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CÂMPUS DE JABOTICABAL

AVALIAÇÃO DO POTENCIAL DE INIBIÇÃO DO DESENVOLVIMENTO VEGETAL CAUSADO PELA AUXINA PRODUZIDA POR CONSÓRCIOS BACTERIANOS

Laiana Lana Bentes Lobo

Engenheira Agrônoma

2023

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Tese apresentada à Faculdade de Ciências Agrárias e Veterinárias – Unesp, Câmpus de Jaboticabal, como parte das exigências para a obtenção do título de Doutor em Microbiologia Agropecuária.

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DADOS CURRICULARES DO AUTOR

Laiana Lana Bentes Lobo – Nascida em 14 de setembro de 1990, na cidade de Belém-PA, Brasil. Filha de Cristiano Bernardo da Cruz Lobo Filho e Lia Silvia Bentes da Silva. Em junho de 2016 se graduou como Engenheira Agrônoma pela Universidade Federal do Amazonas (UFAM – Manaus). Em Agosto do mesmo ano ingressou no Curso de Mestrado da Pós-graduação em Microbiologia Agropecuária da Faculdade de Ciências Agrárias e Veterinárias (FCAV) / Unesp – Jaboticabal, sob orientação do Prof. Dr. Everlon Cid Rigobelo, onde defendeu sua dissertação de Mestrado intitulada “Potencial de bactérias endofíticas na promoção de crescimento em plantas de milho” em março de 2018. Em março de 2019 iniciou o curso de Doutorado no programa de Pós-graduação em Microbiologia Agropecuária, na mesma instituição e sob a mesma orientação. Durante esse período, como aluna de pós-graduação e pesquisadora participou de eventos e projetos apresentando trabalhos e realizando experimentos junto ao Laboratório de Microbiologia do Solo – LSM, além de publicações de artigos científicos em revistas internacionais oriundos do trabalho de mestrado, doutorado e parcerias.

Ainda que eu tenha o dom de profecia, saiba todos os mistérios e todo o conhecimento e tenha uma fé capaz de mover montanhas, se não tiver amor, nada serei. (1Co 13 2)

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AVALIAÇÃO DO POTENCIAL DE INIBIÇÃO DO DESENVOLVIMENTO VEGETAL CAUSADO PELA AUXINA PRODUZIDA POR CONSÓRCIOS BACTERIANOS

RESUMO - As bactérias promotoras de crescimento de plantas (BPCP) possuem diversas habilidades diretas e indiretas para garantir o desenvolvimento e crescimento vegetal. Sendo a síntese de auxinas, principalmente o ácido 3-indol-acético (AIA), uma das habilidades diretas mais importantes. *Bacillus* spp. e *Azospirillum brasilense* são BPCP amplamente utilizadas na agricultura como bioinoculantes e relatadas como sintetizadoras de AIA. Entretanto, pouco se sabe se a inoculação consorciada (co-inoculação) destas bactérias pode reduzir o desenvolvimento vegetal devido ao excesso de AIA sintetizado. Sabendo-se que altas doses de AIA na planta pode ser limitante para o seu crescimento. Por outro lado, diante da necessidade de um sistema agrícola mais sustentável é inegável a importância da utilização de bioinoculades a base de BPCP. Sendo assim, estudos com o intuito de elucidar a interação dessas bactérias com a planta, baseados nas suas características e formas de uso, são muito importantes. No caso de hormônios vegetais, plantas modelos tem sido utilizadas em estudos que avaliam a resposta vegetal nesse sentido. No presente estudo, o tomateiro Micro-Tom (MT) e seus mutantes *diageotropica* (*dgt*) e *entire*, com alterações de baixa e alta sensibilidade para auxina respectivamente, foram utilizados para verificar se a co-inoculação de bactérias sintetizadoras de AIA pode ou não prejucar o desenvolvimento vegetal. Para isso, foram realizados dois experimentos, capítulos dois e três, nesta tese. No primeiro foram utilizados tratamentos com inoculações indivuais e conjuntas de *B. velezensis (BV), B. pumillus (BP) e A. brasilense (AZ)* em cada genótipo de tomateiro. Houve aumento para massa seca de raiz com o tratamento BV+BP+AZ em MT, para massa seca de parte aérea em dgt com BV. Não houve aumento para nenhum parâmetro avaliado em *entire*. A co-inoculação de BP+AZ diminuíram os valores para os parâmetros massa seca de parte aérea, massa seca de raiz e área foliar para *dgt* e *entire*. A co-inoculação de BP+AZ prejudicou os parâmetros analisados em dgt e entire ambos divergentes quanto a sensibilidade ao AIA. A co-inoculação de BV+BP+AZ que teoricamente proporcionou a maior concentração de AIA promoveu um aumento de MSR em MT. No segundo experimento foram utilizados tratamentos com inoculações indivuais e conjuntas de *B. subtilis (BS), A. brasilense selvagem (Azw)* e A. brazilense mutante (Azm). Os resultados mostram que o parâmetro vegetal mais sensível à inoculação microbiana é o número de raízes. Nenhum tratamento aumentou os parâmetros massa seca de parte aérea para MT e *dgt*, massa seca de raiz para o MT, altura da planta em MT e *entire*, área e volume radicular em dgt. O tratamento Azm reduziu a altura da planta para *dgt*, os tratamentos BS+Azw e BS+Azm reduziram no genótipo MT e o tratamento Azw+Azm reduziu em *dgt* o diâmetro da planta, os tratamentos BS e BS+Azw reduziram o número de raízes no MT. Conclui-se que a co-inoculação de Bacillus e *A. brasilense* pode reduzir alguns parâmetros de desenvolvimento vegetal, entretanto, esse efeito não está relacionado a quantidade de AIA sintetizado pelas bactérias.

Palavras-chave: *Bacillus*, *Azospirillum brasilense*, Tomate, fitormonios, auxina.

EVALUATION OF THE POTENTIAL FOR INHIBITION OF VEGETABLE DEVELOPMENT CAUSED BY AUXIN PRODUCED BY BACTERIAL CONSORTIA

ABSTRACT - Plant growth promoting bacteria (PGPB) have several direct and indirect abilities to ensure plant development and growth. Being the synthesis of auxins, mainly 3-indole-acetic acid (IAA), one of the most important direct skills. *Bacillus* spp. and *Azospirillum brasilense* are PGPB widely used in agriculture as bioinoculants and reported as EIA synthesizers. However, little is known whether the combined inoculation (co-inoculation) of these bacteria can reduce plant development due to the excess of synthesized AIA. Knowing that high doses of AIA in the plant can be limiting for its growth. On the other hand, given the need for a more sustainable agricultural system, the importance of using bioinoculates based on PGPB is undeniable. Therefore, studies with the aim of elucidating the interaction of these bacteria with the plant, based on their characteristics and forms of use, are very important. In the case of plant hormones, model plants have been used in studies that evaluate the plant response in this regard. In the present study, the Micro-Tom (MT) tomato plant and its *diageotropic* (*dgt*) and *entire* mutants, with changes of low and high sensitivity for auxin respectively, were used to verify whether the co-inoculation of AIA-synthesizing bacteria can or cannot harm plant development. For this, two experiments were carried out, chapters two and three, in this thesis. In the first, treatments with individual and joint inoculations of *B. velezensis* (BV), *B. pumillus* (BP) and *A. brasilense* (AZ) were used in each tomato genotype. There was an increase in root dry mass with the BV+BP+AZ treatment in MT, for shoot dry mass in *dgt* with BV. There was no increase for any parameter evaluated in *entire*. The co-inoculation of BP+AZ decreased the values for the parameters shoot dry mass, root dry mass and leaf area for *dgt* and *entire*. Co-inoculation of BP+AZ impaired the parameters analyzed in dgt and between both divergent in terms of sensitivity to AIA. Co-inoculation of BV+BP+AZ which theoretically provided the highest concentration of AIA promoted an increase in root dry mass in MT. In the second experiment, treatments with individual and joint inoculations of *B. subtilis* (BS), wild *A. brasilense* (Azw) and mutant *A. brazilense* (Azm) were used. The results show that the plant parameter most sensitive to microbial inoculation is the number of roots. No treatment increased shoot dry mass for MT and *dgt*, root dry mass for MT, plant height for MT and *entire*, root area and root volume for dgt. The Azm treatment reduced the plant height for *dg*t, the BS+Azw and BS+Azm treatments reduced the MT genotype and the Azw+Azm treatment reduced the plant diameter in dgt, the BS and BS+Azw treatments reduced the number of roots in Mt. It is concluded that the co-inoculation of *Bacillus* and *A. brasilense* can reduce some plant development parameters, however, this effect is not related to the amount of AIA synthesized by the bacteria.

Keywords: *Bacillus*, *Azospirillum brasilense*, Tomato, phytormons, auxin.

CAPITULO 1 – Considerações gerais

1. Introdução

As bactérias promotoras do crescimento de plantas (BPCP) possuem habilidades diretas e indiretas para promover o crescimento e desenvolvimento vegetal. Os mecanismos diretos estão relacionados ao aumento da disponibilidade de nutrientes e produção de fitohormônios e os mecanismos indiretos envolvem a inibição de patógenos e indução de resistência sistêmica na planta (Ramakrishna et al., 2019; Li et al., 2021)

Na busca por uma produção agrícola mais sustentável e com redução de custos em insumos as BPCP são uma alternativa viável em muitos sistemas e culturas agrícolas. Tal desempenho, tem sido descritos em diversos estudos sobre as habilidades dessas bactérias em promoverem o crescimento vegetal, manter o estado nutricional da planta conciliando com a redução do uso de insumos químicos (Aloo et al., 2019; de Andrade et al., 2023).

Nesse sentido, a síntese de fitohormonio pelas bactérias é uma habilidade importante, pois tem como resultado o aumento radicular e consequentemente maior eficiência na captação de água e nutrientes exploração de volume de solo permitindo assim uma redução da dose de fertilizantes (Nabrdalik et al., 2018; Safara et al., 2022).

Um grande numero de BPCP, rizosfericas e endofiticas, são relatadas como produtora de ácido 3-indol-acético (AIA), sendo o aminoácido L -triptofano (Trp) o principal precursor de sua biossíntese (Loper e Schroth 1986; Patten e Glick 1996). Dentre estas, os gêneros *Bacillus* e *Azospirillum* são bem conhecidos como produtores desse fitormonio.

Para entender o papel dos hormônios na resposta vegetal o uso de plantas modelo tem sido fundamentais no avanço de estudos nesse sentido (Koornneef; Santos 2016; Gaion, 2017). Carvalho et al. (2011) introgrediram em Micro-Tom uma

série de mutações hormonais, tanto em biossíntese como em sensibilidade, assim possibilitando o estudo das principais classes hormonais e suas respostas a estresses bióticos e abióticos no tomateiro.

Nem sempre é possível correlacionar a concentração de AIA e o crescimento das plantas de forma linear, sendo que, as plantas têm níveis endógenos ótimos desta auxina para o seu melhor desenvolvimento (Duca et al., 2014). O AIA é portanto uma molécula orgânica que dependendo da concentração promove ou inibe o crescimento da planta (Fahad et al., 2015; Mishra et al., 2021).

Sendo assim, é importante entender a melhor forma de aplicação de BPCP, sintetizadoras de AIA, nas culturas agrícolas, uma vez que o uso destas vem crescendo e diversas formas de aplicação são indicadas, como a utilização individual (inoculação) e/ou consórcios bacterianos (co-inoculação), sem considerar a quantidade de AIA sintetizado por estas bactérias.

Neste contexto, o objetivo do presente estudo foi verificar se a co-inoculação de bactérias sintetizadoras de AIA, *Bacillus* spp. e *Azospirillum. brasilense*, prejudica ou não o crescimento e o desenvolvimento de plantas de tomateiro Micro-Tom e seus mutantes em auxina.

2. Revisão de Literatura

2.1. Utilização de BPCP para uma agricultua sustentável

Atualmente os problemas ambientais, redução dos recursos da terra e o esgotamento da riqueza biológica, demandam por uma agricultura mais sustentável, sem reduzir o rendimento e a produtividade das culturas agrícolas (Kesavan e Swaminathan, 2018).

No entanto, para atender a necessidade massiva de produção de alimentos ainda os agricultores dependem da utilização de fertilizantes químicos e pesticidas em práticas agrícolas intensivas (Singh et al. 2020)

Sendo assim, a adoção de práticas agrícolas sustentáveis que envolvam a redução gradual do uso de agroquímicos sintéticos, o aumento da utilização de substâncias derivadas de resíduos biológicos e o aproveitamento do potencial biológico e genético de plantas e micróbios é uma estratégia viável para combater a rápida degradação ambiental, garantir alta produtividade agrícola, e melhorar a saúde do solo (Basu et al. 2021).

As BPCP são amplamente estudas e conhecidas como um grupo de bactérias benéficas para o solo e plantas, colonizando a superfície da raiz ou habitando o solo rizosférico, sendo conhecidas como bactérias rizosféricas e/ou muitas das vezes colonizando os tecidos vegetais e sendo conhecidas como bactérias endofíticas. Ambas promovem o crescimento e a saúde das plantas por meio de múltiplos mecanismos, como a disponibilidade de nutrientes e produzindo fitormonios estimulando o desenvolvimento das raízes e protegendo as plantas de doenças e parasitas. Devido as suas características essas bactérias benéficas possuem o potencial para melhorar o crescimento das plantas reduzindo o uso de fertilizantes e pesticidas sintéticos, além de melhorar a saúde do solo em sistemas agrícolas (De Andrade et al. 2023; Dos Santos et al., 2020).

O uso de bactérias benéficas capazes de sintetizar AIA tem efeito de biocontrole na bio-estimulaçao das plantas, sendo uma alternativa para a redução do uso de agroquímicos. Embora alguns produtos biológicos a base de bactérias não informe sua atividade bioquímica, incluindo a habilidade de produzir AIA, essa habilidade deveria ser esperada. Uma vez que estes produtos biológicos promovem o desenvolvimento de raízes laterais e primárias e também um aumento no número e no comprimento das raízes. Inoculações com esses microrganismos favorece o sistema radicular e consequentemente aumenta a absorção de água e nutrientes, o que leva as plantas a suportar condições adversas. Entretanto, faz-se necessário que as concentrações de AIA sejam ótimas, uma vez que, o excesso de AIA pode inibir o crescimento e o desenvolvimento das raízes (Lata et al., 2018)

2.2. A síntese de AIA e sua importância nas plantas e bactérias

O ácido 3-indol-acético é uma molécula, classificada como um dos principais fitormonios da classe das auxinas, envolvido em muitos aspectos do crescimento e desenvolvimento das plantas, como a divisão e alongamento celular, desenvolvimento das raízes, frutos e senescência (Phillips et al., 2011; Grossman, 2010)

As plantas são organismos típicos produtores de AIA e sua ação dar-se devido a sua presença de forma livre em algumas moléculas, enquanto em outras moléculas o ácido está conjungado com aminoácidos, proteínas, açúcares e assim essas moléculas não estão ativas nas plantas.

As vias de síntese de auxinas nas plantas e o gene *auxin/AIA* regulam a organogenesis, crescimento e as respostas ambientais. A regulação do processo de expressão de *auxin/AIA* é pouco conhecido, embora alguns fatores estejam bem descritos (Safara et al., 2022). Shani et al. (2017) descreveram fatores de transcrição de 38 famílias de genes que diretamente promoveram a transcrição dos genes *AIA5* e *AIA19* em reações de estresse abiótico. As mutações recessivas que foram obtidas desses genes resultaram em uma diminuição da tolerância aos estresses ambientais mostrando um papel importante da auxina nos estresses abióticos. Esses resultados mostram porque os genes de *auxin/AIA* são importantes fatores genéticos e de adaptação ambiental que influenciam o desenvolvimento e a fisiologia da planta (Shani et al., 2017; Poveda e González-Andrés, 2021)

O desenvolvimento das plantas é afetado por este fitormonio de maneira favorável ou prejudicial e muitas bactérias têm a capacidade de sintetiza-lo, incluindo bactérias benéficas e fitopatogênicas (Abadi et al., 2021; Duca et al. 2014). As bactérias produtoras de AIA são geralmente membros da biota endofítica, bem como as bactérias de vida livre e bactérias epífitas e as bactérias de vida livre que vivem na água. A maioria das bactérias produtoras de AIA são associadas as plantas e são membros de muitos filos como as Proteobacteria e os Bacteriodetes (Huang et al., 2021). Podendo usar o AIA como uma molécula sinalizadora para e assim garantir seu recrutamento e colonização. Essas bactérias colonizadoras também exercem um efeito protetor nas plantas hospedeiras (Potters et al., 2007). Por outro lado,

além dos microrganismos benéficos, os fitopatógenos também utilizam o AIA nos processos infecciosos (Costacurta e Vanderleyden, 1995; Ludwig-Müller, 2015).

Para as bactérias o mais importante papel do AIA é sua ação como molécula que permite a interação planta-microrganismo, especialmente como Kim et al. (20cx 11), sugeriram que o papel do AIA seria aumentar a sobrevivência das bactérias. Resultados descritos por Bianco et al. (2006) demonstraram que o AIA preserva a bactéria em condições de estresses ambientais como o excesso da radiação ultravioleta, acidez, alcalinidade, elevadas e baixas temperaturas. Bianco et al. (2009) também observou que em *Sinorhizobium melilotti*, o AIA estimulou a produção intracelular de trealose, um carboidrato fonte de carbono, sendo um componente osmotolerante para essa bactéria. A atividade osmototelarante da trealose promove uma melhor proteção da bactéria contra a dessecação e o congelamento. Matsukawa et al. (2007) observaram que baixas concentrações desta molécula pode regular a formação de estruturas semi-miceliais e atividades antibióticas em algumas cepas de *Streptomyces*, protegendo melhor o nicho de colonização contra outros microrganismos (Matsukawa et a., 2007).

A síntese bacteriana de AIA resulta também na atividade de biocontrole por algumas dessas bactérias. O *B. amyloliquefaciens,* quando inoculados em sementes de milheto, *in vitro*, mostraram atividades inibidoras de alguns fungos patogênicos, incluindo o *Fusarium oxysporium*, *Alternaria* sp e a *Sclerotinia homoeacarpa* (Verma e White, 2018). *A. brasilense* uma das espécies bacterianas descritas como sintetizadora de AIA, também possuem ação no biocontrole e resistência contra antracnose em plantas de morango, além de promover o aumento da tolerância à salinidade e ao estresse hídrico (Galazka et al., 2015).

Sendo assim, as auxinas são fatores importantes na resistência das plantas contra doenças bacterianas e seus sistemas de defesa. O modo de ação é baseado no fato das proteínas repressoras do gene *Auxin/AIA* não serem decompostas, reprimindo os genes de respostas as auxinas. Isto significa que os genes de defesa das plantas são *up-regulated* e as plantas podem usar suas formas de defesa contra ataques de patógenos (Duca et al., 2014). Por outro lado, as auxinas podem induzir

a expressão de muitos genes que alteram a reconstrução celular, na qual, a célula favorece o patógeno (Ludwing-Müller, 2015; Hardoim et al., 2015).

BPCP endofíticas podem promover o crescimento vegetal, mesmo a planta estando em condições de estresse ambiental como a seca, elevadas temperaturas, elevada salinidade e baixa disponibilidade de nutrientes. Os mecanismos moleculares para a tolerância dos estresses nas plantas promovidos pelas bactérias endofíticas, incluem a expressão de genes e de moléculas reativas de oxigênio (Lata et al., 2018).

2.3. Inoculação de BPCP sintetizadoras de AIA

O AIA é considerado o mais importante fator bacteriano que estimula o crescimento das plantas (Spaepen et al., 2007). Paradoxalmente, ambos, patógenos e BPCP são capazes de sintetizar AIA, como visto anteriormente.

Positivamente a produção de AIA exercida por bactérias de gêneros como *Azospirillum*, *Bacillus* e *Bradyrhizobim* tem apresentado diversos resultados, melhorando o desenvolvimento e o crescimento vegetal em diversas culturas de importância agrícola. Como o aumento na produtividade e concentração de açúcar em beterraba (Shi et al. 2010). Desenvolvimento e crescimento de plantas de milho aumentando em cerca de 250% e 140% no peso seco das raízes em condições controladas (Batista et al., 2018, Batista et al., 2021). Aumentou da massa seca e conteúdo de clorofila em plantas de arroz, além da redução no período de germinação das sementes de sete para apenas um dia (Greetatorn et al. 2019)

A produção de fitohormônios pelas bactérias tem como consequência a estimulação das raízes nas plantas, proporcionando assim, maior absorção de nutrientes orgânicos e inorgânicos do solo. Verma e White (2018) identificaram quatro cepas produtoras de AIA com efeito de promoção de crescimento, estas foram aplicadas via tratamento de sementes, sendo elas: *Curtobacterium* sp, *Microbacterium* sp e *Methylobacterium* sp e *Bacillus amyloliquefaciens.* Essas cepas

aumentaram o desenvolvimento vegetal, com o aumento na formação de pêlos radiculares, comprimento das raízes e aumento dos conteúdos fotossintéticos.

Alguns estudos demonstram que a forma de aplicação dessas bactérias pode trazer muitos benefícios para as plantas, sendo as sementes uma via de inoculação eficiente, além de ser uma forma viável para os agricultores (Shi et al., 2011). A inoculação direta ao solo também é utilizada como método de inoculação, mesmo acreditando-se que a inoculação na semente proporcione maior colonização. Khan et al. (2014) usaram bactérias endofíticas *Sphingomonas* em suspensão inoculada diretamente no solo e como resultado dessas inoculações houve um aumento das plantas de tomate, aumento do comprimento das raízes, aumento da quantidade de clorofila e da massa da raiz e de parte aérea. Essa cepa foi capaz de produzir uma quantidade de 11,23 $\mu g\ mL^{-1}$ de AIA, mas sua atividade foi somada à sua habilidade de também produzir giberelinas (Ruckdaschel e Klingmuller, 1992)

Pitzschke (2016) sugeriu que as sementes de quinoa (*Chenopodium quinoa*) que foram colonizadas por *Bacillus* spp tiveram sua taxa de germinação e vigor aumentado e também suportaram melhor as condições de estresses ambiental comparado com as sementes que não receberam. Shi et al. (2009) usaram sementes desinfectadas mergulhando em uma solução bacteriana por seis horas para a inoculação de bactérias endofíticas antes de semear. Segundo Galazka et al. (2015) as inoculações das sementes de milho, trigo, cevada, sorgo e milheto resultaram em efeitos positivos para o seu crescimento e desenvolvimento. Marathe et al. (2017) suplementaram sementes de soja com *Pseudomonas fluorescens* e reportaram um aumento na taxa de germinação, um aumento nos teores de massa seca de raiz e parte aérea. Figueira et al. (2019) mostraram que a colonização de *Salicornia ramosíssima* e *Bacillus aryabhattai* nas raízes dobrou a taxa de germinação das sementes no solo sob condições de elevada salinidade.

Rhizobium spp., *Azotobacter* spp. e *Azospirillum* spp. são os principais biofertilizantes fixadores de nitrogênio disponíveis no mercado (Marks et al., 2015). Sendo também microrganismos descritos como promotores de crescimento e produtores de AIA. O uso comercial da bactéria *Azospirillum brasilense* cepa Ab-V5 em milho vem crescendo exponencialmente no Brasil (Santos et al., 2021; Fukami et

al., 2017; Fukami et al., 2016; Hungria et al., 2010). O primeiro inoculante contendo A. brasilense produzido no país foi lançado no mercado em 2009 e, dez anos depois, estima-se que cerca de 10,5 milhões de doses de inoculantes carreando Ab-V5 foram aplicadas em culturas de cereais no Brasil (Fergunson et al. 2019; Soumare et al. 2020).

Como biofertilizantes as BPCP devem ser capazes de colonizar suficientemente as raízes das plantas hospedeiras, criar uma rizosfera adequada para o crescimento das plantas, aumentan a biodisponibilidade de nutrientes e a saúde da planta (Vessey, 2003; Vejan et al. 2016). As BPCP devem possuir características específicas para sua utilização como um bioinoculante eficiente e bem-sucedido. Devem ser capazes de sobreviver no solo, serem compatíveis com a cultura em que forem inoculados e interagir com a comunidade já existente no solo. Medidas necessárias devem ser tomadas para evitar qualquer efeito não-alvo do bioinoculante e estabilizá-los nos sistemas agrícolas. Essas medidas garantirão a durabilidade do efeito de crescimento das plantas e o bom desempenho das BPCP introduzidos como bioinoculantes (Basu et al, 2021). A variabilidade entre os estudos sobre o impacto do inoculante na planta e no microbioma do solo também pode estar correlacionada com a concentração do inoculante, o método de aplicação e as diversas condições ambientais, incluindo o tipo de solo (Fukami et al. 2016; Marks et al., 2015).

Apesar da produção de auxina ser reconhecida como o principal mecanismo de promoção do crescimento vegetal causado por BPCP, o efeito final das auxinas depende de um bom equilíbrio de seu conteúdo na planta, esse resultado dependerá de todas as fontes de compostos de auxinas no sistema. Além das auxinas produzidas pelas bactérias inoculadas, a própria planta produz seus próprios hormônios como parte de processos fisiológicos complexos, variando em quantidade e sensibilidade. Além disso, a matéria orgânica do solo exibe atividade de auxina, causando respostas nas plantas exatamente como aquelas produzidas por auxinas exógenas. Portanto, a inoculação de bactérias produtoras de auxina pode causar uma ampla variedade de respostas na planta, estas podem ser desde a promoção efetiva do crescimento até a restrição do crescimento, dependendo do conteúdo total de auxina. (Pantoja et al. 2023)

2.4. O tomateiro Micro-Tom e seus mutantes em auxina

O uso de plantas modelos tem sido fundamentais para o avanço em estudos relacionados aos processos e mecanismos genéticos, fisiológicos e bioquímicos, responsáveis pelo crescimento e desenvolvimento das plantas (Lucio 2020; Gaion 2017)

Nesse contexto o tomateiro Micro-Tom (*Lycopersicum esculentum* cv Micro-Tom) lançado em 1989 por J. Scott, inicialmente como ornamental, passou a ser utilizado como planta modelo em muitos estudos, pois apresenta características desejáveis, se assemelhando a *Arabidopsis* o primeiro e mais bem estudado modelo vegetal utilizado em pesquisas biológicas. Além de suas características, como porte pequeno, ciclo curto, frutos carnosos, o tomateiro também tem grande importância agronômica, sendo uma ferramenta ideal para estudos neste sistema (Koornneef, 2010).

Neste tomateiro, Carvalho et al. (2011) indrogrediram uma serie de mutações, 0criando uma coleção de mutantes bastante úteis principalmente no estudo do papel de hormônios em respostas vegetais frente a estresses bióticos e abióticos. Dentre estes mutantes para auxina tem-se o *diageotropica* (*dgt*) (baixa sensibilidade à auxina/ gene defectivo para a biossíntese de uma ciclofinilina) e *entire* (com alta biossíntese de auxina).

Em *entire* devido a sua mutação na síntese de auxina, sua morfologia foliar foi alterada e folhas compostas de tomate foram convertidas em folhas simples. (Zhang et al. 2007). Em *dgt* as plantas com alelo mutado possuem baixa sensibilidade a auxina exógena e tal efeito é associado ao sitio primário de percepção e ação da auxina (Kelly e Bradford 1986). O *dgt* apresenta folhas hiponásticas, raízes plagiotrópicas com menos ramificações e caules delgados (Kelly e Bradford 1986).

3. Referências

Abadi V, Sepehri M, Rahmani H, Dolatabad M, Shamshiripour M, Khatabi B (2021) Diversity and abundance of culturable nitrogen-fixing bacteria in the phyllosphere of maize. **Journal of Applied Microbiology** 131**:** 898-912.

Aloo BN, Makumba BA, Mbega ER (2019) The potential of Bacilli rhizobacteria for sustainable crop production and environmental sustainability. **Microbiological Research 219**: 26–39.

Basu A, Prasad P, Das SN, Kalam S, Sayyed RZ, Reddy MS, El Enshasy H (2021) Plant Growth Promoting Rhizobacteria (PGPR) Green Bioinoculants: Recent Developments, Constraints, and Prospects. **Sustentabilidade** 13(3):1140.

Batista BD, Dourado MN, Figueredo EF et al. (2021) The auxin-producing Bacillus thuringiensis RZ2MS9 promotes the growth and modifies the root architecture of tomato (*Solanum lycopersicum* cv. Micro-Tom). **Arch Microbiol** 203, 3869–3882 .

Batista BD, Lacava PT, Ferrari A, Teixeira-Silva NS, Bonatelli ML, Tsui S, Mondin M, Kitajima EW, Pereira JO, Azevedo JL, Quecine MC (2018) Screening of tropically derived, multi-trait plant growth-promoting rhizobacteria and evaluation of corn and soybean colonization ability. **Microbiol Res** 206:33–42.

Bianco C, Imperlini E, et al. (2006) Indole-3-Ace-tic Acid Regulates the Central Metabolic Pathways in *Escherichia coli*. **Microbiology** 152:2421–2431.

Bianco C, Defez R (2009). Medicago truncatula improves salt tolerance when nodulated by an indole-3-acetic acid-overproducing *Sinorhizobium meliloti* strain. **Journal of experimental botany,** *60*(11):3097-3107.

Carvalho RF, Campos ML, Pino LE et al (2011) Convergence of developmental mutants into a single tomato model system: “Micro-Tom” as an effective toolkit for plant development research. **Plant Methods** 7:18.

Costacurta A, Vanderleyden J (1995) Synthesis of phytohormones by plant-associated bacteria. **Critical reviews in microbiology** 21**:** 1-18.

De Andrade LA, Santos CHB, Frezarin ET, Sales LR, Rigobelo EC (2023) Plant Growth-Promoting Rhizobacteria for Sustainable Agricultural Production. **Microorganisms** 11(4):1088.

Dos Santos RM, Díaz PAE, Lobo LLB, Rigobelo EC (2020). Use of plant growth-promoting rhizobacteria in maize and sugarcane: characteristics and applications. **Frontiers in Sustainable Food Systems** 4:136.

Duca D, Lorv J, Patten C. L, Rose D, Glick BR (2014). Indole-3-acetic acid in plant–microbe interactions. **Antonie Van Leeuwenhoek** *106*(1): 85-125.

Fahad S, Nie L, Chen Y, Wu C, Xiong D, Saud S, et al (2015). Crop plant hormones and environmental stress. **Sust. Agric. Rev**. 371–400.

Ferguson BJ, Mens C, Hastwell AH, Zhang M, Su H, Jones CH, Chu X, Gresshoff PM (2019) Legume nodulation: The host controls the party. **Plant. Cell Environ** 42:41–51.

Figueira C, Ferreira MJ, Silva H, Cunha A (2019). Improved germination efficiency of Salicornia ramosissima seeds inoculated with *Bacillus aryabhattai* SP1016-20. **Annals of Applied Biology** 174(3): 319-328.

Fukami J, Ollero FJ, Megías M & Hungria M (2017). Phytohormones and induction of plant-stress tolerance and defense genes by seed and foliar inoculation with *Azospirillum brasilense* cells and metabolites promote maize growth. **AMB Express**, 7, 1-13.

Fukami J, Nogueira MA, Araujo RS & Hungria M (2016). Accessing inoculation methods of maize and wheat with *Azospirillum brasilense*. **Amb Express**. 6, 1-13.

Gaion LA (2017) Uso de um mutante em giberelinas na enxertia de tomateiro para estudar o papel desse hormônio na sinalização entre a raiz e parte aérea durante o déficit hídrico. **Tese** (Doutorado em Agronomia-Produção vegetal) - UNESP, Jaboticabal.

Gałązka J, Słomnicka R, Góral-Radziszewska K, Niemirowicz-Szczytt K (2015) From pollination to DH lines–verification and optimization of protocol for production of doubled haploids in cucumber. **Acta Scientiarium Polorum Hortorum Cultus** *14*(3):81-92.

Grossmann K (2010). Auxin herbicides: current status of mechanism and mode of action. **Pest Management Science** 66: 113–120.

Greetatorn T, Hashimoto S, Sarapat S, Tittabutr P, Boonkerd N, Uchiumi T, Teaumroong N (2019). Empowering rice seedling growth by endophytic *Bradyrhizobium* sp. SUTN 9-2. **Letters in applied microbiology** 68(3):258-266.

Hardoim PR, Van Overbeek LS, Berg G, Pirttilä AM, Compant S, Campisano A Sessitsch A (2015). The hidden world within plants: ecological and evolutionary considerations for defining functioning of microbial endophytes. **Microbiology and molecular biology reviews** 79(3):293-320.

Huang Y, Dai Z, Lin J, Li D, Ye H, Dahlgren RA, Xu J (2021) Labile carbon facilitated phosphorus solubilization as regulated by bacterial and fungal communities in Zea mays. **Soil Biology and Biochemistry** 163**:**108465.

Hungria M, Campo RJ, Souza EM & Pedrosa FO (2010). Inoculation with selected strains of Azospirillum brasilense and A. lipoferum improves yields of maize and wheat in Brazil. **Plant and soil**, 331, 413-425.

Kelly MO, Bradford KJ (1986) Insensitivity of the diageotropica tomato mutant to auxin. **Plant Physiol** 82:713–717.

Khan AL, Waqas M, Kang SM, Al-Harrasi A, Hussain J, Al-Rawahi A, Lee IJ (2014) Bacterial endophyte *Sphingomonas* sp. LK11 produces gibberellins and IAA and promotes tomato plant growth. **Journal of Microbiology** 52(8):689-695.

Kim JI, Murphy AS, Baek D, Lee SW, Yun DJ, Bressan RA, Narasimhan ML (2011) YUCCA6 over-expression demonstrates auxin function in delaying leaf senescence in *Arabidopsis thaliana*. **Journal of experimental botany** 62(11):3981-3992.

Koohkan H, Maftoun M (2016) Effect of Nitrogen–Boron Interaction on Plant Growth and Tissue Nutrient Concentration of Canola (*Brassica napus* L.). J**ournal of Plant Nutrition**. 39: 922-931.

Koornneef M, Meinke D (2010) The development of *Arabidopsis* as a model plant. **The Plant Journal.** 61: 909-921.

Lata R, Chowdhury S Gond SK, White Jr (2018) Induction of abiotic stress tolerance in plants by endophytic microbes. **Letters in Applied Microbiology** 66: 268-276.

Li H, La S. Zhang X, Gao L, and Tian Y (2021). Salt-induced recruitment of specific root-associated bacterial consortium capable of enhancing plant adaptability to salt stress. **The ISME Journal** 15:2865-2882.

Loper JE, Schroth MN (1986) Influence of bacterial sources of indole-3-acetic acid on root elongation of sugar beet. **Phytopathology** 76:386–389

Lúcio JCB (2020) Auxinas dependem de peróxido de hidrogênio nas respostas das raízes de tomateiro à deficiência de nitrogênio. **Dissertação** (Mestrado em Agronomia e produção vegetal) - UNESP, Jaboticabal, 41 p.

Ludwig-Müller J (2015) Bacteria and fungi controlling plant growth by manipulating auxin: balance between development and defense. **Journal of plant physiology** 172:4-12.

Marathe R, Phatake Y, Shaikh A, Shinde B, Gajbhiye M (2017). Effect of IAA produced by *Pseudomonas aeruginosa* 6a (bc4) on seed germination and plant growth of Glycin max. **Journal of Experimental Biology and Agricultural Sciences**. 5:351-358.

Marks BB, Megías M, Ollero FJ, Nogueira MA, Araujo RS, Hungria M (2015) Maize growth promotion by inoculation with *Azospirillum brasilense* and metabolites of rhizobium tropici enriched on lipo-chitooligosaccharides (LCOs) **AMB Express**, 5:71

Matsukawa E, Nakagawa Y, Iimura Y, Hayakawa M (2007) Stimulatory effect of indole-3-acetic acid on aerial mycelium formation and antibiotic production in *Streptomyces* spp. **Actinomycetologica** 21(1):32-39.

Mishra J, Singh R, and Arora NK (2017). Alleviation of heavy metal stress in plants and remediation of soil by rhizosphere microorganisms. **Front. Microbiol.** 8:1706.

Nabrdalik M, Moliszewska E, Wierzba S (2018) Importance of endophytic strains pantoea agglomerans in the biological control of rhizoctonia solani. **Ecol. Chem. Eng.** 25: 331–342.

Pantoja-Guerra M, Valero-Valero N & Ramírez CA (2023) Nível de auxina total no sistema solo-planta como fator modulador da eficácia de inóculos de PGPR: uma revisão. **Chem. Biol. Tecnol. Agrícola** 10:6.

Patten CL, Glick BR (1996) Bacterial biosynthesis of indole-3-acetic acid. **Can J Microbiol** 42:207–220.

Phillips KA, Skirpan AL, Liu X, Christensen A, Slewinski TL, Hudson C, Barazesh S, Cohen JD, Malcomber S, McSteen P (2011). vanishing tassel encodes a grass-specific tryptophan aminotransferase required for vegetative and reproductive development in maize. **Plant Cell** 23: 550–566.

Pitzschke A (2016) Developmental peculiarities and seed-borne endophytes in quinoa: omnipresent, robust bacilli contribute to plant fitness. **Frontiers in Microbiology** 7:2.

Potters G, Pasternak TP, Guisez Y, Palme KJ, Jansen MA (2007) Stress-induced morphogenic responses: growing out of trouble? **Trends in plant science** 12**:**98-105.

Poveda J, González-Andrés F (2021) Bacillus as a source of phytohormones for use in agriculture. **Applied Microbiology and Biotechnology** 105**:**8629-8645.

Ramakrishna W, Yadav R, & Li K (2019). Plant growth promoting bacteria in agriculture: Two sides of a coin. **Applied Soil Ecology** 138, 10-18.

Ruckdaschel E, Klingmuller W (1992) Analysis of IAA biosynthesis in *Azospirillum lipoferum* and Tn5 induced IAA mutants. **Symbiosis** 13:1-3.

Safara S, Harighi Bahramnejad B, Ahmadi S (2022) Antibacterial activity of endophytic bacteria against sugar beet root rot agent by volatile organic compound production and induction of systemic resistance. **Frontiers in Microbiology**

Santos MS, Nogueira MA & Hungria M. (2021). Outstanding impact of Azospirillum brasilense strains Ab-V5 and Ab-V6 on the Brazilian agriculture: Lessons that

farmers are receptive to adopt new microbial inoculants. **Revista Brasileira de Ciência do Solo**, 45.

Santos LCN (2016) Uso de um mutante em auxina no estudo da desordem nutricional de nitrogênio em tomateiro. **Tese** (doutorado) - Universidade Estadual Paulista, Faculdade de Ciências Agrárias e Veterinárias – Jaboticabal.

Scott JW, Harbaugh BK (1989) Micro-Tom: a miniature dwarf tomato. Florida: Florida Agricultural Experimental Station Circular, 1989, 6 p. (University of Florida, **Agricultural Experimental Station**, S-370).

Shani E, et al. (2017) Plant Stress Tolerance Requires Auxin-Sensitive Aux/IAA Transcriptional Repressors. **Current biology** 27(3):437–444.

Shi Y, Lou K, Li C. (2009). Promotion of plant growth by phytohormone-producing endophytic microbes of sugar beet. **Biology and Fertility of Soils** 45(6):645-653.

Shi Y, Lou K, Li C (2010) Growth and photosynthetic efficiency promotion of sugar beet (*Beta vulgaris* L.) by endophytic bacteria. **Photosynthesis Research** *105*(1):5-13.

Shi Y, Lou K, Li C (2011). Growth promotion effects of the endophyte *Acinetobacter johnsonii* strain 3-1 on sugar beet. **Symbiosis** 54(3):159-166.

Singh TB, Sahai V, Goyal D, Prasad M, Yadav A, Shrivastav P, Ali A, Dantu PK (2020) Identification, characterization and evaluation of multifaceted traits of plant growth promoting rhizobacteria from soil for sustainable approach to agriculture. **Curr. Microbiol.** 77:3633–36

Soumare A, Diédhiou AG, Arora NK, Tawfeeq Al-Ani LK, Ngom M, Fall S, & Sy MO (2021). Potential role and utilization of plant growth promoting microbes in plant tissue culture. **Frontiers in Microbiology** 12:649878.

Spaepen S, Vanderleyden J, Remans R, (2007) Indole-3-acetic acid in microbial and microorganism-plant signaling. **FEMS Microbiology Reviews** 31:425–448

Vejan P, Abdullah R, Khadiran T, Ismail S, Nasrulhaq Boyce A (2016) Role of plant growth promoting rhizobacteria in agricultural sustainability - A review. **Molecules** 21:573.

Verma SK, White JF (2018). Indigenous endophytic seed bacteria promote seedling development and defend against fungal disease in browntop millet (*Urochloa ramosa* L.). **Journal of applied microbiology** 124(3):764-778.

Vessey JK (2003) Plant growth promoting rhizobacteria as biofertilizers. **Plant Soil** 255:571–586.

Wang J, Li R, Zhang H, Wei G, Li Z (2020) Beneficial bacteria activate nutrients and promote wheat growth under conditions of reduced fertilizer application. **BMC Microbiol.** 20:1–12.

CAPITULO 2 - The Negative Effect of Coinoculation of Plant Growth-Promoting Bacteria Is Not Related to Indole-3-Acetic Acid Synthesis[1]

Abstract - The use of coinoculations of indole-3-acetic acid-synthesizing bacteria has become increasingly common in agriculture. Different bacterial isolates are capable of synthesizing IAA. Depending on the amount, IAA can promote, inhibit, or modify the growth and development of plants. However, little is known about the effect of auxins on plants under coinoculation conditions. The present study aimed to verify whether coinoculation with indole-3-acetic acid (IAA)-synthesizing bacteria may or may not harm plant development in three tomato genotypes with different sensitivities to auxins: Micro-Tom (MT), *diageotropica* (*dgt*) (low auxin sensitivity) and *Entire* (high auxin biosynthesis). The experiment was conducted in a completely randomized design with six replicates and eight treatments: control; *Bacillus velezensis* (BV); *B. pumilus* (BP); *Azospirillum brasilense* (AZ); *B. velezensis* + *Bacillus pumilus* (BV + BP); *B. velezensis* + *A brasilense* (BV + AZ); *B. pumilus* + *A brasilense* (BP + AZ); *B. velezensis* + *Bacillus pumilus* + *A. brasilense* (BV + BP + AZ), for each tomato genotype. The parameters analyzed were dry shoot and root weight, plant height, and leaf area. The results showed an increase in root dry weight with BV + BP + AZ treatment in the MT genotype, an increase in shoot dry weight in the *dgt* genotype with BV and no increase in any parameter in the *Entire* genotype for the analyzed parameters. The results also showed that coinoculation with BP + AZ decreased the shoot dry weight, dry root weight, and leaf area values of the *dgt* and *Entire* genotypes. Coinoculations with BP + AZ reduced the parameters analyzed in the *dgt* genotype, which is insensitive to the IAA and *Entire* genotypes and is sensitive. In addition, coinoculation with BV + BP + AZ, which provided the highest IAA concentration, increased the dry root weight in the MT genotype. These results suggest that the reduction in plant development caused by coinoculation is not related to IAA synthesis produced by bacteria but is probably due to negative interference in the interaction mechanisms between plants and bacteria and might also be due to negative bacteria–bacteria interactions.

Keywords Plant growth · Tomato · Growth promotion · Phytormons

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Introduction

Indole-3-acetic acid (IAA) is the main auxin acting as a phytohormone in many plant development processes. The ability to synthesize IAA is widely associated with plants and various plant growth-promoting rhizobacteria (PGPR). Several studies have been published on the potential of PGPR application to improve plant development through the enhancement of its main processes (Defez et al., 2019). The phytohormone auxin controls almost every aspect of plant growth and development (Halliday et al. 2009; Grossmann 2010). The most important and most common auxin is indole-3-acetic acid (IAA), which is produced by bacteria, plants and fungi. In plants, this phytohormone plays a central role in cell division, elongation, fruit development flowers (Phillips et al. 2011). Particularly in dicotyledons, IAA induces lateral root formation, whereas in monocots, IAA induces adventitious root formation (McSteen 2010). Endogenous IAA concentrations vary widely in diferente plant tissues, depending on plant species and organs. Typically, the IAA concentration is low (5–100 ng/g fresh weight) in leaves and 10–100 times lower in roots (Reid et al. 2011). Even at low concentrations, IAA is the main regulator of plant development (Chen and Baluska 2013). Different bacterial isolates are capable of synthesizing IAA, including soil, epiphytic, endophytic, marine, methylotrophic bacteria and cyanobacteria (Sergeeva et al. 2002). Some of these bacteria are harmful to plant health, while others are beneficial. Plants can harbor thousands of IAAproducing bacteria from different genera, such as *Pseudomonas*, *Rhizobium*, *Azospirillum*, *Enterobacter*, *Azotobacter*, *Klebsiella*, *Alcaligenes*, *Pantoea*, *Streptomyces* and *Bacillus* (Apine and Jadhav 2011). Bacterial IAA stimulates the formation of root hairs, increasing the number and length of lateral and primary roots when its concentration is within the optimal range. However, at higher concentrations, bacterial IAA can also inhibit primary root growth (Davies 1995). *Bacillus velezensis* was previously grouped with *B. subtilis* and *B. amyloliquefaciens*; in recent years, several isolates of this bacterium have received attention due to their potential in disease control (Adeniji et al., 2019; Bem fan et al., 2017). Previous studies have determined that *B. velezensis* has the ability to produce IAA in pepper plants (Jiang et al. 2019); in addition, metabolites have been shown to have

antagonistic activity against bacterial and fungal pathogens under laboratory and greenhouse conditions in tomato crops (Cao et al. 2018). *Bacillus pumilus* is a species with the potential to be used as a plant growth promoter, which is an IAA and gibberellin producer and has been shown to stimulate the growth and development of plants (Probanza et al., 2002; Santos et al., 2018). *Azospirillum brasilens*e is the most studied gram-negative plant growth-promoting bacterium. Many studies have shown improvement in the growth and development of plants inoculated with *A. brasilense* due to the production of auxins, cytokinins and gibberellins. Plant inoculation with this bacterium has promoted morphological changes such as increased root surface through greater production of root hairs, which increases nutrient absorption (Rondina et al., 2020). Coinoculation with plant growth-promoting bacteria is becoming increasingly common. This practice has some advantages, such as an increase in the successful establishment of bacteria in the rhizosphere and in the condition of interaction with plants (Bulgarelli et al., 2013). Keswani et al. (2020) published a fascinating review regarding the plant growth-promoting rhizobacteria and fungi that secrete IAA to promote plant growth. However, the coinoculation with bacteria and its effect related to IAA produced on the plants were not approached. Little is known about the advantages of coinoculation of plant growth-promoting bacteria in relation to IAA synthesis. The present study aimed to verify whether coinoculation with indole-3-acetic acid (IAA)-synthesizing bacteria may or may not harm plant development. For this verification, tomato genotypes with different auxin sensitivity levels were used in conjunction with inoculations with bacteria that synthesize different IAA concentrations.

Materials and Methods

Experimental Design

The experiment was carried out in a completely randomized design with eight treatments for each tomato genotype and six replicates (Table 1).

Bacterial Isolates

The following bacterial isolates were used: *B. velezensis* (MZ133757), *B. pumilus* (MZ133755), and *A. brasilense* (11,005), belonging to the collection of the Laboratory of Soil Microbiology, FCAV-UNESP, campus of Jaboticabal. For experimental use, bacteria were routinely streaked on plates containing NA (nutrient agar) culture medium.

Tomato Genotypes

Tomato seeds (*Solanum lycopersicum* L.) The Micro-Tom variety and its *dgt* (low sensitivity to auxin/defective gene for cyclophilin biosynthesis) and *Entire* (with high auxin biosynthesis) mutants belong to the Laboratory of Plant Physiology, Department of Biology Applied to Agriculture Table 1 Description of greenhouse treatments with *diageotropica* (dgt), *Entire* (e) and Micro-Tom tomato genotypes The treatments were carried out with six repetitions each

Table 1 Description of greenhouse treatments with *diageotropica* (dgt), *Entire* (e) and Micro-Tom tomato genotypes

Treatments
Control
***Bacillus velezensis* (BV)**
***Bacillus pumilus* (BP)**
***Azospirillum brasilense* (AZ)**
***B. velezensis* + *Bacillus pumilus* (BV + BP)**
***B. velezensis* + *A. brasilense* (BV + AZ)**
***B. pumilus* + *A. brasilense* (BP + AZ)**
***B. velezensis* + *Bacillus pumilus* + *A. brasilense* (BV + BP + AZ)**

at FCAV-UNESP, campus of Jaboticabal. Seeds of MT, *dgt* and *Entire* genotypes were multiplied to maintain the laboratory genotype bank. In the *Entire* tomato genotype (*Solanum lycopersicum* L), locus (e) controls leaf morphology, and the dominant (e) recessive allele and allele of the locus produce pinnate compounds and

complex reduced leaves. The leaf morphology of these transgenic lines was similar to the leaf morphology of the *Entire* tomato mutant (Zhang et al., 2007). Figure 1 shows the three tomato genotypes. In the *diageotropica* genotype (*dgt*), the mutant gene was *Solyc01g111170*, and plants with the mutated allele had low sensitivity to auxin. The tomato genotype (*dgt*)-dgt has hyponastic leaves, plagiotropic roots with fewer branches and slender stems (Albert et al., 2004).

Greenhouse Experiment

In the greenhouse, seeds were germinated in trays containing a 1:1 mixture of commercial substrate (Plantmax HT) Eucatex producer-Brazil with expanded vermiculite supplemented with 1 g L− 1 10:10 NPK and 4 g L− 1 commercial dolomitic limestone (Ca + Mg). After 15 days, under the same substrate conditions, plants were transferred to pots with a capacity of 1 L, remaining for 90 days to collect fruits and seeds used in the experiment.

In Vitro Auxin Biosynthesis

The analysis of in vitro indole-3-acetic acid production was performed using HPLC. Each bacterial colony and mixtures were applied to 20 mL of dextrose yeast glucose sucrose (DYGS) medium containing (in g L−1): glucose, 2; peptone, 1.5; yeast extract, 2; potassium dihydrogen phosphate, 0.5; magnesium sulfate heptahydrate, 0.5 (Rodriguez Neto et al., 1986), supplemented with 5 mM L-tryptophan and incubated for 48 h at 28 °C under constant stirring at 120 rpm in the absence of light (Andrade, 2012). After growth, 5 mL of each culture was centrifuged at 10,000 rpm for 10 min. The samples (15 mL) were collected for centrifugation (Sorvall centrifuge at 16,266 × g for 30 min at 4 °C) and then concentrated under vacuum using a centrifugal evaporator to a volume of 1 mL. Then, they were filtered through a cellulose ester filter (Millipore Corp.) with a pore size of 0.45 µm and injected in triplicate into an HPLC equipped with an RID detector (Shimadzu RID model 10A). The injected samples were eluted at 20 µL with a mobile phase of acetonitrile:water (75:25 v:v) under the following chromatographic conditions: 35 °C injection

temperature, 20 µL injection volume, 1.0 mL/min flow. The culture medium at time zero was used as a negative control. Four concentrations (50, 25, 12.5, 6.25 µmol/L) were prepared for each indole compound standard indole-acetic acid, indole-pyruvic acid, indole-lactic acid, and indole-acetaldehyde for quantification and then analyzed by HPLC. A Shimadzu Class-VP chromatography data system was used for data acquisition and data analysis. Means and standard deviations were calculated, and SigmaPlot 11.0 was used to generate graphs.

Inoculations in Seeds and Plants

For inoculations, each bacterial isolate was cultivated in a 125 mL Erlenmeyer flask containing 50 mL of nutriente broth (meat extract: 1.0 g/L, yeast extract: 2.0 g/L, peptone: 5.0 g/L, NaCl: 5.0 g/L, pH: 6.8 • } 0.2), and cultures wereincubated at 28 °C for 24 h for standardized growth of 108 colony-forming units (CFU) per mL. The seeds were sterilized prior to inoculation. The seeds were washed with water tap and deionized water and dried on absorbent towels. Then, 2 g of seeds was transferred aseptically to a sterile beaker, washed two times with sterile distilled water and sterilized using 0.2 g% HgCl2 for 30 s. Then, the seeds were washed six times with distilled water (Ladha et al., 1997). After sterilization, seeds were immersed in bacterial suspensions

Genótipo Micro-Tom | Genótipo *dgt* | Genótipo *Entire*

Fig. 1 Tomato genotypes used in this experiment

and stirred at 180 rpm and 28 °C for approximately 30 min. Control treatments consisted of seeds immersed only in the culture medium, free from bacteria, under the same conditions. Throughout the experiment, four inoculations were carried out using 2 mL of each bacterial inoculum according to the treatments in the plants. The period between the inoculations was 15 days.

Dry Shoot and Root Weight, Plant Height and Leaf Area

Plant height was measured using a measuring tape. After measurements, plants were removed from pots, and shoots and roots were separated, with roots being washed and packed in paper bags. To obtain dry weight, after the end of previous analyses, the collected material was kept in a paper bag and placed in an oven with forced air circulation at 65 °C for 72 h. Subsequently, root and shoot weights were obtained using na analytical scale (Denver Instrument Company AA-200) with a precision of 0.0001 g. To measure leaf area, a Marconi AM350 leaf area meter was used.

Bacillus sp. and Azospirillum brasilense Reisolation from Pot Soil

The number of CFUs for both *Bacillus* sp. and *A. brasilense* was estimated by serial dilution (Wollum, 1982). *Bacillus* spp. isolation was performed following a selective isolation method based on thermal shock proposed by Rothfuss et al. (1997) and Vieira and Nahas (2000). *A. brasilense* was isolated using Burks N-free media (Martinez-Toledo et al., 1985). Soil samples (10 g) were obtained from potting soils without tomato plants, and after 48 h of inoculation, 2 g soil samples were added to 250 mL Erlenmeyer flasks containing 18 mL of Burks liquid medium. Samples were incubated for 48 h at 28 °C. Each culture was subcultured five times after 48 h of incubation, and enriched cultures were plated on Burks solid medium and then incubated (28 °C for 48 h). All bacterial strains were selected and counted after Gram staining.

Analysis of Results

From the six repetitions of each treatment, the mean and standard error of each treatment were obtained. Data normality and heteroscedasticity were assessed using the Shapiro– Wilk and Bartlett tests, respectively. Analysis of variance (ANOVA) was performed for each experiment, and when differences were detected, means were compared by Duncan's test at the 5% significance level. Principal componente analysis (PCA) was used to describe the relationship between plant biometric and morphological variables and their association with treatments. PCA was performed after data standardization (to avoid the influence of different units of means of response variables) using Pearson's correlation matrix. Hierarchical clustering on principal componentes (HCPC) was built with the similarity matrix among samples using the Euclidean distance, and the linking of groups was conducted by Ward's method.

Compatibility Test (Dual Culture Assays) with Bacterial Strains Under In Vitro Conditions

The *A. brasilense*, *B. pumilus*, and *B. velezensis* strains were analyzed for their ability to inhibit each other's growth in vitro. They were previously grown in nutrient broth medium in 5-mL test tubes individually for 24 h at 28 °C. For the test, nutrient agar (NA) medium was added to a plate, 1 mL of the bacterial suspension of each strain was added individually, and the others were dripped (10 µL) into the AN and individual suspension plates so that two were inoculated into each plate. Strains different from the specified strains were added in suspension to the medium. All interaction possibilities were evaluated, and the test was performed in triplicate. The plates were incubated for 24 h at 28 °C, and the growth inhibition halo formed around the strain with antagonistic activity was observed.

Results

The indole-acetic acid (IAA) production from each isolate and their combinations was measured in vitro. The values for each isolate used in this experiment were 17.86 µg IAA mL−1 for *A. brasilense,* 17.79 µg IAA mL−1 for *B. pumilus* and 7.40 µg IAA mL−1 for *B. velezensis*, 22.13 µg IAA mL−1 for BV + BP, 19.45 µg IAA mL−1 for BV + AZ, 18.74 µg IAA mL−1 for BP + AZ and 24.71 µg IAA mL−1 for BV + BP + AZ. For the Micro-Tom genotype, there was no difference ($p > 0.05$) among treatments for dry shoot weight, with values ranging from 0.40 to 0.50 g (Fig. 2A). There was also no difference in plant height ($p > 0.05$) between the treatments and the control, with values ranging from 9 to 11 cm (Fig. 2B). Regarding dry root weight, the highest treatment (0.14 g) and the only treatment that differed ($p < 0.05$) from the control (0.07 g) and from the others was BV + BP + AZ (Fig. 2C). There was no difference ($p > 0.05$) between the treatments and the control for leaf area, with values ranging from 90 to 125 cm2 (Fig. 2 D). Treatments did not increase the parameters analyzed; however, the BP + AZ treatment harmed plant development. Cluster analysis showed the formation of four distinct groups. Interestingly, the BP + AZ treatment group was far from the control group (Fig. 2E). Principal component analysis (PCA) showed that dry root

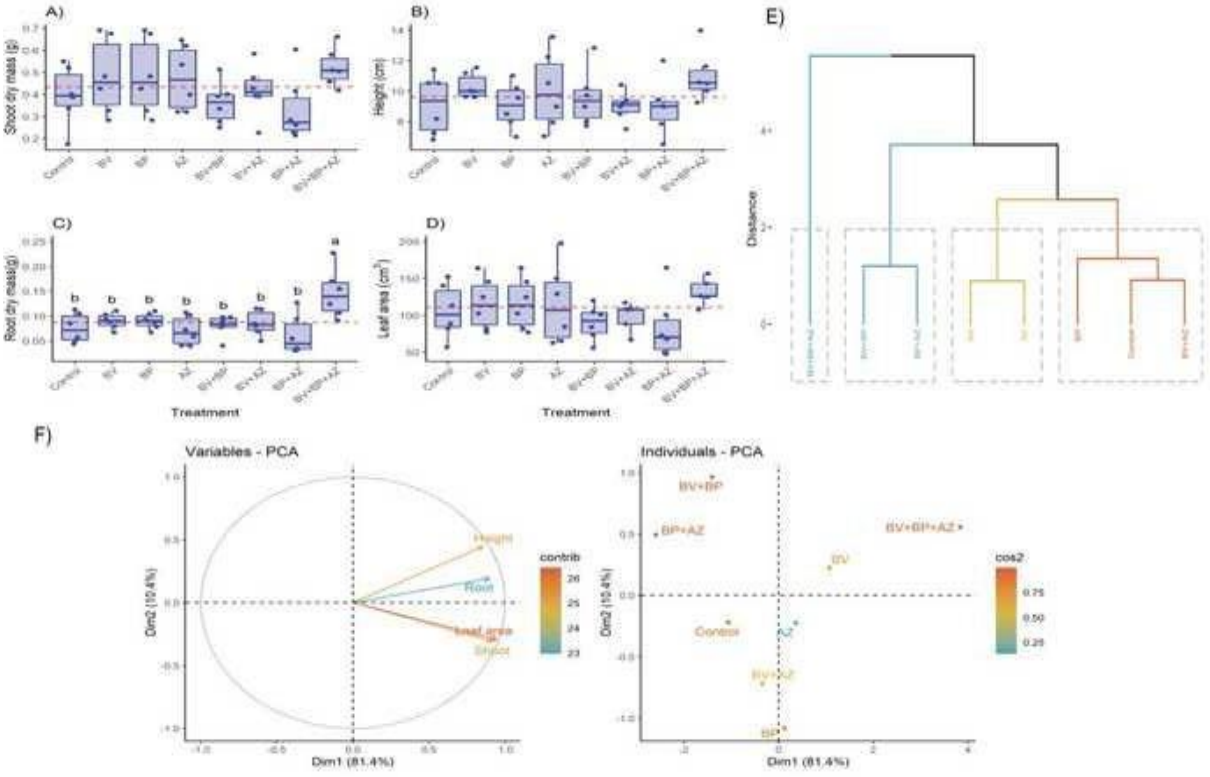

Fig. 2 Biometric data of Micro-Tom tomato genotype **A** dry shoot weight (DSW) and **B** plant height, **C** dry root weight, **D** leaf area, inoculated with *B. velezensis* (BV), *B. pumilus* (BP), and A*. brasilense* (AZ). Means followed by the same lowercase letter do not differ by Duncan's test ($p \leq 0.05$). **E** Hierarchical clustering of principal components (HCPCs) of inoculations tested. **F** Principal componente analysis (PCA) for variables and treatments (individuals). Values on Axes 1 and 2 represent the percentage of total variance explained by axes

weight and leaf area parameters had a negative correlation with the BP + AZ treatment (Fig. 2F). For the *diageotropica* genotype (*dgt*), the highest dry shoot weight value was found for the BV treatment (0.58 g) ($p < 0.05$), differing from the control (0.45 g) ($p < 0.05$). The lowest value found was for the BP + AZ treatment (0.2 g), differing from the control ($p < 0.05$) and from the other treatments (Fig. 3A). The highest plant height value was found for the BV treatment (12 cm), differing only from the BP (10 cm) ($p < 0.05$) and BP + AZ (8.0 cm) treatments. The lowest plant height value found was for the BP + AZ treatment, differing ($p < 0.05$) from all other treatments (Fig. 3B). The highest dry root weight value was found for the BV treatment (0.9 g), which differed from the BP (0.06 g) ($p < 0.05$), AZ (0.05 g), BV +

BP (0.05 g), BP + AZ (0.03 g) and BV + BP + AZ (0.04 g) treatments. The lowest value was found for the BP + AZ (0.03 g) treatment, differing from the control (0.07 g) and BP (0.06 g) treatments (Fig. 3C). The lowest leaf area value (60 cm2) and the only one that differed from the control (131.63 cm2) was in the BV + AZ treatment. The other treatments did not differ ($p > 0.05$) (Fig. 3D). The analysis of hierarchical clusters showed that five distinct groups were formed in relation to treatments and that the BP + AZ treatment was distinct from the control group (Fig. 3E). Principal component analysis (PCA) showed that dry root weight and leaf area were indirectly related to the BP + AZ treatment, which was located on the opposite side of the two-dimensional plane (Fig. 3F). For the *entire* genotype, the lowest values that differed from the control (0.36 g) for dry shoot weight were obtained for BP (0.12 g), BV + AZ (0.2 g), and BV + BP + AZ (0.2 g). The BV + BP (0.33 g) and BP + AZ (0.34 g) treatments did not differ from the control ($p > 0.05$) (Fig. 4A). For plant height, there was no difference ($p > 0.05$) among treatments, with values ranging from 8.5 to 9.6 cm (Fig. 4B). For dry root weight, no treatment presented a value higher than the treatment of the control (0.07 g) ($p > 0.05$). The lowest values were found for the BP (0.032 g), BV + AZ (0.034), and BV + BP + AZ (0.036) treatments (Fig. 4C). For leaf area, no treatment presented a value greater than the value of the control (87.46 cm2); however, treatments that had reduced values ($p < 0.05$) were BP (58.14 cm2), AZ (64.12 cm2), BV + AZ (53.01 cm2), and BV + BP + AZ (59.00 cm2) (Fig. 4D). The analysis of hierarchical clusters showed that five distinct groups were formed in relation to treatments and that the BV + AZ treatment was distant from the control group (Fig. 4E). Principal component analysis (PCA) showed that the parameters dry root weight and leaf

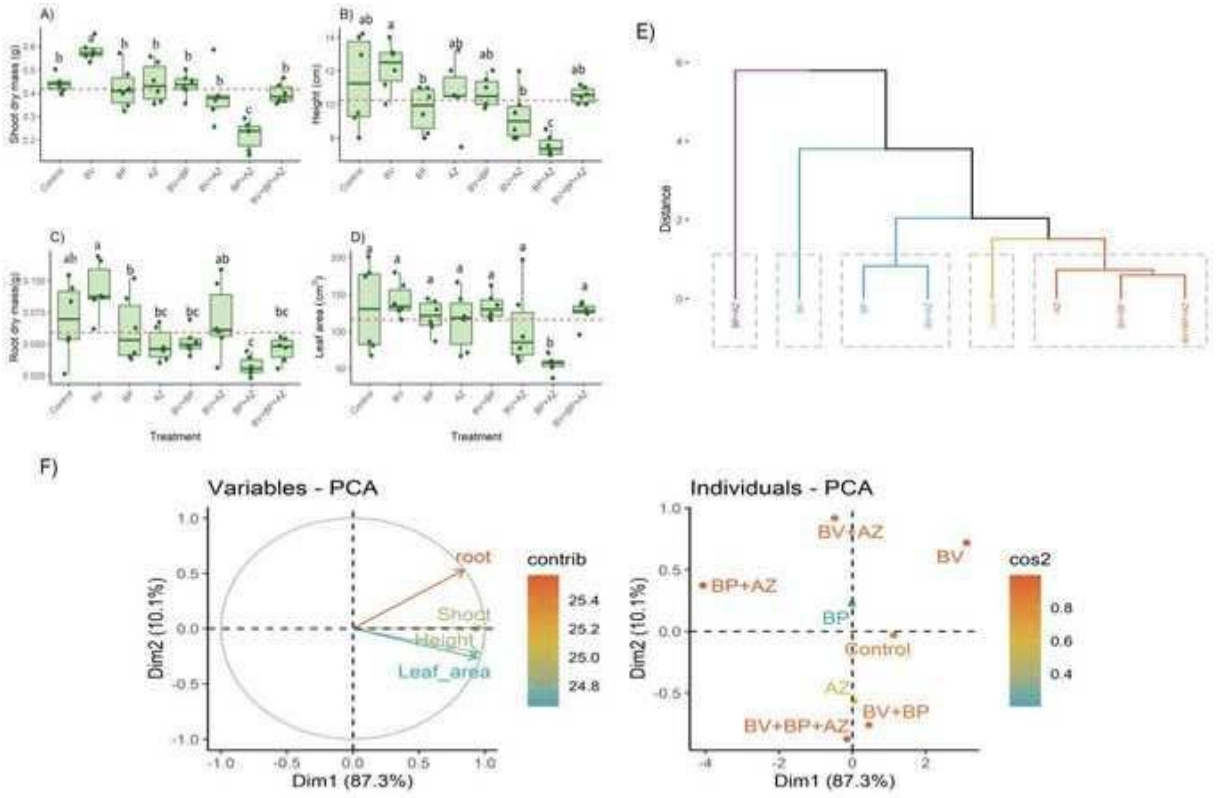

Fig. 3 Biometric data of the *diageotropica* tomato genotype (*dgt*) **A** dry shoot weight (DSW) and **B** plant height, **C** dry root weight, **D** leaf area inoculated with *B. velezensis* (BV), *B. pumilus* (BP), and *A. brasilense* (AZ). Means followed by the same lowercase letter do not differ by Duncan's test ($p \leq 0.05$). **E** Hierarchical clustering of principal components (HCPCs) of inoculations tested. **F** Principal componente analysis (PCA) for variables and treatments (individuals). Values on Axes 1 and 2 represent the percentage of total variance explained by axes

area were in the opposite plane in relation to the BP + AZ treatment (Fig. 4F). The compatibility among the bacteria was evaluated. *B. velezensis* showed slight antagonism with *A. brasilense* and *B. subtilis* (Fig. 5).

Discussion

The results suggest that the damage to plant development caused by coinoculation is not related to IAA synthesis produced by bacteria but is related to plant–bacteria interactions. Furthermore, bacterial inoculations behaved neutrally in MT growth reduction to the other genotypes, which demonstrates changes in plant behavior against inoculated strains (Fig. 6). The decrease in plant development can be caused

by many factors. It is not easy to measure the degree of interference of both bactéria in the plant microorganism interaction. Rhizosphere communication is established in a highly sophisticated manner and controlled through wide-ranging specialized metabolites and exudates and results in altered gene expression in one of both interacting partners. This mutual communication results in alterations in plant growth, inhibition of soil pathogens, nutrient availability, biofilm development and accumulation of soil microbes (Li et al.,2013; Mommer et al., 2016; Sasse et al., 2017). Some studies on the combination of PGPR have found that most of the microorganisms used in mixtures did not interfere negatively with each other. Korer et al. (2017) showed that coinoculation of *B. subtilis* and *Rhizobium* sp. has a synergistic effect on bean growth. Kaur et al. (2015) verified a synergistic effect of coinoculation between *Mesorhizobium* and *Pseudomonas* sp. Significant improvement in growth and symbiotic parameters was observed with coinoculation. Ferreira et al. (2020) evaluated the effects of coinoculation of nodule endophytic strains of the genera *Bacillus*, *Paenibacillus*, *Burkholderia* and *Pseudomonas* with *Rhizobium tropici*. The results showed that the use of rhizobacteria combined with rhizobia contributed synergistically to the promotion of growth and the control of damping off in the common bean. However, few reports agree with our results, indicating that certain mixtures of microbial strains do not show synergistic or at least comparable effects on plant growth promotion with respect to the separate application of microorganisms.

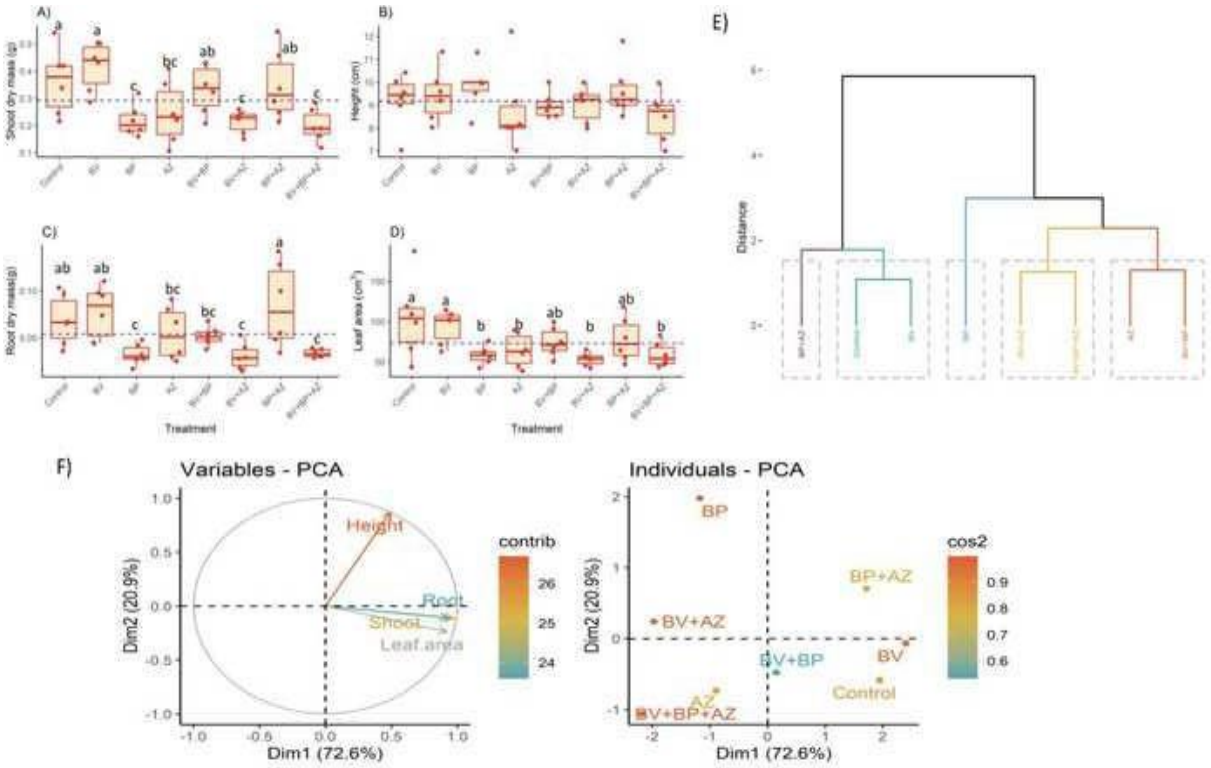

Fig. 4 Biometric data of the *Entire* tomato genotype **A** dry shoot weight (DSW) and **B** plant height, **C** dry root weight, **D** leaf área inoculated with *B. velezensis* (BV), *B. pumilus* (BP), and *A. brasilense* (AZ). Means followed by the same lowercase letter do not differ by Duncan's test ($p \leq 0.05$). **E** Hierarchical clustering of principal components (HCPCs) of inoculations tested. **F** Principal componente analysis (PCA) for variables and treatments (individuals). Values on Axes 1 and 2 represent the percentage of total variance explained by axes

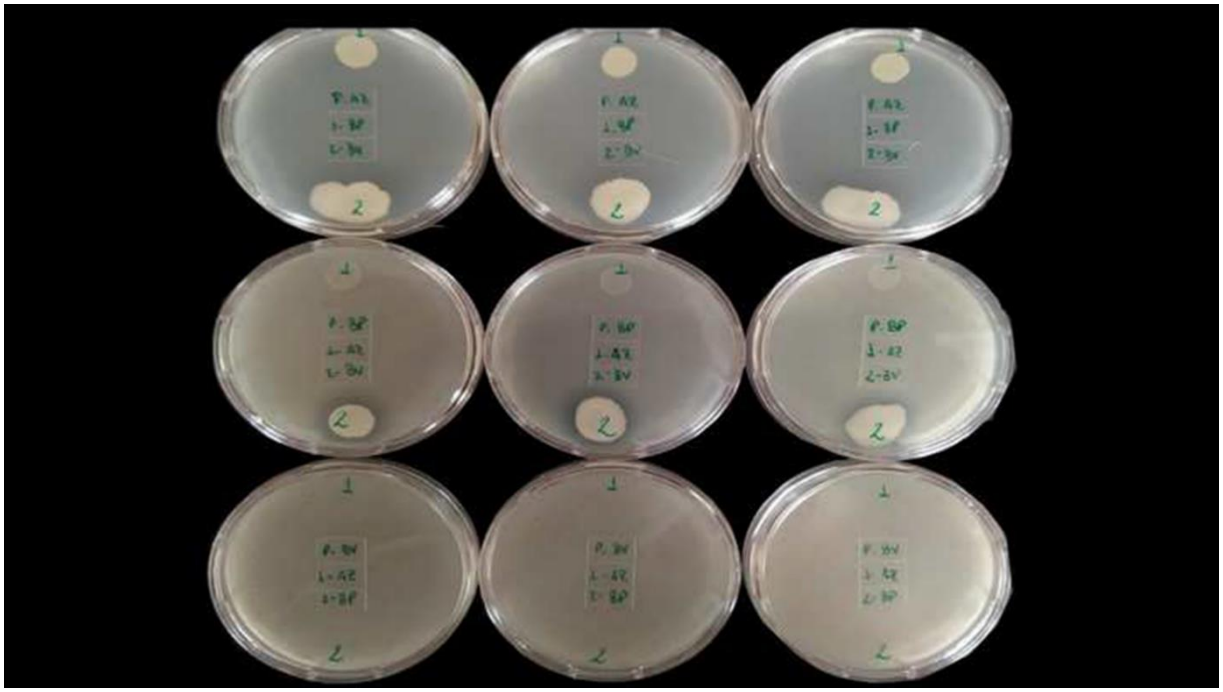

Fig. 5 Compatibility test (culture assays) with bacterial strains under in vitro conditions. The bacteria evaluated were *B. pumilus* (BP), *A. brasilense* (AZ) and *B. pumilus* (BP)

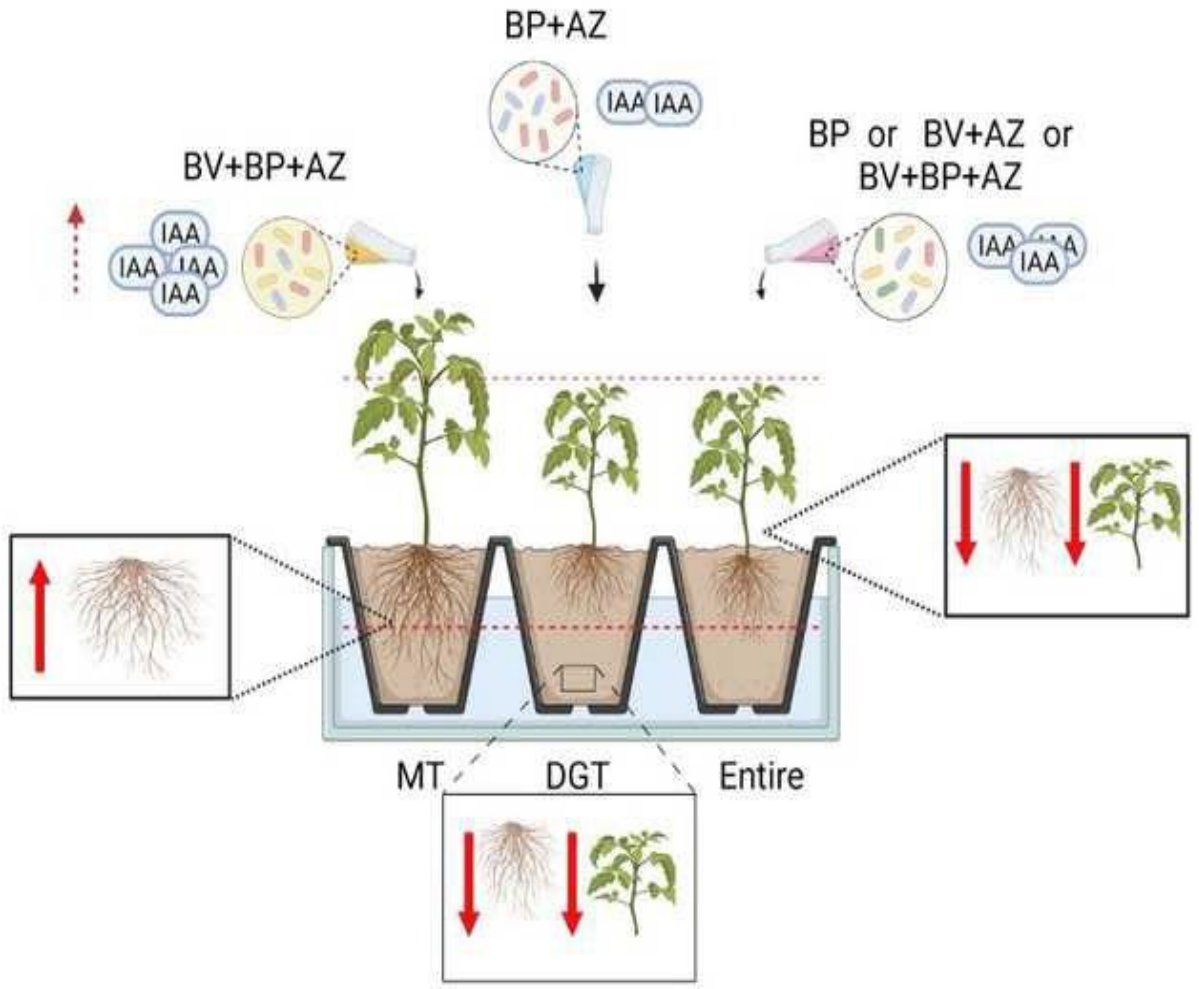

Fig. 6 Scheme showing that coinoculation with BV + BP + AZ promoted an increase in root dry matter in the Micro-Tom genotype. The coinoculation of BP + AZ promoted a decrease in all parameters analyzed in the genotype *dgt*. Coinoculation with BV + AZ promoted a decrease in root and shoot dry matter in the *Entire* genotype. The results show that the harm to plant development is not related to IAA concentration

Felici et al. (2008) used the combination of *B. subtilis* and *A. brasilense* in tomato plants and found that the combination of the two bacteria did not improve plant growth compared to the inoculation of single bacteria. These authors support the hypothesis that these two bacteria might operate differently in modulating root growth. In plantlets coinoculated with *B. subtilis* and *A. brasilense*, activation of the quiescente center was not observed, and primary root elongation was strongly inhibited. This inhibitory effect also induced a morphogenesis resistance process in which several conflicting intercellular signals were involved (Potters et al., 2007; Werner et al., 2003). Therefore, Felici et al. (2008) concluded that the copresence of

the two microorganisms may alter, directly or indirectly, the internal hormone content of the root, interfering with the normal morphogenesis of the root itself. Vestberg et al. (2004) evaluated a mixture of several microorganisms, including fungi and bacteria, and a mixture of *B. subtilis* + *Glomus mosseae*, *B. subtilis* + *Trichoderma harzianum, B. subtilis* + *Pseudomonas fluorescens* and *B. subtilis* + *Gliocladium catenulatum* in strawberry crops. Although *B. subtilis* had the best performance in promoting plant growth, there was no improvement in plant growth due to coinoculation of *B. subtilis* with the aforementioned microorganisms. Coinoculation of plant growth-promoting rhizobacteria (PGPR) and microbial pest control agents is considered na innovative approach in phytosanitary management and for improving crop yield and quality (Janisiewicz, 1996; Marimuthu et al., 2002). In fact, the use of formulated preparations consisting of a single microbial species or isolates as inoculants (i.e., a single antagonist against a single pathogen) has often resulted in inconsistent performance in agriculture and, consequently, in low representation in the world inoculant market. One of the reasons for such failure may be that a single microbial agent is unlikely to be active in all soil environments (in the presence of diferente biotic and abiotic stressors) or against all pathogens attacking the host plant (the latter is also seen as an advantage from the point of view of risk management). One way to overcome this problem is to include different species or strains of beneficial microbes in the same microbial formulation. The application of binary or multiple mixtures would mimic the natural situation more closely and could broaden the spectrum of biocontrol activity (Raupach and Kloepper, 1998). However, excess IAA is known to harm plant development, and little is known about the effect of these inoculations. In the present study, as coinoculations with BP + AZ affected the parameters analyzed in the *dgt* genotype insensitive to IAA and in the *Entire* genotype, which is sensitive, these results strongly suggest that the reduction in plant development caused by coinoculation is not related to IAA synthesis produced by bacteria and that changes in the root architecture induced by microbial combination may involve signaling pathways independent from the two bacterial species. Inoculations did not have a negative effect on the MT genotype, which had no genetic alterations in pathways involved with auxin and draws attention to the fact that genes involved in auxin signaling in plants can be fundamental for interaction

with soil microorganisms, modulating the plant response to bacterial inoculations, regardless of IAA concentration produced by strains. Hypothetically, *B. subtilis*, as well as *A. brasilense*, might produce IAA or another phytohormone by plantregulated mechanisms and might induce phytohormone production by means of other molecular signals (Felici et al., 2007). The reduction in plant development might be a result of a change in the hormone content of the indirect effect of the mixture of bacteria on the ability of plants themselves to produce phytohormones, including cytokinins and abscisic acid (ABA). Perhaps the coinoculation of *B. subtilis* and *A. brasilense* neutralizes their mechanisms of phytostimulation.

Conclusions

Many studies report the urgent need to develop new Technologies with an eco friendly approach to supply food production without yield reduction or environmental impact. However, inconsistent results in the field application of biologicals still hinder their successful use by farmers (Keswani et al., 2020). In this regard, the present study collaborates to better understand the use of coinoculation of PGPR concerning IAA in plant production.

References

Adeniji AA, Babalola OO (2019) *Bacillus velezensis*: phylogeny, useful applications, and avenues for exploitation. Appl Microbiol Biotechnol 103:3669–3682

Albert M, Werner M, Proksch P, Fry S, Kaldenhoff R (2004) The cell wall-modifying xyloglucan endotransglycosylase/hydrolase LeXTH1 is expressed during the defense reaction of tomato against the plant parasite *Cuscuta reflexa*. Plant Biol 6:402–407

Apine O, Jadhav J (2011) Optimization of medium for indole-3-acetic acid production using *Pantoea agglomerans* strain PVM. J Appl Microbiol 110:1235–1244

Bulgarelli D, Schlaeppi K, Spaepen S, Van Themaat EVL, Schulze- Lefert P (2013) Structure and functions of the bacterial microbiota of plants. Ann Rev Plant Biol 64:807–838

Cao Y, Pi H, Chandrangsu P, Li Y, Wang Y, Zhou H, Xiong H, Helmann JD, Cai Y (2018) Antagonism of two plant-growth promoting Bacillus velezensis isolates against Ralstonia solanacearum and *Fusarium oxysporum*. Sci Reports 8:1–14

Chen R, Baluška F (2013) Polar auxin transport. Springer

Davies PJ (2013) Plant hormones: physiology, biochemistry and molecular biology. Springer Science & Business Media

Fan B, Blom J, Klenk H-P, Borriss R (2017) Bacillus amyloliquefaciens, Bacillus velezensis, and Bacillus siamensis form an “operational group B. amyloliquefaciens” within the B. subtilis species complex. Front Microbiol 8:22

Felici C, Vettori L, Giraldi E, Forino LMC, Toffanin A, Tagliasacchi AM, Nuti M (2008) Single and coinoculation of Bacillus subtilis and Azospirillum brasilense on Lycopersicon esculentum: effects on plant growth and rhizosphere microbial community. Appl Soil Ecol 40:260–270

Ferreira LDVM, De Carvalho F, Andrade JFC, Oliveira DP, De Medeiros FHV, Moreira FMDS (2020) Coinoculation of selected nodule endophytic rhizobacterial strains with *Rhizobium tropici* promotes plant growth and controls damping off in common bean. Pedo 30(1):98–108

Grossmann K (2010) Auxin herbicides: current status of mechanism and mode of action. Pest Manag Sci 66:113–120

Halliday KJ, Martinez-Garcia JF, Josse E-M (2009) Integration of light and auxin signaling. Cold Spring Harb Perspect Biol 1:a001586

Janisiewicz W (1996) Ecological diversity, niche overlap, and coexistence of antagonists used in developing mixtures for biocontrol of postharvest diseases of apples. Phytopathol 86:473–479

Jiang C-H, Yao X-F, Mi D-D, Li Z-J, Yang B-Y, Zheng Y, Qi Y-J, Guo J-H (2019) Comparative transcriptome analysis reveals the biocontrol mechanism of Bacillus velezensis F21 against Fusarium wilt on watermelon. Front Microbiol 10:652

Kaur N, Sharma P, Sharma S (2015) Coinoculation of Mesorhizobium sp. and plant growth promoting rhizobacteria Pseudomonas sp. as bioenhancer and biofertilizer in chickpea (Cicer arietinum L.). Legume Res. An Int J 38(3):367–374

Keswani C, Singh SP, Cueto L, Garcia-Estrada C, Mezaache-Aichour S, Glare TR, Borriss R, Singh SP, Blazquez MA, Sansinenea E (2020) Auxins of microbial origin and their use in agriculture. Appl Microbiol Biotechnol 104(20):8549–8565

Korir H, Mungai NW, Thuita M, Hamba Y, Masso C (2017) Coinoculation effect of rhizobia and plant growth promoting rhizobacteria on common bean growth in a low phosphorus soil. Front Plant Sci 8:141

Ladha, J, Barraquio, W, Revilla, L (1997) Isolation of endophytic diazotrophic bacteria from wetland rice. In *Opportunities for Biological Nitrogen Fixation in Rice and Other Non-Legumes*. Springer, 15–24.

Li X-g, Zhang Tl, Wang XX, Hua K, Zhao L, Han ZM (2013) The composition of root exudates from two different resistant peanut cultivars and their effects on the growth of soil-borne pathogen. Int J Biol Sci 9(2):164

Marimuthu S, Subbian P, Ramamoorthy V, Samiyappan R (2002) Synergistic effect of combined application of *Azospirillum* and *Pseudomonas fluorescens* with inorganic fertilizers on root rot incidence and yield of cotton/Synergistische Wirkung der

kombinierten Anwendung von *Azospirillum* und Pseudomonas fluorescens mit anorganischen Dungern auf die Wurzelfaule und den Ertrag von Baumwolle. Zeitschrift fur Pflanzenkrankheiten und Pflanzenschutz/J Plant Dis Protect 109(6):569–577

Mcsteen P (2010) Auxin and monocot development. Cold Spring Harb Perspect Biol 2:a001479

Mommer L, Kirkegaard J, van Ruijven J (2016) Root–root interactions: toward a rhizosphere framework. Trends Plant Sci 21(3):209–217

Phillips KA, Skirpan AL, Liu X, Christensen A, Slewinski TL, Hudson C, Barazesh S, Cohen JD, Malcomber S, Mcsteen P (2011) vanishing tassel2 encodes a grass-specific tryptophan aminotransferase required for vegetative and reproductive development in maize. Plant Cell 23:550–566

Potters G, Pasternak TP, Guisez Y, Palme KJ, Jansen MA (2007) Stress-induced morphogenic responses: growing out of trouble? Trends Plant Scie 12(3):98–105

Probanza A, Garcıa JL, Palomino MR, Ramos B, Manero FG (2002) Pinus pinea L. seedling growth and bacterial rhizosphere structure after inoculation with PGPR Bacillus (B. licheniformis CECT 5106 and B. pumilus CECT 5105). Appl Soil Ecol 20(2):75–84

Raupach GS, Kloepper JW (1998) Mixtures of plant growth-promoting rhizobacteria enhance biological control of multiple cucumber pathogens. Phytopathol 88:1158–1164

Reid AE, Kim SW, Seiner B, Fowler FW, Hooker J, Ferrieri R, Babst B, Fowler JS (2011) Radiosynthesis of C-11 labeled auxin (3-indolyl [1-11C] acetic acid) and its derivatives from gramine. J Label Comp Radiopharm 54:433–437

Rondina ABL, Dos Santos Sanzovo AW, Guimaraes GS, Wendling JR, Nogueira MA, Hungria M (2020) Changes in root morphological traits in soybean coinoculated with Bradyrhizobium spp. And Azospirillum brasilense or treated with A. brasilense exudates. Biol Fert Soils 56:1–13

Santos RM, Kandasamy S, Rigobelo EC (2018) Sugarcane growth and nutrition levels are differentially affected by the application of PGPR and cane waste. MicrobiologyOpen 7(6):e00617

Sarwar M, Kremer R (1995) Determination of bacterially derived auxins using a microplate method. Lett Appl Microbiol 20:282–285

Sasse J, Martinoia E, Northen T (2018) Feed your friends: do plant exudates shape the root microbiome? Trends Plant Sci 23(1):25–41

Sergeeva E, Liaimer A, Bergman B (2002) Evidence for production of the phytohormone indole-3-acetic acid by cyanobacteria. Planta 215:229–238

Vestberg M, Kukkonen S, Saari K, Parikka P, Huttunen J, Tainio L, Devos N, Weekers F, Kevers C, Thonart P (2004) Microbial inoculation for improving the growth and health of micropropagated strawberry. Appl Soil Ecol 27:243–258

Werner TS, Motyka V, Laucou V, Smets R, Van Onckelen H, Schmulling T (2003) Cytokinin-deficient transgenic Arabidopsis plantsshow multiple developmental alterations indicating opposite functions of cytokinins in the regulation of shoot and root meristema activity. Plant Cell 15(11):2532–2550

Zhang J, Chen R, Xiao J, Qian C, Wang T, Li H, Ouyang B, Ye Z (2007) A single-base deletion mutation in SlIAA9 gene causes tomato (Solanum lycopersicum) entire mutant. J Plant Res120:671–678

CAPITULO 3 – Efecct of índole-3-acetic acid on tomato plant growth[1]

Abstract - Plant growth-promoting bacteria have several abilities to promote plant growth and development. One of these skills is the synthesis of indole-3-acetic acid (IAA), which mainly promotes root and shoot development. The bacteria *Bacillus subtilis* and *Azospirillum brasilense* have been widely used in agriculture with this function. However, little is known about whether the joint inoculation of these bacteria can reduce plant development by the excess of IAA produced as a result of the joint inoculation. The objective of the present study was to verify the effect of IAA on the inoculation of *B. subtilis* and *A. brasilense* in three tomato genotypes. The Micro-Tom genotype without mutation for IAA synthesis, *Entire*, has high sensitivity to IAA, and the *diageotropic* genotype (*dgt*) has low sensitivity to IAA. The results show that the plant parameter most sensitive to microbial inoculation is the number of roots. No treatment increased the shoot dry mass parameters for the Micro-Tom genotype and *dgt*, root dry mass for the Micro-Tom genotype, plant height for the Micro-Tom and *Entire* genotypes, root area and root volume for the genotype *dgt*. The Azm treatment reduced plant height compared to the control in the *dgt*, the BS + Azw and BS + Azm treatments in the Micro-Tom genotype and the Azw + Azm treatment in the *dgt* genotype reduced the plant diameter compared to the control. BS and BS + Azw reduced the number of roots in the Micro-Tom. The results strongly support that the mixture of *B. subtilis* and *A. brasilense* can reduce some parameters of plant development; however, this effect is possibly an interference in the mode of action of growth promotion of each isolate and is not related to excess of IAA produced by the bacteria.

Keywords: *Bacillus subtilis*; *Azospirillum brasilense*; tomato

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Introduction

Plant growth-promoting bacteria (PGPB) have several abilities that promote plant growth and development. These abilities may be the synthesis of phytohormones such as indole-3-acetic acid (IAA)[1], phosphorus solubilization[2], nitrogen fixation[3], production of siderophores[4] and induction of systemic resistance[5].

The bacteria capable of producing IAA belong to the numerous genera *Aeromonas, Azospirillum, Enterobacter, Erwinia, Pantoea, Pseudomonas, Flavobacterium, Shortbacterium, Microbacterium, Bacillus, Bradyrhizobium, Streptomyces, Azotobacter, Klebsiella, Alcaligenes, Brevebacteria, Rhodocystia, Rhodocyst* and *Dickeya*. These bacteria live as free-living microorganisms in the soil or as endophytes, colonizing plant tissue[1,6].

Most endophytic bacteria are capable of producing IAA. This phytohormone plays an important role as a molecule of interaction between the plant and the microorganism, in addition to improving the survival of bacteria[7,8]. IAA increases the survival rate of bacteria in situations of environmental stress, such as excessive ultraviolet rays, salinity, acidity, and high and low temperatures. IAA also plays an important role in the chemotaxis of bacteria, a movement used for the bacteria to reach the plant roots[9,10].

PGPB can be inoculated into agricultural crops individually or as a mixture of microorganisms. Both forms bring benefits and losses. Sometimes the failure in the establishment of microorganisms with plant growth promotion abilities occurs due to the noninteraction between the microorganism and the host plant due to the lack of connection between the cell membrane receptors of the microorganisms and the host

cells [11,12]. In this sense, products containing more than one microorganism in their formulations would increase the chance of at least one microorganism of the formulation interacting with the host plant. On the other hand, more studies are needed to answer this question. Is it possible that products based on mixtures of various microorganisms, many of them phytohormone producers, do not hinder the growth and development of plants due to the excess of indole-3-acetic acid?[13].

IAA is a phytohormone and, like all phytohormones, is an organic molecule that, depending on the concentration, promotes or inhibits plant growth[14,15]. Some studies have shown the benefits and effects of promoting plant growth in the use of biological products based on mixtures of various microorganisms[16,17]. Other studies show that these mixtures of microorganisms can cause no effect or even damage in plant growth[13,18].

Objective

The objective of the present study was to verify whether inoculation with a mixture of *B. subtilis* and *A. brasilense*, both indole-3-acetic acid-synthesizing bacteria, impairs the growth and development of tomato plants of three different genotypes.

Materials and Methods

Bacterial Isolates

The following bacterial isolates were used: *Bacillus subtilis* (GenBank deposit number) MZ133755; *Azospirillum brasilense* wild - MZ133758; *Azospirillum brasilense* mutant-MZ133759, belonging to the collection of the Laboratory of Soil Microbiology, FCAV-UNESP, campus of Jaboticabal. For experimental use, bacteria were routinely streaked on plates containing NA (nutrient agar) culture medium.

Tomato Genotypes

Tomato seeds (*Solanum lycopersicum* L) with the Micro-Tom variety and its *dgt* (low sensitivity to *auxin/defective* gene for cyclophilin biosynthesis and genotype *Entire* (with high auxin biosynthesis) mutant belong to the Laboratory of Plant Physiology, Department of Biology Applied to Agriculture at FCAV-UNESP, campus of Jaboticabal. Seeds of MT, *dgt* and *Entire* genotypes were multiplied to maintain the laboratory genotype bank.

In the *Entire* tomato genotype (*Solanum lycopersicum* L), locus (e) controls leaf morphology, and the dominant (e) recessive allele and allele of the locus produce pinnate compounds and complex reduced leaves. The leaf morphology of these transgenic lines was similar to the leaf morphology of the *Entire* tomato mutant[19].

In the *diageotropic* genotype (*dgt*), the mutant gene was *Solyc 01g111170,* and plants with the mutant allele had low sensitivity to auxin. The tomato *dgt* genotype has hyponastic leaves and plagiotropic roots with fewer branches and slender stems[20].

Greenhouse Experiment

New seeds of each tomato genotype were produced to be used in the experiment. For this purpose, in the greenhouse, seeds from the laboratory collection were germinated in trays containing a 1:1 mixture commercial substrate (Plantmax HT) Eucatex producer -Brazil with expanded vermiculite supplemented with 1 g L^{-1}; 10:10 NPK and 4 g L^{-1} commercial dolomitic limestone (Ca + Mg). After 15 days, under the same substrate conditions, plants were transferred to pots with a capacity of 1 L, remaining for 90 days to collect fruits and seeds used in the experiment. After that, the new seeds were used in the experiment as described above. Table 1 shows the description of the treatments in which the control treatment (without inoculation) consisted of *B. subtilis* (BS), *A. brasilense* wild (Azw) without transformation, *A. brasilense* mutant (Azm) and the mixtures *B. subtilis* + *A. brasilense* wild (BS + Azw), *B. subtilis* + mutant *A. brasilense* (BS + Azm), *B. subtilis* + exogenous auxin 400 µg mL^{-1} (BS + IAA Azw), *A. brasilense* + exogenous auxin (30 µg mL^{-1}) (Azw + IAA BS) and *A. brasilense* wild + *A. brasilense* mutant (Azw + Azm)

Table 1. Description of treatments in the greenhouse with tomato genotypes Micro-Tom, *diageotropic* (*dgt*) and *Entire*.

Description of Treatments
Control (no inoculation)
Bacillus subtilis (BS)
Azospirillum brasilense wild (Azw)
Azospirillum brasilense mutant (Azm)
B. subtilis + *A. brasilense* wild (BS + Azw)
B. subtilis + *A. brasilense* mutant (BS + Azm)
B. subtilis + IAA Azw (BS + IAA Azw)
A. brasilense + IAA BS (Azw + IAA BS)
A. Brasilense wild + *A. brasilense mutant* (Azw + Azm)

BS + IAA Azw means *B. subtilis* + 400 µg mL^{-1} exogenous auxin;
AZW + IAA BS means *A. brasilense* + 30 µg mL^{-1} exogenous auxin

Exogenous Auxin

The bacteria *B. subtilis*, wild-type *A. brasilense* and mutant *A. brasilense* were grown in culture medium for 24 hours at 28 °C. After bacterial growth, the media were filtered and centrifuged. In the centrifuged medium and in the supernatant, the concentrations of IAA were measured with the aid of HPLC. In the *B. subtilis* + IAA Azw mixture, Azw was replaced by the amount of IAA found in the extract extracted from the growth medium of this bacterium, approximately 400 µg mL $^{-1}$. The same was done in the Azw + IAA BS treatment, replacing the corresponding extract with 30 µg mL $^{-1}$ IAA for *B. subtilis*. These treatments were performed to measure the effect of IAA-producing bacteria and IAA alone.

In Vitro Auxin Biosynthesis

The in vitro indole-3-acetic acid production analysis was performed using HPLC. Each bacterial colony and mixture were applied to 20 mL of dextrose yeast glucose sucrose (DYGS) medium containing (in g L $^{-1}$): glucose, 2; peptone, 1.5; yeast extract, 2; potassium dihydrogen phosphate, 0.5; magnesium sulfate heptahydrate, 0.5[21], supplemented with 5 mM L-tryptophan and incubated for 48 h at 28 °C under constant stirring at 120 rpm in the absence of light. After growth, 5 mL of each culture was centrifuged at 10,000 rpm for 10 min. The samples (15 mL) were collected for centrifugation (Sorvall centrifuge at 16.266 × g for 30 min at 4 °C) and then concentrated under vacuum using a centrifugal evaporator to a volume of 1 mL.

Then, they were filtered through a cellulose ester filter (Millipore Corp.) with a pore size of 0.45 µm and injected in triplicate into an HPLC equipped with an RID detector (Shimadzu RID model 10A). The injected samples were eluted at 20 µL with a mobile phase of acetonitrile:water (75:25 v:v) under the following chromatographic conditions: 35 °C injection temperature, 20 µL injection volume, 1.0 mL/min flow. The culture medium at time zero was used as a negative control. Four concentrations (50, 25, 12.5, 6.25 µmol/L) were prepared for each indole compound standard indole-acetic acid, indole-pyruvic acid, indole-lactic acid, and indole-acetaldehyde for quantification and then analyzed by HPLC. A Shimadzu Class-VP chromatography data system was used for data acquisition and data analysis. Means and standard deviations were calculated, and SigmaPlot 11.0 was used to generate graphs.

Inoculations in Seeds and Plants

For inoculations, each bacterial isolate was cultured in a 125 mL Erlenmeyer flask containing 50 mL of nutrient broth (meat extract: 1.0 g L^{-1}, yeast extract: 2.0 g L^{-1}, peptone: 5.0 g L^{-1}, NaCl: 5.0 g L^{-1}, pH: 6.8 ± 0.2), and cultures were incubated at 28 °C for 24 h for standardized growth of 10^{8} colony-forming units (CFU) per mL. The seeds were sterilized prior to inoculation. The seeds were washed with water tap and deionized water and dried on absorbent towels. Then, 2 g of seeds was transferred aseptically to a sterile beaker, washed two times with sterile distilled water and sterilized using 0.2 g% HgCl2 for 30 s. Then, the seeds were washed six times with distilled water[22]. After sterilization, seeds were immersed in bacterial suspensions and stirred at 180 rpm and 28 °C for approximately 30 min. Control

treatments consisted of seeds immersed only in the culture medium, free from bacteria, under the same conditions. Throughout the experiment, four inoculations were carried out using 1 mL of each bacterial inoculum according to the treatments in the plants. The period between the inoculations was 15 days.

Azospirillum brasilense mutant

A. brasilense and mutant *A. brasilense* (IAA knockout) for auxin/IAA gene strains were used, which belong to the collection of microorganisms of the Laboratory of Soil Microbiology (LSM) - FCAV/UNESP, campus of Jaboticabal. Both *A. brasilense* strains were gently transformed by Dr. Carl Bauer from the Indian University in the United States.

The mutation method used for the transformation of mutant *A. brasilense* was conjugation, using the *E. coli* HB101 strain to donate the suicidal plasmid pGS9[23] carrying the Tn5 transposon kanamycin resistance genes for Sp. *F94*. Conjugation was performed using the filter membrane method, and the transformed isolates were selected on Luria Bertani agar supplemented with kanamycin and rifamycin both at a concentration of 40 µg mL $^{-1}$.

Experimental Design

The experiment was carried out in a completely randomized design with nine treatments for each tomato genotype and six replicates (Table 1).

The seeds were planted in each pot according to the treatment. The duration of the experiment was 60 days.

Plant height and diameter

After 60 days, the plants of each pot were collected, the plant height was measured with a ruler, and the plant diameter was measured with the aid of a caliper,

Root length, area and density

The roots were separated from the shoots, washed and kept in a test tube containing alcohol (30%). The roots were spread in a layer of water in a transparent tray (30 cm x 20 cm), and images were captured at 400 dpi with a professional Epson Expression 11000XL scanner system. The images were analyzed for root length, root area, root volume and number of roots using WinRHIZO ^{TM} Arabidopsis software (Reagent Instruments Inc).

Shoot and root dry mass

To obtain dry mass, the material was kept in a paper bag and placed in a forced air oven at 55 °C for 72 hours. Subsequently, the root dry mass and shoot dry mass were obtained using an analytical scale (Denver Instrument Company AA-200) with an accuracy of 0.0001 g.

Results

The amounts of indole compounds measured in the supernatant and in the concentrated extract by HPLC were 76.34 µg IAA mL $^{-1}$ and 416.41 µg IAA mL $^{-1}$ for Azw, 8.41 µg IAA mL $^{-1}$ and 140.89 µg IAA mL $^{-1}$ for Azm, and 4.86 and 30.56 µg IAA mL $^{-1}$ for Azw, respectively.

Micro-Tom Genotype

For the Micro-Tom tomato genotype, there was no significant difference ($p > 0.05$) in the shoot dry mass parameter between treatments (Fig. 1A). There was also no difference between the treatments and the control ($p > 0.05$) for the parameters plant height, root dry mass and root volume (Fig. 1B, 1D and 1G). For the plant diameter, the lowest values were found in the BS + Azm and BS + AZm treatments ($p < 0.05$) compared to the control, and there was no difference between the other treatments ($p > 0.05$) (Fig. 1C). For the root length parameter, the highest values were found in the Azm and Azw + IAA BS treatments ($p < 0.05$), and the other treatments did not differ from the control ($p > 0.05$) (Fig. 1E). For the root area parameter, the highest value was found for the Azm treatment ($p < 0.05$). All other treatments did not differ from the control ($p > 0.05$) (Fig. 1F). For root volume, there was no significant difference between the treatments and the control (Fig. 1G). For the parameter number of roots, the BS treatment had the lowest value compared to the control, and the only treatment that showed the highest value compared to the control and the other treatments was Azw + IAA BS (Fig. 1H).

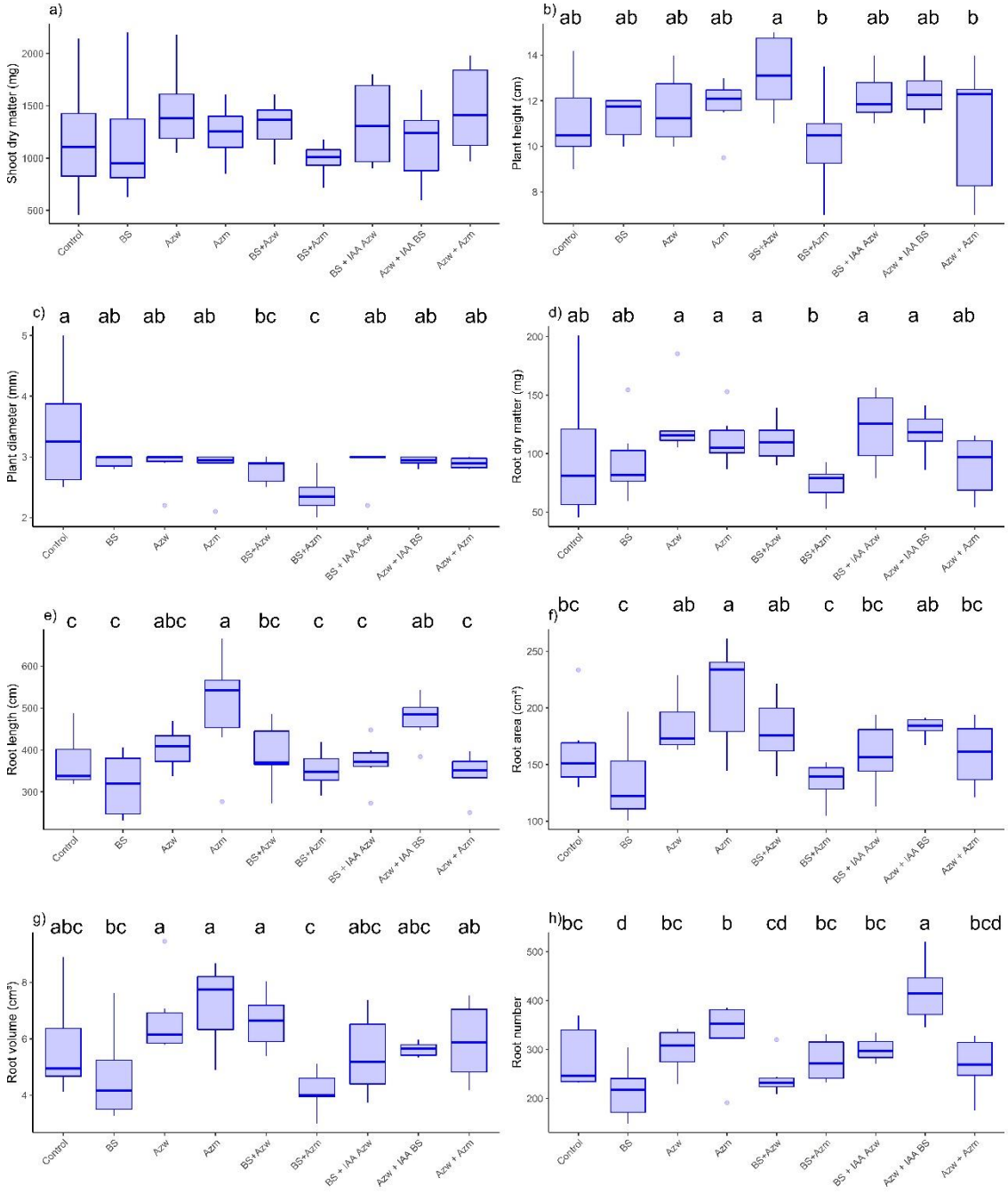

Figure 1. Boxplots for growth evaluation of Micro-Tom tomato plants inoculated with auxin-synthesizing bacteria. a) Shoot dry mass, (b) plant height, (c) plant diameter, (d) root dry matter, (e) root length, (f) root area, (g) root volume, (h)) number of roots. Means followed by the same lowercase letter or absence of letters do not differ by Tukey's test ($p < 0.05$). Control, without inoculation; BS, *B. subtilis;* Azw*, A. brasilense* wild*;* Azm*, A. brasilense* mutant**;** BS + Azw*, B. subtilis* + *A. brasilense* wild; BS + Azm, *B. subtilis* + *A. brasilense* mutant; BS + IAA Azw, *B. subtilis* + exogenous auxin (400 µg mL $^{-1}$); Azw + IAA BS, *A. brasilense* + exogenous auxin (30 µg mL $^{-1}$); Azw + Azm*, A. brasilense* wild + *A. brasilense* mutant.

In the principal component analysis, the Azw + IAA BS treatments had a positive correlation with the parameters root number, root length, root area and root volume. The Azw, BS + IAA Azw, BS + IAA Azw, BS + Azw, and Azw + Azm treatments had a positive correlation with the parameters plant height and root diameter (Fig. 2).

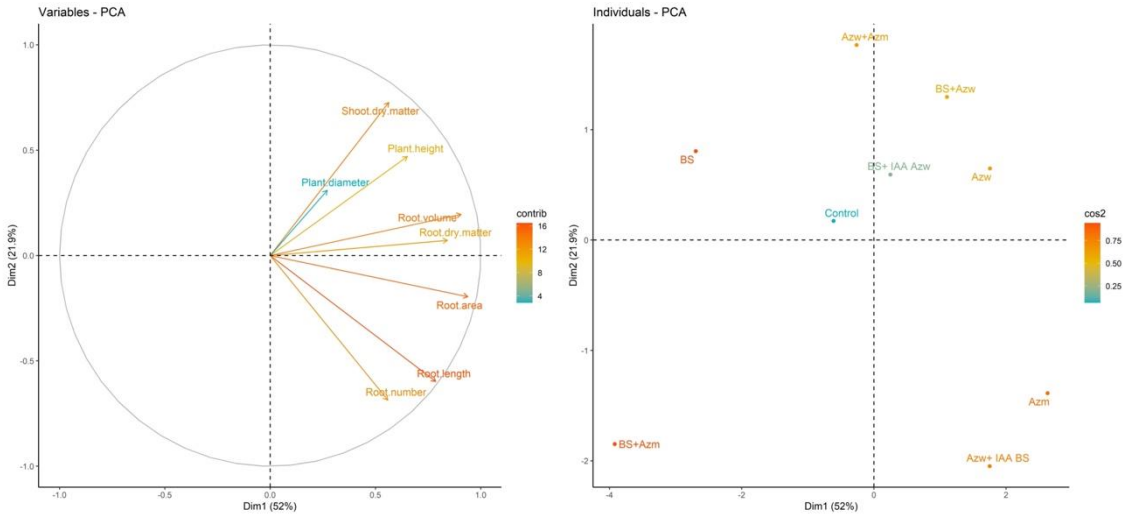

Figure 2. Main component analysis (PCA) for variables and treatments (individuals). Values on Axes 1 and 2 represent the percentage of total variance explained by axes for the Micro-Tom genotype.

The analysis of the hierarchical component shows the formation of eight groups, with the treatments Azw, BS + IAA Azw, Azw + Azm and BS + Azw being the closest and BS + Azm being the most distinct (Fig. 3).

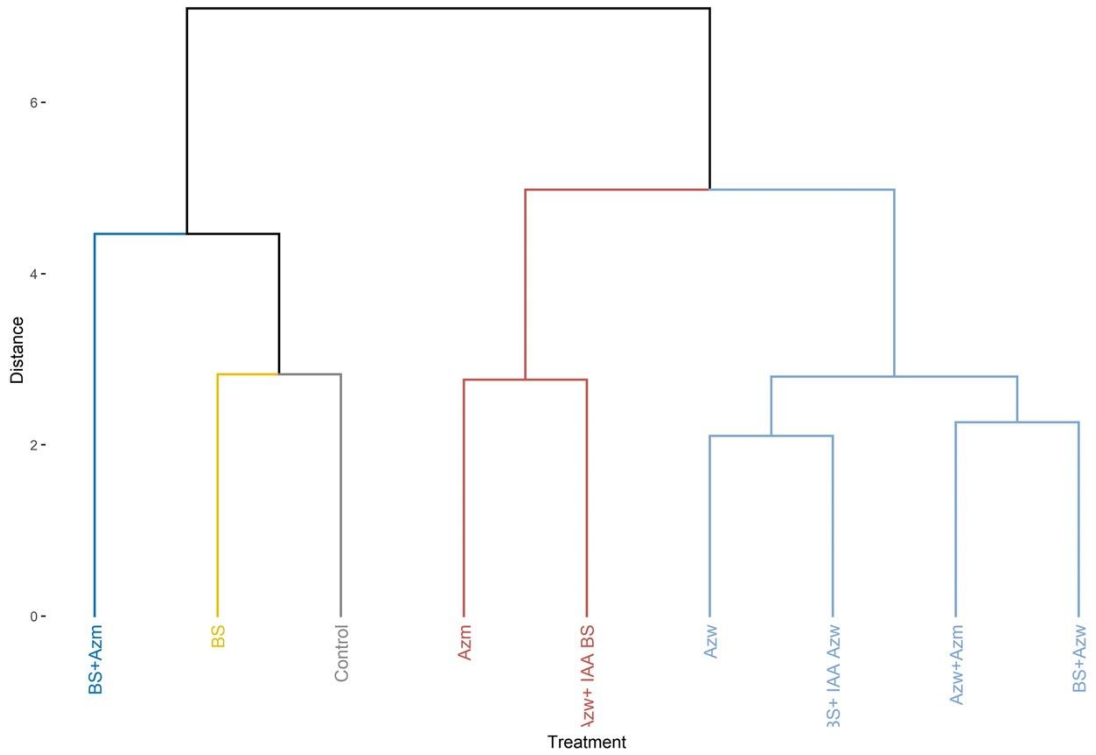

Figure 3. Hierarchical clustering of principal components (HCPCs) of inoculations tested.

Entire Genotype

For the *Entire* tomato *genotype* for the shoot dry mass parameter, the highest values were found for the Azw + IAA BS and Azw + Azm treatments ($p < 0.05$). The other treatments did not differ from the control or from each other ($p > 0.05$) (Fig. 4A). For the plant height parameter, there was no significant difference between the treatments and the control (Fig. 4B). For the plant diameter, the highest values were found in the Azw, BS + Azw, Azw + IAA BS and Azw + Azm treatments ($p < 0.05$). The other treatments did not differ from the control. (Fig. 4C). For the root dry weight parameter, the highest and only value that differed from the control and from the

other treatments was Azw + IAA BS ($p < 0.05$). The other treatments did not differ from each other ($p > 0.05$) (Fig. 4D). For the root length parameter, the highest values were found in the BS + IAA Azw, Azw + IAA BS and Azw + Azm treatments ($p < 0.05$). There was no significant difference between the other treatments ($p > 0.05$) (Fig. 4E). For the root area parameter, the highest values were found in the BS + IAA Azw, Azw + IAA BS and Azw + Azm treatments (Fig. 4F). For the root volume parameter, the highest values were found in the Azw + IAA BS and Azw + Azm treatments. The other treatments did not differ from each other ($p > 0.05$) (Fig. 4G). For the parameter root number, the treatments that did not differ from the control were only BS and BS +Azm ($p < 0.05$), and the other treatments showed higher values than the control ($p < 0.05$) (Fig. 4H).

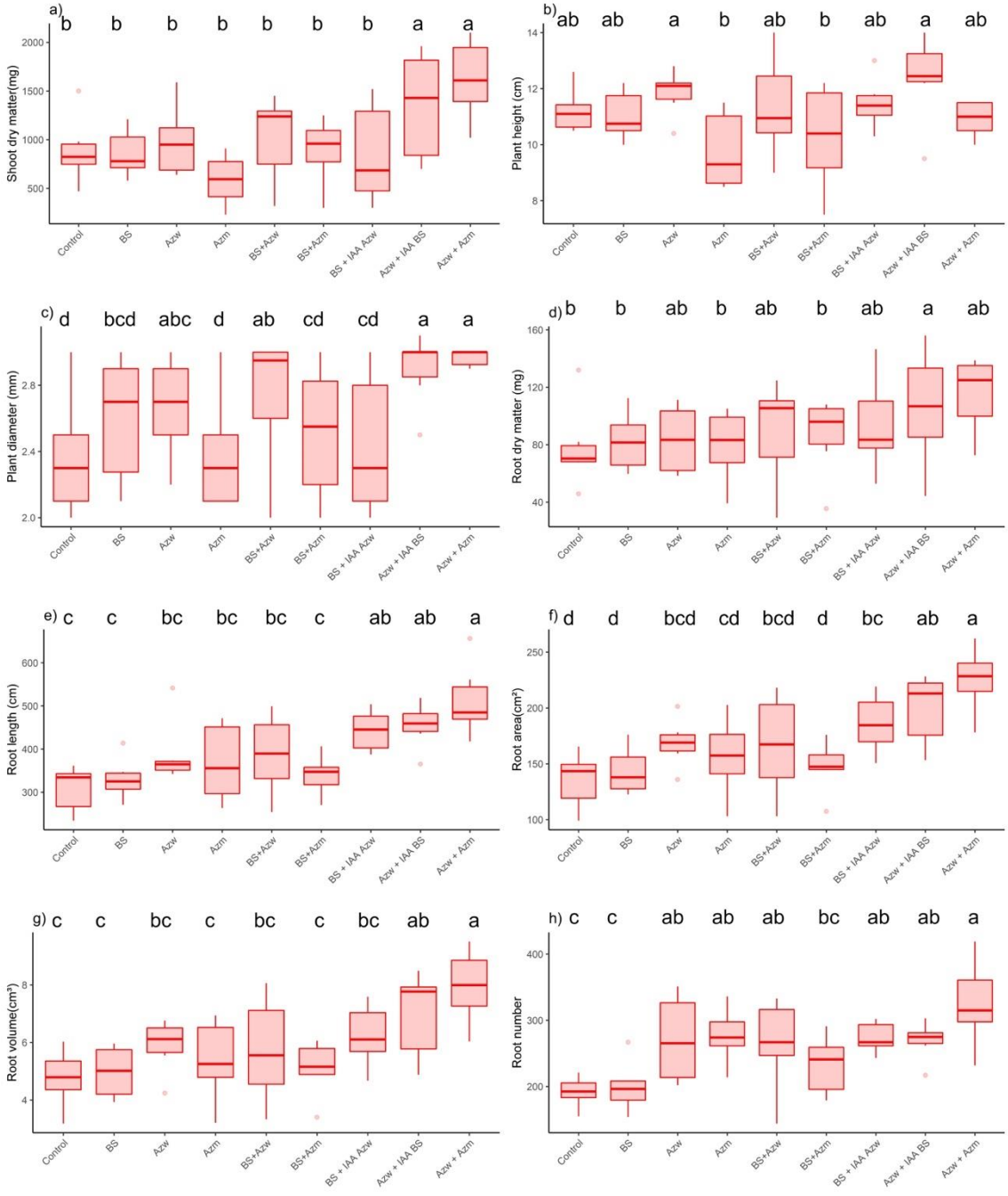

Figure 4. Boxplots of the *Entire* tomato plant growth evaluations inoculated with auxin-synthesizing bacteria. a) Shoot dry mass, (b) plant height, (c) plant diameter, (d) root dry matter, (e) root length, (f) root area, (g) root volume, (h)) number of roots. Means followed by the same lowercase letter or absence of letters do not differ by Tukey's test ($p < 0.05$). Control, without inoculation; BS, *B. subtilis;* Azw*, A. brasilense* wild*;* Azm*, A. brasilense* mutant**;** BS + Azw*, B. subtilis* + *A. brasilense* wild; BS + Azm, *B. subtilis* + *A. brasilense* mutant; BS + IAA Azw, *B. subtilis* + exogenous auxin (400 µg mL $^{-1}$); Azw + IAA BS, *A. brasilense* + exogenous auxin (30 µg mL $^{-1}$); Azw + Azm*, A. brasilense* wild + *A. brasilense* mutant.

In the principal component analysis, the Azw + IAA, Azw and BS + Azw treatments had a positive correlation with the parameters plant height, root dry mass, plant diameter and shoot dry mass. The BS + IAA, Azw, and Azw + Azm treatments had a positive correlation with the parameters root volume, root area, root length and number of roots (Fig. 5).

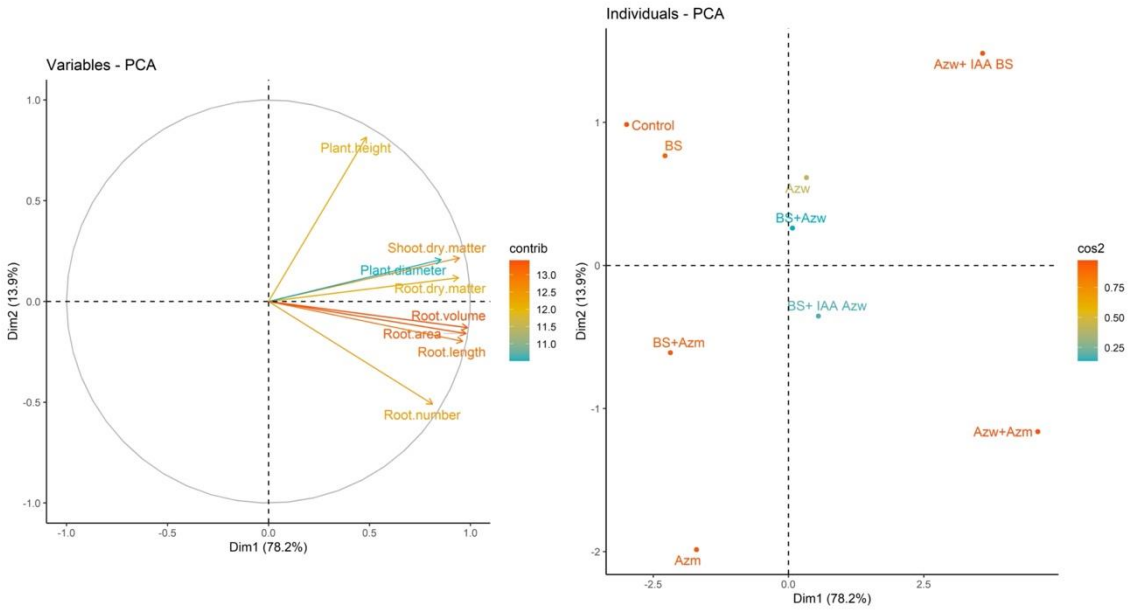

Figure 5. Main component analysis (PCA) for variables and treatments (individuals). Values on axes 1 and 2 represent the percentage of total variance explained by axes for the *Entire genotype*.

In the hierarchical clustering analysis, the Azw + IAA BS and Azw + Azm treatments were the most distinct compared to the other treatments (Fig. 6).

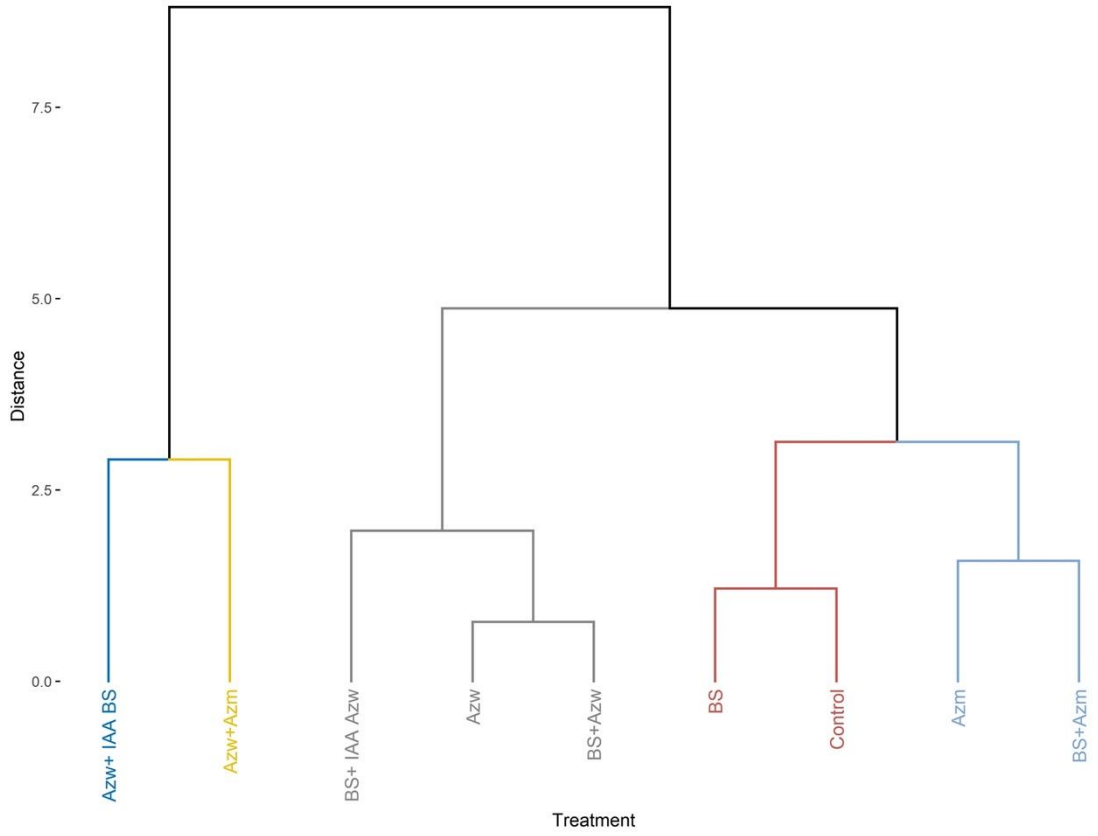

Figure 6. Hierarchical clustering of principal components (*HCPCs*) of inoculations tested for the *Entire* genotype.

Genotype dgt

There was no difference in the *diageotropic* genotype (*dgt*) for the shoot dry mass parameter (p> 0.05) between the control treatment and the other treatments (Fig. 7A). For the plant height parameter, the highest value was found in the BS + IAA Azw treatment (p <0.05), and the lowest value and only treatment that differed from the control was Azm (p <0.05). There was no significant difference (p> 0.05) between the other treatments (Fig. 7B). For the parameter plant diameter, the lowest value and the only treatment that differed from the control was Azw + Azm (Fig. 7C).

For the root dry mass parameter, the highest value and the only parameter that differed from the control was BS + IAA Azw ($p < 0.05$). There was no difference between the other treatments (Fig. 7D). For the root length parameter, the highest values were found in the Azw and BS + IAA Azw treatments compared to the control ($p < 0.05$). There was no significant difference ($p > 0.05$) between the other treatments (Fig. 7E). For the root area parameter, there was no difference between the treatments and the control (Fig. 7F). For the root volume parameter, there was no difference between the control and the other treatments ($p < 0.05$) (Fig. 7G). For the parameter number of roots, the highest values were found in the Azw and Azw + Azm treatments. There was no difference between the other treatments and the control (Fig. 7H).

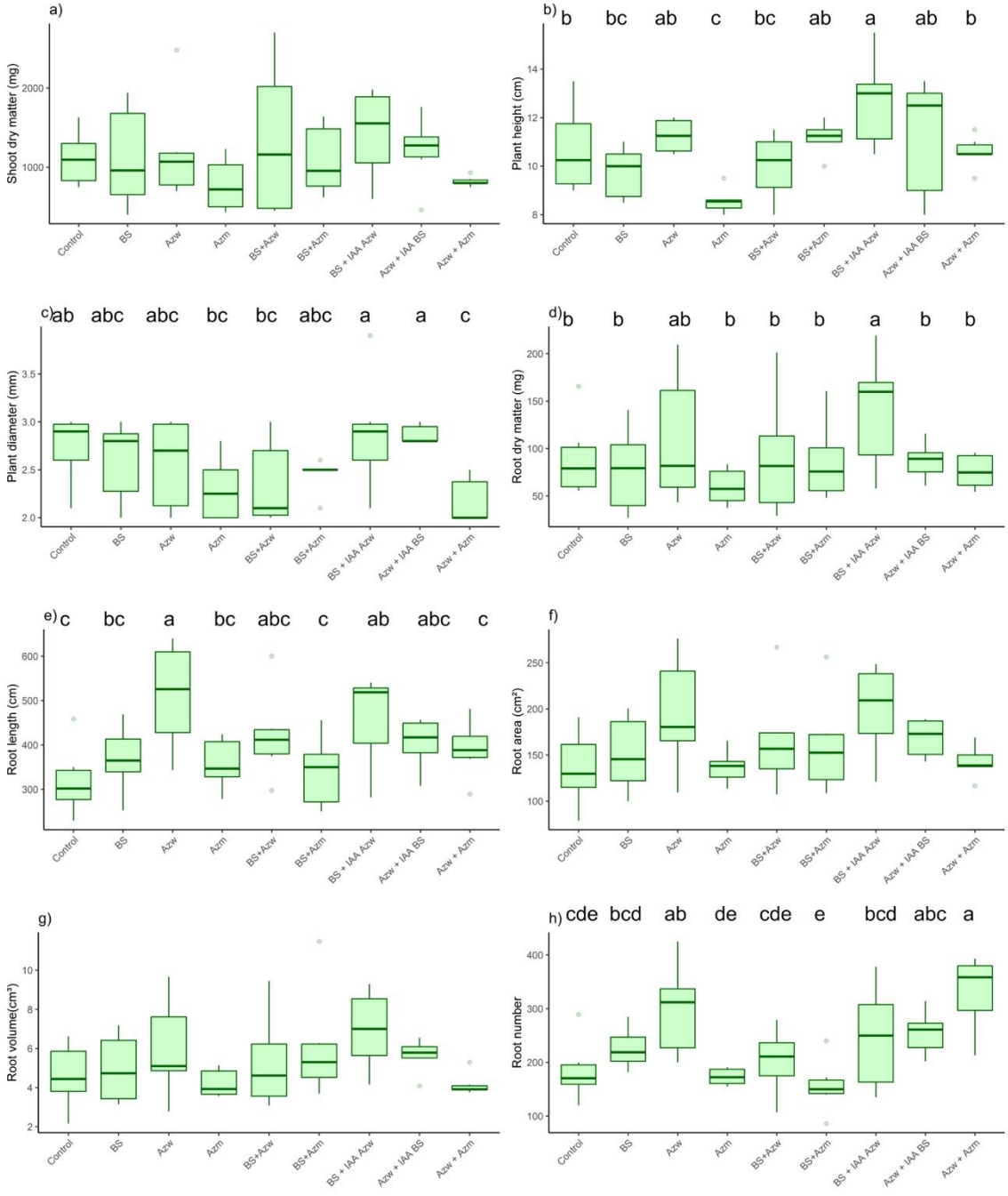

Figure 7. Boxplots for growth evaluation of *dgt* tomato plants inoculated with auxin-synthesizing bacteria. a) Shoot dry mass, (b) plant height, (c) plant diameter, (d) root dry matter, (e) root length, (f) root area, (g) root volume, (h) number of roots. Means followed by the same lowercase letter or absence of letters do not differ by Tukey's test ($p < 0.05$). Control, without inoculation; BS, *B. subtilis;* Azw*, A. brasilense* wild*;* Azm*, A. brasilense* mutant**;** BS + Azw*, B. subtilis* + *A. brasilense* wild; BS + Azm, *B. subtilis* + *A. brasilense* mutant; BS + IAA Azw, *B. subtilis* + exogenous auxin (400 µg mL^{-1}); Azw + IAA BS, *A. brasilense* + exogenous auxin (30 µg mL^{-1}); Azw + Azm*, A. brasilense* wild + *A. brasilense* mutant.

The principal component analysis shows that the Azw treatment had a positive correlation with the parameters number of roots, root length, and root area and that the Azw + IAA BS and BS + IAA Azw treatments had a positive correlation with the parameter plant height.), root dry mass, root volume, shoot dry mass and plant diameter (Fig. 8).

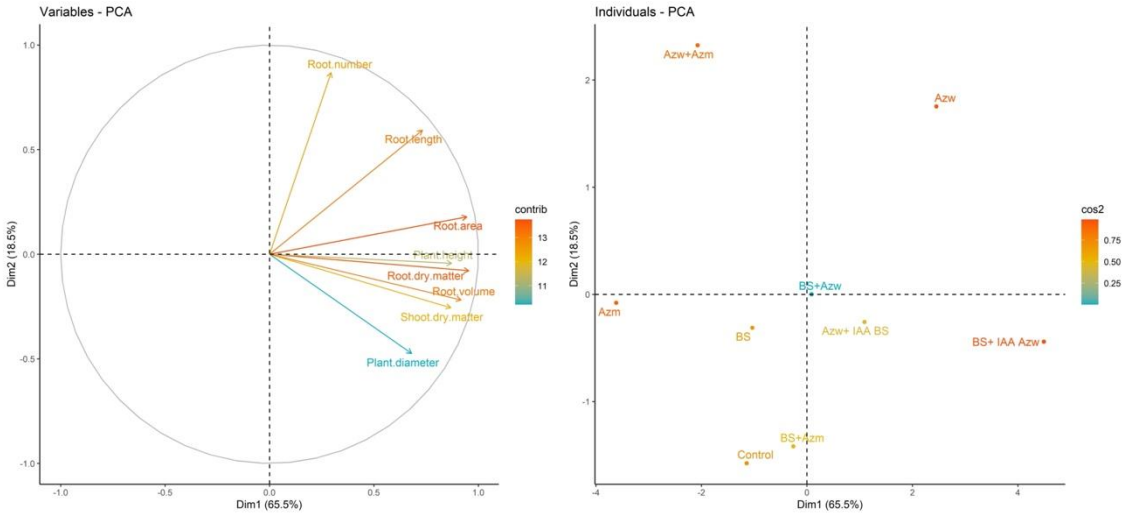

Figure 8. Main component analysis (PCA) for variables and treatments (individuals). Values on Axes 1 and 2 represent the percentage of total variance explained by axes for the *dgt* genotype.

In the hierarchical cluster analysis, the closest groups were Azm and Azw + Azm. The next groups were BS + Azm, BS + Azw, Azw + IAA BS, BS and control. The groups most distinct from the others were Azw and BS + IAA Azw (Fig. 9).

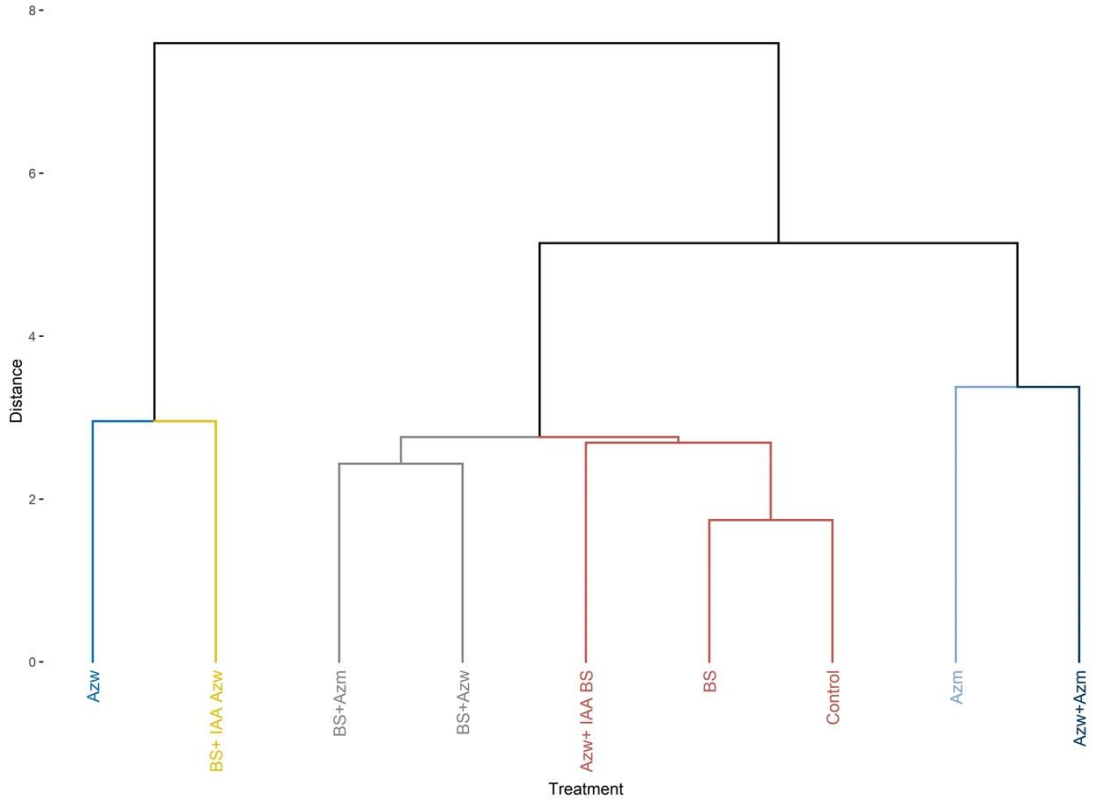

Figure 9. Hierarchical clustering of principal components (HCPCs) of inoculations tested.

Discussion

The inoculations with the bacteria *B. subtilis*, *A. brasilense* wild and *A. brasilense* mutant promoted increases in most of the measured plant parameters compared to the control treatment that did not receive inoculation. However, it is important to note that the inoculation of PGPB synthesizing IAA did not differ from the control treatment for the parameters shoot dry mass in the Micro-Tom and *dgt* genotypes, root dry mass for the Micro-Tom genotype, plant height for the genotypes Micro-Tom and *Entire*, root area for the *dgt* genotype and root volume for the Micro-Tom and *dgt* genotypes. The inoculation of the mixtures of these bacteria also promoted the reduction of the development of some parameters, such as the

reduction in plant diameter in the BS +Azw and BS +Azm treatments for Micro-Tom and Azw +Azm in the *dgt* genotype, and there was a reduction in the height of the inoculated plant with Azm and BS reduced the number of roots in the Micro-Tom genotype (Table 2).

Table 2. Effects of inoculation of the various treatments that differed from the control treatment in relation to the plant parameters analyzed in the tomato genotypes Micro-Tom, *Entire* and *dgt*.

Plant Parameter	Genotype	Effect	Treatments
Shoot Dry Mass	Micro-Tom	No differences	- -
	Entire	Increased	Azw + IAA BS Azw + Azm
	dgt	No differences	- -
Root Dry Mass	Micro-Tom	No differences	- -
	Entire	Increased	Azw + IAA BS
	dgt	Increased	BS + IAA Azw
Plant Height	Micro-Tom	No differences	- -
	Entire	No differences	- -
	dgt	Reduced	Azm
		Increased	BS + IAA Azw
Plant Diameter	Micro-Tom	Reduced	BS + Azw BS + Azm
	Entire	Increased	Azw BS + Azw Azw + IAA BS Azw + Azm
	dgt	Reduced	Azw + Azm

Root Length	Micro-Tom	Increased	Azm Azw + IAA BS
	Entire	Increased	BS + IAA Azw Azw + IAA BS Azw + Azm
	dgt	Increased	Azw BS + IAA Azw
Root Area	Micro-Tom	Increased	Azm
	Entire	Increased	BS + IAA Azw Azw + IAA BS Azw + Azm
	dgt	No differences	- -
Root Volume	Micro-Tom	No differences	- -
	Entire	Increased	Azw + IAA BS Azw + Azm
	dgt	No differences	- -
Number of Roots	Micro-Tom	Reduced	BS
		Increased	Azw + IAA BS
	Entire	Increased	Azw Azm BS + Azw BS + IAA Azw Azw + IAA BS Azw + Azm
	dgt	Increased	Azw Azw + Azm

The main effect of inoculation of IAA-producing bacteria in plants is usually an increase in root development and, as a consequence, an increase in the efficiency of obtaining water, nutrients, and soil volume exploitation, thus allowing a reduction in the dose of fertilizers [24,25]. However, some studies have shown that inoculation of a mixture of IAA-producing microbial isolates may also not effectively respond to the replacement of an agricultural input because it does not promote or even decrease plant development. Felici [18] used a mixture of *B. subtilis* and *A. brasilense* bacteria in tomato plants and found that the combination of the two bacteria did not improve plant growth compared to inoculation of the bacteria separately. These authors support the hypothesis that these two bacteria may operate differently in the modulation of root growth. In seedlings inoculated with the mixture of *B. subtilis* and *A. brasilense*, a strong inhibition of primary root elongation was observed. This inhibitory effect also induced a process of resistance to morphogenesis[26,27]. Therefore,[18] concluded that the presence of the two microorganisms can directly or indirectly alter the internal hormonal content of the root, interfering with its normal morphogenesis. Similar results were found by[13]. These studies suggest that the reduction of some plant parameters promoted by the mixture of these bacteria is not related to the excess of IAA produced by the bacteria but by an interference in the mode of action of the isolates.

The synthesis of IAA is not only used for plant growth and development, but low concentrations of IAA produced by bacteria can interfere with the response of certain microorganisms; for example, they can regulate the formation of semimycelial structures and antimicrobial activity in some strains of bacterium *Streptomyces*. Thus, the secretion of IAA in the rhizosphere produced by bacteria and plants is a signal for

Streptomyces spp. to improve their antimicrobial production activity, improving their efficiency in establishing their colonization niche against other microorganisms[28,29].

The results of the present study reinforce the suggestions of previous studies and show that although the mixture of bacteria has reduced the plant development of some parameters, this effect is not related to the synthesis of IAA. For example, the BS + Azw and BS + Azm mixtures reduced the plant diameter in the Micro-Tom genotype, and the BS + Azm mixture produced almost three times less IAA than BS + Azw (Fig. 1E). On the other hand, for the root dry mass parameter, there was no difference for the same treatments BS + Azw (30.56 µg IAA mL^{-1} + 416.41 µg IAA mL^{-1}) and BS + Azm (30.56 µg IAA mL^{-1} + 140.89 µg IAA mL^{-1}), even though the latter produced less IAA (Fig. 1D). These results show that the concentration of IAA is not the only determining factor but that each plant parameter responds differently to inoculations. Another interesting result occurred in the *Entire* genotype (which is sensitive to IAA) for the shoot dry mass parameter. There was no difference between the BS + Azw treatments that would theoretically produce (30.56 µg IAA mL^{-1} + 416.41 µg IAA mL^{-1}**)** or BS + Azm (30.56 µg IAA mL^{-1} + 140.89 µg IAA mL^{-1}), and there was a difference between BS + IAA Azw (30.56 µg IAA mL^{-1} + 400.00 µg IAA mL^{-1}) and Azw + IAA BS (416.41 µg IAA mL^{-1} **+** 30 µg IAA mL^{-1}**)** (Fig. 4A). These treatments have the ability to produce the same amount of IAA in the plant or less, and even so, there was no difference in a sensitive genotype, which is the *Entire* genotype. The application of plant growth-promoting microorganisms, including IAA-producing bacteria, is an excellent alternative to address the challenges of global agriculture due to the possibility of reducing production costs, doses of mineral

fertilizers and environmental impacts [30,31]. However, in some situations, these microorganisms may fail for this purpose. This failure occurs due to the failure of the plant growth-promoting microorganism to colonize the rhizosphere and subsequently the plant tissues to express the effect growth promoter. In this sense, the use of formulations containing more than one microorganism would increase the chances of successful colonization [12]. However, it is necessary to better understand whether these mixtures or combinations of different IAA-producing microorganisms do not reduce plant development due to excess IAA. The results of the present study show that this reduction may occur but is not related to IAA but is most likely the interference of each microorganism in the mode of action and in the growth promotion effect.

Conclusion

The inoculation of the mixture of *B. subtilis* and *A. brasilense* bacteria can reduce some plant parameters, such as plant height and stem diameter and the number of roots; however, this negative effect is not related to the excess of IAA produced by the bacteria but is probably an interference of each bacterium in the mode of interaction and in the growth promotion effect with the plant.

References

1. Poveda, J.; González-Andrés, F. *Bacillus* as a source of phytohormones for use in agriculture. *Appl. Microbiol. Biotechnol.* **2021**, *105*, 8629-8645.

2. Huang, Y.; Dai, Z.; Lin, J.; Li, D.; Ye, H.; Dahlgren, R.A.; Xu, J. Labile carbon facilitated phosphorus solubilization as regulated by bacterial and fungal communities in Zea mays. *Soil Biology and Biochemistry* **2021**, *163*, 108465.

3. Abadi, V.; Sepehri, M.; Rahmani, H.; Dolatabad, H.; Shamshiripour, M.; Khatabi, B. Diversity and abundance of culturable nitrogen-fixing bacteria in the phyllosphere of maize. *Journal of Applied Microbiology* **2021**, *131*, 898-912.

4. Soares, E.V. Perspective on the biotechnological production of bacterial siderophores and their use. *Appl. Microbiol. Biotechnol.* **2022**, 1-20.

5. Li, H.; La, S.; Zhang, X.; Gao, L.; Tian, Y. Salt-induced recruitment of specific root-associated bacterial consortium capable of enhancing plant adaptability to salt stress. *The ISME Journal* **2021**, *15*, 2865-2882.

6. Costacurta, A.; Vanderleyden, J. Synthesis of phytohormones by plant-associated bacteria. *Critical reviews in microbiology* **1995**, *21*, 1-18.

7. Kim, Y.C.; Leveau, J.; McSpadden Gardener, B.B.; Pierson, E.A.; Pierson III, L.S.; Ryu, C.-M. The multifactorial basis for plant health promotion by plant-associated bacteria. *Applied and Environmental Microbiology* **2011**, *77*, 1548-1555.

8. Vieira, R.F.; Ferracini, V.L.; Parada Dias da Silveira, A.; Almeida Pazianotto, R.A. Improvement of growth of common bean in phosphorus-deficient soils by phosphate-solubilizing and phytohormone-producing bacteria. *Agronomía Colombiana* **2021**, *39*, 372-380.

9. Duca, D.; Lorv, J.; Patten, C.L.; Rose, D.; Glick, B.R. Indole-3-acetic acid in plant–microbe interactions. *Antonie Van Leeuwenhoek* **2014**, *106*, 85-125.

10. Gamit, H.; Amaresan, N. Role of methylotrophic bacteria in managing abiotic stress for enhancing agricultural production: A review. *Pedosphere* **2022**.

11. Tosi, M.; Gaiero, J.; Linton, N.; Mafa-Attoye, T.; Castillo, A.; Dunfield, K. Bacterial endophytes: diversity, functional importance, and potential for manipulation. In *Rhizosphere Biology: Interactions Between Microbes and Plants*; Springer: 2021; pp. 1-49.

12. Mitter, E.K.; Tosi, M.; Obregón, D.; Dunfield, K.E.; Germida, J.J. Rethinking crop nutrition in times of modern microbiology: innovative biofertilizer technologies. *Front. Sustain. Food Syst.* **2021**, *5*, 29.

13. Lobo, L.L.B.; da Silva, M.S.R.d.A.; Carvalho, R.F.; Rigobelo, E.C. The negative effect of coinoculation of plant growth-promoting bacteria is not related to indole-3-acetic acid synthesis. *J. Plant Growth Regul.* **2022**, 1-10.

14. Fahad, S.; Hussain, S.; Bano, A.; Saud, S.; Hassan, S.; Shan, D.; Khan, F.A.; Khan, F.; Chen, Y.; Wu, C. Potential role of phytohormones and plant growth-promoting rhizobacteria in abiotic stresses: consequences for changing environment. *Environmental Science and Pollution Research* **2015**, *22*, 4907-4921.

15. Mishra, P.; Mishra, J.; Arora, N.K. Plant growth promoting bacteria for combating salinity stress in plants–recent developments and prospects: a review. *Microbiol. Res.* **2021**, *252*, 126861.

16. Kaur, N.; Sharma, P.; Sharma, S. Coinoculation of *Mesorhizobium* sp. and plant growth promoting rhizobacteria Pseudomonas sp. as bioenhancer and biofertilizer in chickpea (Cicer arietinum L.). *Legume Research-An International Journal* **2015**, *38*, 367-374.

17. Korir, H.; Mungai, N.W.; Thuita, M.; Hamba, Y.; Masso, C. Coinoculation effect of rhizobia and plant growth promoting rhizobacteria on common bean growth in a low phosphorus soil. *Frontiers in plant science* **2017**, *8*, 141.

18. Felici, C.; Vettori, L.; Giraldi, E.; Forino, L.M.C.; Toffanin, A.; Tagliasacchi, A.M.; Nuti, M. Single and coinoculation of *Bacillus subtilis* and *Azospirillum brasilense* on Lycopersicon esculentum: Effects on plant growth and rhizosphere microbial community. *Applied Soil Ecology* **2008**, *40*, 260-270, doi:10.1016/j.apsoil.2008.05.002.

19. Zhang, J.; Chen, R.; Xiao, J.; Qian, C.; Wang, T.; Li, H.; Ouyang, B.; Ye, Z. A single-base deletion mutation in SlIAA9 gene causes tomato (*Solanum lycopersicum*) entire mutant. *Journal of plant research* **2007**, *120*, 671-678.

20. Albert, M.; Werner, M.; Proksch, P.; Fry, S.; Kaldenhoff, R. The cell wall-modifying xyloglucan endotransglycosylase/hydrolase LeXTH1 is expressed during the defense reaction of tomato against the plant parasite Cuscuta reflexa. *Plant Biology* **2004**, *6*, 402-407.

21. Ruckdaschel, E.; Klingmuller, W. Analysis of IAA biosynthesis in *Azospirillum lipoferum* and Tn5 induced IAA mutants. *Symbiosis* **1992**.

22. Ladha, J.; Barraquio, W.; Revilla, L. Isolation of endophytic diazotrophic bacteria from wetland rice. In *Opportunities for Biological Nitrogen Fixation in Rice and Other Non-Legumes*; Springer: 1997; pp. 15-24.

23. Hartmann, A.; Singh, M.; Klingmüller, W. Isolation and characterization of *Azospirillum mutants* excreting high amounts of indoleacetic acid. *Canadian Journal of Microbiology* **1983**, *29*, 916-923.

24. Nabrdalik, M.; Moliszewska, E.; Wierzba, S. Importance of endophytic strains P*antoea agglomerans* in the biological control of *Rhizoctonia solani*. *Ecol Chem Eng S* **2018**, *25*, 331-342.

25. Safara, S.; Harighi, B.; Bahramnejad, B.; Ahmadi, S. Antibacterial activity of endophytic bacteria against sugar beet root rot agent by volatile organic compound production and induction of systemic resistance. *Frontiers in Microbiology* **2022**, *13*.

26. Potters, G.; Pasternak, T.P.; Guisez, Y.; Palme, K.J.; Jansen, M.A. Stress-induced morphogenic responses: growing out of trouble? *Trends in plant science* **2007**, *12*, 98-105.

27. Werner, T.s.; Motyka, V.; Laucou, V.; Smets, R.; Van Onckelen, H.; Schmülling, T. Cytokinin-deficient transgenic Arabidopsis plants show multiple developmental alterations indicating opposite functions of cytokinins in the regulation of shoot and root meristem activity. *The Plant Cell* **2003**, *15*, 2532-2550.

28. Matsukawa, E.; Nakagawa, Y.; Iimura, Y.; Hayakawa, M. Stimulatory effect of indole-3-acetic acid on aerial mycelium formation and antibiotic production in *Streptomyces* spp. *Actinomycetologica* **2007**, *21*, 32-39.

29. Kumari, S.; Naraian, R. Enhanced growth and yield of oyster mushroom by growth-promoting bacteria *Glutamicibacter arilaitensis* MRC119. *Journal of basic microbiology* **2021**, *61*, 45-54.

30. Sessitsch, A.; Pfaffenbichler, N.; Mitter, B. Microbiome applications from lab to field: facing complexity. *Trends in plant science* **2019**, *24*, 194-198.

31. Santos, R.M.d.; Desoignies, N.; Rigobelo, E.C. The bacterial world inside the plant. *Front. Sustain. Food Syst.* **2022**, 319.

CAPITULO 4 - Considerações finais

O presente estudo colabora para o melhor entendimento e uso da co-inoculação de *Bacillus* spp. e *A. brasilense* como bioinoculantes na produção vegetal, quando considerada a sua capacidade na sintese de AIA.

Sabendo que o efeito final das auxinas pode ocorrer de forma favorável ou prejudicial para as plantas, sendo este dependente do conteúdo de auxina na planta.

Nesse contexto, a alta biossíntese bacteriana poderia interferir negativamente no desenvolvimento vegetal.

Nossos resultados demonstraram que a co-inoculação bacteriana pode afetar negativamente alguns parâmetros do desenvolvimento vegetal. No entanto, ao utilizarmos modelos vegetais com alterações genéticas de alta biossíntese e de baixa sensibilidade para auxina, demonstramos que o efeito negativo causado não está relacionado a capacida de produção de AIA dos consorcios bacterianos utilizados.

Provavelmente este efeito negativo no desenvolvimento vegetal decorra por interferência de cada bactéria no modo de interação e no efeito de promoção de crescimento com a planta.

O resultado do presente estudo elucida de maneira prática respostas mais consistentes que possibilite a melhor forma do uso de BPCP produtoras de AIA.

Sendo assim, a síntese de AIA bacteriano ainda deve ser vista como uma característica positiva na busca por microrganismos promotores de crescimento de plantas.