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WALDO PEREIRA NETO

EFEITO DA COINOCULAÇÃO DE RIZOBACTÉRIAS *STREPTOMYCES* NO PERFIL DE EXSUDATOS DE RAÍZES E NO CRESCIMENTO DE PLANTAS DE MILHO (*ZEA MAYS* L.)

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Orientadora: Profa. Dra. Eliane Romanato Santarém

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RESUMO

O solo que sofre influência das raízes das plantas é denominado rizosfera. Nessa região, ocorre a deposição de moléculas (rizodeposição), que, entre outras funções, recrutam microrganismos e modulam o microbioma. Esses microrganismos atuam nos processos de reciclagem, solubilização e biodisponibilização de nutrientes do solo para o benefício das plantas, favorecendo o crescimento e a defesa dos vegetais. O presente estudo visou avaliar os efeitos de rizobactérias *Streptomyces* spp. no crescimento e no perfil de moléculas presentes nos exsudatos das plantas de milho (*Zea mays* L.). As sementes de milho foram microbiolizadas com os isolados CLV89, CLV104 e CLV179, individualmente ou em dupla ou tripla coinoculação (10^8 ufc $mL^{-1)}$. Sementes não microbiolizadas e sementes tratadas com o inoculante comercial *Azospirillum brasiliense* foram utilizadas como controle negativo e positivo, respectivamente. O crescimento vegetativo foi analisado após 45 dias nas plantas cultivadas em casa de vegetação ou após 21 dias de cultivo em laboratório. A colonização das raízes foi comprovada por microscopia eletrônica de varredura. Os exsudatos em plantas de 21 dias foram coletados em 100 mL de água filtrada estéril, por 24 h, sob iluminação contínua. Aminoácidos, açúcares redutores, proteínas e compostos fenólicos foram quantificados através de reações colorimétricas. O perfil de compostos fenólicos foi determinado por cromatografia líquida de alta eficiência (CLAE) em 258 nm. Os ácidos gálico, hidroxibenzóico, vanílico, ferúlico, cafeico, cinâmico e cumarina foram os compostos fenólicos analisados. Os isolados de *Streptomyces* podem ser considerados rizobactérias promotoras do crescimento de plantas (PGPR), uma vez que foram capazes de produzir a auxina ácido indolacético e seus metabólitos, além de sideróforos, amônia e fenazinas. O CLV104 promoveu efetivamente o crescimento de folhas e raízes de milho em comparação com os demais isolados. A coinoculação tripla, que incluiu o CLV104, não teve efeito promotor no crescimento das plantas superior àquele encontrado com o isolado CLV104. De fato, levou à redução do crescimento das raízes. A coinoculação CLV89+CLV104 promoveu o crescimento de raízes e folhas. O perfil de exsudação das plantas tratadas com os três isolados foi significativamente maior do que com a inoculação única e aos controles, com exceção dos compostos fenólicos. O perfil de compostos fenólicos exsudados pelas raízes analisados por CLAE variou de acordo com o tratamento. Os resultados indicam que a interação entre raízes e *Streptomyces* é mediada, pelo menos em parte, por exsudatos, e a rizodeposição de compostos fenólicos variou especificamente com o isolado colonizando as raízes. A tripla coinoculação induziu a deposição da combinação de compostos, sugerindo que as rizobactérias estão modulando efetivamente o metabolismo secundário das plantas.

Palavras-chaves: Actinomycetes, Consórcio, Exsudação, PGPR, Rizodeposição.

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CAPÍTULO 1

1. Introdução

1.1 Rizosfera e Rizobactérias

A região adjacente às raízes de uma planta, que sofre alterações causadas pela mesma, é denominada rizosfera (do grego, *rhizo* = raiz e *sphera* = campo). Esse termo foi descrito por Lorenz Hiltner, em 1904, e depois por Pinton et al. (2001) como sendo o volume total de solo colonizado por microrganismos e influenciado pelos compostos secretados pelas raízes, formando assim uma região de grande interação bioquímica (Pinton et al., 2007). A rizosfera pode ser dividida em três regiões: a *endorizosfera*, que se refere ao espaço apoplástico entre o córtex e endoderme, que pode ser colonizado por organismos endofíticos; o *rizoplano*, o qual se refere à zona medial próxima à raiz, incluindo a epiderme, e a *ectorizosfera*, que é a região mais externa que se estende até o solo que não sofre influência das raízes (Basu et al., 2017). A quantidade de bactérias na rizosfera é geralmente maior do que aquela encontrada no solo que não sofre influência direta das raízes (Vejan et al., 2016), caracterizando a rizosfera como uma fonte de bactérias produtoras de metabólitos secundários, como auxina, antibióticos, e enzimas extracelulares, e caracteriza-se como uma região importante para processos relacionados com a nutrição da planta, trocas de O_2 e CO_2, mineralização, amonificação, nitrificação, processos simbióticos e funcionamento dos ecossistemas (Hassan et al., 2019). As bactérias hábeis na colonização da raiz são referidas como rizobactérias, formando uma numerosa e significativa comunidade rizosfera-competente, abrangendo de 10^5-10^7 unidades formadoras de colônias por grama de raiz fresca (Bulgarelli et al., 2013; Vacheron et al., 2013). Entre as rizobactérias, estão aquelas que apresentam características metabólicas que interferem positivamente no crescimento das plantas e são conhecidas como Rizobactérias Promotoras do Crescimento Vegetal (PGPR; do inglês, *Plant Growth-Promoting Rhizobacteria*) (Zhou et al., 2015). Dentre este grupo, destacam-se os filos Actinobacteria e Proteobacteria, os quais contêm diversas espécies reconhecidas como PGPR (Amaresan et al., 2017).

As PGPR podem melhorar o crescimento das plantas atuando por múltiplos mecanismos, promovendo o crescimento das raízes (fitoestimulação), garantindo a disponibilidade e absorção de certos nutrientes para a planta (biofertilização), além de estimular a proteção contra estresses bióticos e abióticos (biocontrole) (Tabassum et al., 2017; Gouda et al., 2018). Especificamente, as PGPR produzem vários compostos, incluindo reguladores de crescimento (fitormônios), sideróforos e ácidos orgânicos, fixam nitrogênio atmosférico, solubilizam fósforo e produzem antibióticos para suprimir microrganismos que comprometem o desenvolvimento vegetal. Essas substâncias afetam diretamente o metabolismo das plantas ou melhoram a capacidade adaptativa das plantas de absorver outros nutrientes do solo (Grobelak et al., 2015; Singh et al., 2015). Os fitormônios, incluindo auxinas, citocininas, giberelinas, etileno e ácido abscísico, ajudam as plantas em processos de crescimento, incluindo alongamento e ramificação de raízes (Kudoyarova et al., 2015). As PGPR também podem atuar em processos mais específicos como detoxificação de metais pesados e degradação de poluentes

ambientais, interferindo assim diretamente no ecossistema do solo (rizodegradação), podendo afetar o crescimento das plantas indiretamente (Vocciante et al., 2022).

A colonização radicular por PGPR é considerada um pré-requisito para a promoção do crescimento das plantas. As rizobactérias se disseminam de uma fonte de inóculo, como tratamentos de sementes, para a região da raiz em crescimento ativo e se multiplicam ou crescem na rizosfera, um processo conhecido como colonização de raízes (Benizri et al., 2001). A dispersão das rizobactérias do local de inoculação para a região de crescimento das raízes ocorre pelo movimento ativo e passivo das bactérias. Um modelo de colonização de raízes, proposto por Newman e Watson (1977), prevê que uma abundância de rizobactérias ocorra próximo da região da ponta da raiz da planta em crescimento.

O sucesso da colonização radicular está condicionado à percepção de sinais por ambos os parceiros da interação biológica. A secreção de moléculas pelas raízes, processo conhecido por exsudação (Singh et al., 2019), tem efeito significativo na composição do rizomicrobioma, pois os exsudatos radiculares são a fonte central de nutrientes na rizosfera, criando um nicho para o crescimento de microrganismos nessa região (Zhalnina et al., 2018).

1.2 Rizodeposição

O processo de depósito de compostos com carbono liberados pelas raízes das plantas na rizosfera é denominado rizodeposição, no qual a matriz vegetal radicular exsuda material intracelular (exsudatos radiculares) (García-Salamanca et al., 2013). Cerca de 200 compostos contendo carbono (C) estão presentes nos rizodepósitos e estima-se que cerca de 11% do C líquido fixado pela fotossíntese ou 27% do C alocado ao sistema radicular é exsudado para a rizosfera pelas raízes, embora esta porcentagem possa variar dependendo da espécie da planta, idade e estado nutricional (Jones et al., 2009; Nakayama e Tateno, 2018). Os rizodepósitos são produzidos pela planta e regulam as relações biológicas e físico-químicas entre o sistema radicular, o solo e os organismos ali presentes, por conseguinte, estruturando o rizomicrobioma (Bais et al., 2008, Baetz e Martinoia, 2014). Além de modularem o ambiente rizosférico, estruturam a interação espécie-específica, principalmente na região do rizoplano (Lugtenberg, 2015), o que garante a colonização seletiva, por recrutamento das bactérias específicas. Assim, os exsudados são componentes importantes no acúmulo de carbono no solo, e com isso contribuem para o crescimento e desenvolvimento vegetal (Vives-Peris et al., 2020).

A rizodeposição é dividida em (1) exsudados hidrossolúveis, com substâncias de baixo peso molecular liberadas das raízes por difusão passiva, (2) secreções, compreendendo substâncias de alto peso molecular com dependência de processos metabólicos para sua liberação, (3) excreções, incluindo dióxido de carbono, íons de bicarbonato, prótons, elétrons, etileno, (4) lisados, compreendendo o conteúdo das células descamadas e raízes inteiras e (5) mucilagem, que cobre as raízes de muitas plantas e é composta principalmente de polissacarídeos e ácidos poligalacturônicos de alto peso molecular (Vranova et al., 2013).

Os exsudados são oriundos dos metabolismos primário e secundário das plantas (Dietz et al., 2020). Os metabólitos primários são comumente produzidos em maior quantidade; em geral são compostos por carboidratos, ácidos orgânicos e aminoácidos. A Tabela 1 apresenta alguns metabólitos primários encontrados em *Arabidopsis thaliana* L. (Vives-Peris et al., 2020). Esses compostos servem como fonte energética para diversos microrganismos e reestruturam a rizosfera, alterando o rizomicrobioma (Bhattacharyya e Jha, 2012; Sasse et al., 2018).

Os metabólitos secundários são formados a partir dos metabolitos primários e apresentam maior complexidade molecular (Kessler e Kalske, 2018). São formados em menor quantidade e atuam de maneira direta sobre a nutrição vegetal, como por exemplo, a escopolamina, que quando secretada, age como sideróforo, auxiliando na absorção de ferro em *A. thaliana* (Dimkpa et al., 2016). De forma indireta, metabólitos secundários, como os flavonoides, sinalizam e recrutam bactérias do grupo dos rizóbios, como *Bradyrhizobium* sp., em leguminosas, assim como estrigolactona, que atua como sinalizador para certas espécies de micorrizas (Venturi e Keel, 2016). Exemplos de metabólitos secundários estão listados na Tabela 1.

A composição dos metabólitos depositados pela raiz é variável. Normalmente, a concentração de ácidos orgânicos e aminoácidos nas raízes é de 10 a 20 mM (1– 4% da matéria seca total), e a concentração de açúcares está em torno de 90 mM (Jones et al., 1998). Além disso, as moléculas produzidas por plantas C3 são diferentes das plantas C4. Em milho, por exemplo, encontram-se os açúcares (glicose, frutose, arabinose, xilose, sacarose, galactose, inositol, eritritol), ácidos orgânicos (os ácidos oxálico, succínico, tartárico, málico, fumárico, transaconítico, cítrico) e diversos aminoácidos (ácido glutâmico e aspártico, ácido g-aminobutírico, leucina e isoleucina, alanina, glicina, prolina, serina, lisina, valina, arginina, histidina). Da mesma forma, dependendo da região da raiz, observa-se alteração na composição dos metabólitos. Em *Paspalum notatum* Flüggé, a concentração de aminoácidos e açúcar secretados pelas raízes jovens é maior em comparação com raízes mais antigas (Nabais et al., 2011).

1.3 Actinomycetes e *Streptomyces* spp. como PGPR

Os actinomicetos, um dos principais grupos de bactérias com populações presentes na rizosfera, têm importância ecológica na ciclagem de nutrientes do solo (Elliot e Lynch 1995), além de promover o crescimento de plantas (Merzaeva e Shirokikh, 2006). Pertencente à Ordem Actinomycetales e à Classe Actinobacteria, o gênero *Streptomyces* tem sido referido como PGPR. Esse gênero compreende bactérias aeróbicas Gram-positivas, com mais de 500 espécies descritas (Amaresan et al., 2017). Este gênero tem origem com o desenvolvimento das raízes em plantas vasculares, há mais de 400 milhões de anos (Chater, 2016). São conhecidas por seu complexo ciclo de vida, o qual inclui crescimento de micélio e produção de esporos e por sintetizar a maioria dos antibióticos usados em medicina (Charlop-Powers et al., 2016). As bactérias *Streptomyces* spp. são reconhecidas como PGPR por sua ação na

disponibilização de nutrientes e na produção de fitormônios, bem como por auxiliar nas estratégias de fitoremediação (Amaresan et al., 2017). São microrganismos que apresentam tolerância a condições ambientais adversas, como salinidade, metais pesados e seca (Abbasi et al., 2020; Nozari et al., 2021; Zade et al., 2018). Além disso, foi demostrado que isolados de *Streptomyces* são agentes de biocontrole. *Streptomyces* sp. AcH50 teve papel importante na resistência contra o fungo *Erysiphe alphitoides*, causador de uma doença característica em carvalhos (Kurth et al., 2014) e *S. rochei* induziu tolerância em *Cicer arietinum* contra *Sclerotinia sclerotiorum* (Srivastava et al., 2014).

Tabela 1. Metabólitos primários e secundários encontrados em exsudados de *Arabidopsis thaliana*. Modificado de Vives-Peris et al. (2020).

Aminoácidos	Alanina, arginina, asparagina, cisteína, glutamina, histidina, leucina, isoleucina, metionina, ornitina, prolina, serina, treonina, tirosina, triptofano e valina.
Açúcares	Arabinose, frutose, galactose, glicose, maltose, manose, rafinose, ribose, sacarose e desoxirribose.
Ácidos orgânicos	Acético, ascórbico, benzoico, butírico, cítrico, fumárico, láctico, oxalacético, tartárico, *p*-hidroxibenzoico.
Ácidos graxos	Ômega-3, ômega-6, oleico, palmítico e esteárico.
Esteróis	Campesterol, colesterol, sitoesterol e estigmaesterol.
Fatores de crescimento e vitaminas	Ácido aminobenzoico, biotina, colina, inositol, ácido nicotínico, niacina, piridoxina, riboflavina, estrigolactonas e tiamina.
Enzimas	Amilase, invertase, peroxidase, fosfatase ácida/alcalina, protease, poligalacturonase.
Nucleotídios/purinas	Adenina, guanina e uridina/citidina.
Flavonoides	Chalcona, cumarina, flavona, flavanona, flavonois e isoflavonas.

Nos trabalhos desenvolvidos no Laboratório de Biotecnologia Vegetal da PUCRS, a promoção do crescimento de plantas e o aumento de resistência a doenças mediados por isolados *Streptomyces* spp. têm sido demonstrados. Plantas de *Araucaria angustifolia* tiveram seu crescimento promovido quando tratadas com *Streptomyces* spp. (PM1, PM4 e PM9) (Dalmas et al., 2011). A caracterização bioquímica de seis isolados de *Streptomyces* (PM1, PM3, PM4, PM5, PM6 e PM9) demostrou que estes isolados são capazes de produzir AIA, sideróforos, solubilizar fosfato, além de apresentar antibiose contra *Pectobacterium carotovorum* var. *brasiliense* (Dias et al., 2017). Plantas de *Eucalyptus globulus* e *E. grandis* apresentam modulação do metabolismo secundário quando tratadas com *Streptomyces* spp. (Salla et al., 2014), além de apresentar redução da doença mofo cinzento quando pré-tratadas com *Streptomyces* spp. e desafiadas por *Botrytis cinerea* (Salla et al., 2016). *Streptomyces* CLV27, CLV42 e CLV45 apresentaram características de PGPR e estimularam o crescimento vegetativo de soja

(Horstmann et al., 2020) e a CLV45 modulou o metabolismo de defesa de plantas de soja quando desafiadas por *Xanthomonas axonopodis* var. *glycines* (Horstmann, 2017). Dez isolados de *Streptomyces* mostraram tolerância à salinidade (100 e 300 mM NaCl), mantendo o crescimento bacteriano e produzindo compostos indólicos nessas condições. Esses isolados foram promotores do crescimento de plantas de milho e, dentre eles, os isolados CLV95, CLV97, CLV178 e CLV179 atenuaram os efeitos da salinidade no crescimento de plantas dessa espécie (Nozari et al. 2021; Nozari, 2022).

1.4 Coinoculação de raízes com rizobactérias

As formulações comerciais de PGPR utilizadas na agricultura são frequentemente produzidas a partir de cepas únicas com ação biológica, como agentes de controle de fitopatógenos, estratégia que é eficiente, sustentável e de baixo custo (Sundaramoorthy et al., 2012). Dos produtos disponíveis no mercado, poucos são constituídos por bactérias *Streptomyces* (revisado por Olanrewaju e Babalola, 2019; Tabassum et al., 2017) e na totalidade são aplicados como agentes de biocontrole. No entanto, em busca da maximização do potencial biótico das PGPR, novas abordagens vêm sendo empregadas, propondo formulações com múltiplas bactérias, na perspectiva de que o consórcio entre PGPR seja mais eficiente e versátil que a inoculação com um único microrganismo (Molina Romero et al., 2017; Zeffa et al., 2020). Isso é decorrência da possibilidade dessas bactérias agirem sinergicamente, influenciando de maneira positiva o crescimento e desenvolvimento da planta (Singh et al., 2013; Zoppellari et al., 2014).

Embora apresente potencial na agricultura, o sucesso dessa estratégia ainda é restrito (Syed et al., 2020). No entanto, observa-se um aumento no uso da alternativa de consórcio, ou coinoculação, de bactérias para promover crescimento de plantas. O uso de consórcio ou misturas de PGPR pode ser exemplificado em milho. Como exemplo, o consórcio entre fungos micorrízicos, *Bacillus* spp. e *Pseudomonas* spp., resultou em alterações na transpiração, na atividade estomática e no consumo de CO_2 de plantas de milho, e promoveu tolerância à salinidade, indicando o efeito sistêmico do sobre as plantas (Zoppellari et al., 2014). Em trigo (*Triticum aestivum* L.), Emami e colaboradores (2020) demonstraram que o consórcio entre bactérias tem efeito sinergético, promovendo o crescimento e o desenvolvimento das plantas. Da mesma forma, a coinoculação de diferentes cepas de *Streptomyces* na cultura de mandioca (*Medicago sativa*) resultou em aumento do número de ramos e de raízes secundárias de plantas micropropagadas (Lopes et al., 2019), enquanto em feijão de corda (*Vigna unguiculata*) houve aumento nos níveis de prolina quando realizada a coinoculação de *Streptomyces* spp. com *Bradyrhizobium* sp. (Santos et al., 2014). Além disso, o consórcio das cepas *Streptomyces* KPS-E004 e KPS-A032 foi efetivo contra nematoides em *Capsicum* spp. (Nimnoi e Ruanpanun, 2020).

1.5. A cultura do milho

O milho (*Zea mays* L.) é uma espécie da Família Poaceae, e uma das culturas mais antigas do mundo; registros de seu cultivo data de, no mínimo, sete mil anos. Evidências arqueológicas indicam que a domesticação do milho iniciou com o seu ancestral teosinto há, pelo menos, 9 mil anos (Landau et al., 2020). Por ser originário das Américas, mais especificamente do México, o milho já era conhecido pelos índios brasileiros na ocasião da chegada dos portugueses. Após o descobrimento da América, o milho foi levado para a Europa, onde era cultivado em jardins, até que seu valor alimentício se tornou conhecido nesse continente (Kaush et al., 2021).

Atualmente, o milho é a cultura agrícola mais importante do mundo, a única cuja produção já ultrapassou a marca de 1 bilhão de toneladas. Apesar de o seu principal uso ser a alimentação animal, é possível produzir uma gama de produtos com o cereal, com destaque para combustíveis (Andrade et al., 2020). Muito energético, o milho traz em sua composição vitaminas A e do complexo B, proteínas, gorduras, carboidratos, cálcio, ferro, fósforo e amido, além de ser rico em fibras. Cada 100 g do alimento tem cerca de 360 Kcal, sendo 70% de glicídios, 10% de protídeos e 4,5% de lipídios (CIB, 2006). É o terceiro cereal mais importante do mundo, amplamente usado na alimentação humana e animal (Gavilanes et al., 2020). No Brasil, a safra 2021/2022 deverá atingir 115,2 milhões de toneladas, 32,3% superior à safra 2020/2021, com previsão de 77,1 milhões de toneladas para consumo interno e 37 milhores de toneladas para exportações (CONAB, 2022).

Os avanços tecnológicos na área agronômica vêm auxiliando no manejo da cultura do milho, e são cruciais para a sua produtividade e desempenho (Marini et al., 2015). Nesse sentido, o rendimento da cultura pode ser limitado pelo seu alto requerimento de N (Gotosa et al., 2019). Atualmente, a inoculação de sementes de milho com a rizobactéria *Azospirillum brasiliense* oferece uma estratégia para a redução da adubação nitrogenada (Pereira-Defilippi et al., 2017).

1.6 O uso de PGPR na agricultura: uma alternativa para a sustentabilidade dos cultivos

Na última década, houve um aumento na população mundial a uma taxa alarmante, com previsão de atingir mais de 9 bilhões de pessoas até 2050. O crescimento e a produtividade das plantas em todo o mundo são limitados por estresses ambientais, como seca, salinidade, temperaturas extremas, disponibilidade limitada de nutrientes e presença de metais pesados (Basu et al., 2017). Consequentemente, a produção de alimentos agrícolas precisa ser aumentada consideravelmente para contemplar a população em crescimento. Embora o desenvolvimento de cultivares tolerantes a estresses esteja em desenvolvimento, o processo de produção de tais variedades vegetais é oneroso e demorado. Desta forma, o uso de microrganismos não patogênicos que colonizam a rizosfera das plantas e, portanto, favorecem o crescimento direta ou indiretamente, vem ganhando prioridade no manejo dos estresses ambientais. Além da produção adequada de alimentos, há preocupação da população excesso do uso de fertilizantes e agrotóxicos, que levam a sérios danos ao ambiente e à saúde dos organismos

não-alvo (Vejan et al., 2016). Nesse sentido, as PGPR desempenham importante serviços ecossistêmicos na agricultura, essencialmente aumentando suprimento de nutrientes e a fertilidade do solo e reduzindo os efeitos danosos de patógenos ou estresses abióticos nas plantas, com menor contaminação ambiental. O uso de microrganismos na agricultura é, portanto, uma alternativa para o desenvolvimento de uma agricultura sustentável e ecologicamente correta (Calvo et al., 2014; Prasad et al., 2019).

2. Hipóteses

- A inoculação com mais de um isolado da rizobactéria *Streptomyces* promove o crescimento de plantas de milho, em valor superior ao obtido pelos isolados individuais e ao controle não microbiolizado.

- Há alteração no perfil de exsudados das raízes de milho dependente do isolado de rizobactéria utilizado para colonização.

3. Objetivos

3.1 Objetivo geral

Avaliar o crescimento de plantas de milho (*Zea mays* L.) e o perfil metabólico dos exsudados de raízes mediante tratamento de rizobactérias *Streptomyces*, isoladas ou em consórcio.

3.2 Objetivos específicos

- Verificar a compatibilidade de interação entre três isolados de *Streptomyces* através da técnica de cocultivo *in vitro*;

- Determinar o efeito promotor do crescimento de plantas de milho mediante o consórcio de rizobactérias, comparando com os efeitos individuais dos isolados de *Streptomyces* e com o inoculante comercial;

- Determinar o perfil de exsudados de raízes de milho quando são colonizadas de forma isolada ou com mais de um isolado de *Streptomyces*;

- Confirmar a colonização das raízes de milho pelas rizobactérias *Streptomyces* após cultivo em solo.

CAPÍTULO 2

Co-inoculation with *Streptomyces* isolates promotes growth of maize plants and affects the profile of metabolites exudated by the roots

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Abstract

Microorganisms, individually or in consortium, present in the rhizosphere, promote plant growth and their interaction with the roots are mediated by root exudates. The present study evaluated the growth of maize plants (*Zea mays* L.) and the metabolic profile of root exudates by treating seeds with *Streptomyces* rhizobacteria, isolated or in co-inoculation. Maize seeds were bacterized with isolates CLV89, CLV104 and CLV179, individually or in dual or triple co-inoculation. Non-bacterized seeds and seeds treated with the commercial inoculant *Azospirillum brasiliense* were used as negative and positive controls, respectively. Vegetative growth was analyzed, and root colonization was confirmed by scanning electron microscopy. Exudates from 21-day-old plants were collected in 100 mL of sterile filtered water during 24 h under continuous light. Amino acids, reducing sugars, proteins and phenolic compounds were quantified through colorimetric reactions. The profile of phenolic compounds was determined by HPLC. The *Streptomyces* isolates produced indoleacetic acid auxin and its metabolites, siderophores, ammonia and phenazines. CLV104 effectively promoted the growth of maize compared to the other isolates. Triple co-inoculation, which included CLV104, showed similar effect on plant growth to that found with the single inoculation with CLV104, although reduced root growth was observed. Co-inoculation CLV89+CLV104 promoted root and leaf growth. The exudation of amino acids, proteins and sugars of plants treated with the three isolates was significantly higher than that of the single inoculation and the controls. The profile of phenolic compounds exuded by the roots varied according to the treatment, indicating that the interaction between roots and *Streptomyces* is mediated, at least in part, by exudates, and the rhizodeposition of phenolic compounds varied specifically with the isolate colonizing the roots.

Key words: Actinomycetes, consortium, PGPR, phenolic compounds; rhizodeposition

1. Introduction

Crop production has become highly dependent on synthetic fertilizers and pesticides. Undoubtedly, chemical fertilizers are of paramount importance, as they replace the nutrients in the soil that were consumed by plants during their development and contribute to the production of high yields and quality. However, inappropriate practices in the use of chemical fertilizers cause negative impacts on the environment, contaminating soils and water bodies through the leaching of these compounds, leading to the eutrophication process, which precedes a trophic imbalance in the ecosystem (Guan et al., 2015). Alternatives with less environmental impact are necessary to mitigate the negative effects of agrochemicals, especially fertilizers. The bio-input market is a promising alternative and a target of research for the formulation of commercial products (Shaikh et al., 2018), and therefore, the use of microorganisms with the objective of increasing agricultural production and reducing the use of chemical fertilizers has increased in recent years (Vejan et al., 2016).

Acting as biofertilizers for plants, rhizosphere bacteria, known as Plant Growth Promoting Rhizobacteria, have enormous potential to assist in the development of more sustainable agriculture (Kour et al., 2020), due to the production and secretion of hormones and molecules that increase the availability of nutrients, such as phosphorus and iron, in the soil (Shaikh et al., 2018). In comparison with synthetic fertilizers, microbial inoculants have advantages such as less impact on the environment and on human health, as well as improving soil fertility (Adedeji et al., 2020). Indeed, they often tolerate unfavorable environmental conditions such as water deficit, saline stress, lack of nutrients, and heavy metal pollution (Fasusi et al., 2021). Among the known applications in the use of biofertilizers and biopesticides, bacteria of the genus *Bacillus*, *Pseudomonas*, *Bradyrhizobium*, and *Azospirillum* have been highlighted as commercial products for application in crops, including soybeans and maize (Bettiol et al., 2012; Portugal et al., 2016).

Most works report plant growth promotion and biocontrol using single PGPR strains. However, the formulation of PGPR mixtures is a strategy to address multiple modes of action of these rhizobacteria and consequently broaden the spectrum of target crops. New technologies, such as rhizosphere engineering, for creating synthetic microbial communities with either different bacterial strains or a mixture of bacteria and fungi to promote plant growth, disease resistance, and stress tolerance are evolving and becoming more accessible (Ahkami et al., 2017; Adedeji et al., 2020; Zeffa et al., 2020; Nacoon et al., 2021). Studies using microbial consortia or mixtures of bacterial strains have revealed positive effects on plant growth and defense (Jain et al., 2015a; Jain et al., 2015b). However, minimizing parasitism and competition among microbes while maximizing beneficial effects and cooperation are the main challenges of this technology (Ahkami et al., 2017).

Root colonization by microbes from the rhizosphere depends on organic compounds secreted by the roots, which can serve as chemoattractants for microorganisms in addition to maintaining nutritional requirements for soil bacterial growth and metabolic function (Adedeji et al., 2020).

Compounds released by the roots, called root exudates (REs), include various water-soluble metabolites such as amino acids, proteins, sugars, and signal peptides (Ankati and Podile, 2018). These exudates create a selective environment for beneficial microbes, thus creating an interconnection between plants and microorganisms.

Streptomyces spp., an actinomycete, is one of the predominant microorganisms present in the soil. Its properties as PGPR have often been reported in recent years (Amaresan et al., 2018; Kaur and Manhas, 2022; Silambarasan at al., 2022). Our previous studies demonstrated that several strains of *Streptomyces* spp. exhibited traits related to plant growth promotion, including production of indole-3-acetic acid and siderophores, solubilization of phosphate, phenazines production, and single inoculation with these isolates promoted the growth of tomato (Dias et al., 2017), soybean (Horstmann et al., 2020) and maize (Nozari et al., 2021). Tolerance to extreme salinity levels was also recorded (Nozari et al., 2021). Most of the recent studies with co-inoculation of *Streptomyces* isolates refer to their influence and action as remediation agents for environmental pollution (Saez et al., 2015; Fuentes et al., 2017; Ali et al., 2021; Jeyasundar et al., 2021; Saez et al., 2022; Soumeya et al., 2022). Studies on co-inoculation with *Streptomyces* aiming plant growth promotion are still scarce (Nimnoi and Ruanpanun, 2020; Shariffah-Muzaimah et al., 2020; Abbasi et al., 2022).

This work aimed to characterize *Streptomyces* rhizobacteria as PGPR and demonstrate the effectiveness of using a mixture of these bacteria in the vegetative growth of maize plants. We also analyzed the profile of exudates in roots colonized by the bacteria.

2. Material and Methods

2.1 Identification and cultivation of *Streptomyces* spp.

Three isolates of rhizobacteria *Streptomyces* spp. were previously obtained from the rhizosphere of *Phaseolus vulgaris* L. (CLV89 and CLV104) and *Cucumis melo* L. (CLV179), deposited in the Bacteria Collection of the Laboratory of Plant Biotechnology (CLV) – PUCRS, and used in the experiments. The initial identification of bacteria as belonging to the genus *Streptomyces* was carried out on morphological characteristics typical of the group *Streptomyces* (Dhanasekaran and Jiang, 2016). The 16S rDNA sequence of CLV179 is deposited in the GenBank database under the accession number MN461009. Isolates CLV89 and CLV104 were taxonomically identified by sequencing the rRNA16S gene. Bacterial genomic DNA was extracted by the Wizard® Genomic DNA Purification Kit (Promega, USA). PCR amplification of the 16S rRNA gene was performed using the universal primers 9F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1542R (5'-AGAAAGGAGGTGATCCAGCC-3'), with an initial denaturation at 94 °C for 5 min, followed by 30 cycles at 94 °C for 45 s, 55 °C for 45 s and 72 °C for 60 s, and one last cycle at 72 °C for 6 min, using the Taq Platinum Enzyme Kit (Invitrogen™). The PCR-amplified fragments were subject to Sanger sequencing by Macrogen Inc. (Republic of Korea)

using two sets of primers: 9F-1542R and MG3F (5'-CAGCAGCCGCGGTAATAC-3') and 800R (5'-TACCAGGGTATGTAATCC-3'). The obtained nucleotide sequences (over 1,400 bp) were compared with the sequences in the GenBank database from the National Centre for Biotechnology information (NCBI). The phylogenetic analysis was carried out using the sequences and another 21 *Streptomyces* 16S rRNA sequences from different species retrieved from the NCBI. All sequences were aligned using CLUSTAL W (Larkin et al. 2007). A phylogenetic tree was constructed through the maximum Maximum likelihood Likelihood (ML) method and Tamura-Nei model, using MEGA X (Kumar et al., 2018). The topology of the phylogenetic trees was evaluated by bootstrap resampling (1,000 replications).

For the analysis of the efficiency of the bacterial consortium, the isolate CLV179 was used as the main element of the combination, having been chosen due to the potential for promoting the growth of maize plants in previous experiments (Nozari et al., 2021).

The isolates were cultivated in ISP4 liquid medium (Shirling e Gottlieb, 1966), incubated at 27 ºC and 120 rpm, for 3 days. From this pre-inoculum, 100 µl of bacterial suspension was inoculated in ISP4 medium. The cultures with 10^8 colony units forming (CFU) were then used for the experiments on PGPR-characterization and seed bacterization. The period and conditions of culture varied according to the experiment.

2.2 Compatibility test

The compatibility between the three *Streptomyces* isolates to be used in the co-inoculation experiments was investigated by the co-culture assay. The isolates were cultivated in the liquid ISP4 medium according to item 2.1. The co-culture was established by placing drops (20 µL) of the bacterial suspension of each isolate 0.5 cm apart from each other, in an ISP4 agar plate. Dual or triple combinations (CLV89+CLV104; CLV89+CLV179 and CLV104+CLV179) and (CLV89+CLV104+CLV179) were tested.

2.3 Characterization of *Streptomyces* isolates

Streptomyces isolates were selected for the experiments of co-inoculation based on the information obtained with the compatibility assay. The isolates were then characterized for production of indolic compounds, siderophores, and phenazines and the ability of solubilizing phosphates.

Production of indolic compounds (IC) by the Stm isolates was determined by the Salkowski method, following Horstmann et al. (2020). Briefly, bacterial cultures (4 days old) were centrifuged (2,500 ×g) for 10 min and the supernatant was used in the Salkowski reaction (1:1; v/v). Absorbances were determined at 530 nm using a Spectramax 190 Multimode Microplate Reader. Culture medium

served as a blank. The concentration of IC was determined based on the calibration curve of indole 3-acetic acid (IAA) (from 5 to 200 µg of IAA mL^{-1}) and expressed in µg of IAA mL^{-1}. Quantifications were performed in three replicates. Indole 3-acetic acid (IAA) and its precursor, Indole-3-acetaldehyde (IAAld), and metabolites Indole-3-carboxylic acid (ICA) and Indole-3-lactic acid (ILA) were determined by HPLC (Sykam Research HPLC S600). Separation of analytes was carried out in a MetaSil ODS column (5 µm; 250 × 4.6 mm), with a C18 guard column, using a mobile phase consisting of 0.1% of formic acid (A) and acetonitrile with 0.1% formic acid (B), at a flow rate of 0.5 mL min^{-1}. UV/VIS detector was adjusted to 367 nm. The separation was obtained in gradient mode, at 30 °C, programmed to start with 30% B and ramped to 95% B after 2.5 min. This condition was maintained for 2 min, after which the column was re-equilibrated to the initial condition for an additional 1.5 min. The injection volume was 20 µL. The concentration of the compounds in the supernatant of the bacterial suspension was determined by a calibration curve for each compound analyzed.

Production of siderophores by *Streptomyces* isolates was evaluated using pelletized bacteria from 4-day old cultures following Nozari et al. (2021). The production of siderophores was determined by culturing the bacterial cells in Chrome Azurol S (CAS) agar plates for 5 days at 26 ± 2 °C (Lakshmanan et al., 2015). Siderophore production was estimated by measuring the width of the halo border (mm) and the isolate was considered a siderophore producer when an orange-yellowish halo (≥ 2 mm) formed around the bacterial colony (Dias et al., 2017). Each plate contained three wells and three plates were evaluated for each isolate, totaling nine repetitions. The phosphate solubilization assay was carried out on the medium developed by Pikovskaya (1948). In each plate, three 5-mm wells were inoculated with 100 µL of suspension of each isolate. Plates were incubated at 27 °C for 10 days. Bacterial colonies showing halo zones ≥ 2 mm were considered positives for P solubilization, and nine measurements were taken for each isolate. Production of NH_3 by the isolates was determined through a colorimetric reaction with Nessler reagent. Supernatants were collected from 5-day old cultures followed by cultivation without nitrogen source for 3 days. Absorbances were determined at 425 nm. A calibration curve was established with NH_4Cl (0.18 to 25 µg mL^{-1}).

Phenazines (1-hydroxyphenazine, 1-OH-Phz; phenazine 1-carboxylic acid, PCA; and pyocyanin) were determined in the bacterial supernatant of 4-day-old cultures. Analysis was performed using HPLC (Sykam Research HPLC S600) at 40 °C, with a UV/VIS detector adjusted to 367 nm. The linear gradient consisted of 0–15% of eluent B for 2 min, 15–83% of B for 12 min, 83–0% of B for 2 min, and 0% of B for 4 min at a flow rate of 1 mL min^{-1}, following Kern and Newman (2014), with modifications. Quantification was based on calibration curves of the standards (Sigma-Aldrich™). The concentration was expressed as µg of phenazine mL^{-1}.

2.4 Experiments involving a mixture of *Streptomyces* isolates

Streptomyces isolates were cultivated in ISP4 medium, at 27 ºC, in duplicate, for four days. Bacterial suspensions were filtered using a 20 mL syringe with 1 g of cotton (density 0.32g/cm^2), obtaining cells and spores. The exception to this protocol was CLV104, which was filtered by a syringe filter without membrane due to the great amount of extracellular polymeric substances produced by this isolate. The spore-containing filtrate was serially diluted tenfold in sterile distilled water and 100 µL of each dilution was plated in ISP4 medium for CFU counting (procedure adapted from the protocol available in https://openwetware.org/wiki/*Streptomyces*:Protocols/Spore_Prep). Plates were incubated at 28 ºC for 72 h and CFU mL^{-1} was calculated. To standardize the application of the isolates to the seeds, a suspension with 10^8 spores of each isolate was prepared and used for the following experiments.

Bacterial suspensions were used for the bacterization of maize seeds (*Zea mays* L.; Refugio Max RR2 AG3700; Bayer), and treatments consisted of (*i*) triple co-inoculation (CLV89+CLV104+CLV179), (*ii*) dual co-inoculation CLV89+CLV104; CLV89+CLV179 and CLV104+CLV179); (*iii*) the controls CLV89, CLV104, CLV179; (*iv*) absolute control (non-bacterized seeds, NB), and (*v*) relative control, in which the seeds were treated with the commercial inoculant *Azospirillum brasiliense* (AZO). Absolute control was carried out with seeds treated with sterile water. At bacterization, Silvet^{TM} adhesive (0.1%; v/v) was added to all treatments. The process of bacterization was carried out for 16 h, under agitation (100 rpm). Seeds were then air dried and sown in commercial organic soil in 2 L-plastic pots, and irrigation was performed with tap water to field capacity every two days. Twenty seeds were used per treatment. The analysis of plant growth took place at stage V5 (approximately 45 days after sowing) and consisted of the analysis of length (cm) and fresh and dry mass (g) of shoots and roots separately. Length of roots were determined measuring the longest root from the root system and measurement of shoot refers to the longest leaf. Dry mass was determined after drying leaves and roots in an oven at 70 °C until mass was constant. Root colonization by *Streptomyces* isolates was confirmed by Scanning Electronic Microscopy (SEM) using excised portions of the distal root region, fixed in glutaraldehyde (2.5% in 0.1 M sodium phosphate buffer; pH 7.4) and processed for analysis by the Microscopy Center and Microanalyses-PUCRS.

For collecting exudates, seeds were bacterized with the *Streptomyces* isolates (10^8 spores) and the treatments described above were used. Bacterized seeds (four per pot) were sown in a sterile vermiculite substrate, in plastic pots (1.5 L volume). Irrigation was performed every 2 days with sterile tap water. Cultivation was carried out in a growth chamber at 26 °C and a photoperiod of 16 h. After emergence, plantlets were thinned, and two plants were kept per pot. Four plants were used per treatment. Twenty-one days after emergence, plants were removed from the substrate and roots were carefully washed in running water to remove the adhered substrate. Plants were placed in glass flasks and the root system was completely covered with sterile filtered tap water (100 mL). Root exudates were collected following adapted procedure Oburger and Jones (2018). Roots were allowed to exudate for 24 h in a growth chamber at 26 °C, 16 h-photoperiod. The exudates were filtered on Whatman paper

Grade 1 and lyophilized. The powder was resuspended in 3 mL of Milli-Q water and stored at -20 °C until colorimetric and chromatographic analysis. Roots were dried in oven until constant mass was achieved.

Characterization of the exudates was carried out by concentration of phenolic compounds, total soluble amino acids, proteins, and total reducing sugars. Phenolic compounds were analyzed by Folin-Ciocalteau reaction and absorbances were measured at 765 nm. The concentration of phenolic compounds was determined based on the calibration curve established with gallic acid (0.008 to 0.125 mg mL^{-1}). The phenolic compounds cinnamic acid, coumarin, gallic acid, caffeic acid, coumaric acid, hydroxybenzoic acid, ferulic acid, and vanillic acid were analyzed by HPLC, using a Sikam S600 chromatograph, with a UV/VIS detector set at 280 nm. Chromatography was performed using gradient eluents (A) methanol and (B) 0.5% formic acid, at a flow of 0.5 mL min^{-1} and 25 °C. Concentrations are expressed as mg of phenolics compounds mg^{-1} root dry mass.

Soluble amino acids were analyzed through ninhydrin assay (Sun et al., 2006) with modifications. The reaction was prepared with sodium acetate/acetic acid 0.1M buffer (pH 4.3) and ninhydrin (5% in ethanol), and heated for 15 min at 95 °C. Absorbances were read at 570 nm. The calibration curve was established with glycine (0.075 mM to 0.25 mM). Concentrations are expressed as µM of soluble amino acids mg^{-1} root dry mass.

Total polysaccharides were determined by the anthrone method at 620 nm, with glucose as the standard (Cavero-Olguin et al., 2019). Protein concentration was determined by Bradford assay with bovine serum albumin as standard, at 595 nm (Bradford, 1976). Spectrophotometric measurements were made using a Spectramax 190 Multimode Microplate Reader. Concentrations were also expressed as concentrations of compounds in reference to the mg^{-1} root dry mass.

2.5 Statistical analysis

Results were expressed as mean ± standard deviation of the analyzed parameters. Each sample was evaluated in triplicate. The data obtained were submitted to the tested for normality by Shapiro-Wilk test ($p \geq 0.05$) and for variance homogeneity by Levene's test ($p \leq 0.05$). Data were analyzed by ANOVA and means were separated by the Duncan test ($p \leq 0.05$). Analyses were performed using the software SPSS Statistics v. 22.

3. Results

3.1. Identification of *Streptomyces* isolates

Three actinomycetes from the Library of Microorganisms – Laboratory of Plant Biotechnology (CLV) were identified as *Streptomyces* by the typical morphology of the Actinomycetes group (Dhanasekaran and Jiang, 2016) and confirmed as positive Gram-bacteria by the Gram test. Isolates were able to grow on ISP4 and the morphology is shown in the Figure 1 A-C. All three isolates were confirmed as belonging to the genus *Streptomyces* based on the 16S rDNA gene partial sequences. The *Streptomyces* sp. 16S rDNA sequences were deposited in GenBank database and accessions numbers were assigned as: CLV89- ON723947, CLV104 - ON723950, and CLV179-MN461009. The phylogenetic tree is shown in Figure 1D. Phylogenetic analysis revealed that the isolates were separated into two clades. CLV89 was grouped with S. *vinaceus* and *S. cirratus* with 31% similarity. CLV104 showed 76% similarity with *S. californicus*. High similarity (93%) was found between CLV179 and *S. cellulosae* and *S. bellus*.

3.2 Compatibility among *Streptomyces* spp.

The interaction among *Streptomyces* isolates was analyzed through the co-culture method. Co-cultivation of *Streptomyces* spp. revealed no growth inhibition of one isolate over the other. The bacterial morphology in the double or triple inoculation was similar to the individual growth (Figure 2). The *Streptomyces* CLV89, CLV104, and CLV179 were compatible and were then characterized and used for the co-inoculation experiments.

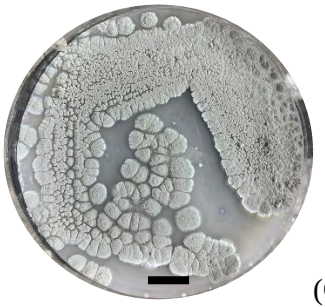
3.3 Characterization of *Streptomyces* spp. isolates as PGPR

The rhizobacteria identified as *Streptomyces* spp. showed traits that characterize them as PGPR. The CLV179 was proved PGPR in previous work (Nozari et al., 2021) and it was then used as a reference for this study. The three isolates were able to produce indolic compounds, ammonia, siderophores, and phenazines, although contrasting results among the isolates were recorded. The ability of solubilizing phosphates was not detected in any *Streptomyces* isolate. Quantitative analysis of the *Streptomyces* culture supernatant showed variable production of indolic compounds (IC) (Table 1). The CLV104 produced the highest concentration of IC, followed by isolate CLV89. The lowest concentration of IC was obtained with CLV179, which produced 2.2 times less IC than CLV104 (Table 1). Contrastingly, CLV179 produced the highest amount of NH_3, approximately 1.7 and 3.4 more than CLV104 and CLV89, respectively. CLV89 produced significantly more siderophores than the other two isolates (Table 1). The profile of phenazines also differed among the isolates. Pyocyanin was

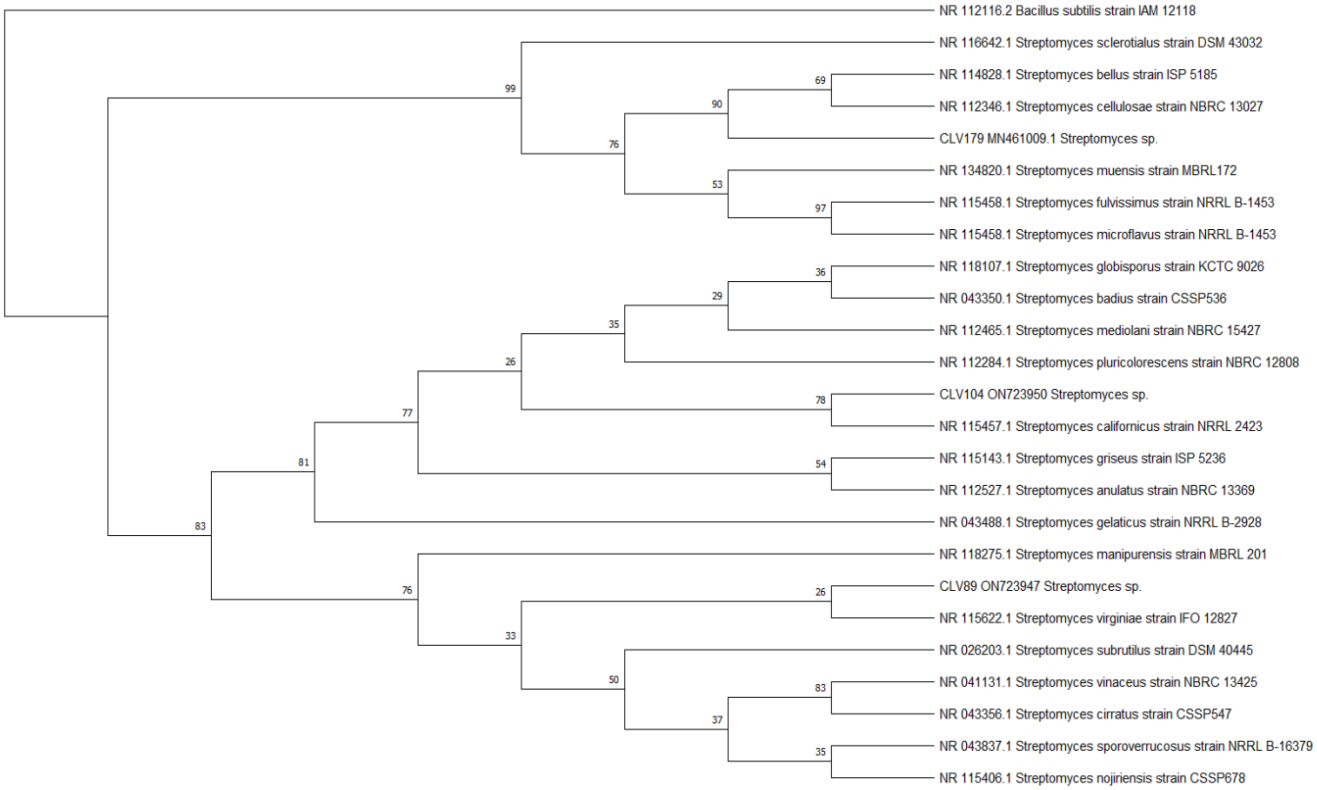
detected in the cultures of the three isolates, being CLV179 the most productive. CLV89 and CLV179 were also able to produce 1-OH-PHZ (Table 1).

(A)

(B)



(C)



(D)

Figure 1 – Colony morphology of *Streptomyces* isolates CLV89 (A), CLV104 (B), and CLV179 (C). Bars= 1 cm. (D) Taxonomic identification of bacterial isolates of *Streptomyces*. Phylogenetic analyses were performed with reference sequences obtained from the NCBI GenBank database. Phylogenetic tree was constructed using the maximum likelihood method and Tamura-Nei model based on 16S rRNA partial gene sequences. Bootstrap percentages based on 1000 replications are shown at branch points.

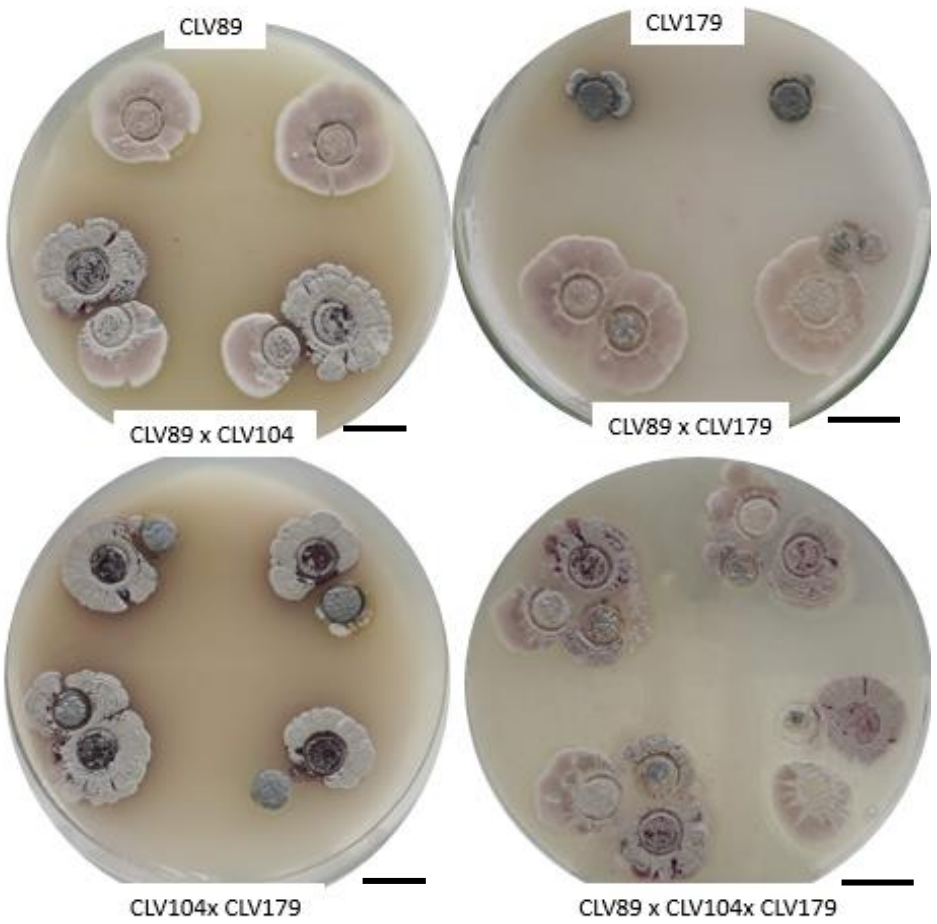


Figure 2 – Colony morphology and growth behavior of *Streptomyces* isolates CLV89, CLV104 (B), and CLV179 (C) analyzed through co-culture method. Bar= 1.5 cm.

Table 1 – Metabolic characteristics of *Streptomyces* isolates.

Isolates	Indolic compounds (mg mL^{-1})	NH_3 (µg mL^{-1})	Siderophore (cm)	Phenazines (µg mL^{-1})		
				PYO	OH-PHZ	PCA
CLV89	4.39 ± 0.51 b	1.95 ± 0.57 c	1.48 ± 0.07 a	201.7 ± 84.1 b	22.2± 1.2 a	ND
CLV104	6.50 ± 0.54 a	3.94 ± 0.50 b	0.46 ± 0.02 b	276.23± 5.5 b	ND	ND
CLV179	2.98 ± 0.16 c	6.61 ± 0.51 a	0.44 ± 0.14 b	560.2± 61.2 a	21.7± 3.6 a	ND

Indolic compounds and NH_3 were evaluated from at least 5 replications. Values of siderophore are the average width of the halo border (cm) of at least three replicates.
Phenazines PYO: Pyocyanin; OH-PHZ: 1-hydroxyphenazine PCA: phenazine 1- carboxylic acid.
Means followed by the different letters in the columns indicate significant difference according to Duncan's multiple range test (P = 0.05).
ND: not detected

Analysis of IC utilizing HPLC allowed identification and quantification of IAA and its precursor, IAAld, and the metabolites ILA and ICA in the supernatants of *Streptomyces* spp. cultures (Table 2). The three isolates produced IAA, IAAld, and the metabolite ILA. ICA was not detected under the culture conditions. The compound quantified in greater quantity, independent of the isolate, was the IAA-precursor, IAAld. Indole-3-Lactic acid (ILA) was the metabolite found in the lowest amounts produced by the isolates (Table 2).

Table 2 - Indole 3-acetic acid and metabolites of the IAA-pathway [Indole-3-lactic acid (ILA), Indole-3-carboxylic acid (ICA), and the precursor Indole-3-acetaldehyde (IAAld)] produced by *Streptomyces* isolates (7-day old culture).

	IAA (µg mL^{-1})	ILA (µg mL^{-1})	ICA (µg mL^{-1})	IAAld (µg mL^{-1})
CLV89	11.55± 1.81	2.46± 0.12	ND	802.67± 244.77
CLV104	11.66± 1.67	2.60± 0.05	ND	1592.16± 223.52
CLV179	21.53±7.08	3.93±1.13	ND	500.94±92.87

ND – non detected

3.4 Growth of plants co-inoculated with *Streptomyces* isolates

Under greenhouse conditions, the growth of maize plants was evaluated from seeds bacterized either with single isolates or the mixture of two or three *Streptomyces* spp. The comparison was established with non-bacterized roots as well as the commercial inoculant *Azospirillum brasiliense*. (Figure 3).

Considering single inoculations, the CLV104 promoted the length and accumulation of biomass in leaves and roots. The treatment was superior to the control without bacteria and the commercial inoculant (Figures 3A and B). The lowest efficiency was observed with CLV89, in which no difference was observed in biomass of leaves and roots, as well as root length, compared to the controls. A small, but significant, increase was observed in leaf length compared to controls (Figure 3A).

Co-inoculation with two or three isolates affected the growth of maize plants. The triple co-inoculation resulted in equal shoot growth in relation to any other combinations in which CLV104 was present. However, plants grew 1.8 and 1.3 times more than NB and *A. brasiliense*, respectively (Figure 3A). Biomass accumulation was superior in plants co-inoculated with the three isolates and with the dual co-inoculation CLV89+CLV104 (Figure 3B). Root length was negatively affected by the triple co-inoculation, whereas the dual inoculation CLV89+CLV104 resulted in higher root growth, being 1.6 and 1.3 longer than NB and *A. brasiliense*, respectively (Figure 3A). Interestingly, dual inoculation with CLV104+CLV179 resulted in shorter roots with the highest dry biomass (Figure 3A, C). It is noteworthy that regardless of the treatment, the seed bacterization with Streptomyces spp. mostly resulted in a significant difference compared to plants derived from non-bacterized seeds (NB). Indeed, treatment with the commercial inoculant did not outperform the plant growth efficiency obtained with most Streptomyces isolates, except for root length with CLV89 treatment. However, *A. brasiliense* promoted plant growth as length and fresh biomass compared to NB plants (Figure 3A-C).

In the laboratory experiment, the inoculation with the mixture of the three *Streptomyces* isolates promoted plant growth, considering shoot and root length, and total plant fresh mass (Figure 4). Contrary to the effect of *Streptomyces* on plant growth observed in the greenhouse conditions, plants co-inoculated with CLV104 and any other isolate did not result in efficient growth promotion when compared to CLV104 alone (Figure 4). On the other hand, co-inoculation with consortia including CLV104 promoted leaf growth when compared to the NB plants (Figure 4).

The analysis of the roots by scanning electron microscopy (SEM) showed colonization by bacteria at the end of 45 days of cultivation under greenhouse conditions, both in single inoculations and in the co-inoculated plants (Figure 5).

3.5 Analysis of root exudates

Root exudates (RE) were analyzed and quantified through the contents of amino acids, proteins, reducing sugars, and the secondary metabolites phenolic compounds (Figure 6). The highest amount of soluble amino acids and proteins was observed in the exudates of roots co-inoculated with the isolate CLV89 (Figure 6A, B). On the other hand, exudation of reducing sugars was significant higher when roots were colonized by a consortium of *Streptomyces*, followed by colonization with CLV89 (Figure 6C). Similar to the concentration of the soluble amino acids and proteins, phenolic compounds analyzed as gallic acid equivalent revealed that, in general, exudates from CLV89-colonized plants contain more phenolic compounds than the other treatments, although some similarity may by observed among the CLV89 and both CLV104 and roots treated with the commercial inoculant *Azospirillum* (Figure 6D).

When phenolic compounds were analyzed by liquid chromatography, the diversity of molecules was evident (Table 3; Figure 7). Ferulic acid was the only phenolic detected in all RE samples, and its concentrations varied from 6.64 to 88.65 mg mg^{-1} root DM. Moreover, cinnamic acid was found in most of the samples analyzed except CLV89+CLV179. In the exudate, concentrations of this molecule varied from 5.76 to 123.38 mg mg^{-1} root DM. The highest concentrations were found to be produced by the roots of CLV179-treated plants. On the other hand, vanillic acid was found in the lowest concentrations, varying from 0.52 to 5.22 mg mg^{-1} root DM (Table 3). Considering the compounds evaluated, hydroxybenzoic avid was detected only when exudates were obtained from roots treated with the consortium of the three isolates (Table 3). Exudates from Azo-treated roots contained only the acids caffeic, ferulic, and cinnamic (Table 3).

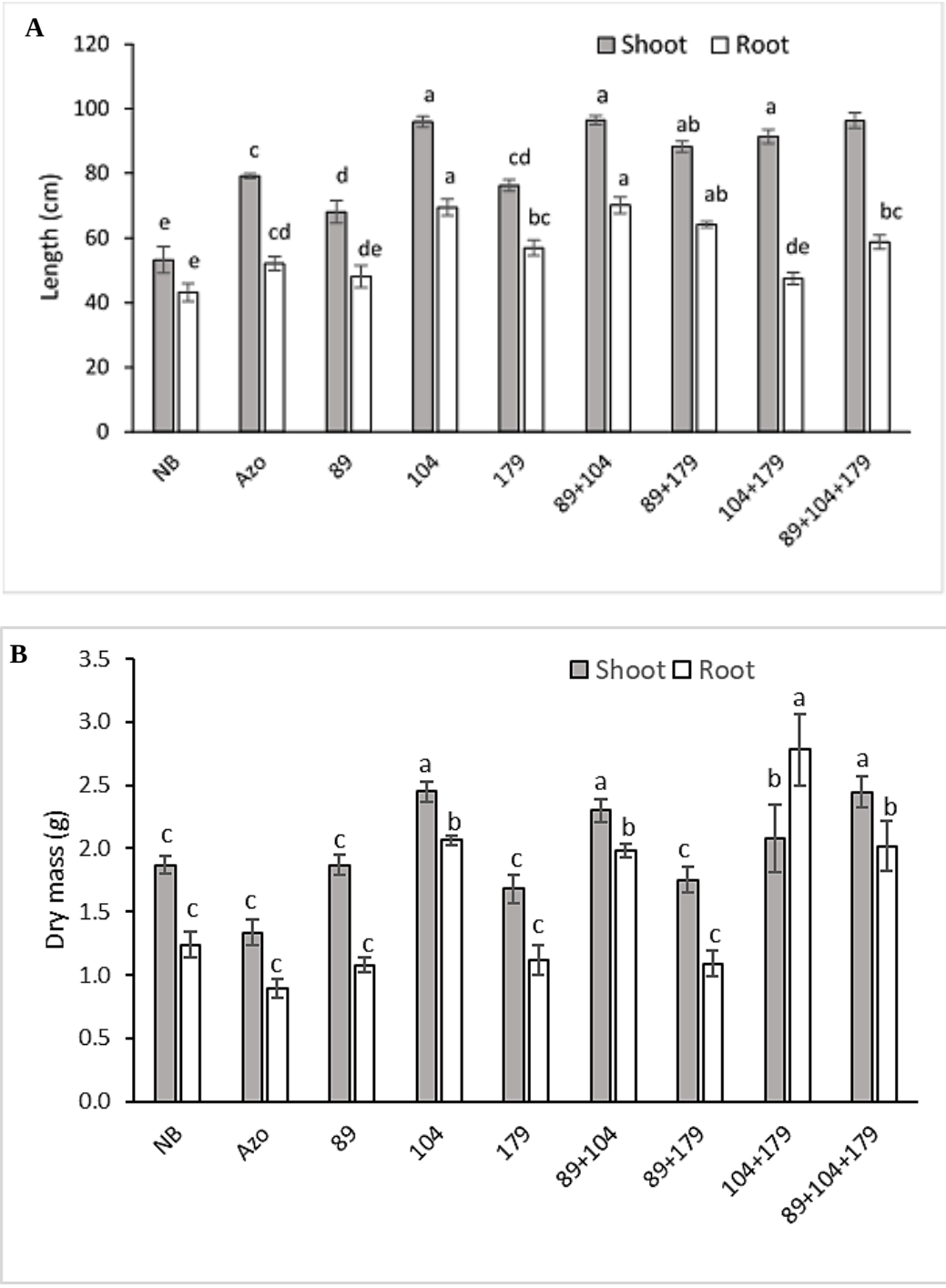


Figure 3 – Growth of shoot and root of maize plants from bacterized seeds in greenhouse. *Streptomyces* isolates were used as single or mixture inoculation. (a) Length (cm); (b) Dry mass (g); NB: non-bacterized seeds; Azo: *Azospirillum brasiliense*. Means followed by the different letters in the columns (shoots or roots) indicate significant difference according to Duncan's multiple range test ($P = 0.05$).

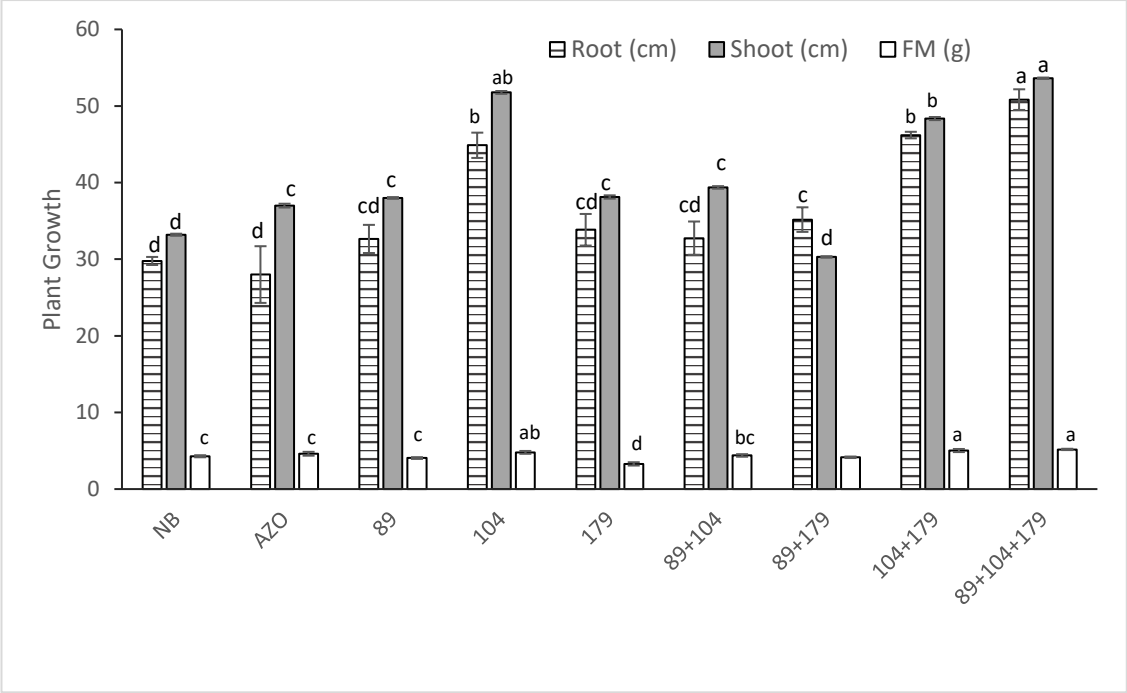


Figure 4 - Growth of shoot and root of maize plants from bacterized seeds in laboratory conditions. *Streptomyces* isolates were used as single or mixture inoculation. Length (cm) of shoots and roots and total fresh mass (g) were the evaluated parameters. NB: non-bacterized seeds; Azo: *Azospirillum brasiliense*. Means followed by the different letters in the columns for each parameter indicate significant difference according to Duncan's multiple range test ($P = 0.05$).

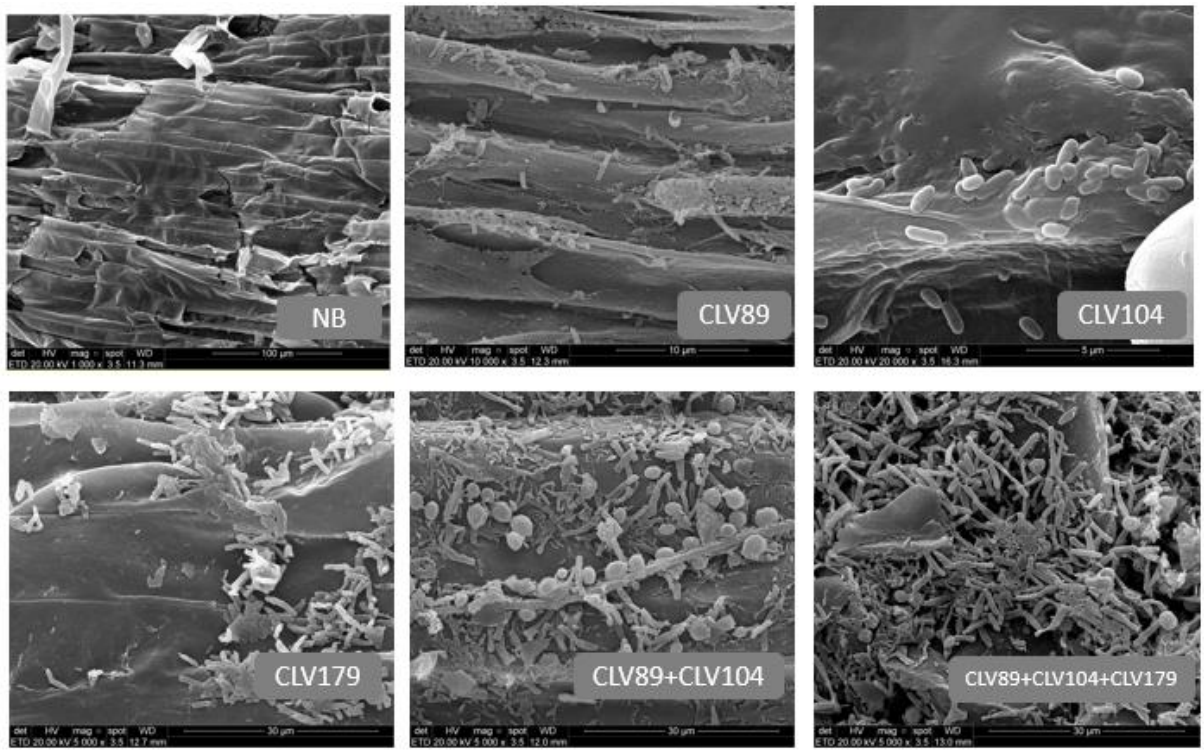


Figure 5 – Root of maize colonized by *Streptomyces* CLV89 (10,000x), CLV104 (20,000x), CLV179, CLV89+194 and the triple co-inoculation (5,000x). NB; roots non-bacterized (1,000x).

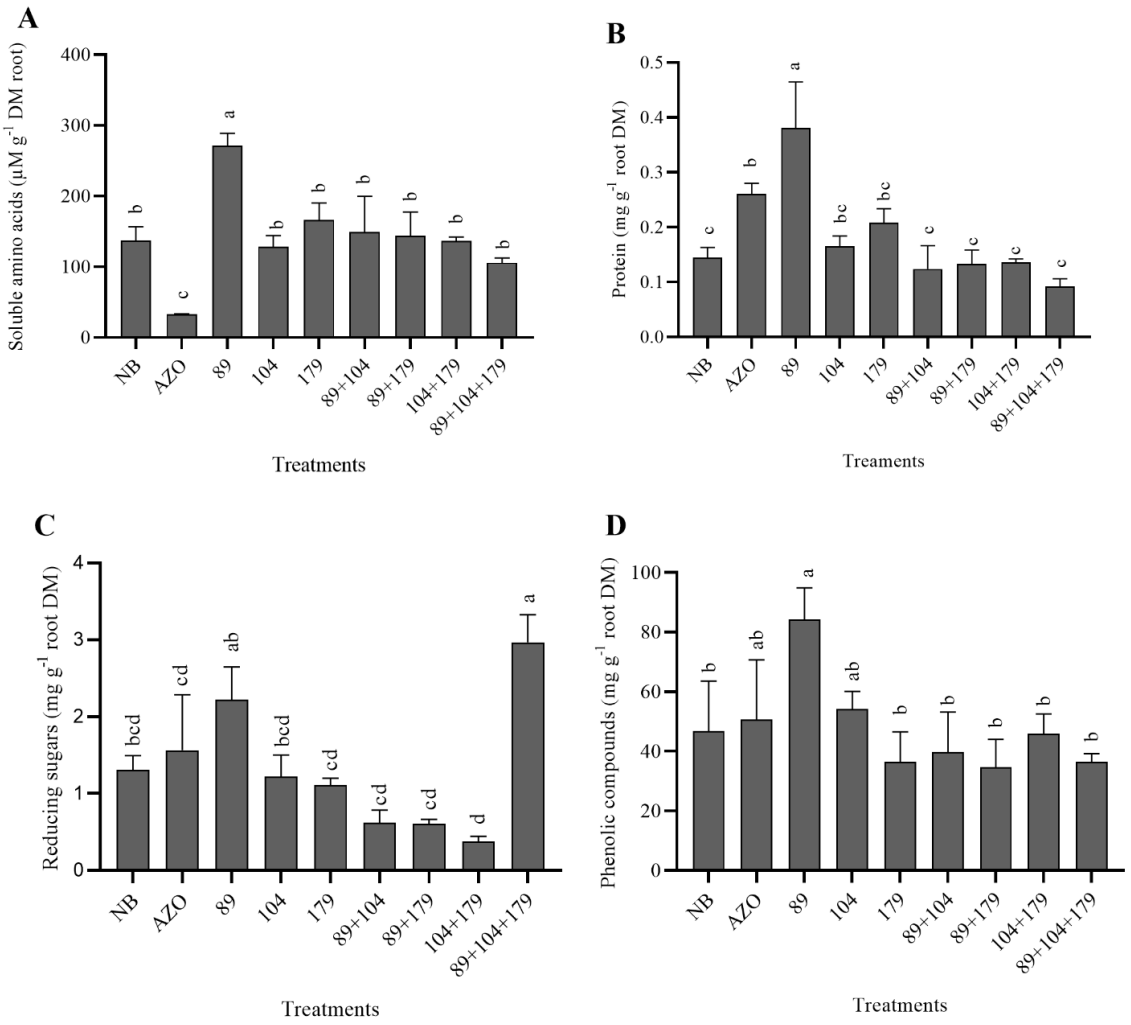


Figure 6 – Root exudates from CLV-treated plants. (A) amino acids (µM), (B) proteins (mg mL^{-1}), reducing sugars (mg mL^{-1}), (D) Phenolic compounds and (E) Flavonoids (mg mL^{-1}). Means followed by the different letters in the columns indicate significant difference according to Duncan's multiple range test ($P = 0.05$).

Table 3 – Profile of phenolic compounds in the exudates obtained from maize roots colonized with rhizobacteria *Streptomyces* spp. Identification of compounds was carried out through HPLC analysis and concentration were determined based on calibration curves of each molecule. Data are expressed as mg of phenolic compounds mg^{-1} root dry mass (DM)

	Phenolic Compounds (mg mg^{-1} root DM)							
	Gallic acid	Hydroxybenzoic acid	Vanillic acid	Caffeic acid	Ferulic acid	Coumaric acid	Coumarin	Cinnamic acid
NB	ND	ND	2.35	12.97	18.73	6.87	7.91	80.31
AZO	ND	ND	ND	10.64	17.08	8.44	ND	13.31
CLV89	30.10	ND	5.22	9.03	88.65	15.86	19.50	89.36
CLV104	42.07	ND	ND	0.30	19.46	7.84	19.04	7.50
CLV179	23.57	ND	ND	ND	31.97	NQ	8.46	123.38
CLV89+104	22.39	ND	0.52	0.39	9.52	NQ	9.44	4.17
CLV89+179	16.44	ND	ND	ND	16.68	NQ	NQ	ND
CLV104+179	NQ	ND	ND	ND	6.64	20.28	13.23	13.07
CLV89+104+179	7.91	7.03	1.77	0.36	36.18	NQ	5.71	5.76

ND: non detected

NQ: below limit of quantification

Depending on the bacteria used for the seed treatment, the profile of the phenolic compounds varies amongst the root exudates (Fig Suppl. 1). Figure 7 illustrates the variation taking into consideration all compounds found in the chromatogram. Eight phenolic compounds were identified through calibration curves established with commercial standards. In the graphs, it is possible to compare the percentage of each molecule identified to those with no standard was available. In RE from roots colonized by CLV89 most of the compounds analyzed were found, and 16.6% of the compounds was caffeic acid, followed by 10.8% of ferulic acid (Figure 7 C). CLV104 exudates showed 15.9% of gallic acid (Figure 7 D). Moreover, CLV179 seemed to induce the exudation of cinnamic acid (19.4%) among the known compounds (Figure 7 E), although only four compounds were identified in the RE from this treatment. Co-inoculation evidenced alteration on the phenolic compounds' profile (Figure 7 F-I). Type and concentration of phenolics were different from those observed when roots were colonized by single isolates. Nevertheless, some patterns may be detected. For example, gallic acid was abundant in RE from CLV104 and CLV179 and was detected in high amounts in the RE from the CLV89+CLV104 and CLV89+CLV179 combinations (Figure 7 F, G). Double inoculation with CLV194+CLV179 showed 13,3% coumaric acid, found in RE-CLV104 (3.3%) but not in CLV179-RE (Figure 7 H). The triple-inoculation resulted in RE with the eight compounds identified, including HB, which was absent in RE from single or double inoculations (Figure 7I). Indeed, the ferulic acid, although identified in all other samples, corresponded to 14.7% of the compounds in the RE-consortium. Such percentage was higher than in the other treatments. Compared to the commercial inoculant *A. brasiliense*, the profile of phenolic compounds exuded by the CLV-treated roots contained more compounds.

4. Discussion

Rhizobacteria *Streptomyces* spp. are abundant in soil and have recognized properties that favor plant growth, induce plant defense, and alter the bioavailability of heavy metals and other environmental pollutants (Chaurasia et al., 2018; Guo et al., 2019; Ali et al., 2021). In this study, the *Streptomyces* CLV89, CLV104, and CLV179 were characterized as PGPR, showing properties of production of indolic compounds, siderophores, and NH_3. Each isolate presented a different profile of metabolite secretion. Although all the isolates studied here efficiently produced indolic compounds, CLV104 was the one with the highest levels. The detailed analysis of the IAA and its metabolites by HPLC showed that this result represents the adding amounts of the IAA and its precursor, IAAld. The production of IAA by bacteria favors the formation of adventitious roots, altering the root architecture and promoting increased water and nutrient uptake (El-Tarabily, 2008; Singh et al., 2019). Regardless of the isolate, the production of the IAAld intermediate was much higher than the concentration of IAA itself, suggesting that the bacteria were in an active process of synthesizing that hormone. It is important to emphasize that when secreted by rhizobacteria, intermediates of the IAA-pathway such as IAAld, can also be used by plants to produce IAA and modulate root development (George et al., 2008). Solubilization of phosphates is also a trait found in *Streptomyces* PGPR (Hamdali et al., 2008; Singh et al., 2019). However, the isolates studied here did not show such capacity. It has been shown that CLV179 only presents this trait when subjected to saline stress above 300 mM NaCl (Nozari, 2022). In addition, all selected isolates were identified as ammonia-producers, and CLV104 and CLV179 were the most productive isolates. Indeed, the ammonia synthesis by PGPR can supply nitrogen to the host plant and thus help protein synthesis (Singh et al., 2019).

Another important metabolite found in *Streptomyces* is phenazine. Phenazines are molecules primarily isolated from *Pseudomonas* sp. that are extensively known as antimicrobial agents (Laursen and Nielsen, 2004). All isolates *Streptomyces* showed production of PYO in the supernatant at seven days of cultivation, and both CLV89 and CLV179 also produced 1-OH PHZ. The phenazine PCA was not identified as metabolites of the isolates. A possible explanation for this result is that PCA is the precursor of PYO synthesis (Dong et al., 2020). In soil, phenazines can facilitate bacteria and plant access to iron and nutrients such as phosphate, trace minerals, and organic compounds associated with mineral soil phases (Hernandez et al., 2004), beside their known antimicrobial proprieties against soil-borne pathogens (Mazurier et al., 2009; Mavrodi et al., 2010). In addition, phenazines may contribute to competitiveness and long-term survival of the producers in natural habitats (Mavrodi et al., 2010). Our previous work has not identified phenazines produced by CLV179; however only four day-cultures were analyzed (Nozari et al., 2021). Thereby, there is likely a timepoint to analyze these metabolites in culture. Therefore, these evaluated PGPR traits in *Streptomyces* isolates makes them beneficial for the promotion of maize plant growth.

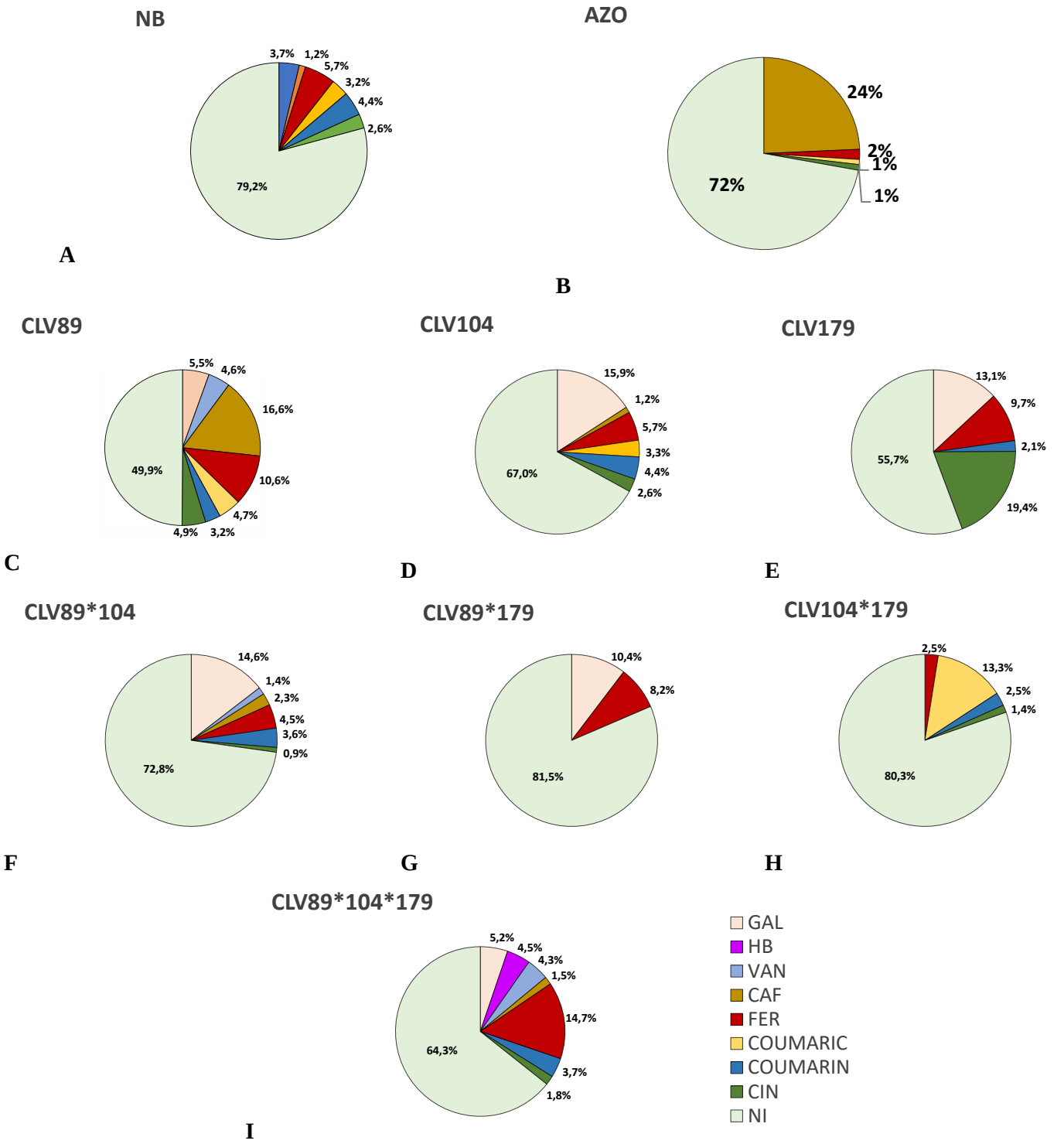


Figure 7 – Profile of phenolic compounds in root exudates from *Streptomyces* CLV-treated plants. GAL: Gallic acid; HB: Hydroxybenzoic acid; VAN: Vanillic acid; CAF: Caffeic acid; FER: Ferulic acid; COUMARIC: Coumaric acid; COUMARIN: Coumarin; CIN: Cinnamic acid; NB: non-bacterized; NI: non-identified. Data were expressed in percentage of the content of each phenolic compound in relation to the total phenolics found by chromatography.

Once characterized as PGPR, the isolates were tested for growth compatibility in co-culture, with the objective of co-inoculation in plants. CLV89, CLV104, and CLV179 were compatible, as they maintained the growth rate and morphology of each isolate without inhibiting growth. Besides, each isolate contributed with different metabolites, which seems advantageous when root colonization is concerned. Since the ability to grow without hindering each other's growth is a requirement for developing microbial consortia (Jian et al., 2020), *Streptomyces* CLV89, CLV104, and CLV179 were suitable to be tested as growth promoters of maize plants in single, dual, and triple co-inoculation.

With single inoculation, *Streptomyces* CLV104 was the isolate that promoted the growth of maize plants in all parameters analyzed. It was even more efficient in shoot and root growth when compared to the commercial inoculant *A. brasiliense*. These results were found both in the greenhouse and laboratory experiments. The growth-promoting effect was also evidenced in comparison with CLV179, which had previously been identified as an effective PGPR in maize (Nozari et al., 2021). Likely, the traits of production of indolic compounds, IAA and IAAld, and NH_3 found in CLV104 may account for the efficiency on promoting growth. Sharma et al. (2014) reported that *Streptomyces* could enhance root formation and density, thus facilitating an efficient nutrient absorption from the soil.

When co-inoculation was applied to maize seeds, the resulting plants showed positive effects on growth when treated with the three isolates or with the dual inoculation CLV89+CLV104 compared to non-bacterized roots. Apparently, the relationship between bacteria, even if of the same genus, but with different metabolic profiles, adds to the rhizosphere important metabolites for plant growth. In the triple inoculation, the higher production of auxin, NH_3 and siderophores by the isolates CLV104, CLV179, and CLV89, respectively, promoted mainly shoot growth, while root growth did not stand out. The large amount of auxin that the three bacteria combined secreted into the rhizosphere may have inhibited root growth, since high concentrations of auxin induce the formation of the hormone ethylene, which inhibits root growth (Chardwick and Burg, 1970). On the other hand, when seed inoculation was performed with CLV89+CLV104, root length seemed to be stimulated. In addition to the amount of auxin being lower in total, there is still the contribution of siderophores, which can indirectly promote root growth (Kumar et al., 2022). However, the evaluated growth criteria were almost equally influenced by the CLV104 isolate and by the triple co-inoculation, suggesting that when in the consortium composition, one bacterium is much more effective as PGPR than the others, the effect can be driven by the dominant rhizobacteria. It is noteworthy that the accumulation of biomass in the roots was improved by the dual inoculation with CLV104+CLV179, combination that did not promote any other criteria evaluated. With this treatment, the roots were shorter but with a high amount of accumulated biomass. The IAA-producing potential of these two isolates may have inhibited root growth in length while promoting the multiplication of secondary roots.

PGPR can enhance plant growth and protection by employing multiple strategies (Suárez-Moreno et al., 2019; Kumar et al., 2022). *Streptomyces* strains have been widely described for their PGPR potential (El-Tarabily, 2008; Anwar et al., 2016; Dias et al., 2017; Nozari et al., 2021). However,

few studies proved their efficiency when consortia are established among *Streptomyces* themselves or when inoculated with other PGPR (Htwe et al., 2018; Ankati et al., 2021; Hata et al., 2021).

Beneficial microbial consortia can promote plant growth and crop yield when carefully formulated and able to colonize target crops. Like human probiotics, these would serve as beneficial colonizers in the ecosystem, popularly referred to as environmental probiotics (Naik et al, 2019). The beneficial effects on plants inoculated with bacterial formulations (individual strains or in a consortium) could be affected by several factors, such as the bacterial genotype used for the formulation and the plant variety. Soe and Yamakawa (2013) tested single and dual inoculation with *Bradyrhizobium yuanmingense* and *S. griseoflavus* in soybean varieties, and the result showed that dual inoculation increased the nodulation and nitrogen fixation in some soybean varieties but not in other varieties. Molina-Romero et al. (2021) showed that the application of the consortium with *Azospirillium brasilense, Pseudomonas putida, Acinetobacter* sp., and *Sphingomonas* sp. was efficient in reducing the use of fertilizers in maize cultivation by 50%. Moreover, consortium of two species of *Streptomyces* in rice increased seed germination, root length, and dry weight comparing to non-bacterized seed, but no difference was observed between consortium and single inoculation regarding plant height and dry weigh (Hata et al., 2021).

The interaction between plants and rhizosphere bacteria are strongly dependent on the quality and quantity of root exudates, which include organic acids, amino acids, vitamins, inorganic ions, flavonoids, and sugars (Zhang et al., 2013; Hao et al 2022). Several studies have shown that phenolic acids, which are universal compounds in plant tissues and soil, when exudated by the roots can be used as source of carbon and energy or act as chemoattractant (Oksinska et al., 2011; Xiong et al., 2020). In our study, ferulic acid was the phenolic detected in all root-microbe interactions, including *A. brasiliensis*. It was also found in RE from non-bacterized roots, suggesting that this compound might be exudated constitutively. However, the pattern of ferulic acid exudation showed variation depending on the bacteria in contact to the root, indicating that it might be important in the process of successful establishment of the plant-microbe interaction. Roots of maize colonized with *Streptomyces* showed the presence of gallic acid in RE and increase on exudation of caffeic, cinnamic and coumaric acids. These phenolic compounds along with p-hydroxybenzoic, vanillic, syringic, chlorogenic, 3,4-dimethoxycinnamic, sinapic, protocatechuic, salicylic and gentisic acids have been reported as chemoattractants and as functional biological control agents of root- and soil-borne pathogenic microbes (Lanoue et al., 2010; Oksinska et al., 2013; Li et al., 2021). Ray et al. (2018) has proved that several phenolics such as gallic, fumaric, ferulic, vanillic acids and the flavonoid quercetin in root exudates of *Abelmoschus esculentus* act as chemoattractants of endophytic *Alcaligenes faecalis* strains.

In conclusion, the *Streptomyces* CLV104 shows a strong PGPR potential in maize, likely due to the production and secretion of IAA and NH_3. Triple co-inoculation, including CLV104, did not promote plant growth in addition to individual use of CLV104. Indeed, it led to reduction of root growth. The dual co-inoculation CLV89+CLV104 promoted root and leaf growth and could be used for

application as a biocontrol due to the ability to produce siderophores. Further studies could test this hypothesis. The interaction between roots and *Streptomyces* is mediated, to some extent, by exudates, and the rhizodeposition of phenolic compounds varied specifically with the isolate colonizing the roots. The inoculation, single or in consortia, induced the rhizodeposition of several compounds, suggesting that the rhizobacteria are effectively modulating the primary and secondary metabolism of the plants.

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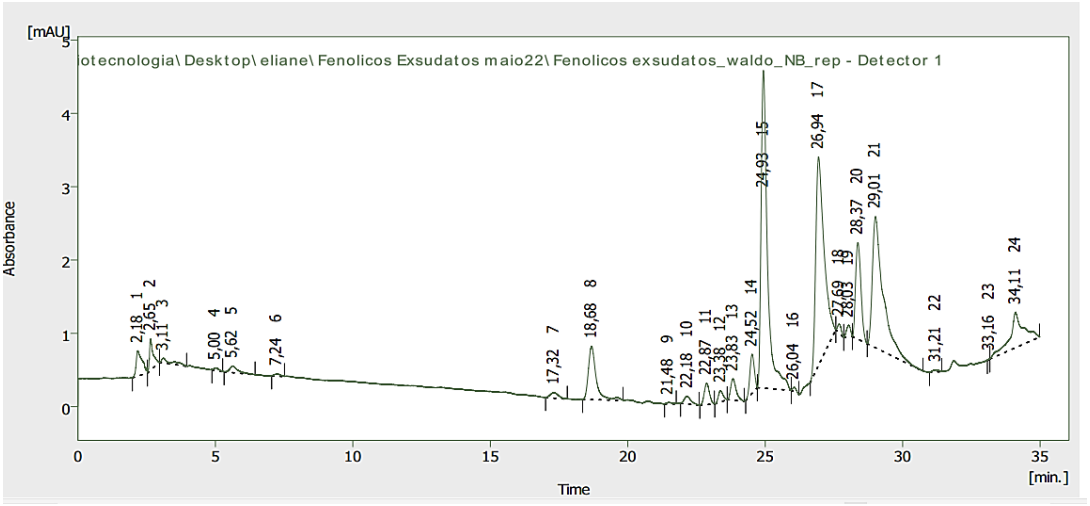

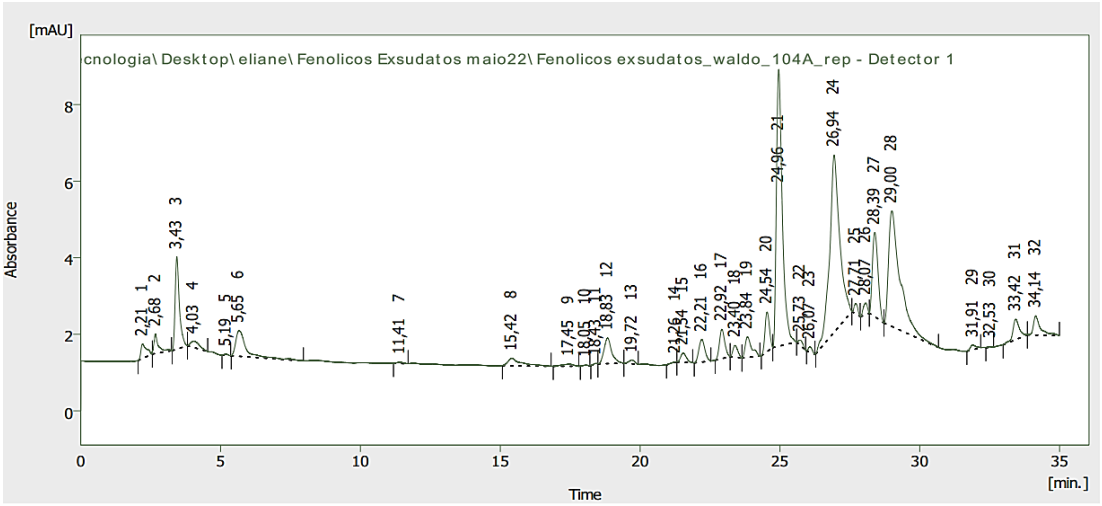

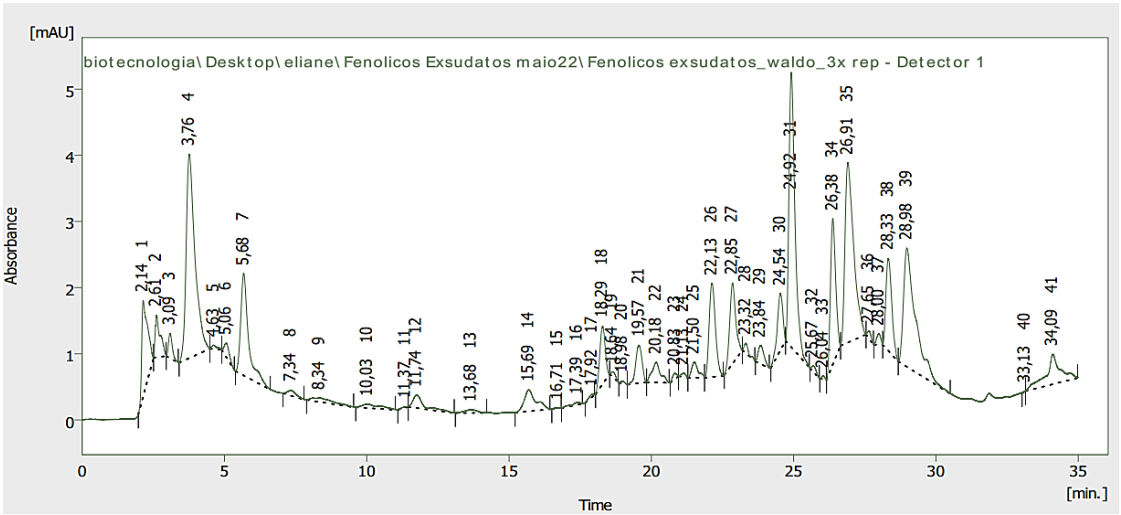

Suppl. Figure 1 – HPLC chromatograms of phenolic compounds from root exudates colonized by Streptomyces. (A) Non-bacterized roots, (B) CLV104, and (C) co-inoculation CLV89+CLV104+CV179.

CONSIDERAÇÕES FINAIS

Os resultados dessa pesquisa permitem considerar que:

- As bactérias *Streptomyces* CLV89, CLV104, CLV179 produzem metabólitos que as classificam como PGPR;
- Em plantas de milho, o isolado mais eficiente como PGPR foi o CLV104, promovendo o crescimento de plantas em níveis superiores àqueles observados com as plantas não microbiolizadas.
- As plantas de milho microbiolizadas com CLV104 apresentaram crescimento maior do que as plantas tratadas com o inoculante comercial *Azospirillum brasiliense.*
- A tripla coinoculação não resultou em crescimento superior ao efeito individual da CLV104 e resultou em redução do crescimento da raiz, provavelmente pela quantidade de auxina produzida pelos isolados;
- A dupla coinoculação CLV89+CLV104 promoveu o crescimento em todos os parâmetros avaliados mostrando efeito semelhante à inoculação com a CLV104;
- As raízes de milho, quando colonizadas por *Streptomyces*, exsudam compostos do metabolismo primário e secundário. A rizodeposição variou de acordo com o isolado que colonizava a raiz, sendo que o perfil de metabólitos exsudados pelas raízes colonizadas por *Streptomyces* (isoladas ou em consórcio) diferiu foi maior do que nas raízes não microbiolizadas.
- O perfil de compostos fenólicos exsudados é modulado pelo isolado de *Streptomyces* em interação com as raízes de milho. O ácido ferúlico pareceu ter síntese e secreção constitutiva pelas raízes, embora a concentração deste composto tenha sido induzida na presença das bactérias. No exsudato das raízes colonizadas pelos três isolados foi detectada a presença do ácido hidroxibenzoico, molécula com reconhecida ação atrativa na interação raiz-microrganismo.

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