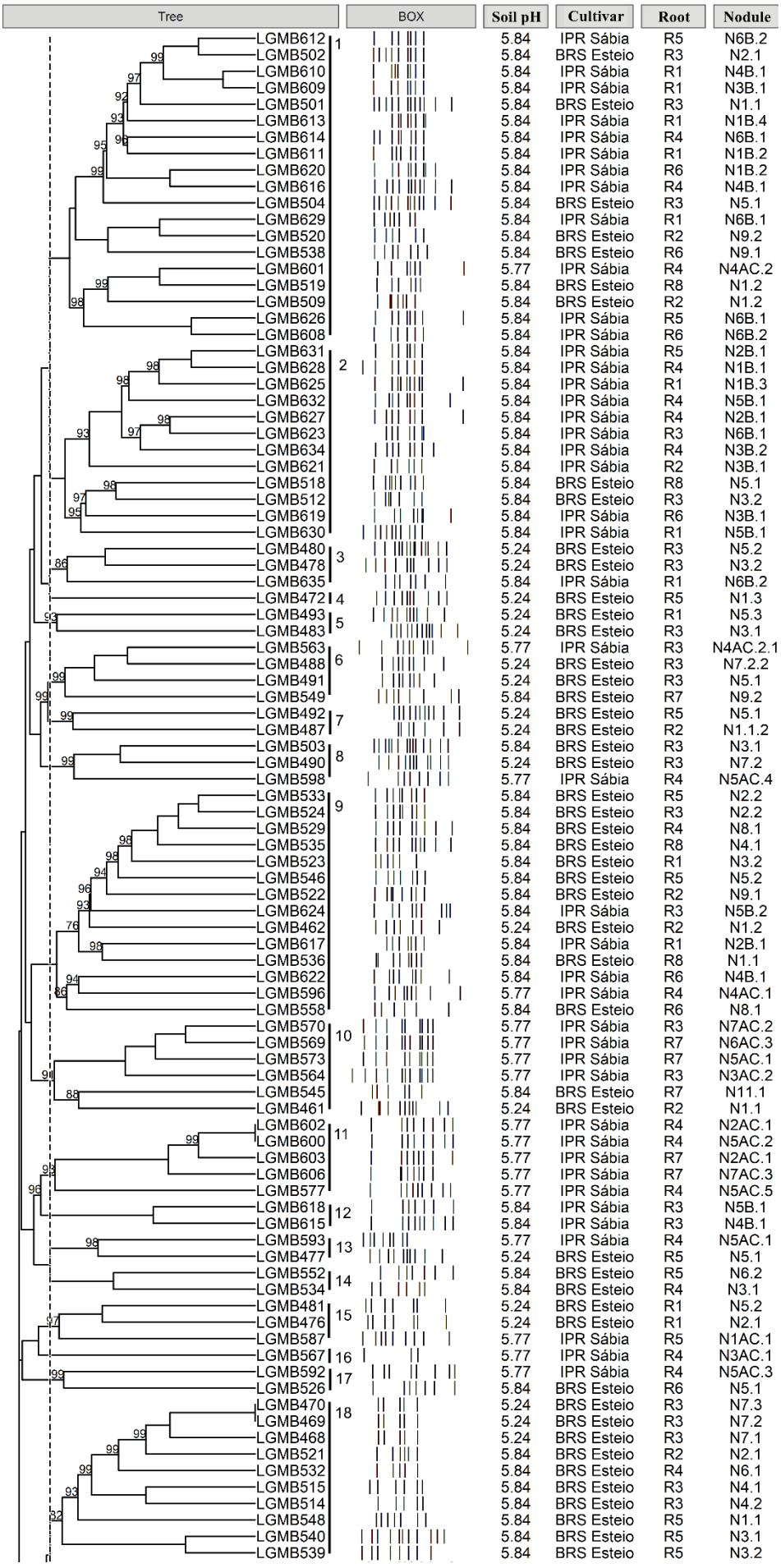
Regarding the morphophysiological characteristics, in YMA Bengal rose dye, all isolates showed pink color, smooth surface, opaque transparency, size ranging from 0.5 mm to 1.2 centimeters after 4-5 days of growth, circular (> 1 mm), and punctiform (≤1 mm), entire border, convex or flattened elevation, and some degree of mucus. All isolates acidified the culture medium with the addition of Bromothymol Blue and did not absorb the Congo red dye. The forms observed by the Gram technique varied amongst gram-negative bacillus, gram-negative coccobacillus, and gram-negative cocci.

The exceptions were the isolates LGMB499 and LGMB500 since they have a smooth surface but with a dry aspect instead of mucous and absorb the Congo red dye. Three isolates basified the medium with Bromothymol Blue, being the LGMB567, LGMB587, and LGMB593. Two isolates maintained the medium's pH with Bromothymol Blue close to neutral, being the isolate LGMB590 and LGMB592. The isolates LGMB567, LGMB587, and LGMB593 also had small and slightly orange colonies.

In general, the data obtained in this morphophysiological characterization corroborate the data obtained by several other studies that involve the identification of *P. vulgaris* L. nodulating bacteria [33, 34, 35, 36, 37, and 38].

Extraction-free BOX-PCR and Cluster analyses

The isolates presented bands of different sizes, but the most frequent were those located between 1500 - 200 bp. The cut-off point at 80% dissimilarity resulted in 33 groups (Figure 2).



Tree	BOX	Soil pH	Cultivar	Root	Nodule
LGMB612	1	5.84	IPR Sábia	R5	N6B.2
LGMB502		5.84	BRS Esteio	R3	N2.1
LGMB610		5.84	IPR Sábia	R1	N4B.1
LGMB609		5.84	IPR Sábia	R1	N3B.1
LGMB501		5.84	BRS Esteio	R3	N1.1
LGMB613		5.84	IPR Sábia	R1	N1B.4
LGMB614		5.84	IPR Sábia	R4	N6B.1
LGMB611		5.84	IPR Sábia	R1	N1B.2
LGMB620		5.84	IPR Sábia	R6	N1B.2
LGMB616		5.84	IPR Sábia	R4	N4B.1
LGMB504		5.84	BRS Esteio	R3	N5.1
LGMB629		5.84	IPR Sábia	R1	N6B.1
LGMB520		5.84	BRS Esteio	R2	N9.2
LGMB538		5.84	BRS Esteio	R6	N9.1
LGMB601		5.77	IPR Sábia	R4	N4AC.2
LGMB519		5.84	BRS Esteio	R8	N1.2
LGMB509		5.84	BRS Esteio	R2	N1.2
LGMB626		5.84	IPR Sábia	R5	N6B.1
LGMB608		5.84	IPR Sábia	R6	N6B.2
LGMB631		5.84	IPR Sábia	R5	N2B.1
LGMB628	2	5.84	IPR Sábia	R4	N1B.1
LGMB625		5.84	IPR Sábia	R1	N1B.3
LGMB632		5.84	IPR Sábia	R4	N5B.1
LGMB627		5.84	IPR Sábia	R4	N2B.1
LGMB623		5.84	IPR Sábia	R3	N6B.1
LGMB634		5.84	IPR Sábia	R4	N3B.2
LGMB621		5.84	IPR Sábia	R2	N3B.1
LGMB518		5.84	BRS Esteio	R8	N5.1
LGMB512		5.84	BRS Esteio	R3	N3.2
LGMB619		5.84	IPR Sábia	R6	N3B.1
LGMB630		5.84	IPR Sábia	R1	N5B.1
LGMB480	3	5.24	BRS Esteio	R3	N5.2
LGMB478		5.24	BRS Esteio	R3	N3.2
LGMB635		5.84	IPR Sábia	R1	N6B.2
LGMB472	4	5.24	BRS Esteio	R5	N1.3
LGMB493	5	5.24	BRS Esteio	R1	N5.3
LGMB483		5.24	BRS Esteio	R3	N3.1
LGMB563		5.77	IPR Sábia	R3	N4AC.2.1
LGMB488	6	5.24	BRS Esteio	R3	N7.2.2
LGMB491		5.24	BRS Esteio	R3	N5.1
LGMB549		5.84	BRS Esteio	R7	N9.2
LGMB492	7	5.24	BRS Esteio	R5	N5.1
LGMB487		5.24	BRS Esteio	R2	N1.1.2
LGMB503	8	5.84	BRS Esteio	R3	N3.1
LGMB490		5.24	BRS Esteio	R3	N7.2
LGMB598		5.77	IPR Sábia	R4	N5AC.4
LGMB533	9	5.84	BRS Esteio	R5	N2.2
LGMB524		5.84	BRS Esteio	R3	N2.2
LGMB529		5.84	BRS Esteio	R4	N8.1
LGMB535		5.84	BRS Esteio	R8	N4.1
LGMB523		5.84	BRS Esteio	R1	N3.2
LGMB546		5.84	BRS Esteio	R5	N5.2
LGMB522		5.84	BRS Esteio	R2	N9.1
LGMB624		5.84	IPR Sábia	R3	N5B.2
LGMB462		5.24	BRS Esteio	R2	N1.2
LGMB617		5.84	IPR Sábia	R1	N2B.1
LGMB536		5.84	BRS Esteio	R8	N1.1
LGMB622		5.84	IPR Sábia	R6	N4B.1
LGMB596		5.77	IPR Sábia	R4	N4AC.1
LGMB558		5.84	BRS Esteio	R6	N8.1
LGMB570	10	5.77	IPR Sábia	R3	N7AC.2
LGMB569		5.77	IPR Sábia	R7	N6AC.3
LGMB573		5.77	IPR Sábia	R7	N5AC.1
LGMB564		5.77	IPR Sábia	R3	N3AC.2
LGMB545		5.84	BRS Esteio	R7	N11.1
LGMB461		5.24	BRS Esteio	R2	N1.1
LGMB602	11	5.77	IPR Sábia	R4	N2AC.1
LGMB600		5.77	IPR Sábia	R4	N5AC.2
LGMB603		5.77	IPR Sábia	R7	N2AC.1
LGMB606		5.77	IPR Sábia	R7	N7AC.3
LGMB577		5.77	IPR Sábia	R4	N5AC.5
LGMB618	12	5.84	IPR Sábia	R3	N5B.1
LGMB615		5.84	IPR Sábia	R3	N4B.1
LGMB593	13	5.77	IPR Sábia	R4	N5AC.1
LGMB477		5.24	BRS Esteio	R5	N5.1
LGMB552	14	5.84	BRS Esteio	R5	N6.2
LGMB534		5.84	BRS Esteio	R4	N3.1
LGMB481	15	5.24	BRS Esteio	R1	N5.2
LGMB476		5.24	BRS Esteio	R1	N2.1
LGMB587		5.77	IPR Sábia	R5	N1AC.1
LGMB567	16	5.77	IPR Sábia	R4	N3AC.1
LGMB592	17	5.77	IPR Sábia	R4	N5AC.3
LGMB526		5.84	BRS Esteio	R6	N5.1
LGMB470	18	5.24	BRS Esteio	R3	N7.3
LGMB469		5.24	BRS Esteio	R3	N7.2
LGMB468		5.24	BRS Esteio	R3	N7.1
LGMB521		5.84	BRS Esteio	R2	N2.1
LGMB532		5.84	BRS Esteio	R4	N6.1
LGMB515		5.84	BRS Esteio	R3	N4.1
LGMB514		5.84	BRS Esteio	R3	N4.2
LGMB548		5.84	BRS Esteio	R5	N1.1
LGMB540		5.84	BRS Esteio	R5	N3.1
LGMB539		5.84	BRS Esteio	R5	N3.2

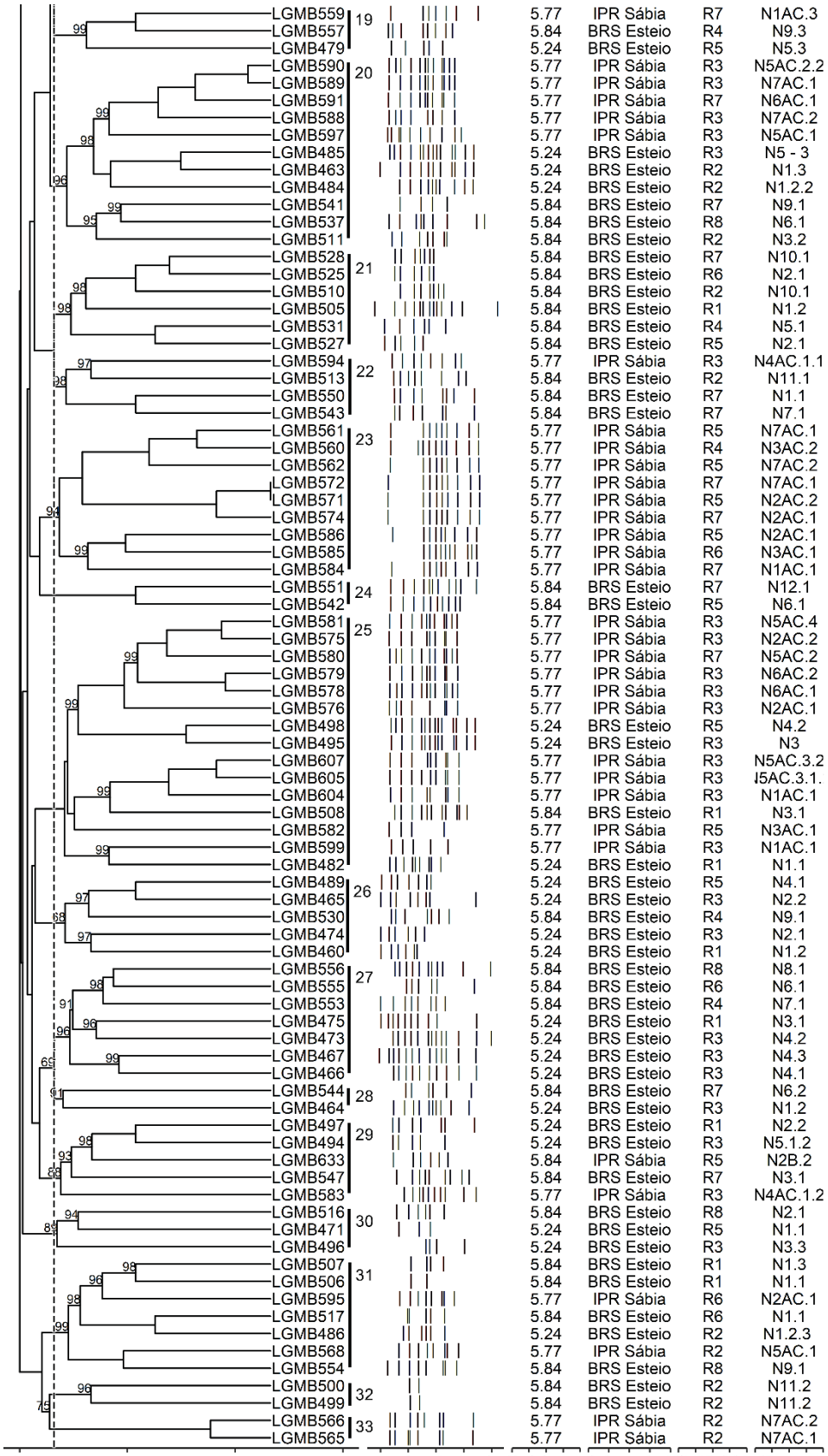


Figure 2. Dendrogram representing the relationships between bacterial isolates. The 33 groups were separated based on 80% dissimilarity indicated by the dotted line. The numbers present between the branches refer to the support values calculated by the

bootstrap with 1000 replicons. The BOX patterns' right-hand side are the soil's pH values, and the data referring to the cultivar, root, and nodules from which the strains were isolated. R = root, N = nodule (for example N4.2 = isolated 2, node 4), AC and B are cv. IPR Sábia codes.

Table 3. Shannon's Diversity Index calculated by cultivar and by pH of the soil from which the cultivar was collected. The values in italics are the highest values found.

Cultivar	Shannon Diversity Index
IPR Sábia Total	1.5137
IPR Sábia pH 5.77	1.0385
IPR Sábia pH 5.84	0.5152
BRS Esteio Total	*2.0078*
BRS Esteio pH 5.84	*1.3152*
BRS Esteio pH 5.24	0.9714

After the formation of the groups, the Shannon Diversity Index was calculated by cultivar and by soil pH. The highest index was obtained by cv. BRS Esteio and by the same cultivar in soil with pH 5.84 (Table 3). Analyzing the guide relations in the dendrogram, it is possible to observe that LGMB499 and LGMB500 (group 32), share all the information, therefore, they were isolated from the root 2 / nodule 11 of the cv. BRS Esteio in soil with pH 5.84. Their origin can also explain the relationships between the LGMB468, LGMB469, and LGMB470 (group 18) since they were all isolated from the root 3 / nodule 7 of the cv. BRS Esteio in soil with pH 5.24. The clade formed by LGMB533 and LGMB524, located in group nine, can be partly explained by these characteristics, as both differ only by the root and consequently the nodule from which they were isolated.

LGMB572 and LGMB571 (group 23) share characteristics of soil, cultivar, but were not isolated from the same root, while LGMB600 and LGMB602 (group 11) share all characteristics, except the nodule from which they were isolated. Most of the relationships described above can be explained by the isolate's origin, although some do not share all the information, mainly root and nodule. Therefore, according to these data, close isolates can inhabit different roots or nodules of the same cultivar.

Analyzing the position of the isolates that differed from the majority in the morphophysiological characterization, the relationship between LGMB499 and LGMB500 is recovered, as already mentioned above. LGMB567, LGMB587, and LGMB593 have band patterns that differ from each other, but still, LGMB567 and LGMB587 are part of the same clade groups fifteen and sixteen. LGMB590 and LGMB592 are in different groups, twenty and seventeen, respectively.

The most recent isolation and identification of diazotrophic bacteria researches are performing polyphasic taxonomy. The isolates' identification takes into account the degree of phenotypic similarity (morphological, physiological, and biochemical) and genotypic characteristics with those described for the type strain. One of these researches was carried out by Chibeba and collaborators [39], who isolated 29 bacteria from *Phaseolus lunatus* (lima bean) nodules. The isolates were grouped in two ways, one considering the phenotypic characteristics and the other based on the results obtained from BOX PCR's genetic characterization. As a result, described by the authors, the phenotypic groups were not fully recovered in the dendrogram resulting from BOX PCR; however, some pairs of isolates formed the same clade in both dendrograms.

Grange and Hungria [40] obtained similar results. The authors verified the genetic diversity of indigenous bacteria isolated from *Phaseolus vulgaris* L. collected in different Brazilian ecosystems, these belonging to the states of Pernambuco (neutral soil) and Paraná (acid soil). The cultivars Aporé and Negro Angel were used as bait plants, 138 and 105 bacteria were isolated from the cultivars, respectively. The isolates were submitted to ERIC PCR and grouped into 34 groups. When clusters were analyzed based on soil and cultivar characteristics, the authors noted that many of the clusters included strains isolated from the same soil type (neutral or acidic), or isolated from the same cultivar (Aporé or Negro Angel) or even same clusters formed between strains from the same cultivar collected in the same type of soil. However, other groupings did not share these characteristics. Using the 16S rDNA gene sequencing, the authors concluded that at least three genera were identified among the isolates, being *Rhizobium*, *Sinorhizobium* e *Mesorhizobium*.

Another research that corroborates with our results is Oliveira et al. [41]. The authors' isolated bacteria from nodules collected from four cultivars representing the two diversification centers of *P. vulgaris* L., being the Mesoamerican – cv Diamante Negro

and cv Ouro Negro, and the Andean - cv Jalo Precoce and Goiano Precoce. The isolates were analyzed separately in two dendrograms, one with Mesoamerican cultivars and the other with Andean cultivars. Nevertheless, in the dendrogram clades, there is a greater presence of strains isolated from the same cultivar. The authors concluded that the diversity of isolates is dependent, in addition to abiotic factors such as soil acidity, of the cultivar origin used as a bait plant, since the Mesoamerican genotypes captured a greater diversity than the Andean genotypes. The Shannon's Diversity Index (Table 3) also points to a differential diversity capture between both cultivars IPR Sábia and BRS Esteio, and between the soils from which they were collected.

Phaseolus is considered a promiscuous genus; that is, legumes belonging to the genus have the capacity to be nodulated by several symbiovars. This characteristic of *P. vulgaris* may be the result of propagation, from the centers of origin and domestication, of the microsimbionts present within its seeds, which when in contact with local strains, carried out the vertical transfer of symbiotic genes [18, 40].

Phylogenetic analysis and Greenhouse experiment

The 33 isolates randomly selected were identified as belonging to 5 different genera (Table 4), being that the most predominant genus in this research was the *Rhizobium*. The position of these isolates in a phylogenetic tree can be seen in the Figure 3, 4, 5 and 6.

Table 4. Bacterial isolated and their respective genera identified by partial sequencing of the 16S rDNA gene.

Genus	Bacterial isolated
Rhizobium	LGMB461, LGMB472, LGMB474, LGMB482, LGMB483, LGMB487, LGMB491, LGMB551, LGMB521, LGMB552, LGMB555, LGMB557, LGMB566, LGMB585, LGMB588, LGMB596, LGMB603, LGMB614 and LGMB619
Bacillus	LGMB476 and LGMB477
Pseudomonas	LGMB490 and LGMB583
Enterobacter	LGMB496, LGMB517, LGMB528, LGMB543, LGMB554, LGMB592, LGMB618 and LGMB635
Herbaspirillum	LGMB499
Unidentified	LGMB567

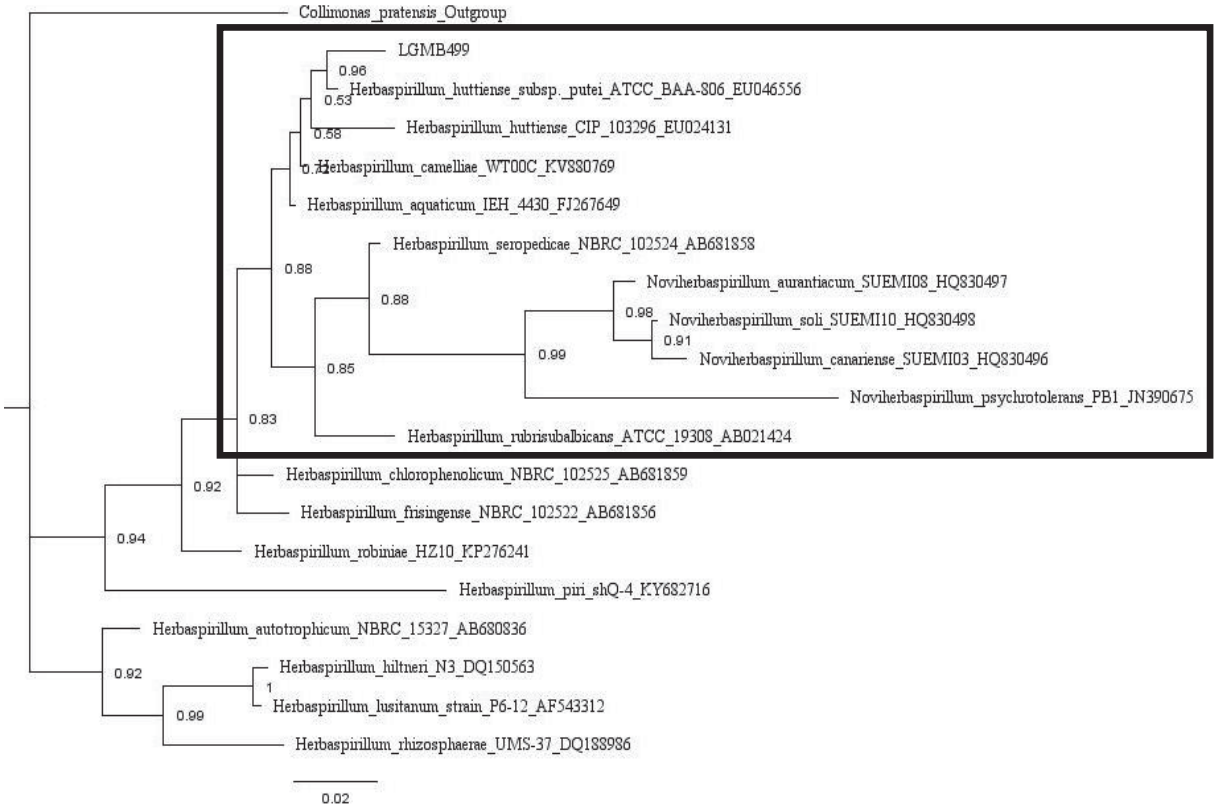


Figure 3. Bayesian inference tree of *Herbaspirillum* species (Betaproteobacteria) based on the 16S rDNA gene. The species *Collimonas pratensis* was used as outgroup. Values on the node indicate the support value of the branches. In the black rectangle the isolate of interest is highlighted.

Figure 4. Bayesian inference tree of *Bacillus* species (Terrabacteria group) based on the 16S rDNA gene. The species *Lederbergia lenta* was used as outgroup. Values on the node indicate the support value of the branches. In the black rectangles are the isolates of interest highlighted.

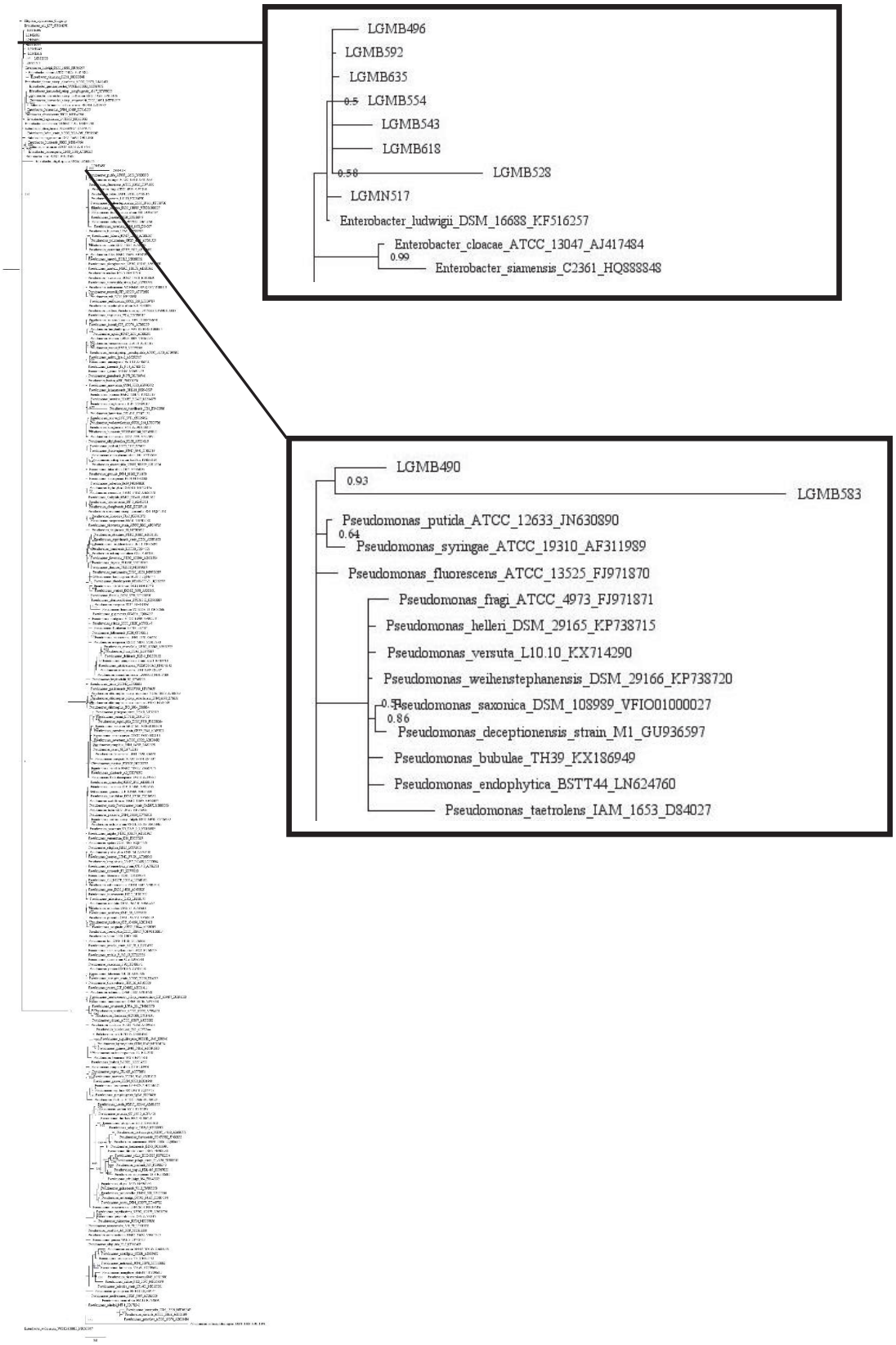


Figure 5. Bayesian inference tree of *Pseudomonas* and *Enterobacter* species (Gammaproteobacteria) based on the 16S rDNA gene. The species *Kluyvera cryocrescens* was used as outgroup. Values on the node indicate the support value of the branches. In the black rectangles are the isolates of interest highlighted.

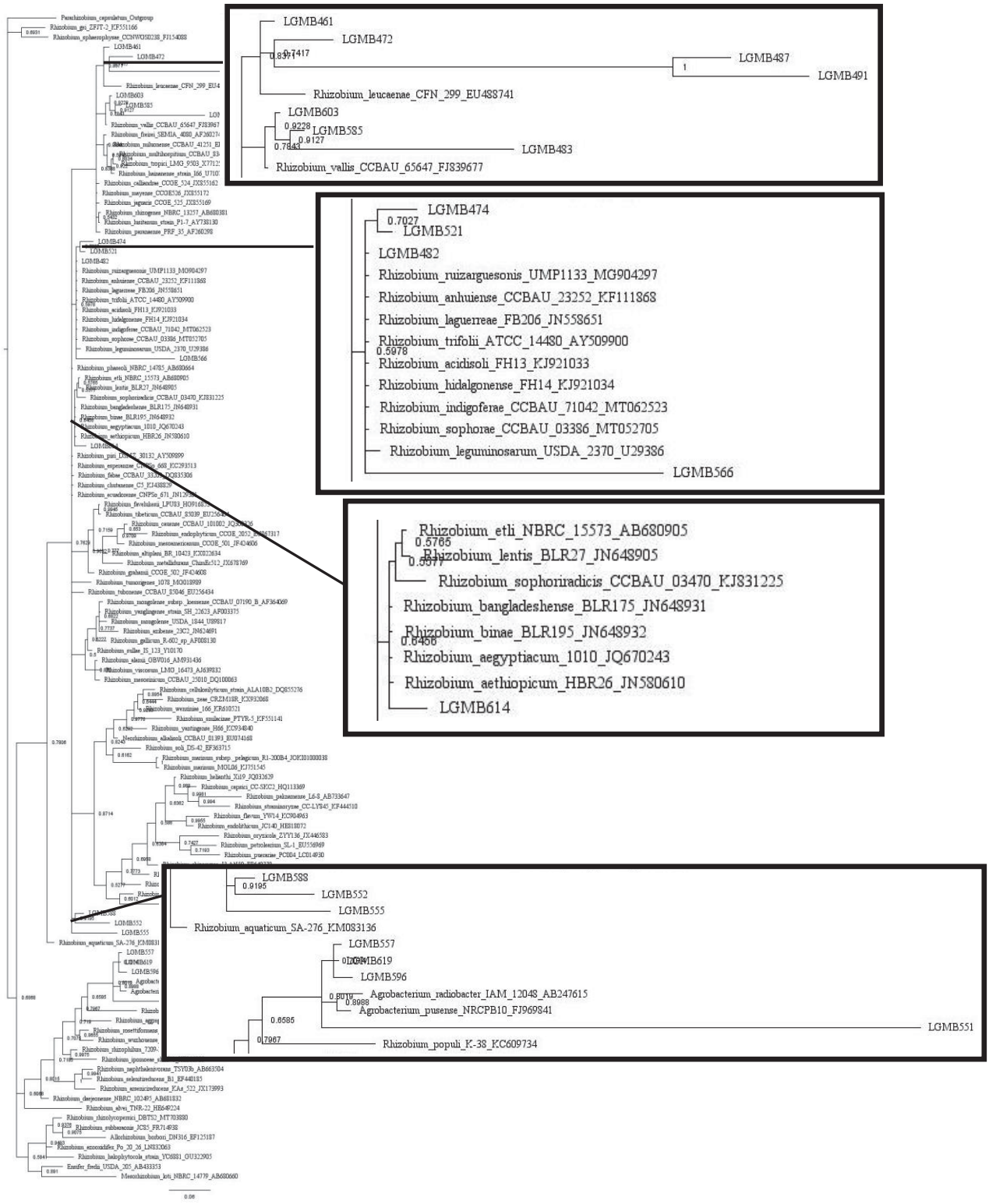


Figure 6. Bayesian inference tree of *Rhizobium* species (Alphaproteobacteria) based on the 16S rDNA gene. The species *Pararhizobium capsulatum* was used as outgroup. Values on the node indicate the support value of the branches. In the black rectangles are the isolates of interest highlighted.

The literature already described that coresident bacterial genera inhabit common bean nodules; some of these have species that are opportunistic pathogens in plants, animals ans humans, for example, *Enterobater* and *Pseudomonas*. *Enterobacter* is found in a variety of environmental habits, has approximately 22 species, and is a member of a group called ESKAPE; this group contains the major resistant bacterial pathogens [42]. *Pseudomonas* species were isolated from different locations, but the soil is the major source, with approximately 30 novel species of this genus isolated from this environment [43]. Some *Pseudomonas* species are human pathogens, as Pseudomonas aeruginosa also has evolved antimicrobial resistance in recent years [44]. Although *Enterobacter* and *Pseudomonas* are genera with pathogenic species, they are also composed of endophytic species.

Enterobacter cloacae is a growth-promoting bacterium from citrus and maize plants [45, 46], *Enterobacter asburiae* is a growth promoter from sweet potato [47] the strain *Enterobacter sp*. J49 is a potential biofertilizer of peanut and maize because it contributes to phosphorus content in plant tissues and soil and promotes these plants' growth [48]. Recent research to describe the results of co-inoculations has shown that strains of *Pseudomonas* (*Pseudomonas brassicacearum* subsp.) when co-inoculated with *Rhizobium*, it induces nodulation superiority, probably due to the great potential for plant growth promoting combined with the ability of *Pseudomonas* to colonize the inside of the nodules and to allocate themselves in a space different from that occupied by *Rhizobium*, thus reducing competition between these bacteria [49]. *Pseudomonas aeruginosa* LSE-2 (KX925973) co-inoculated with *Bradyrhizobium* sp. (LSBR-3) also resulted in a synergistic effect improvement the symbiotic capacity, growth, and soil parameters [50]

Herbaspirillum seropediace can also adhere and colonize *P. vulgaris* roots internally [51], and new species *Herbaspirillum lusitanum* was isolated from nodules of *P. vulgaris* collected in the soil of Serra Peneda in Portugal [52]. *Bacillus* is another genus isolated from nodules of cv. IPR Sábia and BRS Esteio. Chinnaswamy and collaborators [53] isolated *Bacillus megaterium* from root nodules of *Medicago polymorpha*, this isolate also co-inhabit nodules with the symbiotic rhizobia. *Bacillus megaterium* RmBm31 strain was isolated by Dahmani and collaborators [54] from root nodules of *Retama monosperma*, a perennial leguminous, and *Bacillus velezensis* strain S141 was co-inoculated with *Bradyrhizobium diazoefficiens* USDA110 by Sibponkrung and

collaborators [55]. In the latter, the authors found that co-inoculation with *Bacillus* resulted in an increase in nodule number and in the nitrogen fixation efficiency by the production of large soybean nodules.

More than half of the isolates were identified as *Rhizobium*, as previously described in this study, *Rhizobium* is a genus commonly isolated from common bean nodules [31, 32, 33, 35, 37]. It was not possible to identify the LGMB567 isolate, and it is necessary to carry out a more careful analysis of the data referring to this bacterium.

According to the result of the statistical analyzes, only two treatments showed significant differences ($p \leq 0.05$) when compared with other treatments and with the control groups. Treatment 36 (isolated LGMB499, identified as *Herbaspirrilum* sp.) showed significant results with respect to root dry mass, while treatment 22 (isolated LGMB521, identified as *Rhizobium* sp.) showed significant results with respect to root dry mass and shoot dry mass.

Main Conclusions

In this research we identified and verified the potential of a hundred and seventy six bacteria that colonize common bean nodules, of which the most promising bacteria were LGMB499 and LGMB521. However, these bacteria need to be identified at the species level, as well as it is necessary that more investigative works on the influence of these on plant growth be carried out.

Acknowledgements

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14 References

[1] Lindstrom K, Mousavi SA. Effectivenes of nitrogen fixation in rhizobia. Microbial Biotechnology 2020; 13(5), 1314-1335.

[2] Junior FBR, Mendes IC, Hungria M, Capítulo VI Fixação biológica de nitrogênio: fundamentos e aplicações, Azevedo JL, Pamphile JA, Quecine-Verdi MC, Lacava PT (Orgs), Biotecnologia microbiana ambiental Maringá: Editora Eduem, 2018, pp. 125-152.

[3] Vieira RF, Ciclo do Nitrogênio em Sistemas Agrícolas, Brasília, DF: Empresa Brasileria de Pesquisa Agropecuária - Embrapa, 2017.

[4] Masson-Bovin C, Sachs JL. Symbiotic nitrogen fixation by rhizobia – the roots of a success story. Current Opinion in Plant Biology 2018; 7-15.

[5] Dos Santos PC, Fang Z, Mason SW, Setubal JC, Dixon R. Distribution of nitrogen fixation and nitrogenase-like sequences amongst microbial genomes. BMC Genomics 2012; 13-162.

[6] Willems A. The taxonomy of rhizobia: an overview. Plant and Soil 2006, 287:3-14.

[7] De Lajudie PM, Andrews M, Ardley J, Eardly B, Jumas-Bilak E, Kuzmanović N et al. Minimal standards for the description of new genera and species of rhizobia and agrobacteria. International Journal of Systematic and Evolutionary Microbiology 2019; *69*(7),1852-1863.

[8] Taylor BN, Simms EL, Komatsu KJ. More Than a Functional Group: Diversity within the Legume-Rhizobia Mutualism and Its Relationship with Ecosystem Function. Diversity 2020; 12(2), 1-19.

[9] Hungria M, Menna P, Delamuta JRM. Bradyrhizobium, the ancestor of all rhizobia: phylogeny of housekeeping and nitrogen- fixation genes. In: de Bruijn F (ed). Biological Nitrogen Fixation. Hoboken: John Wiley & Sons 2015; 191-202.

[10] Helene LCF, Klepa MS, O'hara G, Hungria M. *Brasyrhizobium archetypum* sp. nov., *Bradyrhizobium australiense* sp. nov. and *Bradyrhizobium murdochi* sp. nov., isolated from nodules of legumes indigenous to Western Australia. International Journal of Systematic and Evolutionary Microbiology 2020; 1-14.

[11] Tong W, Li X, Huo Y, Zhang L, Cao Y, Wang E et al. Genomic insight into the taxonomy of Rhizobium genospecies that nodulate *Phaseolus vulgaris*. Systematic and Applied Microbiology 2018; 300-310.

[12] Sánchez AC, Gutiérrez RT, Santana RC, Urrutia AR, Fauvart M, Michiels J, Vanderleyden J. Effects of co-inoculation of native Rhizobium and Pseudomonas strains on growth parameters and yield of two contrasting *Phaseolus vulgaris* L. genotypes under Cuban soil conditions. European Journal of Soil Biology 2014; 62: 105-112.

[13] Delamuta JRM, Scherer AJ, Ribeiro RA, Hungria M. Genetic diversity of *Agrobacterium* species isolated from nodules of common bean and soybean in Brazil, Mexico, Ecuador and Mozambique, and description of the new species *Agrobacterium fabacearum* sp. nov. International Journal of Systematic and Evolutionary Microbiology 2020; 70: 4233-4244.

[14] Assefa T, Mahama AA, Brown AV, Cannon EKS, Rubyogo JC, Rao IM, Blair MW, Cannon SB. A review of breeding objectives, genomic resources, and marker-assisted methods in common bean (*Phaseolus vulgaris* L.). Molecular Breeding 2019; 1-23.

[15] Torres AR, Brito B, Imperial J, Palacios MJ, Ciampitti IA, Ruiz-Argueso T, Hungria M. Hydrogen-uptake genes improve symbiotic efficiency in common beans (*Phaseolus vulgaris* L.). Antonie van Leeuwenhoek 2020; 687-696.

[16] Shamseldin A, Velázquez E. The promiscuity of *Phaseolus vulgaris* L. (common bean) for nodulation with rhizobia: a review. World Journal of Microbiology and Biotechnology 2020; 1-12.

[17] Fukami J, Cerezini P, Hungria, M. *Azospirillum*: benefits that go far beyond biological nitrogen fixation. AMB Express 2018; 1-12.

[18] Vincent JM. Manual for the practical study for root nodule bacteria. Oxford: Blacwell, 1970. 164 p.

[19] Shannon CE, Weaver W. The Mathematical theory of communication. University Illinois Press: Urbana, 1949.

[20] Raeder U, Broda P. Rapid preparation of DNA from filamentous fungi**.** Letters in Applied Microbiology 1985;17-20.

[21] Weisburg WG, Barns SM, Pelletier DA, Lane DJ. 16S ribosomal DNA amplification for phylogenetic study. Journal of Bacteriology 1991; 697-703.

[22] Menna P, Hungria M, Barcellos FG, Bangel EV, Hess PN, Martínez-Romero E. Molecular phylogeny based on the 16S rRNA gene of elite rhizobial strains used in Brazilian commercial inoculants. Systematic and Applied Microbiology 2006; 315-332.

[23] NCBI Blast. https://blast.ncbi.nlm.nih.gov/Blast.cgi. Accessed on March 4, 2021.

[24] LPSN. https://www.bacterio.net/. Accessed on March 4, 2021.

[25] MAFFT online version 7. https://mafft.cbrc.jp/alignment/software/. Accessed on March 4, 2021.

[26] Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Molecular Biology and Evolution 2011; 2731-2739.

[27] Broughton WJ, Dilworth MJ. Control of leghaemoglobin synthesis in snake beans. Biochemical Journal 1971; 1075-1080.

[28] Kemmitt SJ, Wright D, Goulding KWT, Jones DL. pH regulation of carbon and nitrogen dynamics in two agricultural soils. Soil Biology & Biochemistry 2006; 898-911.

[29] Deora GS, Singhal K. Isolation, biochemical characterization and preparation of biofertilizers using *Rhizobium* strains for commercial use. Bioscience Biotechnology Research Communications 2010; 132-136

[30] Ekundayo FO, Ekundayo EA, Aladesuru L, Salami AA. Effect of some environmental conditions on the growth of *Rhizobium* species. Journal of Biosciences 2018; 15-24.

[31] Martínez-Romero E, Segovia L, Mercante FM, Franco AA, Graham P, Pardo MA. *Rhizobium tropici*, a Novel Species Nodulating *Phaseolus vulgaris* L. and *Leucaena* sp. Tress. International Journal of Systematic Bcateriology 1991; 417-426.

[32] Hungria M, Andrade D de S, Chueire LM de O, Probanza A, Guttierrez-Mañero FJ, Megías M. Isolation and characterization of new efficient and competitive bean (*Phaseolus vulgaris* L.) rhizobia from Brazil. Soil Biology & Biochemistry 2000;1515-1528.

[33] Torres-Gutiérrez R, Granda-Mora KI, Alvarado-Capó Y, Rodriguez AS, Mogollón NGS, de Almeida JR. Genetic and phenotypic diversity of *Rhizobium* isolates from Southern Ecuador. Ciência e Agrotecnologia 2017: 634-647.

[34] Menge EM, Njeru EM, Koskey G, Maingi J. Rhizobial inoculation methods affect the nodulation and plant growth traits of host plant genotypes: A case study of Common bean *Phaseolus vulgaris* L. germplasms cultivated by smallholder farmers in Eastern Kenya. Advances in Agricultural Science 2018: 77-94.

[35] Huo Y, Tong W, Wang J, Wang F, Bai W, Wang E, Shi P, Chen W, Wei G. *Rhizobium chutanense* sp. nov., isolated from root nodules of Phaseolus vulgaris in China. International Journal of Systematic and Evolutionary Microbiology 2019: 2049-2056.

[36] Kawaka F, Makonde H, Dida M, Opala P, Ombori O, Maingi J, Mouma J. Genetic diversity of symbiotic bacteria nodulanting common bean (*Phaseolus vulgaris*) in western Kenya. Plos One 2018: 1-13.

[37] Koskey G, Mburu SW, Kimiti JM, Ombori O, Maingi JM, Njeru EM. Genetic Characterization and Diversity of *Rhizobium* Isolated From Root Nodules of Mid-Altitude Climbing Bean (*Phaseolus vulgaris* L.) Varieties. Frontiers in Microbiology 2018: 1-12.

[38] Kawaka F, Mouma J. Distribution and phenotypic characteristics of common bean (*Phaseolus vulgaris* L.) nodulating bacteria in diverse soils. Acta Agriculturae Scandinavica, Section B - Soil & Plant Science 2020: 1-9.

[39] Chibeba AM, Pereira CS, Antunes JEL, Ribeiro RA, Lopes AC de A, Gomes RLF, Hungria M, Araujo ASF. Polyphasic characterization of nitrogen-fixing and co-resident bacteria in nodules of *Phaseolus lunatus* inoculated with soils from Piauí State, Northeast Brazil. Symbiosis 2020: 279-292.

[40] Grange L, Hungria M. Genetic diversity of indigenous common bean (*Phaseolus vulgaris*) rhizobia in two Brazilian ecosystems. Soil Biology & Biochemistry 2004: 1389-1398.

[41] Oliveira JP, Galli-Terasawa LV, Enke CG, Cordeiro VK, Armstrong LCT, Hungria M. Genetic diversity of rhizobia in a Brazilian oxisol nodulating Mesoamerican and Andean genotypes of common bean (*Phaseolus vulgaris* L.). World Journal of Microbiology and Biotechnology 2011: 643-650.

[42] Davin-Regli A, Lavigne J-P, Pagés J-M. Enterobacter spp. : Update on Taxonomy, Clinal Aspects, and Emerging Antimicrobial Resistance. Clinical Microbiology Reviews 2019; 1-32.

[43] Peix A, Ramírez-Bahena M-H, Velázquez E. The current status on the taxonomy of Pseudomonas revisited: An update. Infection, Genetic and Evolution 2018; 106-116.

[44] Azam MW, Khan AU. Updates on the pathogenicity of Pseudomonas aeruginosa. Drug Discovery Today 2019; 350-359.

[45] Araújo WI, Marcon J, Maccheroni WJ, Elsas D van, Vuurde JWL van, et al. Diversity of endophytic bacterial populations and their interaction with Xylella fastidiosa in citrus plants. Applied Environmental Microbiology 2002; 4906-4914.

[46] Hinton DM, Bacon CW. Enterobacter cloacae is an endophytic symbiont of corn. Mycopathologia 1995; 117-125.

[47] Asis CA, Adachi K. Isolation of endophytic diazotroph *Pantoa agglomerans* and nondiazotroph *Enterobacter asburiae* from sweet potato stem in Japan. Letters Aplied Microbiology 2003; 19-23.

[48] Luduena LM, Anzuay MS, Angelini JG, McIntosh M, Becker A, et al. Genome sequence of the endophytic strain Enterobacter sp. J49, a potential biofertilizer for peanut and maize. Genomics 2019: 913-920.

[49] Pastor-Bueis R, Jiménez-Gómez A, Barquero M, Mateos PF, González-Andrés F. Yield response of common bean to co-inoculation with *Rhizobium* and *Pseudomonas* endophytes and microscopic evidence of different colonized spaces inside the nodule. European Journal of Agronomy 2021; 1-13.

[50] Kumawat KC, Sharma P, Sirari A, Singh I, Gill BS, Singh U, Saharan K. Synergism of *Pseudomonas aeruginosa* (LSE-2) nodule endophyte with *Bradyrhizobium sp.* (LSBR-3) for improving plant growth, nutrient acquisition and soil health in soybean. World Journal of Microbiology and Biotechnology 2019: 35-57.

[51] Schmidt MA, Souza EM, Baura V, Wassem R, Yates MG, Pedrosa FO, Monteiro RA. Evidence for the endophytic colonization of *Phaseolus vulgaris* (common bean) roots by the diazotroph *Herbaspirillum seropedicae*. Brazilian Journal of Medical and Biological Research 2011; 44(3): 182-185.

[52] Valverde A, Velázquez E, Gutiérrez C, Cervantes E, Ventosa A, Igual JM. *Herbaspirillum lusitanum* sp. nov., a novel nitrogen-fixing bacterium associated with root nodules of *Phaseolus vulgaris*. International Journal of Systematic and Evolutionary Microbiology 2003; 53: 1979-1983.

[53] Chinnaswamy A, La Pena TC, Stoll A, Rojo DLP, Bravo J, Rincón A, Lucas MM, Pueyo JJ. A nodule endophytic *Bacillus megaterium* strain isolated from *Medicago polymorpha* enhances growth, promotes nodulation by *Ensifer medicae* and alleviates salt stress in alfalfa plants. Annals of Applied Biology 2018: 295-308.

[54] Dahmani MA, Desrut A, Moumen B, Verdon J et al. Unearthing the Plant Growth-Promoting Traits of *Bacillus megaterium* RmBm31, an Endophytic Bacterium Isolated From Roots Nodules of *Retama monosperma*. Frontiers in Plant Science 2020: 1-15.

[55] Sibponkrung S, Kondo T, Tanaka K, Tittabutr P, Bookerd N, Yoshida K-I, Teaumroog N. Co-inoculation of Bacillus velezensis Strain S141 and Bradyrhizobium Strains Promotes Nodule Growth and Nitrogen Fixation. Microorganisms 2020; 1-24

7 CONCLUSÃO

Este trabalho contribui para a grande área de fixação biológica de nitrogênio em três eixos: a) na compreensão de como os mecanismos epigenéticos regulam a nodulação no feijoeiro comum e na soja, essa compreensão pode nos dar pistas de como aumentar a produção dessas duas culturas de extrema importância econômica e social; b) no aprimoramento de metodologias mais simples e baratas que são amplamente utilizadas para a triagem inicial de isolados bacterianos; c) na descoberta de novos isolados

promissores capazes de aumentar a quantidade de nitrogênio fixado em plantas do feijoeiro comum.

8 REFERÊNCIAS

ADHIKARI, S. & CURTIS, P. D. DNA methyltransferases and epigenetic regulation in bacteria. **FEMS Microbiology Reviews**, 40(5), 575–591, 2016.

AHKAMI, A. H.; WHITE III, R. A.; HANDAKUMBURA, P. P.; JANSON, C. Rhizosphere engineering: Enhancing sustainable plant ecosystem productivity. **Rhizosphere**. v. 3, p. 233-243, 2017.

ALONSO, C; RAMOS-CRUZ, D; BECKER, C. The role of plant epigenetics in biotic interactions. **New Phytologist** 221:731-737, 2019.

ALVAREZ-VENEGAS, R; DE LA PEÑA, C & CASAS-MOLLANO, J. A. Epigenetics in Plants of Agronomic Importance: Fundamentals and Applications, 2014.

ARAÚJO, W. I; MARCON, J; MACCHERONI, W. J; ELSAS, D. VAN; VUURDE, J. W. L. VAN, et al. Diversity of endophytic bacterial populations and their interaction with Xylella fastidiosa in citrus plants. **Applied Environmental Microbiology** 2002; 4906-4914.

ASIS, C. A; ADACHI, K. Isolation of endophytic diazotroph *Pantoa agglomerans* and nondiazotroph *Enterobacter asburiae* from sweet potato stem in Japan. **Letters Aplied Microbiology** 2003; 19-23.

ASSEFA, T.; MAHAMA, A. A.; BROWN, A. V.; CANNON, E. K. S.; RUBYOGO, J. C.; RAO, I. M.; BLAIR, M. W.; CANNON, S. B. A review of breeding objectives, genomic resources, and marker-assisted methods in common bean (*Phaseolus vulgaris* L.). **Molecular Breeding**, 39:20, p. 1-23, 2019.

AZAM, M. W; KHAN, A. U. Updates on the pathogenicity of *Pseudomonas aeruginosa*. **Drug Discovery Today** 2019; 350-359.

BAKER, G.C.; SMITH, J.J.; COWAN D.A. Review and re-analysis of domain-specific 16S primers. **Journal of Microbiological Methods** 55: 541-555, 2003.

BALDRICH, P. & MEYERS, B. C. Bacteria send messages to colonize plant roots. **Science**, 365(6456), 868–869, 2019.

Barraza, A. et al. Downregulation of PvTRX1h increases nodule number and affects auxin, starch, and metabolic fingerprints in the common bean (*Phaseolus vulgaris* L.). **Plant Science**, 274(May), 45–58, 2018.

BARRAZA, A; LUNA-MARTÍNEZ, F; CHÁVEZ FUENTES, J. C. & ÁLVAREZ-VENEGAS, R. Expression profiling and down-regulation of three histone lysine methyltransferase genes (PvATXR3h, PvASHH2h, and PvTRX1h) in the common bean. **Plant OMICS**, 8(5), 429-440, 2015.

BLOW et al. The Epigenomic Landscape of Prokaryotes. **PLoS Genetics**, 12(2), 1–28, 2016.

BILUNG, L. M; PUI, C. F; SU'UT, L; APUN, K. Evaluation of BOX-PCR and ERIC-PCR as molecular tying tool for pathogenic *Leptospira.* Disease Markers, 2018.

BORBA, M. P; BALLARINI, A. E; WITUSK, J. P. D; LAVIN, P; SAND, S. V. D. Evaluation of BOX-PCR and REP-PCR as molecular typing tools for Antarctic *Streptomyces.* **Current Microbiology**. v.77, p.3573-3581, 2020.

BROUGHTON, W. J; DILWORTH, M. J. Control of leghaemoglobin synthesis in snake beans. **Biochemical Journal**; 1075-1080, 1971.

CAI, Z. et al. GmTIR1/GmAFB3 based auxin perception regulated by miR393 modulates soybean nodulation. **New Phytologist**, 215(2), 672–686, 2017.

CASTRO, E. C.; FIDELIS, A. C.; WANDERIOR, A. E. Mercado de cultivares de feijão-comum (*Phaseolus vulgaris)* no Brasil. **IGepec**, v.23, n.1, p.181-198, 2019.

CHENG et al. Histone tales: Lysine methylation, a protagonist in Arabidopsis development. **Journal of Experimental Botany**, 71(3), 793–807, 2020.

CHIBEBA, A. M. et al. Polyphasic characterization of nitrogen-fixing and co-resident bacteria in nodules of *Phaseolus lunatus* inoculated with soils from Piauí State, Northeast Brazil. **Symbiosis**; 279-292, 2020.

CHINNASWAMY, A et al. A nodule endophytic *Bacillus megaterium* strain isolated from *Medicago polymorpha* enhances growth, promotes nodulation by *Ensifer medicae* and alleviates salt stress in alfalfa plants. **Annals of Applied Biology**; 295-308, 2018.

CLÚA, J; RODA, C; ZANETTI, M. E; BLANCO, F. A. Compatibility between Legumes and Rhizobia for the Establishment of a Successful Nitrogen-Fixing Symbiosis. **Genes**. v.9, 1-21, 2018.

CONAB - Companhia Nacional de Abastecimento. A**companhamento da Safra Brasileira de Grãos** v. 6 - Safra 2018/19 - n. 11 - Décimo primeiro levantamento, 2019.

CONAB – Companhia Nacional de Abastecimento. **Perspectivas para a agropecuária.** Safra 2020/2021. Brasília. v. 7, p. 1-31, 2021. Disponível em: http://www.conab.gov.br

COX, M. M., NELSON. D. L. **Princípios de Bioquímica de Lehninger**. 6 ed. Porto Alegre: Artmed, 2014. 1250 p.

CHINNASWAMY, A.; DE LA PEÑA, T. C.; STOLL, A.; ROJO, D. DE LA P.; BRAVO, J.; RINCÓN, A.; LUCAS, M. M.; PUEYO, J. J. A nodule endophytic *Bacillus megaterium* strain isolated from *Medicago polymorpha* enhances growth, promotes nodulation by *Ensifer medicae* and alleviates salt stress in alfafa plants. **Annals of Applied Biology** 172: 295-308, 2018.

CHUEIRE, L. M. O.; BANGEL, E. V.; MOSTASSO, F. L.; CAMPO, R. J.; PEDROSA, F. O.; HUNGRIA, M. Classificação taxonômica das estirpes de rizóbios recomendadas para as culturas da soja e do feijoeiro baseada no sequênciamento do gene 16S rRNA. **Revista Brasileira de Ciência do Solo**. v. 27, p. 833-840, 2003.

CLÚA, J.; RODA, C.; ZANETTI, M. E.; BLANCO, F. A. Compatibility between

Legumes and Rhizobia for the Establishment of a Successful Nitrogen-Fixing Symbiosis. **Genes**. v.9, p. 1-21, 2018.

DAHMANI, M. A; DESRUT, A; MOUMEN, B; VERDON, J. et al. Unearthing the Plant Growth-Promoting Traits of *Bacillus megaterium* RmBm31, an Endophytic Bacterium Isolated From Roots Nodules of *Retama monosperma*. **Frontiers in Plant Science**; 1-15, 2020.

DAMNJANOVIC, D.; HARVEY, M.; BRIDGE, W. J. Applications of colony BOXA2R-PCR for the differentiation and identification of lactic acid COCCI. **Food Microbiology** 82: 277-286, 2019.

DAVIS-RICHARDSON, A. G. et al. Integrating DNA methylation and gene expression data in the development of the soybean-*Bradyrhizobium* N2-fixing symbiosis. **Frontiers in Microbiology**, 7(APR), 1–10, 2016.

DAVIN-REGLI, A; LAVIGNE, J-P; PAGES, J-M. Enterobacter spp. : Update on Taxonomy, Clinal Aspects, and Emerging Antimicrobial Resistance. **Clinical Microbiology Reviews**; 1-32, 2019.

DEL CERRO P.; ROLLA-SANTOS, A. A. P.; GOMES, D. F.; MARKS, B. B.; ESPUNY, M. del R.; RODRÍGUEZ-CARVAJAL, M. Á.; SORIA-DÍAZ, M. E.; NAKATANI, A. S.; HUNGRIA, M.; OLLERO, F. J.; MEGÍAS, M. Opening the "black box" of nodD3, nodD4 and nodD5 genes of *Rhizobium tropici* strain CIAT 899. **BMC Genomics** 16:864. p. 1-10, 2015.

DEL CERRO P.; ROLLA-SANTOS, A. A. P.; GOMES, D. F.; MARKS, B. B.; PÉREZ-MONTAÑO, F.; RODRÍGUEZ-CARVAJAL, M. Á.; NAKATANI, A. S.; GIL-SERRANO, A.; MEGÍAS, M.; OLLERO, F. J.; HUNGRIA, M. Regulatory nodD1 and nodD2 genes of *Rhizobium tropici* strain CIAT 899 and their roles in the early stages of molecular signaling and host-legume nodulation. **BMC Genomics** 16:251. p. 1-13, 2015.

DEL CERRO, P; PÉREZ-MONTAÑO, F.; GIL-SERRANO, A.; LÓPEZ-BAENA, F. J.; MEGÍAS, M.; HUNGRIA, M.; OLLERO, F. J. The Rhizobium tropici CIAT 899 NodD2 protein regulates the production of Nod factors under salt stress in a flavonoid-independet manner. **Scientific Reports** 7:46712. p. 1-10, 2017.

DEL CERRO, P.; MEGÍAS, M.; LÓPEZ-BAENA, F. J.; GIL-SERRANO, A.; PÉREZ-MONTAÑO, F.; OLLERO, F. J.; Osmotic stress activates *nif* and *fix* genes and induces the *Rhizobium tropici* CIAT 899 Nod factors production via NodD2 by up-regulation of the nodA2 operon and the nodA3 gene. **Plos One**. p. 1-23, 2019.

DELAMUTA, J. R. M.; SCHERER, A. J.; RIBEIRO, R. A.; HUNGRIA, M. Genetic diversity of *Agrobacterium* species isolated from nodules of common bean and soybean in Brazil, Mexico, Ecuador and Mozambique, and description of the new species *Agrobacterium fabacearum* sp. nov. **International Journal of Systematic and Evolutionary Microbiology** 70: 4233-4244, 2020.

DE LAJUDIE, P. M.; ANDREWS, M.; ARDLEY, J.; EARDLY, B.; JUMAS-BILAK, E.; KUZMANOVIĆ, N.; LASSALLE, F.; LINDSTROM, K.; MHAMDI, R.; MATÍNEZ-ROMERO, E.; MOULIN, L.; MOUSAVI, S. A.; NESME, X.; PEIX, A.; PUTAWASKA, J.; STEENKAMP, E.; STEPKOWSKI, T.; TIAN, C-F.; VINUSA, P.; WEI, G.; WILLEMS, A.; ZILLI, J.; YOUNG, P. Minimal standards

for the description of new genera and species of rhizobia and agrobacteria. **International Journal of Systematic and Evolutionary Microbiology**, *69*(7), 1852–1863, 2019.

DOS SANTOS, P. C; FANG, Z; MASON, S. W; SETUBAL, J. C; DIXON, R. Distribution of nitrogen fixation and nitrogenase-like sequences amongst microbial genomes. **BMC Genomics**; 13-162, 2012.

DEORA, G. S; SINGHAL, K. Isolation, biochemical characterization and preparation of biofertilizers using *Rhizobium* strains for commercial use. **Bioscience Biotechnology Research Communications**; 132-136, 2010.

EBELING, S. KUNDIG, C. & HENNECKE, H. Discovery of a rhizobial RNA that is essential for symbiotic root nodule development. **Journal of Bacteriology**, 173(20), 6373–6382, 1991.

E. P. E.- EMPRESA DE PESQUISA ENERGÉTICA. Competitividade do Gás Natural: Estudo de caso na indústria de fertilizantes nitrogenados. **Informe técnico**. 18 p., 2019.

Editores HUNGRIA, M.; ARAUJO, R. S. Manual de métodos empregados em estudos de microbiologia agrícola. Empresa Brasileira de Pesquisa Agropecuária, Centro Nacional de Pesquisa de Arroz e Feijão, Centro Nacional de Pesquisa da Soja. Brasília: **Embrapa** - SPI, 1994.

EMBRAPA- Empresa Brasileira de Pesquisa Agropecuária, Centro Ncional de Pesquisa de Arroz e Feijão. BRS Esteio Feijão Preto. **Boletim Informativo**, 2015.Disponível:https://ainfo.cnptia.embrapa.br/digital/bitstream/item/117771/1/CNPAF-2015fd.pdf

EKUNDAYO, F. O; EKUNDAYO, E. A; ALADESURU, L; SALAMI, A. A. Effect of some environmental conditions on the growth of *Rhizobium* species. **Journal of Biosciences**; 15-24, 2018.

FARIA, G; GUIMARAES, R. M; CARVALHO, E. R; SOUSA, D. P. Método para a avaliação da atividade de isoenzimas em sementes de girassol. **Journal of Seed Science**. v.37, n.3, p211-217, 2015.

FELSENFELD, G. A Brief History of Epigenetics. In: Allis CD, Caparros ML, Jenuwein T, Reinberg D (ed) Epigenetics, 2nd edn. Colds Spring Harb Perspect Biology, pp 1-10, 2015.

FIDELIS, R. R.; SALGADO, F. H. M.; ALEXANDRINO, C. M. dos S.; TAVARES, T. C. de O.; CAMPESTRINI, R. Determinação do teor de proteína em genótipos de feijão comum cultivadas em diferentes níveis de nitrogênio. **Ambiência**, v. 15, p. 161-172, 2019.

FIORILLI, V.; CATONI, M.; LANFRANCO, L.; ZABET, R. N. Editorial: Interactions of Plants with Bacteria and Fungi: Molecular and Epigenetic Plasticity on the Host. **Frontiers in Plant Science**, 2020.

FUKAMI, J.; CEREZINI, P.; HUNGRIA, M. *Azospirillum*: benefits that go far beyond biological nitrogen fixation. **AMB Express**. p. 1-12, 2018.

FRANCISCO, P. R. M.; BANDEIRA, M. M.; SANTOS, D.; PEREIRA, F. C., & GONÇALVES, J. L. D. G. Aptidão climática da cultura do feijão comum (*Phaseolus vulgaris*) para o estado da paraíba. **Revista Brasileira de Climatologia**, *19*, 366–378, 2016.

FRANKE, A. C; VAN DEN BRAND, G. J. & GILLER, K. E. Which farmers benefit most from sustainable intensification? An ex-ante impact assessment of expanding grain legume production in Malawi. **European Journal of Agronomy**, 58, 28–38, 2014.

GEPTS, P.; BLISS, F. A.; Phaseolin Variability among Wild and Cultivated Common Beans (Phaseolus vulgaris) from Colombia. **Economic Botany** 40(4). p. 469-478, 1986.

GEPTS, P.; Biochemical Evidence Bearing on the Domestication of Phaseolus (Fabaceae) Beans. **Economic Botany** 44(3). p. 28-38, 1990.

GEPTS, P.; OSBORN, T. C.; RASHKA, K.; BLISS, F. A. Phaseolin-protein Variability in Wild Forms and Landraces of the Common Bean (*Phaseolus vulgaris*): Evidence for Multiple Centers of Domestication. **Economic Botany** 40(4). p. 451-468, 1986.

GRANGE, L.; HUNGRIA, M.; Genetic diversity of indigenous common bean (*Phaseolus vulgaris*) rhizobia in two Brazilian ecosystems. **Soil Biology & Biochemistry** 36: 1389-1398, 2004.

HASANUZZAMAN, M.; MASUD, A. A. C.; BHUIYAN, T. F.; ANEE, T. I. Legumes for Soil Fertility Management and Sustainable Agriculture. **SATSA Mukhapatra - Annual Technical Issue**, p. 29 - 46, 2019.

HASSAN, A. A. & YOUSSEF, M. A. H. Isolation and characterization of *Bacillus thuringiensis* bacteriophages using electron microscope and RAPD-PCR. **Journal of Agricultural Chemistry and Biotechnology**, *2*(12), 343–359, 2011.

HELENE, L. C. F.; KLEPA, M. S.; O'HARA, G.; HUNGRIA, M. *Brasyrhizobium archetypum* sp. nov., *Bradyrhizobium australiense* sp. nov. and *Bradyrhizobium murdochi* sp. nov., isolated from nodules of legumes indigenous to Western Australia. **International Journal of Systematic and Evolutionary Microbiology** p. 1-14, 2020.

HERAS, J; DOMÍNGUEZ, C; MATA, E. et al. A survey of tools for analysing DNA fingerprints. **Briefings in bioinformatics** 17: 903-911, 2016.

HOANG, N. T; TÓTH, K. & STACEY, G. The role od microRNAs in the legume-*Rhizobium* nitrogen-fixing symbiosis. **Journal of Experimental Botany**, 71(5), 1668-1689, 2020.

HOSSAIN, M. S. et al. Characterization of the spatial and temporal expression of two soybean miRNAs identifies SCL6 as a novel regulator of soybean nodulation. **Frontiers in Plant Science**, 10(April), 1–14, 2019.

HUANG, C. Y; WANG, H; HU, P; HAMBY, R. & JIN, H. Small RNAs – Big Players in Plant-Microbe Interactions. **Cell Host and Microbe**, 26(2), 173–182, 2019.

HINTON, D. M; BACON, C. W. *Enterobacter cloacae* is an endophytic symbiont of corn. **Mycopathologia**; 117-125, 1995.

HUNGRIA, M.; VARGAS, M. A. T. Environmental factors affecting N_2 fixation in grain legumes in the tropics, with an emphasis on Brazil. **Field Crops Research**. v. 65, p. 151-164, 2000.

HUNGRIA, M.; ANDRADE, D. de S.; CHUEIRE, L. M. de O.; PROBANZA, A.; GUTTIERREZ-MAÑERO, F. J.; MEGÍAS, M. Isolation and characterization of new efficient and competitive bean (*Phaseolus vulgaris* L.) rhizobia from Brazil. **Soil Biology & Biochemistry**. v. 32, p. 1515-1528, 2000.

HUNGRIA, M.; MENNA, P.; DELAMUTA, J. R. M. Bradyrhizobium, the ancestor of all rhizobia: phylogeny of housekeeping and nitrogen- fixation genes. In: de Bruijn F (editor). Biological Nitrogen Fixation. Hoboken: **John Wiley & Sons**, p. 191–202, 2015.

Huo, Y. et al. *Rhizobium chutanense* sp. nov., isolated from root nodules of Phaseolus vulgaris in China. **International Journal of Systematic and Evolutionary Microbiology**: 2049-2056, 2019.

HOANG, N. T.; TÓTH K & STACEY G. The role od microRNAs in the legume-*Rhizobium* nitrogen-fixing symbiosis. Journal of Experimental Botany. 2020, 71(5), 1668-1689. http:// doi.org/10.1093/jxb/eraa018

IAPAR - Instituto Agronômico do Paraná. Feijão IPR Sábia: Uma opção para alto rendimento. **Boletim Informativo**, 2018. Disponível em: http://www.iapar.br/arquivos/File/Sementes_e_Mudas/IPRSabia.pdf

ICHIDA, H.; MATSUYAMA, T.; ABE, T. & KOBA, T. DNA adenine methylation changes dramatically during establishment of symbiosis. **FEBS Journal**, 274(4), 951–962, 2007.

ICHIDA, H.; YONEYAMA, K.; KOBA, T. & ABE, T. Epigenetic modification of rhizobial genome is essential for efficient nodulation. **Biochemical and Biophysical Research Communications**, 389(2), 301–304, 2009.

Jaccard P. The distribution of the flora in the alpine zone. **New Phytologist**. v. 11, n. 2 (1912).

JI, Z. J.; YAN, H.; CUI, Q. G.; WANG, E. T.; CHEN, W. F.; CHEN, W. X. Competition between rhizobia under different environmental conditions affects the nodulation of a legume. **Systematic and Applied Microbiology**. v. 40, p. 114-119, 2017.

JOHNSTON, A. W. B.; BEYNON, J. L.; BUCHANAN-WOLLASTON, A. V.; SETCHELL, S. M.; HIRSCH, P. R.; BERINGER, J. E. High frequency transfer of nodulating ability between strains and species of *Rhizobium*. **Nature** 276:634-636, 1978.

JUNIOR F. B. R., MENDES, I. C., HUNGRIA, M. Biotecnologia microbiana ambiental. Capítulo VI. Fixação biológica de nitrogênio: fundamentos e aplicações. Maringá: **Editora Eduem**, p. 125-152, 2018.

KASCHUK, G; HUNGRIA, M; SANTOS, J. C. P. & BERTON-JUNIOR, J. F. Genetic diversity of rhizobia associated with common bean (*Phaseolus vulgaris* L.) grown under no-tillage and conventional systems in Southern Brazil. **Applied Soil Ecology**. v.32, n. 2, p. 210-220, 2006.

KAWAKA, F. et al. Genetic diversity of symbiotic bacteria nodulanting common bean (*Phaseolus vulgaris*) in western Kenya. **Plos One** 2018: 1-13.

KAWAKA, F; MOUMA, J. Distribution and phenotypic characteristics of common bean (*Phaseolus vulgaris* L.) nodulating bacteria in diverse soils. Acta Agriculturae Scandinavica, Section B - **Soil & Plant Science** 2020: 1-9.

KEMMITT, S. J; WRIGHT, D; GOULDING, K. W. T; JONES, D. L. pH regulation of carbon and nitrogen dynamics in two agricultural soils. **Soil Biology & Biochemistry**; 898-911, 2006.

KOEUTH, T.; VERSALOVIC, J.; LUPSKI, J. R. Differential Subsequence Conservation of Interspersed Repetitive Streptococcus pneumoniae BOX Elements in Diverse Bacteria. **Genome Research**. p. 408-418, 1995.

KOUKI, S.; SIFI, B. Effect of nitrogen fertilization on the symbiotic rhizobia/Common bean (Phaseolus vulgaris L.). **Journal of New Sciences Agriculture and Biotechnology IABC**, *31*(19). p. 1336–1340, 2016.

KOSKEY, G. et al. Genetic Characterization and Diversity of *Rhizobium* Isolated From Root Nodules of Mid-Altitude Climbing Bean (*Phaseolus vulgaris* L.) Varieties. **Frontiers in Microbiology** 2018: 1-12.

KUMAWAT, K. C. et al. Synergism of *Pseudomonas aeruginosa* (LSE-2) nodule endophyte with *Bradyrhizobium sp.* (LSBR-3) for improving plant growth, nutrient acquisition and soil health in soybean. **World Journal of Microbiology and Biotechnology** 2019: 35-57.

LA PENA, T. C.; FEDOROVA, E.; PUEYO J. J.; LUCAS M. M. The Symbiosome: Legume and Rhizobia Co-evolution toward a Nitrogen-Fixing Organelle? **Frontiers in Plant Science**. v.8, p. 1-26, 2018.

LEE, A.; WONG, E. Optimization and the Robustness of BOX A1R PCR for DNA Fingerprinting Using Trout Lake *E. coli* Isolates. **Journal of Experimental Microbiology and Immunology**. v.13, p. 104-113, 2009.

LINDSTRÖM, K.; ASERSE, A. A.; MOUSAVI, S. A. Evolution and Taxonomy of Nitrogen-Fixing Organisms with Emphasis on Rhizobia. In: de Bruijn F (editor). Biological Nitrogen Fixation. Hoboken: **John Wiley & Sons**, p. 21-38, 2015.

LINDSTROM, K; MOUSAVI, S. A. Effectivenes of nitrogen fixation in rhizobia. **Microbial Biotechnology**; 13(5), 1314-1335, 2020.

LI, H; DENG, Y; WU, T; SUBRAMANIAN, S. & YU, O. Misexpression of miR482, miR1512, and miR1515 increases soybean nodulation. **Plant Physiology**, 153(4), 1759-1770, 2010.

LIU, C-W; MURRAY, J. D. The Role of Flavonoids in Nodulation Host-Range Specificity: An Update. **Plants**. p.1-13, 2016.

LÖHNIS, F.; HANSEN, R. Nodule bacteria of leguminous plants. **Journal of Agricultural Research**. v. 20, p. 543-556, 1921.

LPSN. https://www.bacterio.net/. Accessed on March 4, 2021.

LUDUENA, L. M. et al. Genome sequence of the endophytic strain *Enterobacter* sp. J49, a potential biofertilizer for peanut and maize. **Genomics** 2019: 913-920.

MAFFT online version 7. https://mafft.cbrc.jp/alignment/software/. Accessed on March 4, 2021.

MARTÍNEZ-ROMERO, E.; SEGOVIA, L.; MERCANTE, F. M.; FRANCO, A. A.; GRAHAM, P.; PARDO, M. A. *Rhizobium tropici*, a Novel Species Nodulating *Phaseolus vulgaris* L. and *Leucaena* sp. Tress. **International Journal of Systematic Bcateriology**. v. 41, n. 3, p. 417-426, 1991.

MARTÍN-RODRÍGUEZ, J; LEIJA, A; FORMEY, D. & HERNÁNDEZ, G. The microRNA319d/TCP10 node regulates the common bean – Rhizobia nitrogen-fixing symbiosis. **Frontiers in Plant Science**, 9(August), 1–13, 2018.

MAKARENKOV, V; KEVORKOV, D; LEGENDRE, P. Phylogenetic network construction approaches. **Applied Mycology and Biotechnology.** v. 6, p. 61-97, 2006.

MASSON-BOIVIN, C.; GIRAUD, E.; PERRET, X.; BATUT, J. Establishing nitrogen-fixing symbiosis with legumes: how many rhizobium recipes? **Cell Press: Trends in Microbiology**. v. 17, n. 10, p. 458-466, 2009.

MASSON-BOVIN, C.; SACHS, J. L. Symbiotic nitrogen fixation by rhizobia – the roots of a success story. **Current Opinion in Plant Biology**. v. 44, p. 7-15, 2018.

MENNA, P.; HUNGRIA, M.; BARCELLOS, F. G.; BANGEL, E. V.; HESS, P. N.; MARTÍNEZ-ROMERO, E. Molecular phylogeny based on the 16S rRNA gene of elite rhizobial strains used in Brazilian commercial inoculants. **Systematic and Applied Microbiology**, v.29, n.4, p.315-332, 2006.

MENGE, E. M; NJERU, E. M; KOSKEY, G; MAINGI, J. Rhizobial inoculation methods affect the nodulation and plant growth traits of host plant genotypes: A case study of Common bean *Phaseolus vulgaris* L. germplasms cultivated by smallholder farmers in Eastern Kenya. **Advances in Agricultural Science**: 77-94, 2018.

MESQUITA, F. R.; CORRÊIA, A. D.; ABREU, C. M. P. de; LIMA, R. A. Z.; ABREU, A. de F. B. Linhagens de feijão (*Phaseolus vulgaris* L.): composição química e digestibilidade preotéica. **Ciência e Agrotecnologia**. v. 31, p. 1114-1121, 2007.

MORAES, E. S.; MENELAU, A.S. Análise do mercado de feijão comum. **Revista de Política Agrícola**, n.1, 2017.

MORÓN, B.; SORIA-DÍAZ, M. E.; AULT, J.; VERROIOS, G.; NOREEN, S.; RODRÍGUEZ-NAVARRO, D. N.; GIL-SERRANO, A.; THOMAS-OATES, J.; MEGÍAS, M.; SOUSA, C. Low pH Changes the Profile of Nodulation Factors Produced by *Rhizobium tropici* CIAT899. **Chemistry & Biology**. v. 12, p. 1029-1040, 2005.

MOURA, L; PEREIRA, G; COSTA, S. et al. Cancro do castanheiro na região do Minho: Estrutura populacional e variabilidade genética de populações de *Cryphonectria parasitica*. **Revista de Ciências Agrárias**, *38*(2), 238–247, 2015.

MBENGUE, M. D.; HERVÉ, C.; DEBELLÉ, F. Chapter one: Nod factor signaling in symbiotic nodulation. **Advances in Botanical Research**. v. 94, p. 1-39, 2020.

NCBI Blast. https://blast.ncbi.nlm.nih.gov/Blast.cgi. Accessed on March 4, 2021.

NAIR M. N.; MANICKAVELU A. MicroRNAs as fine-turners of gene regulation in plant-microbe interactions. **Current Science** 2020, v. 119, n. 8, 1282-1290.

NTATSI, G.; KARKANIS, A.; TRAN, F.; SAVVAS, D.; IANNETTA, P. P. M. Which Agronomic Practices Increase the Yield and Quality of Common Bean (*Phaseolus vulgaris* L.)? A Systematic Review Protocol. **Agronomy**. p. 1-10, 2020.

Niyikiza, D. et al. Interactions of gene expression, alternative splicing, and DNA methylation in determining nodule identity. **The Plant Journal**, 2020.

NIZAMPATNAM, N. R; SCHREIER, S. J; DAMODARAN, S; ADHIKARI, S. & SUBRAMANIAN, S. MicroRNA160 dictates stage specific auxin and cytokinin sensitivities and directs soybean nodule development. **Plant Journal**, 84(1), 140–153, 2015.

NOVA-FRANCO, B. et al. The micro-RNA172c-APETALA2-1 node as a key regulator of the common bean-*Rhizobium etli* nitrogen fixation symbiosis. **Plant Physiology**, 168(1), 273–291, 2015.

ORMEÑO-ORRILLO, E.; MENNA, P.; ALMEIDA, L. G. P.; OLLERO, F. J.; NICOLÁS, M. F.; RODRIGUES, E. P.; NAKATANI, A. S.; BATISTA, J. S. S.; CHUEIRE, L. M. O.; SOUZA, R. C.; VASCONCELOS, A. T. R.; MAGÍAS, M.; HUNGRIA, M.; MARTÍNEZ-ROMERO, E. Genomic basis of broad host range and environmental adaptability of *Rhizobium tropici* CIAT 899 and *Rhizobium* sp. PRF 81 which are used in inoculants for common bean (*Phaseolus vulgaris* L.). **BMC Genomics**, 13:735. p. 1-26, 2012.

OLIVEIRA, M. G. de C.; OLIVEIRA, L. F. C.; WENDLAND, A.; GUIMARÃES, C. M.; QUINTELA, E. D.; BARBOSA, F. R.; CARVALHO, M. da C. S.; JUNIOR, M. L.; SILVEIRA, P. M. Conhecendo a Fenologia do Feijoeiro e Seus Aspectos Filotécnicos. Brasília, DF: **Embrapa**, 2018.

OLIVEIRA, J. P. et al. Genetic diversity of rhizobia in a Brazilian oxisol nodulating Mesoamerican and Andean genotypes of common bean (*Phaseolus vulgaris* L.). **World Journal of Microbiology and Biotechnology**: 643-65, 2011.

PACKEISER, H; LIM, C; BALAGURUNATHAN, B. et al. An extremely simple and effective colony PCR procedure for bacteria, yeasts, and microalgae. **Applied Biochemistry and Biotechnology**, 169(2), p. 695–700, 2013.

PASTOR, A. V.; PALAZZO, A.; HAVLIK, P.; BIEMANS, H.; WADA, Y.; OBERSTEINER, M.; KABAT, P. The global nexus of food-trade-water sustaining environmental flows by 2050. **Nature Sustainability**. v. 2, p. 499-507, 2019.

PASTOR-BUEIS, R. et al. Yield response of common bean to co-inoculation with *Rhizobium* and *Pseudomonas* endophytes and microscopic evidence of different colonized spaces inside the nodule. **European Journal of Agronomy**; 1-13, 2021.

PEIX, A.; RAMÍREZ-BAHENA, M. H.; VELÁZQUEZ, E.; BEDMAR, E. J.; Bacterial Associations with Legumes. **Critical Reviews in Plant Science** 34: 17-42, 2015.

PEIX, A; RAMÍREZ-BAHENA, M-H; VELÁZQUEZ, E. The current status on the taxonomy of Pseudomonas revisited: An update. Infection, **Genetic and Evolution**; 106-116, 2018.

PERRONE, A; MARINELLI, F. Plant stress biology in epigenomic era. **Plant Science**, pp 1-15, 2020.

QUICENO-RICO, J. M; CAMAS-REYES, J. A. & ALVAREZ-VENEGAS, R. Molecular cloning and characterization of two Trithorax Group genes from *Phaseolus vulgaris* roots and symbiotic nodules. **Plant OMICS**, 5(2),103–114, 2012.

PUENTES-TÉLLEZ, P. E; SALLES, J. F. Construction of effective minimal active microbial consortia for lignocellulose degradation. **Microbial Ecology**. v.76, p.419-429, 2018.

RAEDER, U. AND BRODA, P. Rapid preparation of DNA from filamentous fungi**. Letters in Applied Microbiology**., n. 1, p.17-20, 1985.

RAJEEV, M; SUSHMITHA, T. J; TOLETI, S. R. & PANDIAN, S. K. Culture dependent and independent analysis and appraisal of earlystage biofilm-forming bacterial community composition in the Southern coastal seawater of India. **Science of the Total Environment**, 666, 308–320, 2019.

RAY, D. K.; MULLER, N. D.; WEST, P. C.; FOLEY J. A. Yield Trends Are Insufficient to Double Global Crop Production by 2050. **Plos One**. v. 8., 2013.

REN, B.; WANG, X.; DUAN, J. & MA, J. Rhizobial tRNA-derived small RNAs are signal molecules regulating plant nodulation. **Science**, 365(6456), 919–922, 2019.

REYERO-SAAVEDRA, M. DEL R. et al. Gene silencing of argonaute5 negatively affects the establishment of the legume-rhizobia symbiosis. **Genes**, 8(12), 1–19, 2017.

ROBERTS, M. R.; SÁNCHEZ A. L. Chapter 2 Plant Epigenetic Mechanims in Response to Biotic Stress. Book: Epigenetics in Plants of Agronomic Importance: Fundamentals and Applications. Editores: Alvarez-Vnegas, R; De-la-Peña, C.; Casas-Mollano J. A. Editora Springer. p. 65-112, 2019.

RUBIO, L. M.; LUDDEN, P. W. Biosynthesis of the Iron-Molybdenum Cofactor of Nitrogenase. **Annual Review of Microbiology** 62: 93-111, 2008.

RUBIO, L. M.; LUDDEN, P. W. Maturation of Nitrogenase: a Biochemical Puzzle. **Journal of Bacteriology**. p. 405-414, 2005.

SALGOTRA, K. AND GUPTA, M. Exploring the Role of Epigenetic in Cereal and Leguminous Crops Exposed to Abiotic Stress. In: Alvarez-Venegas R, De-la-Pena

C, Casas-Mollano JA (ed) Epigenetics in Plants of Agronomic Importance: Fundamentals and Applications, 2nd edn. Spring, 149-170, 2019.

SÁNCHEZ-NAVARRO, V.; ZORNOZA, R.; FAZ, Á.; FERNÁNDEZ, J. A. Comparing legumes for use in multiple cropping to enhance soil organic carbon, soil fertility, aggregates stability and vegetables yields under semi-arid conditions. **Scientia Horticulturae** 246: 835-841, 2019.

SÁNCHEZ-ROMERO, M. A., & CASADESÚS, J. The bacterial epigenome. **Nature Reviews Microbiology**, 18(1), 7–20, 2020.

SÁNCHEZ, A. C.; GUTIÉRREZ, R. T.; SANTANA, R. C.; URRUTIA, A. R.; FAUVART, M.; MICHIELS, J.; VANDERLEYDEN, J. Effects of co-inoculation of native *Rhizobium* and *Pseudomonas* strains on growth parameters and yield of two contrasting Phaseolus vulgaris L. genotypes under Cuban soil conditions. **European Journal of Soil Biology** 62: 105-112, 2014.

SANCHS, J. L.; QUIDES, K. W.; WENDLANDT, C. E. Legumes versus rhizobia: a model for ongoing conflict in symbiosis. **New Phytologist** 219:1199-1206, 2018.

SANTOS, M. S.; NOGUEIRA, M. A.; HUNGRIA, M. Microbial inoculants: reviewing the past, discussing the present and previewing an outstanding future for the use of beneficial bacteria in agriculture. **AMB Express**, p. 1-22, 2019.

SANTOS P. C.; FANG Z.; MASON S. W.; SETUBAL J. C.; DIXON R. Distribution of nitrogen fixation and nitrogenase-like sequences amongst microbial genomes. **BMC Genomics** 2012; 13-162.

SCHMIDT, M. A.; SOUZA, E. M.; BAURA, V.; WASSEM, R.; YATES, M. G.; PEDROSA, F. O.; MONTEIRO, R. A. Evidence for the endophytic colonization of *Phaseolus vulgaris* (common bean) roots by the diazotroph *Herbaspirillum seropedicae*. **Brazilian Journal of Medical and Biological Research**, 44(3): 182-185, 2011.

SCHWEMBER, A. R; SCHULZE, J; DEL POZO, A; CABEZA, R. A. Regulation of Symbiotic Nitrogen Fixation in Legume Root Nodules. **Plants**, 1-18, 2019.

SHANNON, C. E; WEAVER, W. The Mathematical theory of communication. University Illinois Press: Urbana, 1949.

SHAMSELDIN, A.; VELÁZQUEZ, E. The promiscuity of *Phaseolus vulgaris* L. (common bean) for nodulation with rhizobia: a review. **World Journal of Microbiology and Biotechnology**. p. 1-12, 2020.

SIBPONKRUNG, S. Co-inoculation of Bacillus velezensis Strain S141 and Bradyrhizobium Strains Promotes Nodule Growth and Nitrogen Fixation. **Microorganisms** 2020; 1-24.

SU, Z.; WILSON, B.; KUMAR, P. & DUTTA, A. Noncanonical Roles of tRNAs: tRNA Fragments and Beyond. **Annual Review of Genetics**, 54(1): 1–23, 2020.

SUZAKI, T; ITO, M. & KAWAGUCHI, M. Genetic basis of cytokinin and auxin functions during root nodule development. **Frontiers in Plant Science**, 4(MAR), 1–6, 2013.

SONG, X; LI, Y; CAO, X. & QI, Y. MicroRNAs and Their Regulatory Roles in Plant-Environment Interactions. **Annual Review of Plant Biology**, 70, 489–525, 2019.

TAMURA K, PETERSON D, PETERSON N, STECHER G, NEI M, KUMAR S. MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Molecular Biology and Evolution 2011; 2731-2739.

TAYLOR, B. N.; SIMMS, E. L.; KOMATSU, K. J. More Than a Functional Group: Diversity within the Legume-Rhizobia Mutualism and Its Relationship with Ecosystem Function. **Diversity**, 12(2), 2020.

THEY, N. H; FERREIRA, L. M. H; MARINS, L. F. & ABREU, P. C. Bacterial Community Composition and Physiological Shifts associated with the El Niño Southern Oscillation (ENSO) in the Patos Lagoon Estuary. **Microbial Ecology**, *69*(3), 525–534, 2015.

TIWARI, M. et al. Dynamics of miRNA mediated regulation of legume symbiosis. **Plant Cell and Environment**, 1-13, 2020.

TONG, W.; LI, X.; HUO, Y.; ZHANG, L.; CAO, Y.; WANG, E.; CHEN, W.; TAO, S.; WEI, G. Genomic insight into the taxonomy of Rhizobium genospecies that nodulate *Phaseolus vulgaris*. **Systematic and Applied Microbiology** 41:300-310, 2018.

TORRES, A. R.; BRITO, B.; IMPERIAL, J.; PALACIOS, M. J.; CIAMPITTI, I. A.; RUIZ-ARGUESO, T.; HUNGRIA, M. Hydrogen-uptake genes improve symbiotic efficiency in common beans (*Phaseolus vulgaris* L.). **Antonie van Leeuwenhoek**. p. 687-696, 2020.

TORRES-GUTIERREZ, R. et al. Genetic and phenotypic diversity of *Rhizobium* isolates from Southern Ecuador. **Ciência e Agrotecnologia** 2017: 634-647.

TULLIO, L. D.; GOMES, D. F.; SILVA, L. P.; HUNGRIA, M.; BATISTA, J. S. da S. Proteomic analysis of *Rhizobium. freirei* PRF 81^{T} reveals the key role of central metabolic pthways in acid tolerance. **Applied Soil Ecology**. p. 98-103, 2019.

TULLIO, L. D.; NAKATANI, A. S.; GOMES, D. F.; OLLERO, F. J. MAGÍAS, M. HUNGRIA, M. Revealing the roles of y4wF and tidC genes in Rhizobium tropici CIAT 899: biosynthesis of indolic compounds and impact on symbiotic properties. **Archives of Microbiology**. p. 13, 2018.

TURNER, M. et al. Ectopic expression of miR160 results in auxin hypersensitivity, cytokinin hyposensitivity, and inhibition of symbiotic nodule development in soybean. **Plant Physiology**, 162(4), 2042–2055, 2013.

UNITED NATIONS. World Population Prospects, 2019.

Link: https://population.un.org/wpp/Download/Standard/Population/

VALDÉS-LÓPEZ, et al. Argonaute Proteins : Why Are They So Important for the Legume-Rhizobia Symbiosis? **Frontiers in Plant Science**, 1-10, 2019.

VALVERDE, A.; VELÁZQUEZ, E.; GUTIÉRREZ, C.; CERVANTES, E.; VENTOSA, A.; IGUAL, J-M. *Herbaspirillum lusitanum* sp. nov., a novel nitrogen-fixing bacterium associated with root nodules of *Phaseolus vulgaris*. **International Journal of Systematic and Evolutionary Microbiology,** 53: 1979-1983, 2003.

VERSALOVIC, J.; KOEUTH, T.; LUPSKI, J. R.; Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. **Nucleic Acids Research**. v. 19, p. 6823-6831, 1991.

VASCHETTO, L. M. Exploring an emerging issue: crop epigenetics. **Plant Molecular Biology** 33:751-755, 2015.

VINCENT, J. M. Manual for the practical study for root nodule bacteria. Oxford: Blacwell, 1970. 164 p.

VIEIRA, R. F. Ciclo do Nitrogênio em Sistemas Agrícolas. Brasília, DF: Empresa Brasileria de Pesquisa Agropecuária - **Embrapa**, 2017. 165 p.

VITTORI, V. D.; BELLUCCI, E.; BITOCCHI, E. RAU, D. ; RODRIGUEZ, M. ; MURGIA, M. L. ; NANNI, L. ; ATTENE, G. ; PAPA, R. Domestification and Crop History. In: de la Vega, M. P.; Santalla, M; Marsolis, F. (editores). The Common Bean Genome. **Springer**, p. 21–56, 2017.

ZILIO, M.; SOUZA, C. A.; COELHO, C. M. M. Phenotypic diversity of nutrients and anti-nutrients in bean grains grown in different locations. **Revista Brasileirade Ciencias Agrarias**, *12*(4), 526–534, 2017.

ZHAO, LF.; XU, Y.; LAI, X.; Antagonistic endophytic bacteria associated with nodules of soybean (*Glycine max* L.) and plant growth-prmoting properties. **Brazilian Journal of Microbiology** 49: 269-278, 2018.

ZHU, J. Comparison of Four Template Preparation Methods and Optimization of BOX A1R PCR for DNA Fingerprinting of Escherichia coli Isolates. **Journal of Experimental Microbiology and Immunology**. p. 153–160, 2010.

WANG, L. et al. A GmNINa miR172c-NNC1 Regulatory Network Coordinates the Nodulation and Autoregulation of Nodulation Pathways in Soybean. **Molecular Plant**, 12(9), 1211–1226, 2019.

WANG, E. T.; CHEN, W. F.; TIAN, C. F.; YOUNG J. P. W.; CHEN, W. X. Ecology and Evolution of Rhizobia: Principles and Applications. **Springer Nature Singapore**, 2019.

WANG, Q.; LIU, J.; ZHU, H. Genetic and Molecular Mechanisms Underlying Symbiotic Specificity in Legume-Rhizobium Interactions. **Frontiers in Plant Science**. v.9, p.1-18, 2018.

WANG, Q; YUNG, W. S; WANG, Z. & LAM, H. M. The histone modification H3K4me3 marks functional genes in soybean nodules. **Genomics**, (September), 1–13, 2020.

WANG, Y. et al. Soybean miR172c targets the repressive AP2 transcription factor NNC1 to activate ENOD40 expression and regulate nodule initiation. **Plant Cell**, 26(12), 4782–4801, 2014.

WANG, Y. et al. microRNA167 directed regulation of the auxin response factors GmARF8a and GmARF8b is required for soybean nodulation and lateral root development. **Plant Physiology**, 168(3), 101-116, 2015.

WANG, J; MEI, J. & REN, G. Plant microRNAs: Biogenesis, homeostasis, and degradation. **Frontiers in Plant Science**, 10(March), 1–12, 2019.

WASSERMANN, E; MONTECCHIA, M. S; GARAVENTA, V. S; CORREA, O. S; ROMERO, A. M. Virulence and pCM1 plasmid carriage are related to BOX-PCR fingerprint type in strains of *Clavibacter michiganensis* subsp. *michiganensis* that cause bacterial wilt and canker of tomato in Argentina. **Plant Pathology**. 69: 723-732, 2020.

WEISBURG, W.G.; BARNS, S.M.; PELLETIER, D.A.; LANE, D.J. 16S ribosomal DNA amplification for phylogenetic study. **Journal of Bacteriology,** v.173, p.697-703, 1991.

WILLEMS, A. The taxonomy of rhizobia: an overview. **Plant and Soil**, 287:3-14, 2006.

WOESE, C. R. Bacterial Evolution. **Microbiological Reviews**. v. 51, p. 221-271, 1987.

WOODMAN, M. E. Direct PCR of intact bacteria (colony PCR). **Current Protocols in Microbiology**, (SUPPL. 9), 1–6, 2008.

YAN, Z. et al. miR172 regulates soybean nodulation. **Molecular Plant-Microbe Interactions**, 26(12), 1371–1377, 2013.

YAN, Z. et al. Identification and functional characterization of soybean root hair microRNAs expressed in response to *Bradyrhizobium japonicum* infection. **Plant Biotechnology Journal**, 14(1), 332–341, 2016.

YAN, Z. et al. Identification of microRNAs and their mRNA targets during soybean nodule development: Functional analysis of the role of miR393j-3p in soybean nodulation. **New Phytologist**, 207(3), 748–759, 2015.

YOUSSEF, M. A; MANSOUR, A. AND SOLLIMAN, S. S. Molecular Markers for New Promising Drought Tolerance Lines of Rice under Drought Stress via RAPD-PCR and ISSR Markers. **Journal of American Science**, 6(12) 355-363, 2010.

ZENG, J. Cross-Kingdom Small RNAs Among Animals, Plants and Microbes. **Cells**, 8(4), 371, 2019.

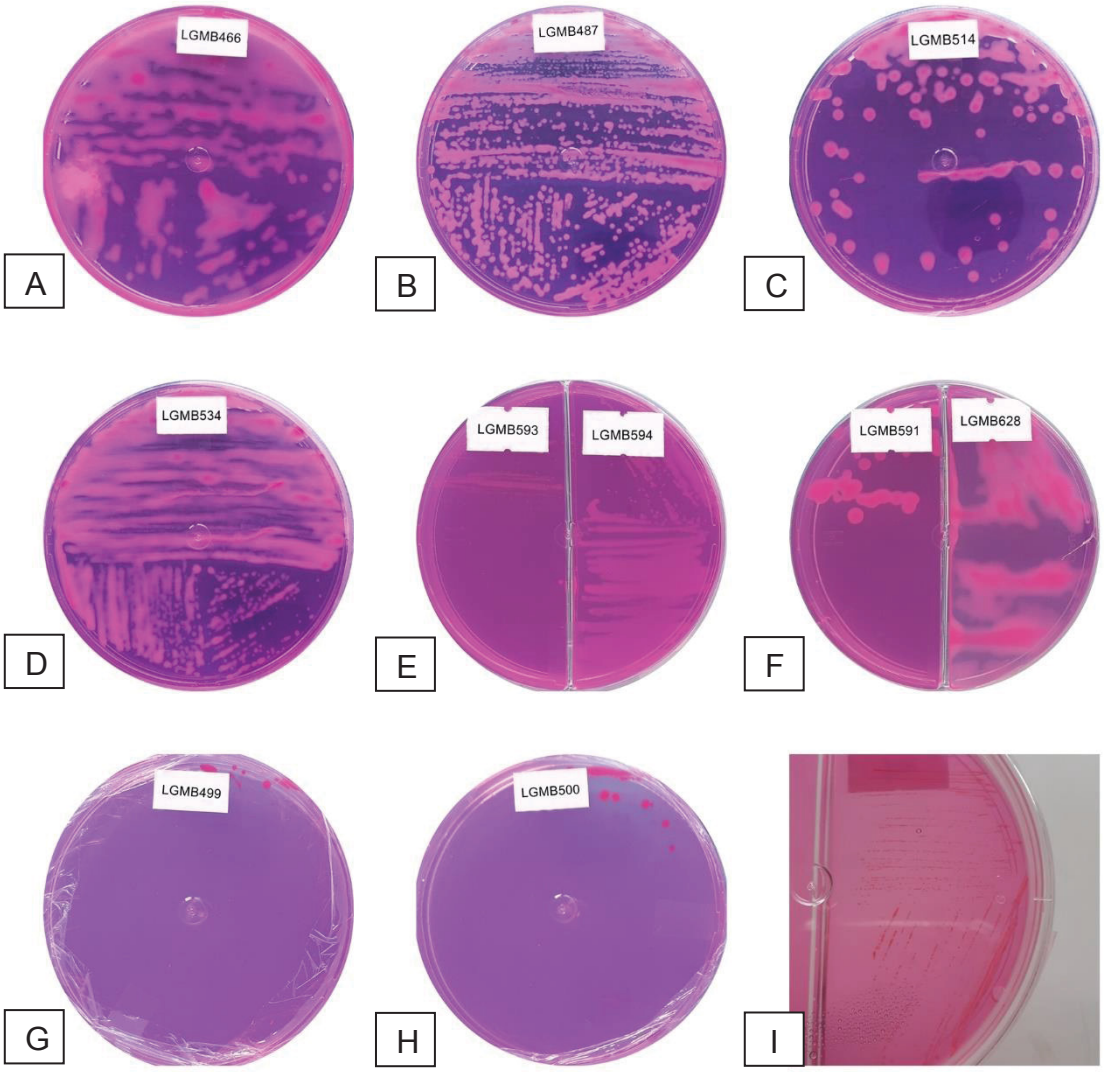
ZHOU et al. The function of histone lysine methylation related SET domain group proteins in plants. **Protein Science**, 29(5), 1120–1137, 2020.

ZHU J. Comparison of Four Template Preparation Methods and Optimization of BOX A1R PCR for DNA Fingerprinting of Escherichia coli Isolates. **Journal of Experimental Microbiology and Immunology**. p. 153–160, 2010.

ZHU, L; XU, H; ZHANG, Y; FU, G; WU, P. Q; LI, Y. BOX-PCR and PCR-DGE analysis for bacterial diversity of a naturally fermented functional food (Enzyme®). **Food Bioscience**. v.5, p115-122, 2014.

9 APÊNDICE

APÊNCICE 1. Diversidade morfológica dos isolados em meio YMA com adição de Rosa de Bengala após 4-5 dias de crescimento a 28 ºC

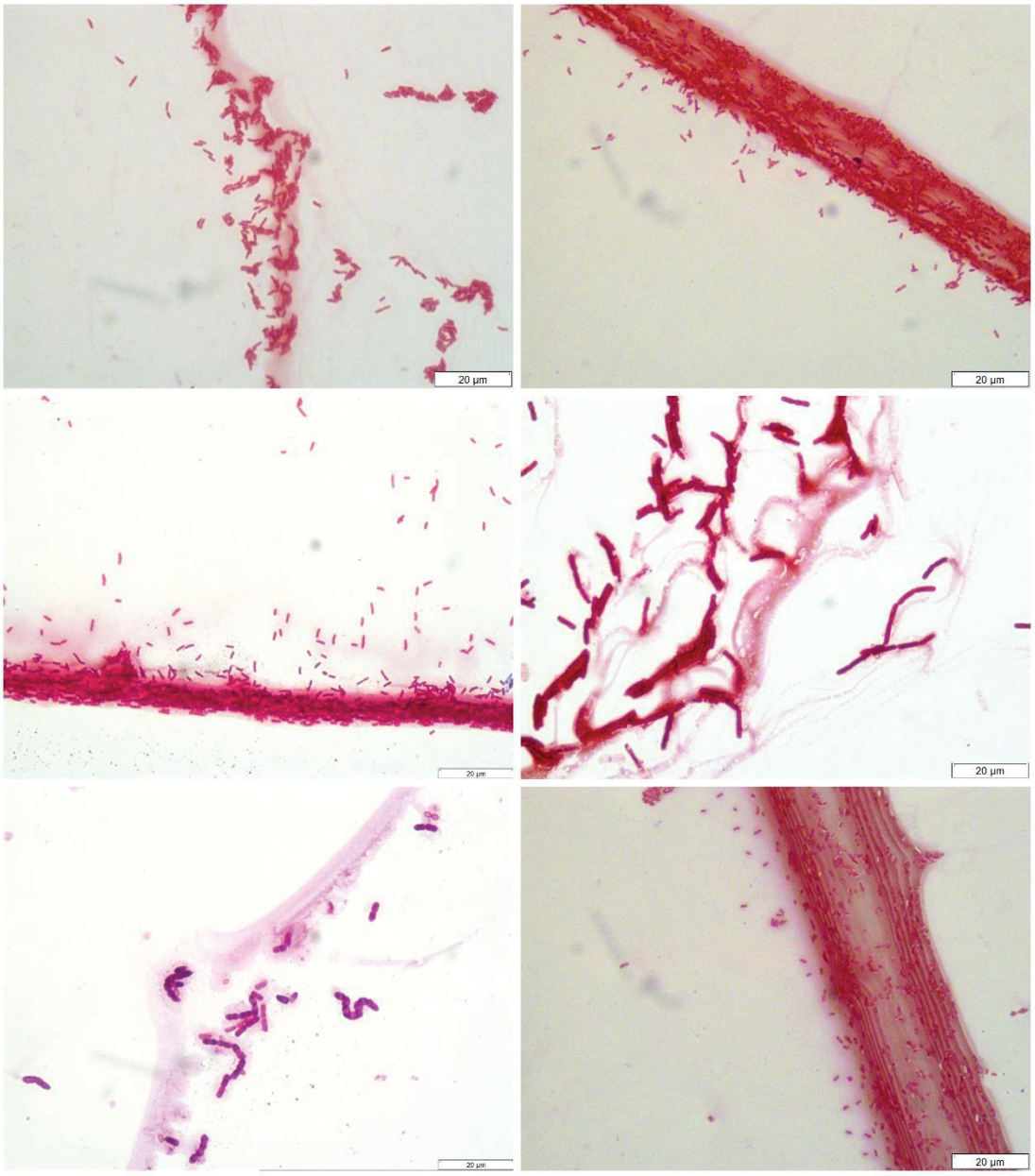


APÊNDICE 1. Diversidade morfológica dos isolados em meio YMA com adição de Rosa de Bengala após 4-5 dias de crescimento a 28 ºC. A) LGMB466, B) LGMB487, C) LGMB514, D) LGMB534, E) LGMB593 e LGMB594, F) LGMB591 e LGMB628, G) LGMB499, H) LGMB500, I) LGMB587. A, B, C, D, G e H foram isolados da cv. BRS Esteio. E, F e I foram isolados da cv. IPR Sábia.

9.1 APÊNDICE 2. Isolados em meio YMA com adição de corante vermelho congo e meio inclinado com indicador de pH após 4-5 dias de crescimento a 28 ºC

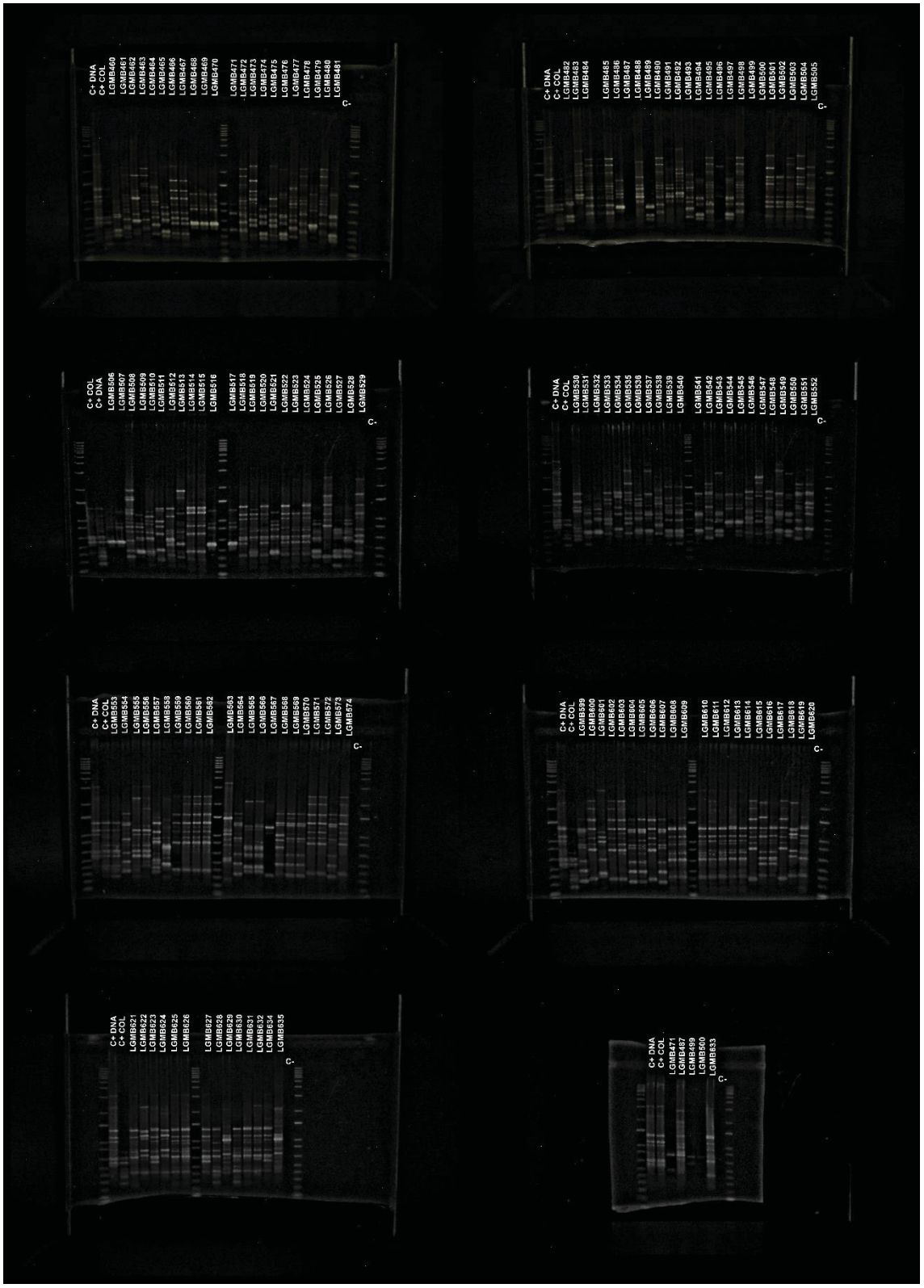
APÊNDICE 2. Isolados em meio YMA com adição de corante vermelho congo e meio inclinado com indicador de pH após 4-5 dias de crescimento a 28 ºC. A) Absorção de corante pelos isolados LGMB500 e LGMB499. C) e D) Acidificação do meio pelos isolados LGMB499 e LGMB500. E), F) e G) Basificação do meio pelos isolados LGMB567, LGMB587 e LGMB593. H) e I) Manutenção do pH do meio pelos isolados LGMB590 e LGMB592.

9.2 APÊNDICE 3. Micrografia dos isolados da cv. BRS Esteio corados pelo método de Gram após cultivo em meio YMA com adição de Rosa de Bengala após 4-5 dias de crescimento a 28 ºC.



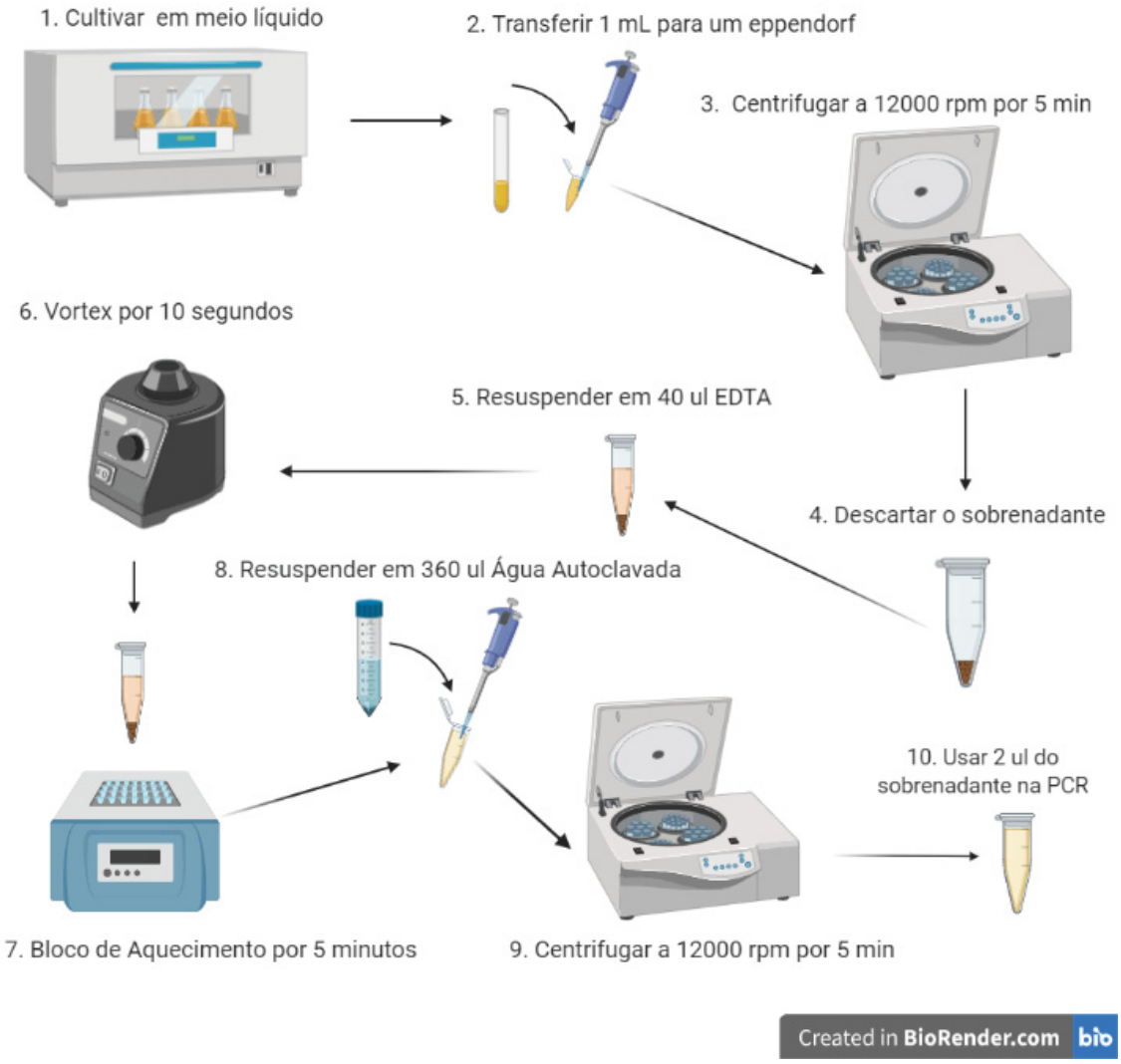
APÊNDICE 3. Micrografia dos isolados da cv. BRS Esteio corados pelo método de Gram após cultivo em meio YMA com adição de Rosa de Bengala após 4-5 dias de crescimento a 28 ºC. A) LGMB469, B) LGMB492, C) LGMB495, D) LGMB499, E) LGMB500, e F) LGMB539. As imagens foram obtidas em Microscópio Olympus BX51 localizado no Centro de Tecnologias Avançadas em Fluorescência – CTA

9.3 **APÊNDICE 4**. Géis resultantes de BOX PCR livre de extração de todos os isolados utilizados neste trabalho

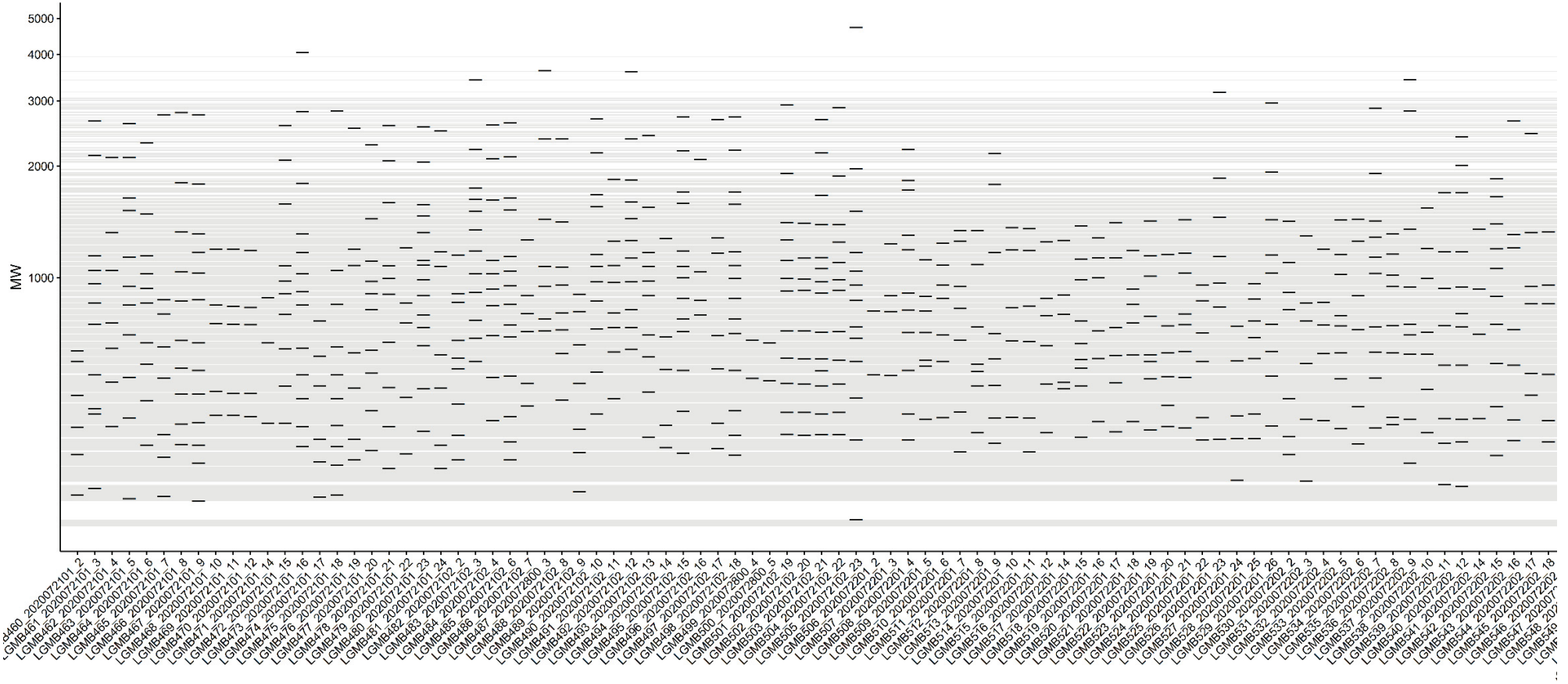


APÊNDICE 4. Géis resultantes da BOX PCR a partir da colônia de todos os isolados utilizados neste trabalho. LGMB é a sigla utilizada pelo Laboratório de Genética de Microrganismos, e o número logo após (460 a 635) representa o isolado dentro da coleção. C+ DNA e C+ COL são, respectivamente, o controle positivo da reação = DNA do isolado LGMB502, e o controle positivo da PCR de colônia = o material proveniente da colônia da bactéria LGMB502. C-, representa o controle negativo da reação.

9.4 APÊNDICE 5. Esquema representativo do passo a passo do protocolo de PCR livre de extração

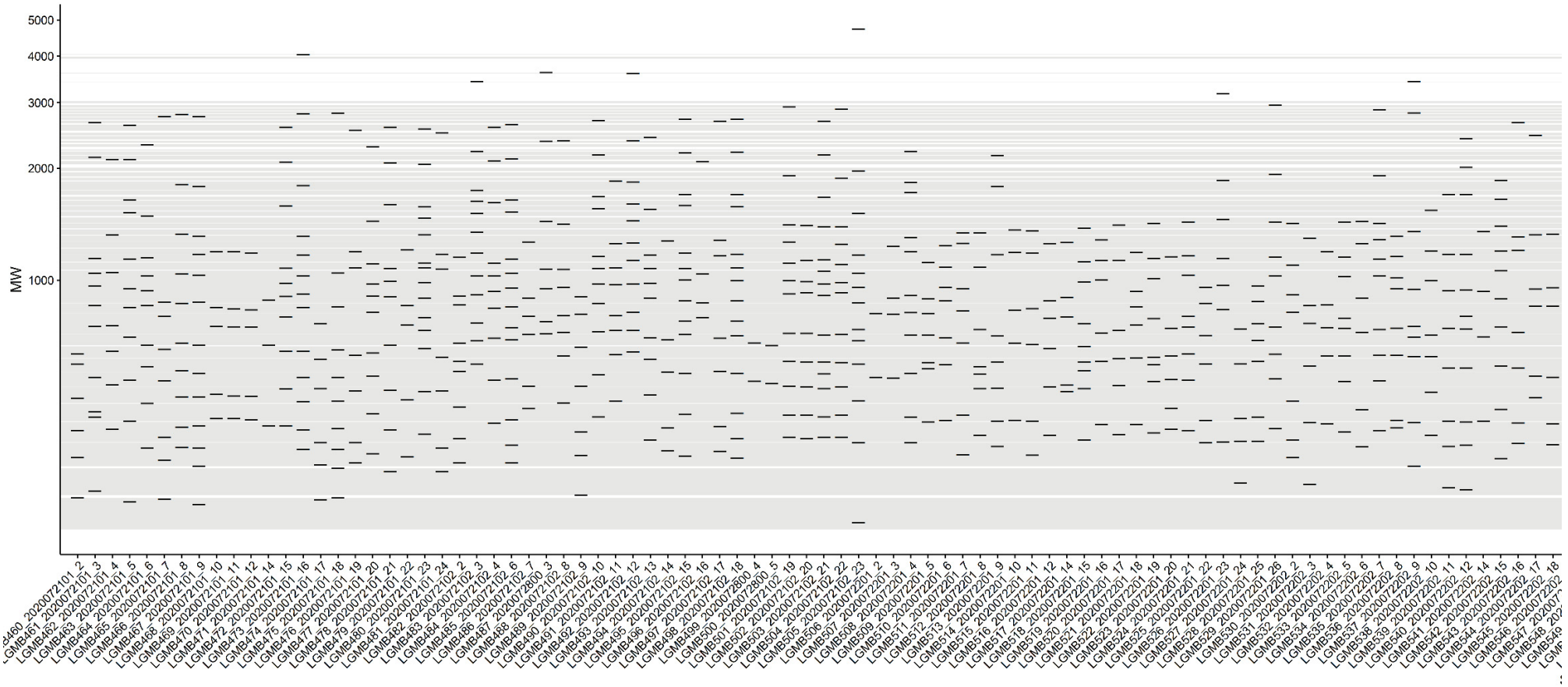


9.5 **APÊNDICE 6**. PADRÕES DE BANDA DE ALGUNS ISOLADOS OBTIDOS ATRAVÉS DE BOX PCR LIVRE DE EXRAÇÃO COM



APÊNDICE 6. Padrões de banda de alguns isolados obtidos através de BOX PCR livre de extração. Cada retângulo cinza representa o limite de 30 pb, assim as bandas que estão dentro de um determinado retângulo são consideradas a mesma banda em cada um dos isolados.

9.6 **APÊNDICE 7**. Padrões de banda de alguns isolados obtidos através de BOX PCR LIVRE DE EXTRAÇÃO COM LIMITE DE 50 PB



APÊNDICE 7. Padrões de banda de alguns isolados obtidos através de BOX PCR de Colônia. Cada retângulo cinza representa o limite de 50 pb, assim as bandas que estão dentro de um determinado retângulo são consideradas a mesma banda em cada um dos isolado.

10 ANEXOS

10.1 **ANEXO 1.** Meios de Cultura

- Meio YMA (VINCENT, 1970)

Manitol	10,0 g
$K_2HPO_4.3H_2O$	0,5 g
NaCl	0,1 g
$MgSO_4.7H_2O$	0,2 g
Extrato de Levedura	1,0 g
Ágar	15,0 g

O volume final foi completado para 1000 mL com água purificada. O pH foi ajustado entre 6,7-6,9 com NaOH 1 M e/ou HCl 1 N conforme a necessidade. O meio foi autoclavado e armazenado em temperatura ambiente.

- Meio YMA- Vermelho Congo

Ao volume final de 1000 mL do meio YMA foram adicionados 2,5 mL do corante Vermelho Congo (1%). O pH foi ajustado entre 6,7-6,9 e o meio foi autoclavado e armazenado à temperatura ambiente.

- Meio YMA-Azul de Bromotimol

Ao volume final de 1000 mL do meio YMA foram adicionados 5 mL do corante Azul de Bromotimol (0,5%). O pH foi ajustado entre 6,7-6,9 e o meio foi autoclavado e armazenado à temperatura ambiente.

- Meio YMA- Rosa de Bengala

Ao volume final de 1000 mL do Meio YMA foram adicionados 0,033 g do fungicida Rosa de Bengala. O pH foi ajustado entre 6,7-6,9 e o meio foi autoclavado e armazenado à temperatura ambiente.

10.2 **ANEXO 2.** Preparo do tampão de corrida TAE, tampão de corrida Sódio – Borato, preparo do marcador Invitrogen 1 Kb Plus DNA Ladder e do Loading Dye para DNA 6X diluição de dNTPs.

- Tão de corrida TAE 50X

Volume: 1 litro

242 g de TRIS BASE

57.1 ml Ácido Acético Glacial

100 ml de EDTA 0.5M pH 8.0

q.s.p. com ddH_2O 100 ml

Autoclavar

Referência: http://labs.icb.ufmg.br/lbem/protocolos/SOLUCAO.html

- Sódio – Borato 50X

Volume: 1 litro

20 g de Hidróxido de Sódio (NaOH)

120 g de Ácido Bórico (H_3BO_3)

Completar o volume com água destilada

Invitrogen 1 Kb Plus DNA Ladder, Catálogo do Fabricante: 10787018:

1 ul de DNA ladder

1 ul de 10x BlueJuice Gel Loading Buffer

8 ul de água destilada autoclavada

Loading Dye para DNA 6X Protocolo #0043.01:

Descrição: Procedimento para 4 diferentes cores

Glicerol 20%		
Reagente	**Conc. Final**	**Volume**
Glicerol 85%	20 %	2,35 mL
Água	--	7,65 mL
	Total	10 mL

Glicerol 20%		
Reagente	**Conc. Final**	**Volume**
Glicerol 100%	20 %	2 mL
Água	--	8 mL
	Total	10 mL

Corante 0,4% em Glicerol 20%		
Reagente	**Conc. Final**	**Volume**
Corante em questão		0,04 g
Glicerol 20%		10 mL

	DNA gel-loading dye (6X)		
	Reagente	**Conc. Final**	**Volume**
	Glicerol 85%	45,8 %	5,4 mL
	SDS 10%	0,5 %	0,5 mL
	EDTA 0,5 mol/L pH 8,0	10 mmol/L	0,2 mL
	Tris-HCl 1mol/L pH 8,0	100 mmol/L	1 mL
em glicerol 20%	Azul de Bromofenol 0,4%	0,02 %	0,5 mL
	Xileno Cianol 0,4%	0,012 %	0,3 mL
	Vermelho de Cresol 0,4%	0,02 %	0,5 mL
	Alaranjado G 0,4%	0,032 %	0,8 mL
	Glicerol dos Corantes	4,2 %	--
	Água	--	0,8 mL
		Total	10 mL

Produza alíquotas e armazene a -20°C. A alíquota em uso pode ser mantida a temperatura ambiente sem detrimento da função. Para a produção dos corantes, realize a pesagem dos mesmos em um falcon de 15 mL, homogeneíze em Vortex e deixe o precipitado não solubilizado no fundo, sem ser carregado ao *loading* final.

- dNTPs

Os quatro desoxirribonucleotideos (dAPT, dTTP, dGTP, dCTP) estoques (100 mM), foram diluídos em água ultra pura a 2,5 mM (solução de uso).