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SANDRA MARA BARBOSA ROCHA

CARACTERIZAÇÃO DE BACTERIAS PROMOTORAS DE CRESCIMENTO EM PLANTAS ISOLADAS EM ÁREAS COM LODO DE CURTUME COMPOSTADO

TERESINA – PI

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Dissertação apresentada ao Programa de Pós-Graduação em Agronomia/ Agricultura Tropical do Centro de Ciências Agrárias da Universidade Federal do Piauí, como parte dos requisitos para obtenção do título de Mestre em Ciências com área de concentração: Agricultura Tropical.

Orientador

Prof. Dr. Ademir Sérgio Ferreira de Araújo

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TERESINA-PI

2018

Aos meus amados pais, Antônio e Solimar.
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Sanderson e ao meu sobrinho Noah.
Ao meu amado companheiro
Danilo Bezerra e ao nosso mais novo amor - Heloísa.

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“A tarefa não é tanto ver aquilo que ninguém viu,
mas pensar o que ninguém ainda pensou
sobre aquilo que todo mundo vê.”
(Arthur Schopenhauer)

RESUMO GERAL

A aplicação de lodo de curtume, um tipo de resíduo produzido pelas indústrias de curtumes, tem promovido o acúmulo de cromo (Cr) no solo e, consequentemente, sua captação pelas plantas. Objetivou-se avaliar a influência de doses de lodo de curtume compostado (LCC) na nodulação e desenvolvimento de plantas de feijão-fava (*Phaseolus lunatus* L.), assim como isolar e realizar caracterizações fisiologica e bioquímica de bacterias promotoras de crescimento e avaliar *in vitro* à tolerância a diferentes concentrações de cromo. Por oito anos, o LCC foi aplicado, continuamente, em cinco doses: 0; 2,5; 5; 10 e 20 Mg ha^{-1} (base seca). Vasos de 2,8L foram preenchidos com solo e posteriomente semeada com feijão-fava, aos 45 dias após emergência foram avaliada a massa seca da parte área (MSPA), nitrogênio total (N-Total), número de nódulos (NN), massa seca dos nódulos (MSN), massa seca da raiz (MSR), tamanho do nódulo (TN), eficiência de fixação de nitrogênio (EFN), nitrogênio aculmulado na parte áerea (NAC), teor de cromo na planta (Cr-plant) e numero de isolados bacterianos (NI). Os testes bioquímicos realizados foram: urease, protease, amilase, lipase, solubilização de fosfato, catalase, gelatinase e produção de ácido indol-3- acético (AIA), teste *in vitro* de tolerância de isolados a diferentes doses de cromo 0; 25; 50; 100 e 200 µg ml^{-1} na forma de $K_2Cr_2O_{7.}$ Plantas de feijão-fava apresentaram maior nodulação em tratamentos com LCC, no entanto, os nódulos encontrados nesses tratamentos apresentaram menor tamanho e diversidade de isolados bacterianos do que os encontrados no tratamento sem LCC. O acúmulo de N aumentou com a aplicação da maior dose (20 Mg ha^{-1}), enquanto a eficiência de fixação de nitrogênio foi maior nas doses de 2,5 e 5 Mg ha^{-1} de LCC. Houve aumento no conteúdo de Cr na parte aérea com o aumento das doses de LCC. Um total de 54 bacterias promotoras de crecimento foi isolado em nódulos, sendo 40%, 37%, 13% e 10% do isolados encontrados nos tratamentos com 2,5; 5; 10 e 20 Mg ha^{-1} , respectivamente a maioria dos isolados forma positivos para o teste de urease, solubilização de fosfato e catalase, enquanto alguns isolados forma positivos para o teste de protease, lipase, carboximetilcelulose, gelatinase e amilase Assim, o uso de lodo de curtume compostado, em longo prazo, aumenta o acúmulo de cromo nas plantas, aumentando a nodulação e diminuindo a diversidade de rizóbios nos nódulos. No entanto, a aplicação das menores taxas melhora a eficiência da fixação biológica de N. Os isolados UFPI-LCC61, UFPI-LCC64 e UFPI-LCC87 apresentaram alta capacidade bioquímica e tolerância ao Cr. No entanto, o isolado UFPI-LCC87 apresentou alta capacidade bioquímica e tolerância a maior concentração de Cr.

Palavras chave: *Phaseolus lunatus*, resíduo industrial, microbiologia do solo, testes bioquímicos.

ABSTRACT

The application of tannery sludge, a type of residue produced by the tanneries, has promoted the accumulation of chromium (Cr) in the soil and, consequently, its uptake by plants. The objective of this study was to evaluate the influence of doses of composted tannery sludge (CTS) on nodulation and development of lima bean plants (*Phaseolus lunatus* L.), as well as to isolate and perform physiological and biochemical characterization of growth promoting bacteria and to evaluate in to different concentrations of chromium. For eight years, CTS was continuously applied in five doses: 0; 2.5; 5; 10 and 20 Mg ha^{-1} (dry basis). The dry mass of the area (MSPA), total nitrogen (N-Total), number of nodules (NN), mass dry mass of nodules (MSN), root dry mass (MSR), nodule size (TN), nitrogen fixation efficiency (EFN), aerial part nitrogen (NAC), chromium content in the plant (Cr-plant) and number of bacterial isolates (NI). The biochemical tests were: urease, protease, amylase, lipase, phosphate solubilization, catalase, gelatinase and indole-3-acetic acid (AIA), in vitro tolerance test of isolates at different doses of chromium 0; 25; 50; 100 and 200 μg ml^{-1} in the form of $K_2Cr_2O_7$. Plants of lima bean presented higher nodulation in treatments with CTS, however, the nodules found in these treatments presented smaller size and diversity of bacterial isolates than those found in the treatment without CTS. The accumulation of N increased with the application of the highest dose (20 Mg ha^{-1}), while nitrogen fixation efficiency was higher at the 2.5 and 5 Mg ha^{-1} doses of CTS. There was an increase in the Cr content in the aerial part with the increase of the doses of CTS. A total of 54 growth promoting bacteria were isolated in nodules, being 40%, 37%, 13% and 10% of the isolates found in the treatments with 2,5; 5; 10 and 20 Mg ha^{-1}, respectively. Most isolates were positive for the urease test, phosphate solubilization and catalase, while some isolates were positive for the protease, lipase, carboxymethylcellulose, gelatinase and amylase assay of composted tannery sludge, in the long term, increases the accumulation of Cr in the plants, increasing the nodulation and decreasing the diversity of rhizobia in the nodules. However, the application of lower rates improves the biological fixation efficiency of N. The isolates UFPI-LCC61, UFPI-LCC64 and UFPI-LCC87 showed high biochemical capacity and tolerance to Cr. However, the isolate UFPI-LCC87 showed high biochemical capacity and tolerance to higher concentration of Cr.

Keywords: *Phaseolus lunatus*; bioremediation, industrial waste, soil microbiology, biochemical tests

SUMÁRIO

1 INTRODUÇÃO

Os metais pesados são considerados uma fonte de poluição ambiental e seus níveis elevados em solos agrícolas causam sérios riscos, não só para o crescimento normal das plantas e para o redimento das culturas, mas também para a saúde humana (GILL et al., 2015; DALCORSO, 2012) e microrganismos do solo. Entre esses metais pesados destaca-se o cromo (Cr), que está presente em altas concentrações no lodo de curtume, um subproduto da indústria curtumeira (CERVANTES et al., 2001).

Recentemente, a compostagem tem sido utilizada como método de tratamento do lodo de curtume, para que possa ser aplicado como potencial corretivo do solo (MIRANDA et al., 2014). O lodo de curtume compostado (LCC) têm seus níveis de Cr reduzido (GONÇALVES et al., 2014), no entanto, a aplicação em longo prazo resulta em aumento da salinidade e alcalinidade do solo, assim como a acumulação de Cr no solo e nas plantas (MIRANDA, et al., 2014).

Embora a aplicação de LCC tenha favorecido o crescimento das plantas e melhorado a fertilidade do solo (SOUSA et al., 2017), o efeito da aplicação permanente e o aumento do acúmulo de Cr nas plantas, microrganismo e solo não são claros. O uso de microrganismos é considerado um método adequado para biorremediação de solos contaminados por metais, pois eles têm diferentes formas de suportar a toxicidade do metal (KARTHIK et al., 2017; OJUEDEIRIE; BABALOLA, 2017)

As bactérias promotoras de crescimento em plantas (BPCP) possuem mecanismos especializados que desempenham um papel fundamental na tolerância ao estresse e na promoção do crescimento e produção das plantas. Essas bactérias desencadeiam na planta a produção de diferentes hormônios de crescimento de plantas, como auxinas, citocinina e giberelina, bem como compostos voláteis (TORRE-RUIZ, et al., 2016). O uso de bactérias é considerado como uma possível maneira de reduzir efeitos tóxicos de metais pesados em plantas e fornece uma abordagem eficaz para a biorremediação de solos (KARTHIK et al., 2017).

Objetivou-se avaliar a influência de doses de lodo de curtume compostado (LCC) na nodulação e desenvolvimento de plantas de feijão-fava (*Phaseolus lunatus* L.), assim como isolar e caracterizar, fisiológica e bioquímicamente bacterias promotoras de crescimento e avaliar *in vitro* à tolerância a diferentes concentrações de cromo.

2 REVISÃO DE LITERATURA

2.1 Lodo de curtume compostado na agricultura

O solo é continuamente impactado por diversos fatores que influenciam gradativamente no crescimento das plantas, alterando diretamente a produtividade da cultura e a fertilidade do solo. Os estresses tanto abióticos como bióticos contribuem com 50 e 30%, respectivamente, para as perdas na produtividade agrícola em todo mundo (CHODAK et al., 2015). Dentre os estresses abióticos encontra-se a contaminação por metais pesados, tais como o cromo. (GODECKE et al., 2012).

Aproximadamente 5,2 bilhões de hectares de terras agrícolas são afetados por processos de degradação do solo, como erosão, salinidade e contaminação por resíduos industriais (NUMAN et al., 2018). Dentre os resíduos industriais, os advindo das atividades químicas, mineração, processamento de metal e couro, estão entre as fontes mais poluidoras (GHANI, 2010). As indústrias curtumeiras, responsáveis pelo processamento de couro bovino são geradoras de uma grande produção de resíduos sólidos, conhecidos por lodo de curtume (PATEL; PATRA, 2015).

A indústria curtumeira representa um importante setor da economia brasileira, processando no ano de 2017 cerca de 34 milhões de couros (IBGE, 2017). No final do processamento do couro nos curtumes, há uma grande quantidade de resíduos, sendo este resíduo a fonte de estudos sugerindo seu uso, viabilidade ou restrição na agricultura (MIRANDA et al., 2014, POSSATO et al., 2014, BERILLI et al., 2014, PATEL; PATRA, 2015). Isso se deve em parte pela composição desse resíduo ser constituído de material orgânico eficaz na fertilização e neutralização de solos ácidos (GODECKE et al., 2012).

O lodo de curtume seco possui mais de 90% de matéria orgânica em sua massa e sua composição é rica em nutrientes essenciais às plantas e microrganismos do solo (SOUSA et al., 2018, SINGH et al., 2013), promovendo a produtividade de diferentes culturas (ARAÚJO et al., 2013, MIRANDA et al., 2014, GUIMARAES et al., 2015). No entanto, o uso continuo contribui para a elevação do pH e a presença de sódio e cromo em altas concentrações, o que pode impossibilitar o uso como fertilizante alternativo (BERILLI et al., 2014).

A utilização desse resíduo na agricultura necessita de um processo de reciclagem, como a compostagem, que tem por finalidade reduzir sua toxidade e favorecer sua decomposição (ARAÚJO et al., 2014). Vig et al., (2014) estudando o efeito da compostagem do lodo de curtume sobre os elementos químicos presentes obtiveram resultados satisfatórios, onde observou-se após o processo de compostagem, um incremento de 66,6% e 44,8% na quantidade dos elementos nitrogênio e fósforo, respectivamente.

Estudos realizados demonstram uma influência do lodo sobre a atividade e estrutura da comunidade microbiana nos solos (NAKATANI et al., 2011; MIRANDA et al., 2014), que pode ocorrer tanto a curto como a longo prazo. Essa influencia está relacionada principalmente ao aumento de N inorgânico e ao pH do solo (NAKATAMI et al., 2011).

Estudos sobre a verificação do efeito do lodo nas propriedades químicas, biológicas do solo, assim como, seu efeito no desenvolvimento das plantas (OLIVEIRA; ARAÚJO e MELO, 2015, ARAUJO et al., 2016) dentre elas soja e milho (FERREIRA et al., 2003), caupi (TEIXEIRA et al., 2006), café (BERILLI et al., 2014), demonstrado um aumento no rendimento das plantas.

Estudos como Sousa et al., (2018) também mostraram que há aumento no teor de Cr no solo e na planta. Este resposta é motivo de preocupação, tendo em vista que esse elemento pode ser absorvido pelas plantas acumulando-se geralmente nas raízes, (MERLINO et al., 2010) ou ser translocado para as partes comestíveis e entrar assim na cadeia trófica (SOUSA et al., 2018).

2.1.1 Cromo

O Cr é encontrado nos solos associados com óxidos de elementos como o chumbo (Pb) e o ferro (Fe). Sua forma trivalente, Cr (III), é a forma natural e mais estável no ambiente, enquanto que sua forma hexavalente, Cr (VI), provém de atividades antrópicas poluidoras sendo 500 vezes mais tóxicas do que a forma trivalente (GUPTA et al., 2010; RIBEIRO, 2013).

A oxidação do Cr (III) para o Cr (VI) ocorre em função do pH, da disponibilidade de óxidos de manganês, da presença de compostos orgânicos com baixa massa molecular e da presença de água no solo. Já a redução do Cr (VI) para Cr(III), dependerá de fatores físico-químicos como o pH, a presença de matéria orgânica e de íons ferrosos, e a quantidade de oxigênio no solo, além, da ação microbiana que por meio da diminuição da concentração de oxigênio e do aumento da concentração de CO_2 pode reduzir Cr(VI) a Cr(III) (CCME, 1999). Algumas bactérias apresentam a capacidade para reduzir Cr (VI) a Cr (III), forma menos tóxica (STAMBULSKA et al., 2018).

O cromo no solo pode ser absorvido pela microbiota benéfica do solo e pelas plantas em crescimento afetando as composições microbianas e suas funções fisiológicas (STAMBULSKA et al., 2018). O uso de microrganismos tolerantes ou desintoxicantes de metais coletivamente chamado de biorremediação oferece uma opção sustentável e de baixo custo para limpar os solos poluídos. Dentre eles as BPCP tolerantes a metais apresentam um papel importante na redução da toxidade desses elementos (SAIF et al., 2017), além de proporcionar remediação, esses microrganismos promovem o crescimento das plantas.

O emprego de BPCP como os rizóbios pode ser uma alternativa eficiente e de baixo custo para a remoção de metais pesados do solo, além de disponibilizar nitrogênio para as plantas (HAO et al., 2014). Alguns grupos de microrganismos têm a capacidade genética e bioquímica para interagirem e sobreviverem aos efeitos tóxicos dos metais, auxiliando o estabelecimento e desenvolvimento vegetal (MALLICK et al., 2018). Estudos relacionados com a capacidade de remediação destes microrganismos oferecem ainda uma grande potencial a ser explorado (DAHMER, 2017).

Os mecanimos envolvidos são: (i) adsorção e acumulação de metais pesados; (ii) secreção microbiana de enzimas e metabolitos bioativos (isto é substâncias poliméricas extracelular, sideróforos e ácidos orgânicos) para diminuir sua toxidade alterando o estado redox dos metais e aumentando a complexação e a biodisponibilidade dos metais; essas ações também podem indiretamente auxiliar a fitorremediação (HAO et al., 2014).

Locais fortemente contaminados com cromo, com níveis elevados de concentrações, afetam não apenas o crescimento das plantas mais também microrganismos benéficos presentes no solo (KARTHIK et al., 2017), desenvolvendo nesses microrganismos mecanismos para sua sobrevivência, tornado-os resistentes (SULOWICZ et al., 2011) e adaptados a essas condições considerando-as condições fisiológicas normais para seu crescimento (ALI et al., 2013, AKPONAH 2013, KUMAR et al., 2014, SMITH et al., 2015), podendo a toxidade do cromo às plantas ser reduzida pela aplicação de bactérias promotoras de crescimento (JOUTEY et al., 2016).

2.2 Bactérias Promotoras de Crescimento de Plantas

As Bactérias Promotoras de Crescimento de Plantas (BPCP) são caracterizadas como microrganismos benéficos que vivem e colonizam a rizosfera produzindo substâncias promotoras de crescimento vegetal (KLOEPPER et al., 2004). Incluem microrganismo de vida livre, simbiônticos (rizobios) e edófiticos, apesar da diferença entre essas bacterias, todas utilizam os mesmo mecanismos promotores de cresciemnto. (GLICK, 2012).

As BPCP influenciam a mobilidade de metais pesados e melhoram o crescimento das plantas através da liberação de fitormônios (auxinas, giberelinas, etileno), solubilização fosfato, fixação de nitrogênio atmosférico, sintetizam sideroforos e produtos antimicrobianos (SALEEM et al., 2007). Embora a contaminação do solo por metais pesados possa afetar a diversidade de bacterias colonizadoras de raízes, a pressão ambiental do solo pode selecionar certas bacterias capazes de desenvolver interações complexas com outros microrganismos do solo e plantas (SALEEM; MOE 2014)

Os principais benefícios diretos das BPCP são: facilitar o fornecimento de nutrientes como fósforo; nitrogênio e ferro, solubilização do fósforo; disponibilizar ferro através da produção de sideróforos; controle dos níveis de fitormônios, diminuindo efeitos negativos de estresses ambientais; produção de citocininas, giberelinas e ácido idolacético (AIA); redução na produção de etileno (GLICK, 2012). Além das atividades de promoção de crescimento muitos BPCP reduzem a toxidade a metais pesados por mecanismos diferentes tais como biossorção, biolixiviação, imobilização, acúmulo intracelular e transformações catalizadas por enzimas (DIXIT et al., 2015), reduzindo assim as concentrações de metais no solo (GLICK, 2012).

De forma geral, supõe-se que a combinação de todos esses eventos beneficiam as plantas em crescimento e sanidade. As BPCP mais estudadas são as *Pseudomonas* spp. não fluorescentes e fluorescentes; espécies de *Bacillus*, *Streptomyces*, *Rhizobium*, *Bradyrhizobium*, *Acetobacter* e *Herbaspirilu*, *Agrobacterium radiobacter*, *Enterobacter cloacae* e *Burkholderia cepacia*, entre outras (MARIANO et al., 2004).

O desenvolvimento da agricultura sustentável e a crescente intenção pela diminuição do uso de fertilizantes químicos tornam a utilização de BPCP uma estratégia importante para o aumento da produtividade (MOREIRA & SIQUEIRA, 2006).

2.3 Propriedades bioquímicas de bactérias promotoras de crescimento

As BPCP são conhecidas por produzir uma grande quantidade de compostos biologicamente ativos, tais como fitormônios, enzimas, ácidos orgânicos que solubilizam o fósforo inorgânico (MARRA et al., 2012, GLICK, 2012). As bactérias que apresentam mais de uma característica para a promoção de crescimento vegetal, são almejadas e rastreadas para uma possível aplicação no campo, objetivando o aumento da produção agrícola (VERMA et al., 2001)

O AIA sintetizado por BPCP desempenha um papel fundamental no crescimento e desenvolvimento de plantas influenciando vários comportamentos fisiológicos, incluindo promoção da germinação das sementes, alongamento e divisão celular, iniciação das raízes e crescimento das plantas (LAMBRECHT et al., 2018), tanto a curto como em longo prazo. A diminuição na produção de AIA em estudos com uso de cromo (VI) pode ser devido ao envolvimento ativo no controle do estresse por metais do que na produção de AIA (KARTHIK et al., 2016).

O fósforo é o segundo macronutriente essencial ao lado do nitrogênio, indispensável em todas as fases de crescimento das plantas. A maioria dos solos agrícolas é deficiente em P e, portanto requerem aplicação de fertilizantes fosfatados para sustentar a produção agrícola (DASH et al., 2017). Os microrganismos solubilizadores de fosfato mineralizam fosfato que reduz a deficiência de P no solo, aumenta a disponibilidade de fosfato solúvel e melhora o crescimento das plantas, a FBN, os metabolismos reguladores do promotor de crescimento e a disponibilidade de elementos como ferro, zinco, boro, cobre, molibdênio, manganês (SABER et al., 2005, PONMURUGAN and GOPI, 2006)

As enzimas constituem o segundo grupo de produtos biológicos mais importantes de necessidade humana, inúmeros processos industriais, sobretudo na área de biotecnologia industrial, ambiental e de alimentos, utilizam a tecnologia das enzimas em várias de suas etapas (PANDEY et al., 1999). As enzimas hidroliticas são aplicadas na degradação de várias substâncias naturais e nos mais diversos processos industriais. As ureases são comumente observadas em ambientes terrestres e aquáticos, nos quais desempenham funções essenciais no metabolismo do nitrogênio, um exemplo são os processos de degradação proteica e substituição de nucleotideos que requerem atividade ureolítica. Na agricultura, o uso de ureia como fertilizante exige o controle da urease no solo para aumentar a sua eficiência e minimizar perdas de nitrogênio (MOBLEY; HAUSINGER, 1989)

O suprimento de carboidrato é um dos principais fatores envolvidos na formação e desenvolvimento de nódulos em leguminosas (ALLISON et al., 2014). Autores surgerem uma possível participação da amilase no estabelecimento da simbiose rizóbio-leguminosa (BERTHELOT; DELMONTE, 1999; GLENN; DILWORTH, 1981; SINGH; SINGH, 1985, VAN SPRONSEN et al., 1994). Juntos, a expressão de todos esses recursos pode melhorar a eficiência da simbiose rizóbio-leguminosas (NAVEED et al., 2015)

2.5. *Phaseolus lunatus* L.: Importância

O feijão-fava (*Phaseolus lunatus* L.) assim como outras leguminosas é amplamente conhecido pela capacidade de estabelecer associações simbióticas com bactérias do solo, comumente conhecidas como rizóbios, que são capazes de realizar a FBN em uma estrutura especializada conhecida como nódulo (ORMENO-ORILLO et al., 2006). O cultivo tem pouca relevância, o principal motivo para o cultivo limitado é a limitação na aquisição de sementes, principalmente por não existir cultivar comercial para produção em grande escala.

Segundo o IBGE (2016), no Brasil foram produzidas 3.637 toneladas de grãos secos do feijão fava, em uma área plantada de 20.209 hectares. Os estados da Paraíba, Ceará, Pernambuco e Piauí, são os maiores produtores, e junto fazem do Nordeste a maior região produtora, com 3.609 toneladas de grãos. No entanto, apesar de sua importância social, tem merecido pouca atenção dos órgãos de pesquisa e extensão, o que tem resultado em limitado conhecimento das características agronômicas da cultura (SANTOS et al., 2002) o que contribui para baixos níveis de produtividade.

O conhecimento da relação simbiótica do feijão-fava vem sendo objeto de estudo ao longo dos anos com trabalhados realizados principalmente no Peru (MATSUBARA; ZUNINGA-DÁVILA, 2015, ORMEÑO et al., 2008, MATOS et al., 1998), México (DURAN et al., 2014, LOPEZ et al., 2013) e Brasil (OLIVEIRA et al., 2008, ANTUNES et al., 2011). As implicações agronômicas dessa simbiose podem promover pesquisas sobre a fixação biológica de nitrogênio e na seleção e caracterização de rizóbios (ORMEÑO-ORRILLO et al., 2006), o que pode proporcionar uma menor dependência no uso de fertilizantes nitrogenado aumento na produtividade dessa cultura.

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CAPITULO I - Nodulation and biological nitrogen fixation in Lima bean in soil with successive application of composted tannery sludge

Abstract

Application of composted tannery sludge can change the soil chemical properties and promote the accumulation of chromium in soil and, thus, influence the nodulation and biological N fixation. This study evaluated the responses of Lima bean (*Phaseolus lunatus* L.) to application of composted tannery sludge, after eight years, on nodulation and N fixation. For eight years, the compost was applied, continuously at five rates: 0, 2.5, 5, 10, and 20 Mg ha^{-1} (dry basis). Plants of Lima bean showed higher nodulation in treatments with compost; however, nodules found in these treatments presented lower size and diversity than those found in treatment without compost. The accumulation of N increased with the application of the highest rate (20 Mg ha^{-1}) while the efficiency of N fixation was highest in the rates of 2.5 and 5 Mg ha^{-1}. There was an increase in chromium content in shoot with the increase in compost rates. Thus, the use of composted tannery sludge, in long-term, increases the accumulation of chromium in plants, increasing nodulation while decrease rhizobia diversity in nodules. However, the application of the lowest rates improves the efficiency of biological N fixation.

Keywords: *Phaseolus lunatus*; industrial waste; soil microbiology

Resumo

A aplicação de lodo de curtume compostado pode alterar as propriedades químicas do solo e promover o acúmulo de cromo no solo e, assim, influenciar a nodulação e a fixação biológica de N. Este estudo avaliou as respostas do feijão-fava (*Phaseolus lunatus* L.) à aplicação de lodo de curtume compostado, após oito anos, sobre nodulação e fixação de nitrogênio. Por oito anos, o composto foi aplicado, continuamente, em cinco doses: 0, 2,5, 5, 10 e 20 Mg ha^{-1} de base seca). Plantas de feijão-fava apresentaram maior nodulação em tratamentos com composto; no entanto, os nódulos encontrados nesses tratamentos apresentaram menor tamanho e diversidade do que os encontrados no tratamento sem composto. O acúmulo de N aumentou com a aplicação da maior dose (20 Mg ha^{-1}), enquanto a eficiência de fixação de N foi maior nas doses de 2,5 e 5 Mg ha^{-1}. Houve um aumento no teor de cromo na parte aérea com o aumento nas taxas de compostagem. Assim, o uso de lodo de curtume compostado, em longo prazo, aumenta o acúmulo de cromo nas plantas, aumentando a nodulação e diminuindo a diversidade de rizóbios nos nódulos. No entanto, a aplicação das menores taxas melhora a eficiência da fixação biológica de N.

Palavras-chave: *Phaseolus lunatus*; Residuo industrial; microbiologia do solo.

1 Introduction

Tannery industries are important for Brazilian economy by processing about 34 million of leather per year (IBGE, 2017). However, during the leather processing, the industries release a solid waste commonly called tannery sludge (TS) which presents high content of organic matter and chemical elements (PATEL; PATRA, 2015) that can be plant nutrients (WONG et al., 2007; GRUPTA; SINHA, 2006). Therefore, there is an effort of using TS in agricultural soil al alternative method for improving soil properties (SOUSA et al., 2017; SHARMA et al., 2017).

On the other hand, TS presents high content of chromium (Cr) that could accumulate in soil and pollute the environment. Thus, it is necessary a previous treatment of TS before its use as soil amendment. Recently, the processing of composting is recognized as a suitable method for processing TS before its application in the soil since this process could decrease TS toxicity and environmental impact (SINGH et al., 2010; SILVA et al., 2014; SOUSA et al., 2018). Previous studies have shown that the application of composted tannery sludge (CTS), in long-term, may contribute for improving the content of organic matter and soil fertility (ARAUJO et al., 2013; SOUSA et al., 2017). In addition, the application of CTS has positively influenced the growth and yield of cowpea and maize (GONÇALVES et al., 2014; SOUSA et al., 2018). Otherwise, the application of CTS favored the soil salinization and alkalinization, and also the Cr accumulation.

Environmental pollutants, such as Cr compounds, can cause damage to rhizobia, legumes, and their symbiosis. In plants, toxic effects of chromium compounds are associated with the increased production of reactive oxygen species (ROS). ROS and oxidative stress development as well as with inhibition of pigment synthesis and modification of virtually all cellular components (STAMBULSKA et al., 2018).

Thus, the use of CTS, in long-term, could affect the biological process, such as biological N fixation (BFN). The BNF is an important process carried out by soil microorganism in the rhizosphere that contributes for the availability of N to plants. This process occurs in root nodules and the nodulation is considered a suitable indicator for evaluating BNF. Also, nodulation and BNF are important parameters used in the evaluation of toxic effects resulting from application of polluting organic wastes (MIRANDA et al., 2014). Thus, on the hand, the application of CTS can favor the plant growth and improve soil fertility. On the other hand, the increase in soil salinity, pH and Cr accumulation after the application of CTS could negatively influence the nodulation, diversity of N fixers, and BNF.

On contrary, a previous study with application of CTS, in short-term, has shown that nodulation and BNF in cowpea were not negatively influenced by the waste (MIRANDA et al., 2014). However, the responses of others legume plants to application of CTS, mainly in long-term, on nodulation and BNF are not clear. Therefore, this study evaluated the responses of Lima bean (*Phaseolus lunatus* L.), a legume plant highly resistant to adverse condition (FOFANA et al., 1997) to application of CTS, after eight years, on nodulation and BNF. The main hypothesis is that there would be a distinct response of Lima bean to application of CTS, after eight years, related with nodulation and biological nitrogen fixation.

2 Material and methods

The experiment was carried out in a greenhouse located at Agricultural Science Center of Federal University of Piaui, Brazil (05º 05' S; 42º 48' W, 75 m). The climate is dry tropical (two distinct seasons: a rainy summer and a dry winter) with an annual temperature of 30 °C and rainfall of 1,200 mm. The soil is classified as a Fluvisol (10% clay, 28% silt, and 62% sand). The soil samples used in this pot experiment were collected from the experimental field with application of application of CTS for eight years and annually cropped with cowpea and maize. The CTS used in this experiment was obtained by mixing tannery sludge with sugarcane straw and cattle manure (ratio 1:3:1; v:v:v), and processed during 90 days. The physicochemical characteristics of the CTS are shown in Table 1.

Table 1. Physical and chemical characteristics of composted tannery sludge used in the experimental area for eight years.

pH	**Umi.**	**COT**	**N**	**P**	**K**	**Ca**	**Mg**	**Na**	**S**	**Cu**	**Ni**	**Cd**	**Cr**	**Pb**
H_2O	%	---------------------g kg^{-1}--------------------								----------mg kg^{-1}----------				
7.5	68	201	15	4.9	2.9	121	7.2	49.1	10	16	23	1.9	1.943	40
MLP*	-	-	-	-	-	-	-	-	-	200	70	3	150	180

* Maximum limit allowed by Brazilian regulations (CONAMA, 2009).

For eight years, CTS was applied, continuously in experimental plots (2 x 5 m) in four replicates, at five rates: 0 (without CTS application), 2.5, 5, 10, and 20 Mg ha^{-1} (dry basis). For this study, pots (2.8 L) were filled with soil collected from experimental plots with CTS. The experiment was arranged in a completely randomized design with four replicates. The soil pH ($CaCl_2$), electric conductivity (EC), exchangeable cations (Ca^{2+}, Mg^{2+}, K^+ and Na^+) and available phosphorus (P) were analyzed according to Donagema et al. (2011). Total organic C (TOC) was determined by the wet method using a mixture of 5 mL of potassium dichromate (0.167 mol L^{-1}) and 7.5 mL of concentrated sulfuric acid, with the application of heat (170 °C for 30 min). Soil Cr was extracted by the DTPA-TEA method and measured using the USEPA-3050 method (USEPA, 1986). The soil properties are shown in Table 2.

Table 2. Chemical properties of the soil after eight years of consecutive application of composted tannery sludge.

Doses LCC	**pH**	**CE**	**COT**	**P**	**K**	**Ca**	**Mg**	**Na**	**Cr**
(Mg ha^{-1})	$CaCl_2$	dS m^{-1}	g kg^{-1}	mg dm^{-3}	--------mmolc dm^{-3}--------				Mg Kg^{-1}
0	5.1	0.5	4.9	4.3	1.9	12.5	5.0	4.4	5.8
2.5	5.4	0.5	5.7	5.0	1.8	17.0	5.8	4.9	27.2
5	5.8	0.5	6.8	6.0	1.9	22.8	7.0	4.9	58.0
10	6.2	0.6	6.6	7.8	1.9	23.5	7.5	4.6	96.6
20	6.6	0.6	7.1	9.5	1.8	25.8	7.0	4.9	165.9

The seeds of lima bean were disinfested superficially by immersion in alcohol (70%, 30 s), followed by immersion in sodium hypochlorite (2%, 5 min) and washed successively 10 times with autoclaved distilled water. (HUNGRIA; ARAÚJO, 1994). At sowing, five seeds were used per pot and, on the seventh day after planting, two plants were left in each pot. All plants were irrigated with Hoagland and Arnon's nutritive solution modified according to Silveira et al. (1998) and without nitrogen, in the amount of 2 mL of the solution per kg of soil. Irrigations were performed according to the need of the crop.

The evaluation was done 45 days after plant emergence (during flowering) the shoots were dried (65°C; 72 h) and weighed to determine the shoot dry weight

(SDW). The shoots were milled to determine the total N (N-Total) using the Kjedahl method, as described by Silva and Queiroz (2006). The nodules were separated from the roots and counted to determine the number of nodules (NN). After the count, the nodules and roots were dried (65 ° C; 72 h) and weighed to determine nodule dry weight (NDW) and root dry weight (RDW). The nodule size was calculated by the relation between nodule dry mass and number of nodules. The nitrogen contents in the shoots were measured using the methods described by Bremner. Based on the aforementioned data, the nitrogen fixation efficiency, shoot dry matter nitrogen content and accumulated nitrogen content were calculated according to Keeney and Nelson, 1982. The chromium content in the aerial part (Cr-plant) was determined according to the procedure described in USEPA-3050 (1986).

For determination of the number of isolates (NI) three nodules were collected at 45 days after emergence of the seedlings. Bacterial isolates were obtained from root nodules of *Phaseolus lunatus*. Root nodules was sterilized in 0.1% (w/v) sodium hypochloride for 5 min immersed in 95% (v/v) ethanol for 10 s, and then washed six times with distilled water and streaked on yeast extract mannitol agar (YMA) medium containing 0.0025% (w/v) congo red (VINCENT, 1970). After 15 days of inoculation at 28ºC, single colony was selected and re-streaked on YMA medium for purification. Microscopy was done by observing morphology and cultural characteristics of the isolates. Accordingly, the colony characteristics (that is, shape, size, color, elevation, and margin of the bacterial colony) were determined by observing the colonies on nutrient agar plates of the overnight grown microorganisms. Physiological characteristics such as growth time; formation of acids and alkalis, presence of polysaccharide; volume of mucus (MELLONI et al., 2006); mucus elasticity and bubble production were evaluated in YMA medium with bromothymol blue.

The data were submitted to analysis of variance (ANOVA), preceded by F test ($P < 0.05$), using the software Assistat 7.6 beta (Silva; Azevedo, 2008). Redundancy analysis (RDA) was used to visualize the differences between the treatments and determine its correlation with environmental parameters. First, the matrices were analyzed using Detrended Correspondence analysis to evaluate the gradient size of the species distribution, which indicated linearly distributed data (length of gradiento), suggesting the RDA as the best-fit mathematical model for the data. Forward selection and the Monte Carlo permutation test were applied with 1000 random permutations to verify the significance of environmental parameters upon the biological variables. RDA plots were generated using Canoco 4.5 software (Biometrics, Wageningen, The Netherlands). We used permutational multivariate analysis of variance (PERMANOVA) (ANDERSON, 2001) to test whether sample clusters harbored significantly differences between treatments. Spearman's rank correlation coefficients were calculated to explore the relationship between biological variables and environmental factors according to the different treatments using the 'multtest' package in R (R Development Core Team, 2007) and the correction was made using Benjamini–Hochberg false discovery rate (BENJAMINI; HOCHBERG, 1995).

3 Results and discussion

Plants of lima bean showed higher number of nodules in treatments with CTS (Figure 1A); while the treatment with the highest CTS rates presented higher nodule dry weight than treatment without CTS (Figure 1B). However, nodules found in these treatments were lower than those found in treatment without CTS (Figure 1C). In addition, the treatments with CTS promoted a reduction of number of bacterial isolates as compared with the treatment without CTS (Figure 1D).

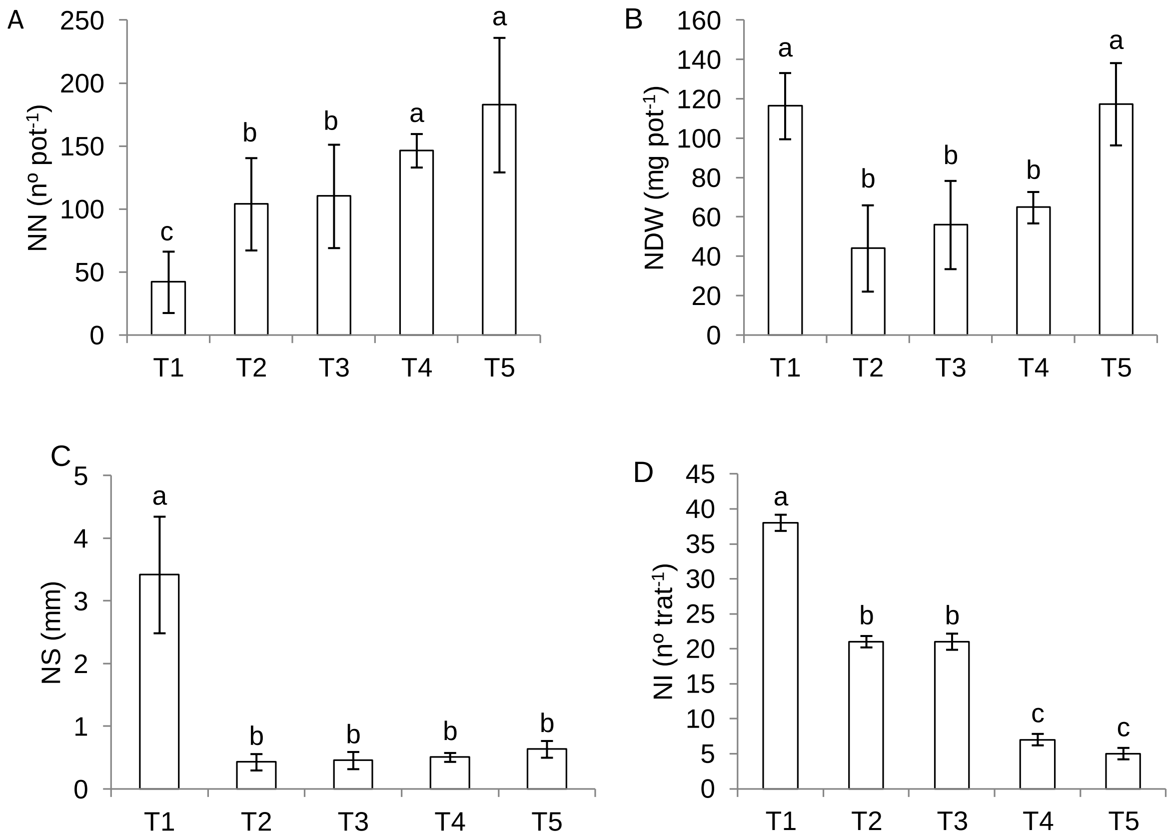

Figure 1. Effect of CTS on nodule number (NN), nodule dry weight (NDW), nodule size (NS) and number of rhizobia isolates (NI) in Lima bean grown in CTS-treated soils. T1= 0 Mg ha^{-1} CTS; T2 = 2.5 Mg ha^{-1} CTS; T3= 5.0 Mg ha^{-1} CTS; T4=10 Mg ha^{-1} CTS; and T5 = 20 Mg ha^{-1} CTS.

The accumulation of N increased with the application of the highest CTS rate (20 Mg ha^{-1}) as compared with the treatment without CTS (Figure 2A). Although, the highest CTS rate has increased the accumulation of N, the efficiency of BNF was highest in the CTS rates of 2.5 and 5 Mg ha^{-1} as compared with the treatment without CTS. Shoot dry weight was higher in treatment with the application of 20 Mg ha^{-1} CTS (Figure 3A). On the other hand, there was an increase in Cr content in shoot with the increase in CTS rates (Figure 3B).

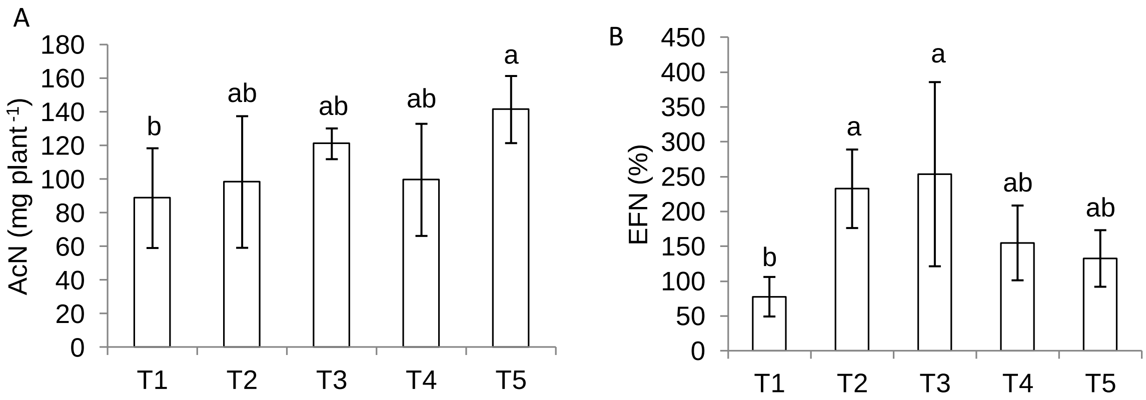

Figure 2. Effect of CTS on accumulation of N (AcN) and efficiency of N fixation (EFN) in Lima bean grown in CTS-treated soils. T1= 0 Mg ha^{-1} CTS; T2 = 2.5 Mg ha^{-1} CTS; T3= 5.0 Mg ha^{-1} CTS; T4=10 Mg ha^{-1} CTS; and T5 = 20 Mg ha^{-1} CTS.

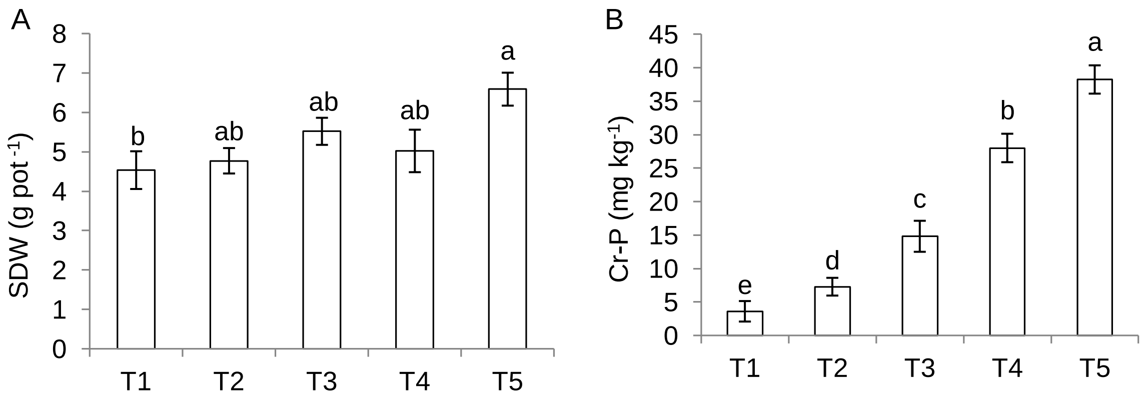

Figure 3. Effect of CTS on shoot dry weight (SDW) and accumulation of Cr in plant (Cr-P) in Lima bean grown in CTS-treated soils. T1= 0 Mg ha^{-1} CTS; T2 = 2.5 Mg ha^{-1} CTS; T3= 5.0 Mg ha^{-1} CTS; T4=10 Mg ha^{-1} CTS; and T5 = 20 Mg ha^{-1} CTS.

The analysis of redundancy has shown that 64.2% of total variation was explained by treatment and chemical properties of soil (Figure 4) and it separated the treatment in three distinct groups. Treatment without CTS (T1) separated from others, while the treatments with 2.5 and 5 Mg ha^{-1} CTS (T2 and T3); and 10 and 20 Mg ha^{-1} CTS formed two distinct groups. Also, RDA indicated electricity conductivity (EC) and pH as the main variables influencing the nodulation and BNF in this study. The analysis of correlation of Spearman (R) showed that the accumulation of Cr in shoot was positively correlated with chemical properties of soil, mainly soil Cr and pH (Table 3). On contrary, the number of isolates showed a significant negative correlation with CTS rates, Cr and pH in soil.

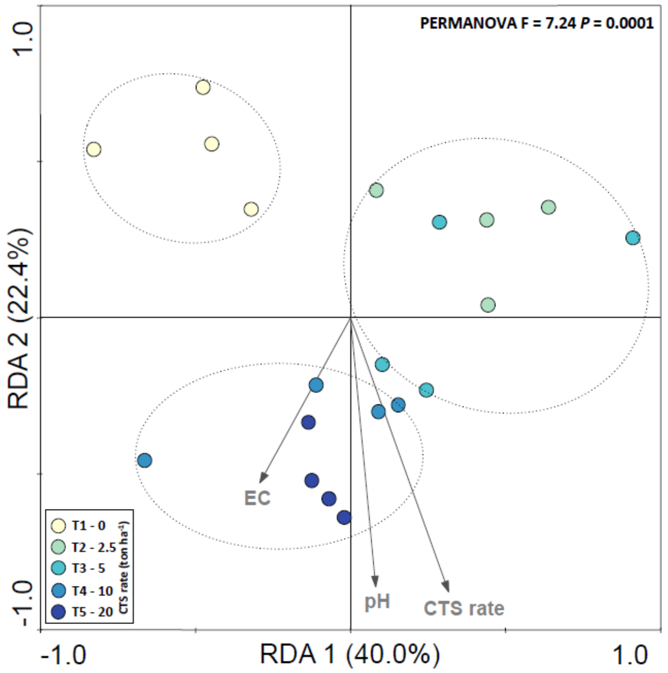

Figure 4. Redundancy analysis (RDA) between treatments (CTS-treated soils) and soil variables. T1= 0 Mg ha^{-1} CTS; T2 = 2.5 Mg ha^{-1} CTS; T3= 5.0 Mg ha^{-1} CTS; T4=10 Mg ha^{-1} CTS; and T5 = 20 Mg ha^{1}

Table 3. Spearman (R) correlation values between plant variables and soil chemical factors. Only significant correlations (P <0.05) are presented in the table.

	Cr-Plant	NI	NN	NS	AcN	SDW	AcN/R DW	NL	NDW
CTS rates	0.975	-0.922	0.797		0.472				
pH	0.963	-0.906	0.754						
EC			0.537						
TOC	0.786	-0.656	0.724		0.490	0.471			
P	0.795	-0.704	0.823		0.456			0.463	
Ca	0.832	-0.745	0.799	-0.486					
Mg	0.587	-0.478	0.686	-0.523			0.453		
Na									-0.444
Cr	0.943	-0.902	0.797						

Treatments with CTS distinctly influence nodulation of lima bean. While soils with CTS had stimulated the number of nodules in lima bean, these nodules were small and with reduced numbers of bacterial isolates. On the hand, these results suggest that CTS stimulates the nodule formation in lima bean, probably because of higher content of P in soil with CTS that contributes for bacterial infection and formation of nodules (SULIEMAN; TRAN, 2015). Also, the results show that the changes in soil chemical properties, after CTS amendment over time, did not negatively influence the formation of nodules. On the other hand, the highest size of nodules found in treatment without CTS can be more important than numbers of nodules in relation to the process of BNF. Therefore, many small nodules found in treatments with CTS could suggest lower efficiency of BNF (ANDRAUS et al., 2016) and also could indicate adverse conditions to native rhizobia (TEIXEIRA et al., 2006). In addition, nodules found in the treatments with CTS presented small numbers of bacterial isolates suggesting a negative effect on rhizobial diversity in soil and, at the same time, an effect of selection of most adapted rhizobia isolates to adverse conditions promoted by the waste.

Although with smallest nodules, treatments with CTS, mainly in the highest rate (20 Mg ha^{-1}), presented higher accumulation of N and it can be explained by the higher content on organic matter found in soils with application of CTS (Table 2) that could have contributed with N to plants. These results agree with Miranda et al. (2014), in cowpea, that observed an increase in the soil organic matter content, after application of CTS, and consequently higher N availability to plants. On the other hand, the N accumulated in plants from soil without CTS resulted from BNF. In this case, the results suggest that, although these plants showed larger nodular size and number of rhizobial isolates, the native bacteria were not efficient in BNF. Indeed, the efficiency of BNF was higher in plants under the CTS rates of 2.5 and 5 Mg ha^{-1} that, although the soils with these CTS rates accumulated less organic matter when compared to the highest CTS rates, they presented accumulation of N similarly to treatments with 10 and 20 Mg ha^{-1}.

The higher accumulation of N presented by plants in the treatments with CTS, mainly at the highest rate (20 Mg ha^{-1}), has contributed to the differences in the shoot dry mass of lima bean when compared to the treatment without CTS. Also, these results suggest that CTS promoted an increase in plant growth due to the availability of plant nutrients in the soil. Similar results were observed by Miranda et al. (2014) and Silva et al. (2010) evaluating the effect of CTS on the growth of cowpea and pepper, respectively.

The increase in Cr content in plants with the increasing of CTS rates reflects the pattern of Cr accumulation in soil after successive applications of CTS in long-term. Moreover, the results indicate that there is a direct absorption of this element by lima bean similarly with the response cowpea and maize to composted tannery sludge in a previous study (SOUSA et al., 2017). Previous studies with application of tannery sludge have shown an accumulation of Cr in the shoot of common bean (LOPEZ-LUNA et al., 2012) and Trigonela (SINHA et al., 2007). According to Sousa et al., 2017 there is influence of response of the crop plant for the accumulation, translocation and export of Cr.

In general, Cr uptake by the plants accumulates in the roots and forms barriers that diminish their translocation to the shoot (MOREIRA et al. 2013). BNF, being a process carried out at the root level, can be influenced by the excessive presence of Cr due to the alteration of the biochemical processes of the microorganisms and direct toxicity on *Bradyrizobium* (HUNGRIA et al., 1994) and rhizobial symbiosis (STAMBULSKA et al. 2018). The biological fixation of nitrogen in the soybean and absorption of P, K, Ca and Mg decreased in cultures with concentrations of Cr^{3+} higher than 20 mg L^{-1}. Cr content in the shoot dry matter of the soybean higher than 5.8 mg Kg^{-1} can be considered phytotoxic.

The redundancy analysis showed that there was an effect of CTS on the variables of nodulation, BNF and plant growth, as reported above. However, there is a separation between treatments with the lowest and highest CTS rates. On the one hand, data show that the application of CTS at 2.5 and 5 Mg ha^{-1} provided higher BNF efficiency and less negative effect on the diversity of isolates. On the other hand, at the rates of 10 and 20 Mg ha^{-1} a negative effect is observed on the nodulation and BNF that are significantly clustered with soil pH and salinity. This information can be confirmed by the Spearman correlation, which showed a significant negative effect of CTS, pH and, also Cr content in soil, on the number of isolates. This can be explained by the negative effect that these factors exert on the diversity of rhizobia, especially in tropical soils (HUNGRIA; VARGAS, 2000). Also, these factors may act on the rhizobia and their host affecting symbiosis, since abiotic factors such as pH and metals, such as chromium, quantitatively and qualitatively alter the rhizobia population in the soil (FIGUEIREDO et al., 2007).

4 Conclusions

The application of CTS, in long-term, increases the Cr accumulation in soil and influences the nodulation and nitrogen fixation in lima bean. Therefore, CTS increased nodulation while decreased rhizobia diversity in nodules. Also, the application of CTS increases the content of Cr in lima bean. On the other hand, the application of the lowest CTS rates improves the efficiency of biological N fixation.

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CAPÍTULO II - Capacidade bioquímica de bactérias promotoras de crescimento de plantas em solo contaminado com cromo após aplicação de lodo de curtume compostado

RESUMO

A aplicação de lodo de curtume compostado, em longo prazo, promove o acúmulo de cromo (Cr) no solo. Esta contaminação por Cr pode selecionar bactérias com capacidade bioquímica e capaz de tolerar alta concentração deste metal. Este estudo avaliou a capacidade bioquímica de bactérias promotoras de crescimento de plantas (BPCP) em nódulos radiculares de *Phaseolus lunatus* cultivados em solo contaminado com Cr com aplicação de lodo de curtume compostado. Um total de 54 BPCPs foram isolados em nódulos, sendo 40%, 37%, 13% e 10% isolados encontrados nos tratamentos com 2,5, 5, 10 e 20 Mg ha^{-1}, respectivamente. A maioria desses isolados foi positiva para o teste de urease, catalase e solubilização de fosfato, enquanto alguns isolados foram positivos para o teste de protease, lipase, carboximetilcelulose, gelatinase e amilase. Este estudo mostrou uma diminuição no número de isolados capazes de tolerar alta concentração de Cr. Os isolados UFPI-LCC61, UFPI-LCC64 e UFPI-LCC87 apresentaram alta capacidade bioquímica e de tolerância ao Cr. No entanto, o isolado UFPI-LCC87 apresentou alta capacidade bioquímica e tolerância à maior concentração de Cr.

Palavras-chave: Biorremediação; testes bioquímicos; resíduo industrial; tolerância.

Biochemical ability of plant growth-promoting bacteria in chromium-contaminated soil after application of composted tannery sludge

ABSTRACT

Application of composted tannery sludge, in long-term, have promoted accumulation of chromium (Cr) in the soil. This Cr contamination could select bacteria with biochemical ability and able to tolerate high concentration of this metal. This study evaluated the biochemical ability of plant growth-promoting bacteria in root nodules of *Phaseolus lunatus* grown in Cr-contaminated soil with application of composted tannery sludge. A total of 54 PGPBs were isolates in nodules, being 40%, 37%, 13% and 10% isolates found in the treatments with 2.5, 5, 10 and 20 Mg ha^{-1}, respectively. The majority of these isolates were positives for the test of urease, catalase, and phosphate solubilization, while some isolates were positives for the test of protease, lipase, carboxymethyl cellulose, gelatinase, and amylase. This study showed a decrease in the number of isolates able to tolerate high concentration of Cr. The isolates UFPI-LCC61, UFPI-LCC64 and UFPI-LCC87 presented high biochemical ability and tolerance to Cr. However, the isolate UFPI-LCC87 showed high biochemical ability and tolerance to the highest concentration of Cr.

Key words: Bioremediation; biochemical tests; industrial waste, tolerance.

1 Introduction

The incorrect disposal of industrial wastes has promoted soil pollution and affected the environment. Especially, the application of tannery sludge, a type of waste produced by tannery industries, has promoted the accumulation of chromium (Cr) in soil and, consequently, its uptake by plants (Sousa et al. 2017). In plants, high levels of Cr may interrupt photosynthesis and respiration processes, lead to oxidative damage and also inhibit enzymatic activity (Singh et al. 2013). Therefore, it is important to find suitable ways to protect the plants against the abiotic stress caused by Cr. Soil microorganisms may be used to protect the plants against Cr since they have different ways to endure metal toxicity (Ojuedeirie and Babalola 2017).

In Cr-contaminated soils, previous studies have reported that some tolerant bacteria can survive in high concentration of this metal (Mishra and Doble 2008; Ilias et al. 2011). Among soil bacteria, plant growth-promoting bacteria (PGPB) release plant-growth regulators, mineral solubilizers, phytohormones, and various secondary metabolites (Ahemad 2015), that can improve the plant growth (Sayel et al. 2014). Also, PGPBs present potential for mitigating plant stress in polluted soils (Tirry et al. 2018). According to Fahad et al. (2014), the use of PGPBs can be an effective and ecological solution to ameliorate the metal stress in plants.

PGPBs involve both symbiotic and free-living bacteria that infect legumes and non-legumes plants (Vargas et al. 2017). In legumes plants, rhizobia belonging to PGPB groups present ability to form root nodules and fix N from atmosphere, to be further converted to ammonia for plant uptake (Lindstrom and Martinez-Romero 2005). Also, rhizobia present potential for supporting contaminated soil with metals through different mechanisms, such as secretion of enzymes and bioactive metabolites (Hao et al. 2014). On the other hand, other non-rhizobia PGPBs are also found inside the nodules that, although they are unable for fixing N, present potential for promoting plant growth, mainly under environmental stress (Hidalgo and Hirsch 2017). Previous studies have been done evaluating tolerance of PGPBs in the presence of metals (Abdel-lateif 2017; Chaudri et al. 2008; Ausili et al. 2002), however it remains unclear the responses of these bacteria in Cr-polluted soil.

In this study, we used soils from an experimental field with long-term application of composted tannery sludge, during eight years, that present high accumulation of Cr. The main hypothesis would be that the accumulation of Cr over time could select tolerant PGPBs with biochemical ability to survive in Cr-polluted soil by producing enzymes with potential for degrading Cr. Therefore, the aim of this study was isolate and evaluate the biochemical ability of PGPBs found in root nodules of *Phaseolus lunatus* grown in Cr-contaminated soil with application of composted tannery sludge.

2 Material and methods

This study was conducted in a greenhouse located at Agricultural Science Center of Federal University of Piaui, Brazil (05º 05' S; 42º 48' W. 75 m) by using pots (2.8 L) with soils collected from the experimental field with application, for eight years, of composted tannery sludge (CTS) in five rates: 0, 2.5, 5, 10, and 20 Mg ha^{-1}; dry basis. The detail of this long-term field experiment with CTS can be found in Sousa et al. (2017). The experimental designed was completely randomized design with four replicates.

The soil pH ($CaCl_2$), electric conductivity (EC), exchangeable cations (Ca^{2+}, Mg^{2+}, K^+ and Na^+) and available phosphorus (P) were analyzed according to Donagema et al. (2011). Total organic C (TOC) was determined by the wet method according to Yeomans and Bremner (1978). Soil Cr was extracted by the DTPA-TEA method and measured using the USEPA-3050 method (USEPA 1986). The chemical properties of the soil are shown in Table 1.

Table 1. Chemical properties of the soil after eight years of consecutive application of composted tannery sludge (CTS).

CTS	pH	EC[1]	TOC[2]	P	K	Ca	Mg	Na	Cr
(Mg ha^{-1})	$CaCl_2$	dS m^{-1}	g kg^{-1}	mg dm^{-3}	--------mmolc dm^{-3}--------				Mg kg^{-1}
0	5.1	0.5	4.9	4.3	1.9	12.5	5.0	4.4	5.8
2.5	5.4	0.5	5.7	5.0	1.8	17.0	5.8	4.9	27.2
5	5.8	0.5	6.8	6.0	1.9	22.8	7.0	4.9	58.0
10	6.2	0.6	6.6	7.8	1.9	23.5	7.5	4.6	96.6
20	6.6	0.6	7.1	9.5	1.8	25.8	7.0	4.9	165.9

[1] Electric conductivity; [2] Total organic carbon

We used Lima bean (*Phaseolus lunatus*) as trap plant because its ability to tolerate adverse conditions (Fofana et al. 1997). Therefore, seeds of Lima bean were superficially disinfested in alcohol 70% for 30 s, and sodium hypochlorite 2% for 5 min, being after washed with autoclaved distilled water (Hungria and Araújo, 1994). At sowing, five seeds were used per pot and, on the seventh day after planting, two plants were left in each pot. All plants were irrigated, according to the crop requirement, with Hoagland and Arnon's N-free nutritive solution (2 mL kg^{-1} soil) (Silveira et al., 1998).

For isolation, characterization and evaluation of biochemical profile, three nodules were collected 45 days after plant emergence (during flowering). The sampled nodules were sterilized with sodium hypochlorite 0.1% (w/v) for 5 min, immersed in ethanol 95% (v/v) for 10 s, and then washed six times with distilled water. Nodules were streaked on yeast-extract mannitol agar (YMA) medium containing 0.0025% (w/v) of Congo red dye (Vincent, 1970). After 15 days, single colony was selected and re-streaked on YMA medium for purification.

For biochemical characterization, isolates were grown in YMA medium. The biochemical tests were: Gram (Yano et al. 1991), production of urease and protease (Dees et al. 1995), amylase (Vedder 1915), lipase (Renwick et al. 1991), catalase and gelatinase (Yano et al. 1991), and indole-3-acetic acid (IAA) (Sarwar and Kremer 1995). Also, the ability of solubilize P was estimated according to (Nautiyal. 1999; Verma et al. 2001).

The effects of different concentrations of Cr on the rhizobia were investigated in YMA culture medium containing the Congo red dye. The concentrations of Cr were: 0, 25, 50, 100 and 200 μg ml^{-1} in the form of $K_2Cr_2O_7$. After incubation at 28°C for 4-7 days, visual evaluations of growth were performed using two parameters: + (positive growth) and - (no growth), as compared with a control without Cr (Milicic et al. 2006).

We used the principal component analysis (PCA) to compare the metabolic profile of the isolates and relate them to the CTS rate. The PCA plot was generated using Canoco 4.5 software (Biometrics. Wageningen. The Netherlands).

3 Results

In this study, a total of 54 isolates were found in nodules of Lima bean grown in soils amended with CTS. From the total, we found 22 (40%), 20 (37%), 7 (13%) and 5 (10%) PGPBs in the treatments with 2.5, 5, 10 and 20 Mg ha^{-1} CTS, respectively. These isolates were assessed for biochemical tests.

The results showed that the majority of isolates were positives for the test of urease (95%), catalase (72%), and phosphate solubilization (46%); while some isolates were positives for the test of protease (37%), lipase (37%), carboxymethyl cellulose (26%), gelatinase (24%), and amylase (18%) (Table 2).

Table 2. Number of bacterial isolates with positive response of biochemical tests.

CTS Mg ha^{-1}	Catalase	Gelatinase	Urease	Protease	Amylase	Lipase	PS[1]	CMC[2]
2.5	12	7	20	8	6	4	5	5
5.0	16	3	19	9	3	12	13	5
10	7	2	7	3	1	4	4	3
20	4	1	5	-	-	-	3	1
Total	39	13	51	20	10	20	25	14

[1] phosphate solubilization; [2] carboxymethyl cellulose

From the total of isolates, we screened 20 that were positive for more than five biochemical tests (Table 3). In these biochemical tests, the results highlight the isolates UFPI-LCC61, UFPI-LCC64, and UFPI-LCC87 as positives for six biochemical tests. The test of tolerance to Cr showed six and eleven isolates with positive growth in the concentration of 100 and 200 ug mL^{-1} Cr, respectively (Table 3). For this test, the isolate UFPI-LCC87 (positive for six biochemical tests) was able to tolerate 200 ug mL^{-1} Cr. Interestingly, all isolates produced IAA, with the maximum production found for the isolate UFPI-LCC50.

Table 3. Biochemical ability and tolerance *in vitro* to Cr of bacterial isolates from soil with aplication of CTS.

Isolates*	Cat[1]	Gel[2]	Ure[3]	Prot[4]	Amy[5]	Lip[6]	PS[7]	CMC[8]	Cr μg mL^{-1}	IAA[9] μg mL^{-1}
UFPI-LCC01	+	+	-	-	-	+	+	+	200	15
UFPI-LCC04	+	-	+	-	-	+	+	+	200	17.3
UFPI-LCC05	+	+	-	-	-	+	+	+	200	22.6
UFPI-LCC41	-	+	+	-	-	+	+	+	100	70.0
UFPI-LCC43	-	+	+	+	+	+	-	-	200	54.5
UFPI-LCC44	+	-	+	-	-	+	+	+	200	8.3
UFPI-LCC45	+	+	+	+	+	-	-	-	50	80.5
UFPI-LCC50	+	+	+	+	+	-	-	+	100	660.7
UFPI-LCC58	+	-	+	+	+	-	-	+	200	4.7
UFPI-LCC61	+	-	+	+	-	+	+	+	100	4.5
UFPI-LCC64	-	+	+	+	-	+	+	+	100	9.9
UFPI-LCC69	+	+	+	-	-	+	+	-	50	3.0
UFPI-LCC71	-	-	+	+	-	+	+	+	200	6.7
UFPI-LCC72	+	-	+	+	-	+	+	-	200	14.7
UFPI-LCC74	+	-	+	+	-	+	+	-	100	8.8
UFPI-LCC75	+	-	+	+	-	-	+	+	200	6.1
UFPI-LCC81	+	+	+	-	-	+	+	-	50	17.1
UFPI-LCC83	+	-	+	+	-	+	+	+	100	5.6
UFPI-LCC84	+	-	+	+	-	+	+	-	200	4.4
UFPI-LCC87	+	-	+	+	-	+	+	+	200	5.7

[1] catalase; [2] gelatinase; [3] urease; [4] protease; [5] amylase; [6] lipase; [7]phosphate solubilization; [8] carboxymethyl cellulose; [9] indole-3-acetic acid

The PCA analysis based on the CTS rate and the biochemical profile of the isolates explained 97% of the data variation in the first two axes of the graph (Figure 1). This analysis grouped the isolates according to the CTS rate. The isolates UFPI-LCC45, UFPI-LCC50, UFPI-LCC43, UFPI-LCC58 and UFPI-LCC48 (from the treatment with 2.5 Mg ha^{-1}) correlated with the production of amylase and gelatinase; while the isolates UFPI-LCC41 and UFPI-LCC44 correlated with the production of protease and lipase.

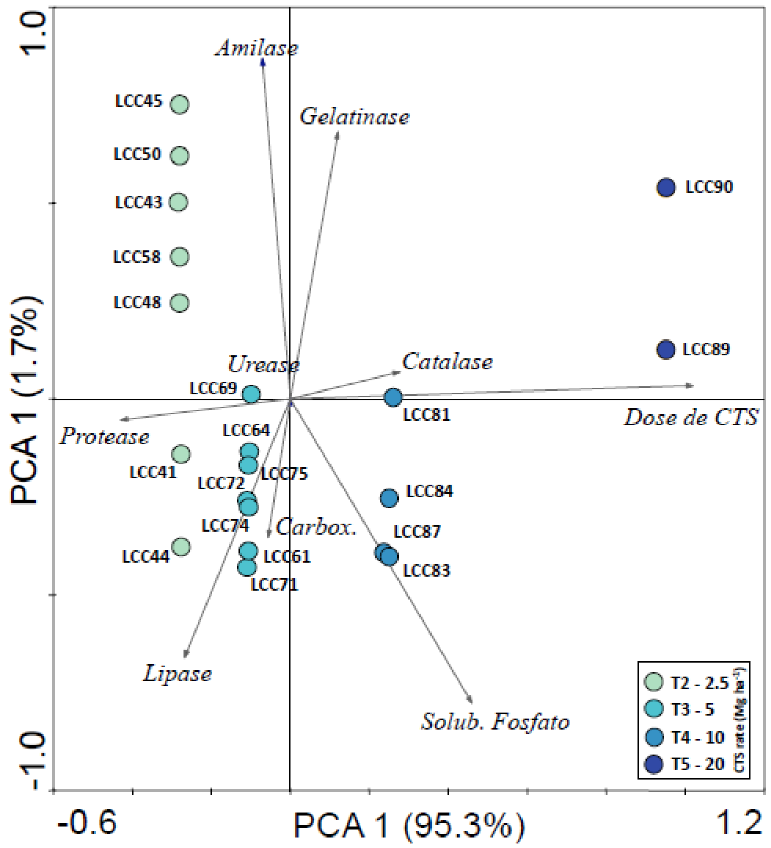

Figure 1. Principal component analysis (PCA) biplot based on the metabolic activity of isolates retrieved from soil treated with different CTS rates.

The isolates from the treatment with 5 Mg ha^{-1} correlated with the production of urease and carboxymethyl cellulose. Interestingly, the isolates from the treatment with 10 Mg ha^{-1} and 20 Mg ha^{-1} correlated with the production of catalase and phosphate solubilization.

4 Discussion

Application of CTS for eight years decreased the diversity of PGPBs. The results show a reduction in the number of isolates from the lowest to the highest CTS rates, which may suggest a negative effect of the long-term application of CTS on PGPBs probably due to the accumulation of Cr in soil. On the other hand, this study may have selected isolates able to tolerate this adverse condition. These results are important since the selection of PGPBs with tolerance to adverse environmental conditions may be an opportunity to find isolates for using in bioremediation of polluted sites (Stambulska et al. 2018).

This study evaluated and found PGPBs with important biochemical abilities, such as urease, catalase and phosphate solubilization activity. Thus, the ability of the majority of isolates in producing and releasing urease and catalase is important for the soil and plants. Firstly, urease activity is important for soil fertility since this enzyme catalyzes the hydrolysis of urea to ammonium that can be uptake by plants (Nosheen and Bano 2014). Secondly, the presence of isolates with catalase activity can be related with the ability of bacteria to protect plants against the oxidative stress. Therefore, these isolates are important for plant growth and can act as inductors of stress tolerance in plants.

In this study, the growth response of these isolates to Cr was also assessed and showed that the bacterial isolates responded to different concentrations of Cr. The results showed a decrease in the number of isolates able to tolerate high concentration of Cr. Indeed, only eleven isolates were able to grow in concentration of 200 ug mL^{-1} Cr. It agrees with Anyanwu and Ezaka (2011) who evaluated the response of bacterial isolates to different concentration of Cr and found a decrease in the growth of isolates as the concentration of Cr increased. These authors also observed that the isolates were slightly inhibited at concentration of 200 µg mL^{-1} Cr.

Thus, this result suggests the possibility of using these isolates as promising PGPBs and also for remediation purposes. These isolates with tolerance to high concentration of Cr may present potential for using in soils contaminated with this metal and also act as plant growth-promoter bacteria. Previous studies have reported that long-term exposure to metals imposes a selection pressure that favors the proliferation of metal tolerant microbes (Hutchinson and Symington 1997; Parameswari et al. 2009). This tolerance may be associated with the ability of these isolates to produce and release enzymes that enable them to thrive the high concentration of Cr. Therefore, the presence of isolates with tolerance to concentrations of Cr in soils with CTS suggest that the permanent application of this waste fosters adaptation and selection of Cr resistant bacteria.

The isolates found in treatments with the highest CTS rates (10 and 20 Mg ha^{-1}) correlated with the production of catalase and phosphate solubilization. Catalase is an enzyme that catalyzes the decomposition of hydrogen peroxide to water and oxygen, being important in protecting the cell from oxidative damage by reactive oxygen species (ROS) (Chelikani et al. 2004). It is known that some bacterial groups have the ability for producing catalase as defense mechanisms to suppress oxidative stress (Nakamura et al. 2012). A previous study with bacterial isolates from soils with Cr has shown that the production of catalase was not affected by this metal (Silva et al. 2012). It may confirm that some bacteria can keep the functional activity of catalase in the presence of Cr and highlights the importance of this enzyme to protect the bacteria against the accumulation of hydrogen peroxide.

The presence of phosphate-solubilizing bacteria in soil is important as they can solubilize soil P and make available to plants. However, some studies have evaluated these bacteria under adverse environmental conditions, such as salinity and high temperature, and found high phosphate solubilization in these environmental stress (Gain and Gaur 1991; Johri et al. 1999). Although it is not clear the effect of Cr on phosphate-solubilizing bacteria, usually these bacteria present abilities for remediating metal contaminated soil through chelation of metals and facilitating phytostabilization (Paul and Sinha 2015). This result suggests that these isolates from soils with CTS have the potential to produce catalase and solubilize phosphates at high concentrations of Cr. These isolates may also be used as potential bacterial strains to restore soils with problems of Cr contamination. Thus, further study should be done for obtaining inoculants that are able to promote the plant growth and protect them against stress.

5 Conclusions

The long-term application of composted tannery sludge, for eight years, promoted the accumulation of Cr in soil and selected bacterial strains with biochemical abilities and tolerance to Cr. This study showed a decrease in the number of isolates able to tolerate high concentration of Cr. The isolates UFPI-LCC61, UFPI-LCC64 and UFPI-LCC87 presented high biochemical ability and tolerance to Cr. However, the isolate UFPI-LCC87 showed high biochemical ability and tolerance to the highest concentration of Cr. Therefore, these isolates present potential to be used in soils contaminated with Cr and also for promoting plant growth.

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