INTERAÇÕES ECOLÓGICAS E POTENCIAL BIOFERTILIZANTE DE ACTINOBACTÉRIAS E BACTÉRIAS DIAZOTRÓFICAS NA AGRICULTURA

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CAMPOS DOS GOYTACAZES - RJ

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Esta tese é dedicada às pessoas que mais amo no mundo, minha família. Em especial, meu amado esposo Rony e minha mãe Juçara.

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“A menos que modifiquemos nossa maneira de pensar, não seremos capazes de resolver os problemas causados pela forma como nos acostumamos a ver o mundo”. (Albert Einstein)

SUMÁRIO

RESUMO GERAL

O gênero *Streptomyces* compreende actinobactérias Gram-positivas filamentosas reconhecidas pela capacidade de produzir compostos bioativos que podem influenciar o crescimento. Já as bactérias diazotrófica são procariotos Gram-negativos cujo potencial em induzir o crescimento vegetal encontra-se amplamente descrito na literatura. No entanto, o estudo da utilização conjunta destes grupos microbianos ainda é incipiente. A presente tese objetivou explorar características relevantes de actinobactérias do gênero *Streptomyces*, enfatizando sua capacidade em beneficiar o crescimento vegetal, bem como estudar a interação de isolados selecionados desse gênero com *Herbaspirillum seropedicae* estirpe HRC54, uma bactéria promotora do crescimento de plantas. No Capítulo 1, foi realizada uma revisão de literatura sobre o gênero, destacando aspectos relacionados a vias metabólicas secundárias, interações com plantas hospedeiras e os recentes avanços na elucidação de mecanismos de promoção do crescimento. No Capítulo 2 foi feita a caracterização fisiológica de estreptomicetos isolados de vermicompostos maturados, além da investigação por propriedades relacionadas à promoção de crescimento vegetal. A inoculação dos isolados ou seus metabólitos em plântulas de milho (*Zea mays* var. UENF 506-11) e tomate (Micro-Tom mutante *DR5::GUS*) foi avaliada. Incrementos significativos foram observados na massa fresca de raiz (27,8 a 77,2%) e parte aérea (20,0 a 58,2%), bem como na massa seca de raiz (26,9 a 111,11%) e parte aérea (53,7 a 119,9%) de plântulas de milho inoculadas com os isolados. Análises histoquímicas revelaram atividade auxínica de compostos metabólitos secretados pelo isolado AC05 em tomate cv. Micro-Tom mutante *DR5::GUS* . No Capítulo 3 a compatibilidade entre *H. seropedicae* estirpe HRC54 e quatro isolados de *Streptomyces* (AC01, AC05, AC06 e AC31) foi avaliada. Plântulas de tomate cv. Santa Clara foram inoculadas com interações compatíveis. A interação entre HRC54 e o isolado AC06 foi estudada quanto a ocorrência de cooperação metabólica para o amido. Por fim, a atividade auxínica de metabólitos secretados por HRC54 e/ou AC06 foi investigada. *H. seropedicae* estirpe HRC54 foi compatível com os isolados AC01, AC06 e AC31. Os isolados AC01, AC06 e a combinação de AC06 com HRC54 foram eficientes em promover o crescimento de plântulas de tomate. Compostos metabólicos produzidos por HRC54 e por AC06 desempenham atividade auxínica, induzindo a atividade da β-glucuronidase em tomate Micro-Tom DR5-GUS, além de estimular a indução de raízes laterais. Os resultados apontam para o potencial de combinações de estreptomicetos e bactérias diazotróficas na promoção de crescimento vegetal.

Palavras-chave: *Streptomyces* spp., *Herbaspirillum seropedicae*, interação microbiana, promoção de crescimento vegetal.

ABSTRACT

The genus *Streptomyces* comprises filamentous Gram-positive actinobacteria recognized for their ability to produce bioactive compounds, which may influence plant growth. Diazotrophic bacteria are Gram-negative prokaryotes whose potential inducing plant growth is widely described in literature. However, the study about combined use of these microbial groups is still incipient. This thesis is dedicated to explore relevant characteristics of *Streptomyces* actinobacteria, emphasizing their ability to benefit plant growth, as well as study the interaction of selected isolates with *Herbaspirillum seropedicae* strain HRC54, a plant growth-promoting bacteria. In Chapter 1, a literature on *Streptomyces* genus was conducted, focused on aspects related to secondary metabolic pathways, interactions with host plants and the recent advances in the understanding of growth-promoting mechanisms. In Chapter 2 streptomycetes isolated from matured vermicomposts was characterized about physiological and plant growth-promoting traits. Inoculation of isolates or its metabolites in maize plantlets (*Zea mays* var. UENF 506-11) and tomato (Micro-Tom mutant *DR5::GUS*) was evaluated. Significant increases were observed in fresh root mass (27.8 to 77.2%) and shoot (20.0 to 58.2%), as well as root dry weight (26.9 to 111.11%) and shoot (53.7 to 119.9%) of maize plantlets inoculated with the isolates. Histochemical analysis revealed auxin-like activity of metabolic compounds secreted by AC05 isolate in tomato cv. Micro-Tom mutant *DR5::GUS*. In Chapter 3, the compatibility between *H. seropedicae* strain HRC54 and four *Streptomyces* isolates (AC01, AC05, AC06 and AC31) was evaluated. Tomato plantlets cv. Santa Clara were inoculated with compatible interactions. The interaction between HRC54 and AC06 was studied searching for metabolic cooperation starch. Finally, auxin-like activity of secreted metabolites by HRC54 and/or AC06 was investigated. *H. seropedicae* strain HRC54 was compatible with AC01, AC06 and AC31 isolates. AC01, AC06 and AC06 combined with HRC54 were effective in improving tomato growth. Metabolic compounds produced by HRC54 and AC06 plays auxin-like activity, inducing β-glucuronidase activity in tomato Micro-Tom DR5-GUS and further stimulated induction of lateral roots. The results point to potential of combined use of streptomycetes and diazotrophic bacteria in plant growth promotion.

Keywords: *Streptomyces* spp., *Herbaspirillum seropedicae*, microbe interaction, plant growth promotion.

INTRODUÇÃO

Projeções para a população mundial em 2050 apontam para quase 10 milhões de habitantes. Como consequência, haverá a necessidade de produzir mais alimentos para atender à crescente demanda. O modelo de produção predominante nos sistemas agrícolas baseia-se no uso de fertilizantes minerais e outros agroquímicos que são, na maioria das vezes, utilizados de forma indiscriminada. O custo econômico, ambiental e social atribuído a este modelo tem repercutido em questionamentos sobre tal forma de produzir e levado à reflexão sobre a lógica do sistema de produção convencional, a partir de uma visão holística da exploração dos recursos naturais. Ergue-se, nesse cenário, o paradigma da sustentabilidade agrícola que propõe uma otimização da produtividade em agroecossistemas, preconizando a segurança e a soberania alimentar.

O melhor entendimento da maquinaria biológica do solo e da sua interação com as diferentes espécies vegetais, ao longo dos anos, tem viabilizado o desenvolvimento de um importante produto biotecnológico capaz de contribuir para a sustentabilidade de sistemas agrícolas: o uso de rizobactérias bactérias promotoras do crescimento de plantas (RPCP), na forma de bioinoculantes, tem sido empregado e tem alcançado aceitação mundial.

A indução do crescimento vegetal por RPCP pode ocorrer por meio de mecanismos de biofertilização, bioestimulação e bioproteção. A biofertilização consiste no aumento da disponibilidade de nutrientes essenciais às plantas, envolvendo atividades como a fixação biológica do nitrogênio, e solubilização de nutrientes. A bioestimulação, por sua vez, resulta da ação fitormonal de compostos metabólicos secretados. Já os mecanismos de bioproteção estão relacionados ao controle de fitopatógenos e redução de danos causados por estresse de natureza abiótica. A ocorrência desses mecanismos é encontrada, por exemplo, em espécies de RPCP pertencente aos grupos das actinobactérias e das bactérias diazotróficas.

Bactérias diazotróficas são assim designadas pela habilidade que possuem de fixar o nitrogênio atmosférico, processo biológico que representa uma das principais vias de fornecimento desse elemento às espécies vegetais. Além da fixação biológica do nitrogênio, estas bactérias Gram-negativas pertencentes ao Filo Proteobacteria são ainda capazes de contribuir para o crescimento vegetal por meio de outros

mecanismos como a solubilização de fosfatos, produção de compostos indólicos e controle de fitopatógenos. Por outro lado, as actinobactérias pertencem ao Filo e à Classe Actinobacteria que apresentam alta proporção de G-C (guanina e citosina) em seu DNA. O gênero *Streptomyces* é o mais estudado, sobretudo, pela capacidade de produção de antibióticos apresentada por diversas espécies. Essa característica confere um elevado desempenho dessas espécies em controlar fitopatógenos. Comumente isolados a partir de solo, nos últimos anos estreptomicetos têm sido encontrados em associações com espécies vegetais, produzindo um conjunto de enzimas extracelulares e de metabólitos secundários, que podem atuar direta ou indiretamente na promoção de crescimento de plantas, o que tem chamado a atenção de pesquisadores.

A despeito da exploração extensiva das potencialidades individuais de bactérias diazotróficas e actinobactérias para promoção do crescimento vegetal, estudos focados no uso combinado destes micro-organismos ainda são escassos. Nesse sentido, o presente trabalho dedicou-se a explorar características relacionadas à promoção de crescimento vegetal em isolados de actinobactérias do gênero *Streptomyces*, e sua interação com *Herbaspirillum seropedicae* estirpe HRC54, uma bactéria notadamente promotora do crescimento de plantas. No Capítulo 1, encontra-se um artigo de revisão, recentemente publicado, sobre o gênero *Streptomyces*, resultante de uma extensa pesquisa sobre aspectos relacionados a vias metabólicas secundárias, interações com plantas hospedeiras e os recentes avanços na elucidação de mecanismos de promoção do crescimento. No Capítulo 2 é apresentado um artigo em que foi realizada a caracterização fisiológica de estreptomicetos isolados de vermicompostos maturados e a investigação por propriedades relacionadas à promoção de crescimento vegetal, em observou-se a eficiência dos isolados em induzir o crescimento de plântulas de milho. No Capítulo 3 são apresentados dados dos estudos de compatibilidade entre *H. seropedicae* estirpe HRC54 e isolados de *Streptomyces* (AC01, AC05, AC06, AC31) e a particular interação entre HRC54 e o isolado AC06 quanto a atividade auxínica de metabólitos secretados e quanto a ocorrência de cooperação metabólica para o amido. Neste trabalho, os isolados AC01, AC06 e a combinação de AC06 com HRC54 foram eficientes em promover o crescimento de plântulas de tomate. Compostos metabólicos produzidos por HRC54 e por AC06 desempenham atividade auxínica, tomate Micro-Tom DR5-GUS.

CAPÍTULO 1

PLANT GROWTH PROMOTION BY STREPTOMYCETES: ECOPHYSIOLOGY, MECHANISMS AND APPLICATIONS
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ABSTRACT

The genus *Streptomyces* comprises filamentous Gram-positive bacteria that are widely recognized for their ability to produce bioactive compounds such as antimicrobial, antiparasitic and immune-suppressing compounds via secondary metabolism. These bioactive compounds represent a third of all commercially available antibiotics. Streptomycetes have been found in beneficial associations with plants where they have improved plant growth and protected against pests, which have attracted the attention of researchers worldwide. This review focuses on the potential of streptomycetes as plant growth-promoting bacteria (PGPS) and considers features related to secondary metabolic pathways, interactions with host plants and recent advances in elucidating plant growth-promoting mechanisms. Such advances in basic knowledge have increased the prospects for streptomycetes to be used as bioinoculants for sustainable agriculture.

Keywords: *Streptomyces*, Actinobacteria, Biotechnological potential.

INTRODUCTION

It has been estimated that the world's population will reach about nine billion by 2050 [1], which will require high levels of yield from agricultural systems. Centered on the Green Revolution model established in the last century, a range of research and technology transfer initiatives have been employed to meet the demand for food, fiber, and energy. However, it has become increasingly clear that the conventional systems of food production have many negative impacts on the environment [2].

To develop food production under environmentally and socially sustainable systems represents one of the twenty-first century's greatest challenges for agricultural researchers. One of the most promising initiatives for a new model of agriculture is based on converting the natural processes that occur in the soil–plant system into biological input technologies. In this context, microorganisms, their products and their processes are essential resources for a new generation of biotechnologies applicable to plant production and protection, and potentially a paradigm shift in agricultural practice.

Using plant growth-promoting bacteria (PGPB) for the benefit of agriculture has received increasing attention and acceptance [3–5]. Besides the well-studied Gram-negative plant-associated bacteria, Gram-positive bacteria can also have beneficial interactions with plants and promote plant growth [6–8]. *Streptomyces* is the most widely studied genus of Gram-positive PGPB and is the central subject of this review. This genus comprises a wide diversity of species that have a high G-C (guanine and cytosine) ratio in its DNA, up to 75 % of its genome [9]. This genus produces a wide variety of biologically active compounds; some with plant growth activity. It has been suggested by some authors that the increasingly intensive surveys of soil-borne microbes have resulted in a decreased frequency of newly discovered compounds [10]. However, the metabolic versatility and cosmopolitan behavior of Streptomyces species have enabled them to be isolated from different environments, some of which have not yet been explored, hence, presenting an opportunity to discover new bioactive compounds.

Morphogenetic and physiological aspects of the *Streptomyces* genus have been reviewed [11, 12]. Other reviews have focused on the biotechnological potential of plant-associated endophytic actinobacteria [10] and free-living plant growth-promoting

actinobacteria [7]. This review presents an overview of the plant growth-promoting ability of various species from the genus *Streptomyces*; it considers historical aspects of their discovery, their physiological features, their plant growth mechanisms and their application as bioinoculants in agriculture.

REVIEW

Historical and taxonomic aspects

Nearly 200 years after Antony van Leeuwenhoek reported the first observation of bacteria in 1684 using his own handcrafted microscope, other pioneers, such as Ferdinand Cohn and Robert Koch, founded modern concepts about bacteriology as a science domain [13]. It took another 200 years to identify and reach the current understanding of actinobacteria. Further, only 204 years after van Leeuwenhoek studies, the first description of a microorganism that eventually became known as an actinobacterium was described, when Armauer Hansen discovered a microorganism in the tissues of leprosy patients, which was later described as the etiologic agent of this disease in 1874 [14].

In 1875, Cohn described the first Actinobacteria species, which he named *Strepthrotrix foersteri*. He isolated this microbe from samples of human tear ducts provided by R. Foerster, a medical friend. Cohn supposed that *Strepthrotrix foersteri* was not associated with any disease, but that it reached the patient's eye through airborne soil particles. Later, he observed that *Strepthrotrix foersteri* had morphological features of fungi and bacteria [14]. However, the proposed nomenclature for the bacterial genus was deemed invalid because *Strepthrotrix* had already been classified as a true fungus by Corda in 1839 [15]. Then in 1877, Carl Otto Harz described the etiologic agent of "lampy jaw". Harz observed structures similar to reproductive bodies and hyphae of fungi; therefore, he considered the microorganism to be a fungus and named it *Actinomyces bovis* [14]. In 1882, Robert Koch discovered another microorganism during his observations using light microscopy that is now recognized as an actinobacterium: the tuberculosis pathogen *Mycobacterium tuberculosis* [16]. Koch observed that the microbes presented morphological characteristics that were similar to those of microorganisms previously described by Hansen associated with leprosy disease [17]. Although there was a clear relationship between these microorganisms, it was not until 1916 that R.E. Buchanan suggested a nomenclature

and classification for this group. Buchanan proposed the order Actinomycetales, containing the family *Actinomycetaceae* and the following genera: *Actinobacillus*, *Leptotrichia*, *Actinomyces*, and *Nocardia* [18]. In 1943, Waksman and Henrici proposed a new classification for the actinomycetes, which was based on their ability to form branching cells. Waksman and Henrici observed that one actinomycete group formed a condensed mat of interlinked branching hyphae that produced reproductive spores. The *Streptothrix* described by Cohn fell into this group, but due to the invalid genus name, Waksman and Henrici named it *Streptomyces*, which means “twisted fungus” [19].

The first proposed nomenclature of actinobacteria was based on sporulation patterns. Although morphological characteristics are typically important for *Streptomyces* identification, some studies have demonstrated that classification based on cell morphology, colony pigmentation, and physiological features do not always reflect the natural phylogenetic relationship between actinobacteria and related organisms [21]. Introduction of the polyphasic taxonomic approach combined molecular and biochemical analyses which elucidated streptomycetes systematics. In addition, increased availability of 16S rRNA sequence data has enabled accurate studies of taxonomic affiliations and phylogenetic relationships [15, 21, 22].

The *Streptomyces* genus belongs to the Streptomycetaceae family and the single order *Streptomycetales* [23]. This order belongs to both Phylum and Class Actinobacteria [23]. The actinobacteria from the *Streptomyces* genus are the most extensively studied mycelial actinobacteria. They are aerobic, Gram-positive bacteria that grow as branching filaments that consist of vegetative mycelia and aerial hyphae [15, 24]. Some morphological and physiological properties, along with the ability to produce a wide range of pigments, have been used not only to classify the *Streptomyces* genus [25, 26], but also to study its ecological distribution and biotechnological potential [27, 28]..

Morphological differentiation and physiology

Streptomycetes have a markedly different cell envelope structure than Gram-negative bacteria, such that *Streptomyces* genus has been identified using cell wall

composition [29, 30]. Similar to other actinobacteria, streptomycetes have no outer membrane and their cell walls have a thick peptidoglycan (or murein) layer [34]. The presence of LL-diaminopimelic (LL-DAP) in the cell wall confers a typical chemotaxonomic characteristic to all members of the *Streptomyces* genus [31, 32], and its presence together with glycine characterizes the cell wall as Type I [33, 34]. Teichoic acids (anionic glycopolymers) are another important cell wall component that confers a negative charge to the cell surface and contributes to physiological functioning and cell co-aggregation [35, 36].

The life cycle is initiated when favorable environmental conditions and nutrient availability promote spore germination [12] (Figure 1). Next, germ tubes grow to form syncytial vegetative or substrate mycelia, which consist of interconnected feeding hyphae that are responsible for nutrient uptake [37]. When nutrients become scarce, or another stress condition occurs, programmed cell death of the substrate mycelia and cell differentiation at the center of the colony result in aerial hyphae [12, 38]. These aerial hyphae are subtly distinguishable from the feeding hyphae, as they are covered by a hydrophobic fibrous layer, perhaps to help the aerial hyphae break the surface tension on air pockets in the soil, whereas the feeding hyphae have a smooth hydrophilic surface [24].

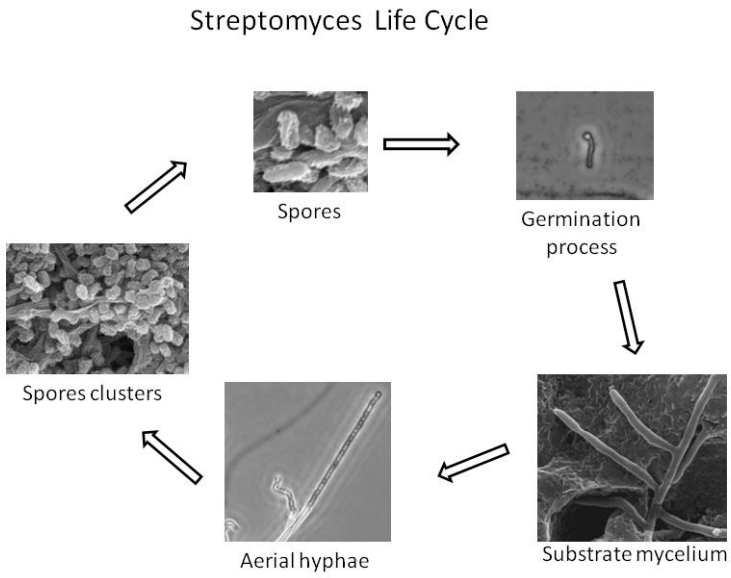

Figure 1: Life cycle of *Streptomyces* [39] modified

The growth of *Streptomyces* involves hyphal tip extension and sub-apical branching [40]. Unlike the process in rod-shaped bacteria where cytokinesis is based on building a cross-wall by depositing murein into lateral walls, *Streptomyces* growth occurs by hyphae production at the cell pole [37]. Although it is not clearly elucidated, this cell growth pattern is regulated by the apical protein complex DivIVA. In *Bacillus subtilis*, DivIVA interacts with the Min system to coordinate division at the middle of the cell. In contrast, in *Streptomyces*, the Min system is absent, thus DivIVA affects division at the cell tip. Another aspect of streptomycetes growth involves the conservation of two groups of proteins, the tubulin homologue FtsZ and several membrane proteins, which are both associated with cytokinetic Z-ring and septal peptidoglycan [11].

The last phase of the *Streptomyces* life cycle consists of the apical cells of the aerial hyphae differentiating into a spore chain [12]. A differentiating apical compartment grows by tip extension and starts synchronous, multiple cell divisions into a developmentally controlled form [41]. Again, there is the participation of FtsZ, which leads to sporulation septa and then these pre-spores assemble thick spore walls by depositing actin [42]. The size of *Streptomyces* spores can range from 0.7 to 1.2 µm [43, 44]. These last two phases of the *Streptomyces* life cycle are closely related to antibiotic production [14]. During programmed cell death of the substrate mycelia, antibiotics are simultaneously produced; perhaps to protect the nutrient sources against competitor microorganisms [45, 46].

Among the 23,000 bioactive secondary metabolites produced by microorganisms, two-thirds are produced by actinobacteria, and *Streptomyces* spp. accounts for more than 70% of these [47]. This production of secondary metabolites is attributed to the development of aerial hyphae as a result of nutrient limitation [48]. The biological activity of these compounds involves inhibitory or microbiocide activity against microorganisms (i.e., antibiotics), toxicity against metazoans, microbial hormone-like activity, and metal transport [49-51]. It has been well demonstrated that the secondary metabolites produced by streptomycetes increases adaptation to biological, physical, and chemical stresses, thus they are recognized as ‘stress metabolites’ [36].

Structural genome studies tell us that most genes involved in regulating secondary metabolite pathways are arranged in clusters. These clusters might encode the highly phosphorylated guanosine nucleotide (p)ppGpp and some regulatory

proteins involved in producing secondary metabolites [52]. In *Streptomyces* species under nutritional stress, alarmone ppGpp plays a role as a regulator of antibiotic production [53, 54]. Members of both the SARP and LAL families of regulatory proteins appear to be confined to actinobacteria, mainly genus *Streptomyces*, and have shown species-specific controls for secondary metabolism pathways [55-57]. Cell-to-cell communication is a determining factor for modulating antibiotic production, and γ-butyrolactones (GBLs) are the main intercellular signaling compound [49].

The overall ecophysiological traits of the *Streptomyces* genus support the concept of cosmopolitan biogeographical behavior. The traits include the ability to form spores under unfavorable abiotic conditions, a competitive ability related to antibiotic production, a broad pH range that is favorable for growth, and a wide pH range that is optimal for growth between different *Streptomyces* species (such as pH 4.3 for the acidophilic *S. yeochonensis* [58], pH 7.0 for the neutrophilic *S. roseus* [59], and pH 10 for the alkaliphilic *S. alkalithermotolerans* [60]. Streptomycetes are typically chemoorganotrophic with a great versatility for metabolizing a wide range of carbon sources including mono- and di-saccharides, polyol, organic acids (glucose, dextrose, fructose, lactose, maltose, mannitol, rhamnose, sucrose, glycerol, and glycolic acid), polysaccharides (including cellulose and starch), and more complex and recalcitrant C-sources (such as humic and fulvic acids) [61-63].

Representatives of the genus *Streptomyces* are well recognized as soil-dwelling bacteria. Nevertheless, many reports have shown that they are widely distributed in both aquatic and terrestrial environments [61]. This cosmopolitan distribution of streptomycetes might be attributed to their production of spores, which are readily spread and thus could explain its presence in different environments (see Table 1). Several members of genus *Streptomyces* have been isolated from various vegetative and reproductive plant parts, such as roots, tubers, stems, leaves, and seeds. There are many questions concerning the ecological and physiological significance of this interaction that would potentially converge for successful practical applications related to plant-growth promoting and plant protection properties [64, 65].

Table 1: Biotechnological potential and cosmopolitan features of *Streptomyces* species.

Species	Strain	Isolated from	Major characteristics	Reference
S. coelicolor	A3(2)	Soil	The best genetically known representative of the genus.	[145]
S. xiamenensis	DSM 41903^T	Mangrove sediment	Not discribe.	[62]
S. axinellae	DSM 41948^T	Mediterranean sponge *Axinella polypoides* (Porifera)	Not discribe.	[42]
S. griseus	DSM 40236^T	Soil	Producer of streptomycin antibiotic.	[19]
S. chumphonensis	KK1-2^T	Marine sediments	Not discribe.	[146]
S. rochei	SM3	Decomposed cow dung	Alleviates the stresses caused by *Sc. sclerotiorum* and salt in chickpea.	[147]
S. fildesensis	GW25-5^T	Antarctic soil	Not discribe.	[148]
S. scabies	ATCC 49173	Potato scab	Pathogen of potato scab.	[149]
S. pseudovenezuelae	ACTA 1383	Rhizosphere of *Ebenus sibthorpii*	Antagonist activity against *Rhizoctonia solani.*	[122]
S. oryzae	S16-07^T	Surface-sterilized stems of rice	Not discribe.	[150]
S. wadayamensis	A23	Plant tissue of *Citrus reticulata*	Antagonist activity against *Xylella fastidiosa.*	[151]
S. kebangsaanensis	SUK12^T	Inner tissue of *Portulaca oleracea* L. stems	Phenazine-1-carboxylic acid producer.	[152]
S. phytohabitans	KLBMP4601^T	Surface-sterilized roots of *Curcuma phaeocaulis*	Not discribe.	[153]
S. diastaticus	UENF AC01	Vermicompost of sunflower cake	Plant growth promoting streptomycete. AIA and catalase producer.	[154, 155]
S. variabillis	UENF AC31	Vermicompost of sunflower cake	Phosphate solubilizer.	[154, 155]

^{T} Type strain

Ecology of *Streptomyces*–plant host interaction

To acquire a better understanding and to manipulate the interactions between plant growth-promoting streptomycetes (PGPS) and their hosts, it is necessary to elucidate those biochemical mechanisms that lead to compatible relationships. As shown above (Table 1), most of these streptomycetes are soil-dwelling bacteria with a free-living life cycle in the soil (i.e., saprophytic competence), and they are able to efficiently colonize the rhizosphere and rhizoplane compartments. Eventually, some PGPS might become endophytic and colonize the inner tissues of the host plant and partly or fully conduct their life cycle within them [66]. Therefore, before discussing plant responses to PGPS, it is necessary to describe the rhizosphere and rhizoplane colonization process.

The rhizosphere is the soil volume under the influence of plant root exudates, secretions, and loose cell deposition [67]. The rhizoplane plays a crucial modulation role on the microbial community structure and microbial diversity that will ultimately influence plant growth and performance into the soil-plant system [68]. Additionally, microbial activity in the rhizosphere soil is markedly influenced by carbon-containing metabolites released from roots via the rhizodeposition process [69]. Rhizodeposition consists of numerous compounds such as ionic secretions, free oxygen and water, enzymes, proteins, mucilage, amino acids, organic acids, sugars, and phenolics [70]. These compounds acts as nutritional resources, chemoattractants, chemorepulsants, and signaling compounds that shape the microbial community structure and activity of different groups of microorganisms and have a significant influence on plant root–microorganism interactions [71].

The PGPS are widely recognized to be good rhizosphere colonizers and their rhizosphere competence may be partially explained by several chemotaxic features, such as bacterial rate multiplication, quorum sensing-controlled gene expression, amino acids, antibiotics, and siderophore synthesis [72]. Initially, soil-borne or introduced streptomycete cells that respond to released compounds are actively attracted to the rhizosphere by chemotaxis [70, 73]. At this point, the versatile nutritional requirements and increased population rates coupled with bioactive compound production and detoxification mechanisms are determinants for successful rhizosphere establishment. The former properties play a pivotal role in overcoming complex competition and can be partially explained by the ability to produce antibiotics

and other bioactive compounds that confer an ecological advantage and allow PGPS to colonize niches. Several biomolecules that are secondary metabolites of *Streptomyces* contribute to biocontrol and successful colonization; e.g., siderophores from *S. coelicolor* [74], the antifungal nigericin and antibiotic geldanamycin from *S. violaceusniger* YCED-9 [75], and chitinase from *S. violaceusniger* YH27A [76].

Some secreted proteins are important for successful root colonization by *Streptomyces* [77]. Differential protein expression was exhibited by *S. coelicolor* when it was cultivated in minimal medium with and without *Lemna minor* fronds. Bacterial enzymes involved in degrading cellulose, alkenes, and amino acids were induced in the presence of plant extracts; suggesting that the carbon and energy were acquired through degrading compounds present in *L. minor* exudates [78].

Isolates of PGPS can be screened for the attribute that only a minority of species are recognized as phytopathogenic agents; namely *Streptomyces scabies*, *S. acidiscabies*, *S. turgidiscabies*, and *S. ipomoeae,* which are all etiologic agents of common scab diseases [79]. *S. scabies* is a model organism for investigating plant host-pathogen interactions in Gram-positive bacteria in which a protein secretion system is essential for its pathogenesis [77]. The effector proteins can be secreted by the Secretory (Sec) and Twin-arginine translocation (Tat) pathway or, additionally, by the specialized Type VII secretion system (T7SS) [80]. Thaxtomin, a family of nitrated dipeptide phytotoxins, is an important pathogenicity factor secreted by *S. scabies* and it plays a role in cellulose biosynthesis inhibition [81]. Nec1 is a protein that is required for root colonization by *S. scabies* [82]. Another important intercellular signal attributed to pathogenic streptomycetes and its host is nitric oxide (NO) [83]. Synthase-derived NO is produced by plant-pathogenic *Streptomyces* in response to cellobiose production, which expands plant tissues, and appears to be involved in the nitration of thaxtomin [84].

In contrast, a wide variety of *Streptomyces* species establish beneficial plant-microbe interactions [85-87]. The ability of some *Streptomyces* species to gain access into root tissues and to establish an endophytic lifestyle without causing visible harm or symptoms in the host plant have been reported (Table 1). These species can be found mainly in the apoplastic compartments that comprise the intercellular spaces and lumen of differentiated dead cells (sclerenchyma and xylem cells) of the host plant organs (roots, stems, leaves, flowers, fruits, and seeds); intracellular occurrences

seem to be less frequent [10]. Using a *Streptomyces* sp. strain EN27 tagged with green fluorescent protein, the endophytic colonization of embryos, endosperm, and emerging radicles of wheat seeds has been reported [88].

Unlike Gram-negative bacteria, *Streptomyces* and other filamentous actinobacteria possess active penetration structures that grow on the plant surface and infect intact cells [89]. The ability to attach and develop an infection point through the cell wall of sweet potato (*Ipomoea batatas* [L.] Lam.) has been reported for *S. ipomoea*. Subsequently, the tip-growth hyphae colonize the interior of parenchyma cells and establish an endophytic interaction [90]. Similarly, using short branches that emerged from the main hyphae, *S. scabies* was observed to penetrate the cell walls of potato plants [91]. Based on the described cell wall penetration process by these short hyphae within a short and uniform distance from the branching point, the authors suggested a specialized penetration function for this structure. Furthermore, *Streptomyces* might enter plant tissues by natural openings such as stomatal apertures, lenticels, hydathodes, wounds, broken trichromes, and root hair cracks formed by lateral root emergence zones [89]. A transmission electron microscope analysis has demonstrated that *S. galbus* MBR-5 entered the leaves of *Rhododendron* sp. seedlings through stomatal openings [92].

Plants exhibit defense responses during infection by PGPS, but they are less aggressive than those expressed during pathogenic interactions [93]. Although the biochemical mechanisms involved in these plant responses are not clearly elucidated, the pivotal role of phytohormones such as salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) has been widely reported in the literature [94-97]. *Arabidopsis thaliana* inoculated with the endophytic *Streptomyces* sp. strain EN27 induced low levels of gene expression related to the JA and ET pathways [94]. Furthermore, plant response to PGPS infection involves generating reactive oxygen species, which are cytotoxic [84]. This plant process can be subverted by enzymatic machinery related to antioxidant activity; e.g., catalase and superoxide dismutase by *S. coelicolor*, and aconitase by *S. viridochromogenes* strain Tü494. This process plays an important role in alleviating the cytotoxic effects induced by reactive oxygen species and in allowing successful plant tissue colonization [98-100].

Endophytic colonization usually represents an important ecological advantage to the microorganism because the protected environment is less susceptible to abiotic

stress (pH, redox, osmotic, and hydrolic variations) and microbial competition compared with the rhizosphere–soil system. Moreover, the intimate contact between plant host cells and microbial cells might be more effective for the bidirectional exchange of signals, functional metabolites, and nutritional sources for a successful beneficial interaction, which may promote growth of the host plant [89, 101]. The mechanisms involved in plant growth-promoting activity by both endophytic and rhizosphere streptomycetes will be discussed below.

Plant growth promotion by streptomycetes

Most of the fundamental and applied studies of beneficial plant-microbe interactions relate to Gram-negative bacteria [102-104]. Although less often studied, many representative groups of Gram-positive bacteria, particularly those belonging to genus *Streptomyces*, exhibit a range of traits that may improve plant growth by using different mechanisms [7]. As quoted for other beneficial interactions, the plant growth promoting effects related to *Streptomyces*-plant interactions can be divided into biofertilization, biostimulation, and bioprotection [105].

Biofertilization effects. Due to the mineralogical and electrochemical properties of many soils, some essential mineral nutrients are unavailable to plants because they are present in insoluble forms [106]. Biofertilization consists of a direct mechanism that improves macro or micronutrient acquisition (uptake and assimilation) by plants. Nutrients sequestered in the crystalline lattice of the mineral fraction of soil can be solubilized and released into solution by organic acids (such as gluconic acid, citric acid, succinic acid, and oxalic acid) that are secreted by different microorganisms [107]. Jog and colleagues [65] reported the release of free phosphate by acidification resulting from the release of malic acid and gluconic acids by *Streptomyces* mhcr0816 and *Streptomyces* mhce0811, respectively. These authors also observed an increase in the number of branches and lateral roots, shoot length, and mineral content of Fe, Mn, and P in wheat plants inoculated with these streptomycetes. Soil treated with a phosphate-solubilizing strain of *Streptomyces* that was isolated from a wheat field, increased N, Fe, P, and Mn content in shoots of wheat [108]. In addition, solubilization of rock-phosphate by *S. youssoufiensis* has been reported [109, 110]. Streptomycetes can further promote mineral supply by synthesizing siderophores and siderophore

uptake systems [108]. *Streptomyces* sp. GMKU 3100, a siderophore-producing endophytic streptomycete, was capable of promoting the growth of rice and mungbean, whereas its siderophore-deficient mutant did not differ from the uninoculated control [87]. Until now, there is no convincing evidence for free-living or endophytic *Streptomyces* specie able to fix nitrogen, since the controversial report related to *S. thermoautotrophicus* by Ribbe and colleges [111] was recently refuted [112]

Biostimulation effects. Plant growth can be directly improved by the microbial production of metabolic compounds with phytohormonal activity at micromolar to nanomolar concentrations [65]. Auxin and auxin-like compounds regulate many aspects of plant growth and development including cell plasticity, tissue elongation, embryogenesis, tip dominance, and emergence of lateral roots [113]. The production of indole-3-acetic acid (IAA) by *Streptomyces* spp. has been quoted in several reports [108, 114, 115]. For example, *S. atrovirens* ASU14 utilized tryptophan and produce 22 µg/mL of the IAA [116]. An auxin-like activity due to pteridic acid A was produced by *S. hygroscopicus* TP-A0451 isolated from *Pteridium aquilinum* (bracken) stems [117]. This compound stimulated root elongation and induced the formation of adventitious roots in *Phaseolus vulgaris* (kidney bean) hypocotyls. Another class of phytohormones that have an important function in plant growth is gibberellin. This phytohormone is involved in physiological process that includes seed germination, growth of stems and leaves, floral induction, and growth of flowers and fruits [118, 119]. *Streptomyces* species isolated from a marine environment exhibited the ability to produce a range of phytohormones, including gibberellic acid, and enhanced the agronomic performance of eggplant (*Solanum melongena*) by influencing its growth parameters, including root length and fresh or dry root weight [120].

Bioprotection. Plant-associated microbes might have the ability to minimize the challenges imposed by phytopathogen. This biocontrol activity can manifest as antibiosis, nutrient and space competition, parasitism, predation, hypovirulence, and induced systemic resistance [70, 121], which are indirect mechanisms of plant growth promotion. The versatile production of secondary metabolites involved in biocontrol is primarily a competitive strategy to successfully colonize the root zone [72]. The genus *Streptomyces* is widely recognized as being able to synthesize several bioactive metabolites that act to control phytopathogen and to confer an advantage to rhizosphere or endophytic colonization [122]. It has been reported that antifungal

metabolites produced by 213 *Streptomyces* strains isolated from different habitats exhibited *in vitro* antagonistic activity against *Rhizoctonia solani* [123]. An investigation into the nematicidal property of *Streptomyces roseoverticillatus* CMU-MH021 revealed the production of secondary metabolites that acted against the root-knot nematode *Meloidogyne incognita* [124]. *S. roseoverticillatus* CMU-MH021 produced fervenulin, which decreased the percentage of hatched eggs and increased the percentage mortality of second-stage juveniles; and it produced isocoumarin, which also increased the percentage mortality of second-stage juveniles. A recently released draft genome sequence of the mushroom mycoparasite antagonist *Streptomyces* sp. strain 150FB, with close correspondence to the *S. avermitilis* MA-4680 genome, revealed possible factors related to disease suppression. Namely, a set of genes encoding extracellular enzymes involved in degrading fungal cell wall polysaccharides, disrupting membranes and proteins, and also peroxidases and ribonucleases [125]. In addition, two terpene and two siderophore biosynthetic gene clusters were detected. Streptomycetes can also stimulate plant defense by the *priming* phenomenon, resulting in induced systemic resistance [126]. *Streptomyces* sp. strain AcH505 suppressed oak powdery mildew infection [127]. This streptomycete strain elicited a systemic defense response in oak (*Quercus robur*), inducing the jasmonic acid/ethylene-dependent pathway and the salicylic acid-dependent pathway.

Secondary metabolites from streptomyces species includes not only plant bioprotection effects (scope of the present review), as well as other bioactivity properties related to application in medical science. The table 02 summarize examples of some these compounds and their genes and operon structures. Advances in the genetic basis of secondary metabolites pathways enhance the perspectives for discovering new compounds in ecological surveys under distinct environment conditions.

Plant-associated streptomycetes can also benefit the host plant by mitigating abiotic stress such as heat, cold, drought, and nutrient depletion; thus reducing their negative impacts and consequently increasing plant growth [128]. The application of *Streptomyces filipinensis* no. 15, a 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase and IAA producer, reduced endogenous levels of ACC, the immediate precursor of ethylene, in both roots and shoots and subsequently enhanced plant fitness to the environment [129]. Plant growth-promoting and stress-alleviating

Table 2: Secondary metabolites produced by *Streptomyces* species, main bioactivity property, and their genes and structural organization

***Streptomyces* specie**	**Secondary metabolite**	**Bioactivity**	**Genetic basis**	**Reference**
S. clavuligerus	Clavulanic acid	Antibiotic	*herABCDEFG* genes	[157]
S. coelicolor	Actinorhodin	Antibiotic	*abeABCD;* α-*abeA* genes	[158]
S. griseus	Streptomycin	Antibiotic	*strFGHIK* genes	[159]
S. hygroscopicus	Geldanamycin	antitumor	*ahba-B* locus	[160]
S. hygroscopicus var. ascomyceticus	Ascomycin	Immunosuppressant; Neurotrophic; Antifungal	FK520 gene cluster	[161]
S. thioluteus	Aureothin	Antitumor; Antifungal; Insecticidal	*aur* operon (*aurA* through *aurI*)	[162]
S. venezuelae	Chloramphenicol	Antibiotic	*sven*0916-*sven*0928 gene cluster	[163]
S. violaceusniger	Meridamycin	Neuroprotectant	*merA-D; mere; merP* genes	[164]
S. viridochromogenes Tu¨494	Phosphinothricin tripeptide	Herbicide	*phsB; orfM; phsC; pmi; ppm; ppd; phsA; pat; dea; prpA*	[165]
S. griseus	Griseobactin	Siderophore	*dhbACEBG* operon	[166]
S. scabiei	Indole-3-acetic acid	Auxin	*iaaM; iaaH*	[167]

activities have been demonstrated for a halo-tolerant and ACC deaminase-producing *Streptomyces* sp. strain PGPA39 applied to tomato (*Solanum lycopersicum*) plants under salinity stress [130].

Furthermore, a different approach that is based on the indirect mechanisms of plant growth by PGPS has received particular attention. In a study involving a tripartite culture system [131], streptomycete application seemed to act as a modulator in both mycorrhizal and nitrogen-fixing symbiosis, and raised their ability to induce plant growth [132, 133]. An increased number, size, and vigor of root nodules was observed on young pea (*Pisum sativum*) seedlings cultivated in soil with naturally abundant *Rhizobium* and that was inoculated with *S. lydicus* WYEC108 [134]. *Streptomyces* sp. AcH505 isolated from the hyphosphere of a spruce (*Picea abies*) promoted mycelial growth and the mycorrhization rate by *Amanita muscaria* and *Suillus bovinus*, and it suppressed mycelial extension of the plant pathogens *Armillaria obscura* and *Heterobasidion annosum* [135, 136].

Our group has recently proposed some different methods to enhance the plant-growth promoting ability of the endophytic diazotrophic bacteria *Herbaspirillum seropedicae* strain HRC54 (unpublished results). One method involved axenic studies combining *H. seropedicae* with the *Streptomyces bellus* strain UENF AC06 that was isolated from mature vermicompost. This mixed cultivation increased the population growth and nitrogen fixation rates of *Herbaspirillum* when measured by acetylene reduction activity. Therefore, *S. bellus* strain AC06 may be used to improve the plant growth promotion response by *H. seropedicae* strain HRC54, and we suggest that this mixed cultivation is a potential bioinoculant for agricultural systems.

Biotechnological application of PGPS in agriculture

Using plant growth-promoting bacteria to improve nutrient availability to plants is an important practice for sustainable agriculture and is an alternative to chemical pesticides and fertilizers [137]. PGPS can enhance nutrient availability to plants by biosynthesizing metal chelators and phosphorus solubilizers, producing phytohormones, controlling phytopathogen, and alleviating abiotic stress. Although few *Streptomyces* strains are used as biofertilizers, commercial bioinoculants (microbe-

based products) have been developed to improve plant growth promotion (Table 3) [64].

The first challenge to produce a bioinoculant using PGPS is to find the best strain of streptomycete (single formulation) or its combination with other microbes (mixed formulation). Screening studies involve different experimental assays in the laboratory and greenhouse. Elite strains must be tested under different environmental conditions and for different plant species and different genotypes of a target crop [4]. Following these steps, it is necessary to develop specific bioinoculant formulations, which involve determining the required physical and chemical characteristics of the carrier, and ascertaining which additives and metabolites would increase the viability, activity, and performance of the microorganism when introduced under field conditions [4]. Lastly, research to develop methods of bioinoculant delivery and timing must be carried out in the greenhouse and field to maximize the plant response to the applied bioinoculant. It has been found that applying streptomycetes is different to applying Gram-negative bacteria because better plant responses have generally been obtained when the streptomycete application to the soil or substrate occurred before sowing the seeds, thus allowing the selected strain to colonize and establish [6, 138, 139].

Viability and activity of the population inoculum is often affected by abiotic stress. Since the endophytic compartment represent a more protective niche for plant-bacteria interaction, formulations based on endophytic streptomycete strains had advantages over non endophytic streptomycetes that would guarantee successful positive plant host response [140]. Representative streptomycetes possess the ability to colonize the rhizosphere or endophytic tissues of plants [6, 88], to produce spores resistant to irradiation, heat, and drought [12], and to have an impressive range of metabolites with a variety of biological activities [141]. *Streptomyces* may also convert plant exudates or macromolecules/supramolecules present in the rhizosphere into a form that can be used by other plant growth-promoting microbes. This proposition originated from our research group and represents a technological conversion of the fundamental concept of ecological succession of community members and metabolic cooperation. In this line of research, we combined the non starch-degrading diazotrophic bacterium *Herbaspirillum seropedicae* strain HRC54 with *Streptomyces bellus* strain UENF AC 06, and verified that an increased population of the bacteria was probably sustained by C-sources from a starch degradation by-product. It is noteworthy that *Herbaspirillum*

did not grow when *S. bellus* was absent in the medium and when starch was the sole C-source (unpublished results). It is another example of a simple idea that can be converted into a biotechnological product with a range of applications for sustainable crop production.

Concluding remarks and perspectives

The greatest challenge for agriculture in the current century is to produce food for the increasing world population and to reduce the dependence on non-renewable resources and to reduce environmental impact. The use of plant growth-promoting microbes to enhance crop production has emerged as a sustainable and alternative tool to meet this challenge [142]. The most studied and technologically developed plant growth-promoting microbes mainly include rhizobia and other Gram-negative bacteria. However, besides their widely known ability to produce antibiotics, representatives of the Gram-positive genus *Streptomyces* have been found in beneficial associations with plants, including those of agronomic importance [65, 143-145]. The diversity of bioactive compounds produced by *Streptomyces* spp. includes substances capable of improving plant growth; hence they are recognized as PGPS. Increased knowledge of secondary metabolic pathways involving production of bioactive compounds and mechanisms of plant growth promotion by *Streptomyces* will be assisted by the rapid development of functional genomics and bioinformatics over the coming years. PGPS can promote plant growth by colonizing the rhizosphere or the endophytic plant environment. However, the interactions between rhizosphere PGPS and indigenous microbiota as well as the infection process performed by endophytic PGPS are still not clearly elucidated. Metagenomic approaches and the use of molecular markers such as fluorescent proteins will certainly contribute to the study of dynamic microbial populations in the plant rhizosphere, PGPS inoculation, and streptomycetes endophytic plant colonization. In addition, the use of PGPS as commercial biofertilizers is developing, but research into the design of bioinoculant formulations (such as additives, carriers, and delivery methods) will increase the plant growth and yields and acceptance by farmers around the world.

Table 3: Commercially developed plant growth-promotion products from streptomycetes

Streptomycete active ingrediente or their metabolites	**Comercialized product**	**Target crop**	**Plant growth promotion effects**
Streptomyces lydicus WYEC 108	Actinovate® SP/ Actinovate® AG (seed application)	Grass, ornamentals, vegetables and forest species in greenhouses, nurseries and more.	Biocontrol by soilborne plant pathogens and foliar diseases
	Micro108® soluble/ Micro108® Seed Inoculant	Food and fiber crops, ornamentals, turf grass, landscape plants, including tree seedlings for transplanting to the forest.	Enhances plant vitality and encourages more vigorous root systems
	Actino - Iron®	For indoor/outdoor greenhouse, nursery, turf grass, ornamental plant, and field uses.	Biocontrol by soilborne plant pathogens.
Streptomyces griseoviridis	Mycostop®	Seedling production, vegetables, herbs and ornamentals.	Biocontrol by soil and seed-borne pathogens.
Streptomyces avermitilis	AVID®0.15EC	Shadehouse, greenhouse, fiel-grown ornamentals, foliage plants, Christmas trees, and other woody ornamentals.	Biocontrol of leafminers, mites, and suppression of aphids, whiteflies, and thrips.
	VERMITEC® (additional brands AGRI-MEK®)	Cotton, citrus, pome fruit, nuts and vegetables.	Biocontrol of mites and insects.
	PROCLAIM® (additional brands AFFIRM®, DENIM ™)	Vegetables (*Brassica*, leafy and fruiting vegetables) and cotton.	Lepidoptera. Side effects on mites, leafminers and thrips.
	AVICTA®	Cotton, corn and soybean	Biocontrol of nematodes.

Adapted from Hamedi and Mohammadipanah [63]

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CAPÍTULO 2

MICROBIOLOGICAL AND AGRO-BIOTECHNOLOGICAL CHARACTERIZATION OF HUMIC ACID USING-STREPTOMYCETES ISOLATED FROM MATURED VERMICOMPOST.

ABSRACT

Microorganisms and organic matter play crucial role as sources for a more sustainable agricultural practices. Using humic acid as a sole C-source in solid medium plates it was obtained sixteen actinobacteria-like isolates from matured vermicompost materials. Based on colony morphology, six isolates were selected as representatives for further studies. Colony appearance in AGS medium and light microscopy observation confirmed that all isolates belong to the *Streptomyces* genus from actinobacteria phylum. Partial 16S-rDNA sequence analysis confirmed morphological data and provided species level identification for all isolates that was assigned as *Streptomyces diastaticus* strain AC01 and AC15, *S. hyderabadensis* strain AC05, *S. bellus* strains AC06 and AC09 and *S. variabillis* strain AC31. Clustering analysis based on BIOLOG SFP2^{TM} system had shown 70% similarity level for all isolates with four clusters that correspond to the 16S rDNA phylogenetic assigned to the four species. Nitrogen nutritional requirements were also evaluated in solid medium revealing that arginine, yeast extract, urea, peptone, soybean tryptone and tryptone type I were the best sources for faster growth in comparison with N-inorganic sources, as well showed remarkable influence of the N-source in colony appearance and pigmentation. Most of the isolates were tolerant to 3% sodium chloride added to the medium (except AC15 and AC31) and had no growth under pH 5.5 (except AC01 and AC05), showing optimal pH for growth at 6.5 to 7.0. Related to the production of extracellular enzymes, all isolates were able to produce catalase, most of them were lipase and amylase positive and none had shown cellulose and xylanase activity. Positive plant growth promoting attributes showed that indole compounds were mainly detected in tryptophan added medium, most of the isolates were able to solubilize calcium phosphate and no one able solubilized zinc oxide. Micro-Tom (MT) tomato seedlings linked with DR5-GUS reporter treated with AC05 strains metabolites had shown increased number of mitotic sites and lateral root, as well reporter activity of β-Glucoronidase linked with auxin-responsive genes related to control plants. Gnobiotic studies using maize seeds (var. UENF 506-11) indicated great influence of the metabolites secreted by the *Streptomyces* spp. ranging from 25 to 89 % of seed germination in 96 h assay. Maize biomass accumulation over 10 days was significantly positively influenced by the inoculation related to control plantlets with root and shoot fresh mass respectively ranging from 27.8 to 77.2% and 20 to 58.2%, as well root and shoot dry mass respectively ranging from 26.9 to 111.11 % and 53.7 to 119.9%. We showed that vermicompost represent a potential source for *Streptomyces* humic-acid-users and underlining one of the plant growth promoting mechanisms induced by actinobacteria strains used.

Keysword: *Streptomyces* spp., biofertilization, plant growth promotion.

INTRODUCTION

The exponential growth of human population requires high crop yields in agricultural ecosystems. In global aspects, chemical fertilizers are the most used method to replace nutrients removed from the soil by harvested crops. However, environmental costs of the indiscriminate use of synthetic inorganic fertilizer include freshwater, groundwater and marine contamination, eutrophication of water bodies, depletion of soil quality and increased greenhouse gases emission which overall contribute to environment depreciation and would compromise earth life for future generations [2, 3]. Therefore, scale development of renewable low-energy input based technologies represents a great challenger to fulfilling nutrient demands in global agricultural systems. Plant-beneficial microorganisms and organic matter play crucial role as biological sources for a more sustainable agricultural practices with wide potential as agro-biotechnological designed products.

Several studies have reported the use of vermicompost (stable organic matter from different raw materials produced by earthworm activity) as an alternative technology to improve plant growth [4-6]. Besides the physic-chemical quality and presence of bioactive substances [6, 7], the benefits performed by vermicomposts to induce plant growth are also related to the high diversity of microorganism-associated including fungi, bacteria and actinobacteria [8]. In this sense, even less explored than soil, rhizosphere soil and plant tissue compartments, vermicomposts represent an important natural source for isolation surveys of plant growth-promoting microorganisms, being prospective candidates for agricultural bioinoculants.

More than four hundred million years of co-evolutionary genetic basis for beneficial microbe-plant interactions gave rise to multiples plant-growth promoting (PGP) traits that increases plant fitness by facilitating nutrient resource acquisition into the natural ecosystems. Biological nitrogen fixation, mineral solubilization (P, Zn, K, etc) and organic matter mineralization coupled with nutrient recycling (microbial enzymes) are among the most relevant environmental services that increase nutrient bioavailability for plants (biofertilization effects). Other group of PGP mechanisms emerges from the production of hormone-like compounds and other bioactive molecules that under micromolar to nanomolar concentration modulate uptake, assimilation and distribution of the nutrient over the plant body. Such biostimulation effects have been well described for architectural and biochemical changes in the root

system, which in many cases, affect in a positive manner plant growth and development [9-11].

Currently, microbial inoculants are the main technological approach to at least partially replace the global use of mineral fertilizers. Almost all of the market available bioinoculants are made up with α-proteobacteria or β-proteobacteria species with a broad range of a field plant response and inconsistent results. New formulations with improved inoculation performance would be obtained with wider environmental prospection for microbial diversity. Moreover, systematic screening must to consider soil saprophytic competence, ability to respond to plant rhizosphere exudates and multiples plant growth promotion traits. In this study, we proposed a simple solid medium containing humic acid as a sole C-source to access the culturable microbial community associated to matured vermicompost. Since prompt available carbon sources are scarce in the soil system, the ability to access stable organic matter would represent a competitive advantage related to overall microbial community. Remarkably, actinobacteria-like colonies were predominant and all isolates obtained were confirmed as belonging to *Streptomyces* genus. Six representative isolates were tested for different agro-biotechnological features and tested for effective plant-growth promotion in maize and tomato seedlings under gnotobiotic conditions.

MATERIAL AND METHODS

Actinobacteria isolation procedures in vermicompost material

The microorganisms were isolated from 120-d matured vermicompost using sunflower cake (biodiesel byproduct) and sugarcane filter cake (bio-ethanol byproduct) as raw materials. To perform it, 10 g of each material were collect and diluted in 90 mL of saline solution (0.85% NaCl). The suspension was agitated in an open rotatory shaker at 150 rpm for 1 h (10^{-1} dilution). After that, 1 mL of the suspension was collected with a pipette and dropped into a tube containing 9 mL of saline solution (10^{-2} dilution), being successively diluted until 10^{-6} (serial dilution technique). Aliquots of 0.1 mL were spread on the surface of a solid medium and the Petri plates were incubated at 30 ºC for 7-d. The solid medium was specially formulated for the present study with the mineral composition of the JNFb solid medium, where malate C-source

was replaced by humic acid as sole C-source (extracted from 120d vermicomposted cattle manure), plus NH_4Cl as N-source. The media composition (g per L) was: Humic acid (0.025 g C); K_2HPO_4 (0.6 g); KH_2PO_4 (1.8 g); $MgSO_4.7H_2O$ (0.2 g); NaCl (0.1 g); $CaCl_2.2H_2O$ (0.02 g); Fe•EDTA (solution 1.64%) (4 ml); 2 mL of micronutrients solution [$Na_2MoO_4.2H_2O$ (0.2 g), $MnSO_4.H_2O$ (0.235 g), H_3BO_3 (0.280 g), $CuSO_4$ (0.008 g), $ZnSO_4.7H_2O$ (0.024 g) in distilled water (200 mL)]; 1 mL of vitamin solution [Biotina (0.01 g), Pyridoxol-HCl (0.02 g) and distilled water (100 mL)], NH_4Cl (0.1 g), agar (15 g), and distilled water until complete 1000 mL, pH adjusted to 6.5. Followed the incubation period, colonies were visible on plates at 10^{-3} to 10^{-4} dilution with clear predominance of actinobacteria-like colonies. Later on, actinobacteria colonies were recognized by their both macroscopic and microscopic properties [23]. Selected colonies were then inoculated onto arginine glycerol salt agar (AGS) [16] for purification. Purified colonies were photodocumented and cell morphology obtained using light microscopy. Later on, the isolates were stored in 20% glycerol at -80ºC. Six out sixteen isolated were selected based on morphological features, and initially designated AC01, AC05, AC06, AC09, AC15 and AC31.

Molecular characterization

Actinobacteria genomic DNA of six isolates (AC01, AC05, AC06, AC09, AC15 and AC31) was extracted using the UltraClean® Microbial DNA Isolation Kit (MoBio®) following manufacturer's instructions and quantified in 3000 NanoDrop (Thermo Scientific USA). DNA quality was analyzed using a 0.8% agarose gel stained with gelred. The amplification of 16S rDNA gene was carried out by polymerase chain reaction (PCR) using the primers 27F (5'-AGAGTTTGATC(AC)TG GCTCAG-3') and 1492R (5'-ACGG(CT)TACCTTGTTACGACTT-3'). PCRs mixture used include 1 µL of each primer, 1 µL of dNTPs mix (0.25 mM each), 5 µL of $MgCl_2$ (2.5 mM), 5 µL of 10x PCR buffer, 1 µL of template DNA (50 ng), 1 µL *Taq* polymerase (2.5 U) and 35 µL of sterile water, in total volume of 50 µL. The PCR reaction conditions were as follows: 3 min at 95 ºC for one cycle followed by 30 cycles of 1 min each at 94 ºC, 30 s 55 ºC, and 30 s 72 ºC, and finally one cycle for 10 min at 72 ºC, in 96-well thermocycler Veriti model (Applied Biosystems). PCR products were visualized using 1% agarose gel electrophoresis under UV light. Sequencing reactions were performed using the Big Dye Terminator Sequencing Kit-Cycle Sequencing Ready ABI Prism version 3 (Life

Technologies, USA). Furthermore, six partial sequences were subjected for comparison with the homologous sequences available at GenBank (https://www.ncbi.nlm.nih. gov/genbank/) using BLAST program (http://www.ncbi.nlm.nih.gov/blast/).

Use of different carbon and nitrogen sources

Carbon source use were investigated using BIOLOG SFP2^{TM} plates following manufacturer's instructions, where we tested the ability of the six streptomycetes isolates (AC01, AC05, AC06, AC09, AC15 and AC31) to use 95 carbon sources. To perform it, spore suspensions of each strain were prepared in 0.2% agar-water. Spore concentrations were adjusted to an optical density of 0.20 at 590 nm. An aliquot (100 µL) of a dilution containing 1.5 mL of the spore suspension in 13.5 mL 0.2% agar-water was inoculated into each well of the BIOLOG SFP2^{TM} plate. The inoculation with the same volume of sterilized water in one BIOLOG SFP2^{TM} plate was used as negative growth control. Then, the plates were incubated at 28 ºC for 6 days. After the incubation, the absorbance of each well at an optical density of 590 nm was recorded in spectrophotometer (Shimadzu UV-visible - 1240). The absorbance of the control plate (blank measurement) with water was subtracted from absorbance of each well carbon source. Data was organized in excel spreadsheets recording positive (1) or negative (0) growth for all isolates in each carbon sources. The index "Simple Matching" was obtained by algorithm Unweighted Pair Group Method with Arithmetic Mean (UPGMA) and the actinobacteria strains were clustered and graphically represented by a similarity dendrogram.

The ability to growth using different nitrogen was evaluated by streaking of the six actinobacteria isolates in basal culture media with the following composition: K_2HPO_4 (1 g); NaCl (1 g); $MgSO_4.7H_2O$ (0.5 g); micronutrients solution (1 mL), agar (20 g) and distilled water (1000 mL). Different inorganic nitrogen source (ammonium chloride, ammonium sulphate, urea) and organic nitrogen source (L-arginine, yeast extract, bacteriological peptone, tryptone, tryptone type-1) were added separately at 1.25% (w/v) concentration to the basal media and the pH adjusted to 7.2. The plates were incubated at 28 ºC for 6 days and the growth behavior described as: much growth (+++), medium growth (++), low growth (+) and no growth (-). Colony appearance was

two strains were also photodocumented to show the pigmentation pattern variation under different nutritional resources.

Production of extracellular enzymes

The cellulolytic and xylanolytic activity was determine preparing dishes containing AGS solid culture media supplemented with 1% CMC (carboxymethyl cellulose) or xylan [17]. The inoculum was prepared from spore-forming colonies growth in AGS solid medium. Spore were suspended in sterilized water and adjusted for optical density of 0.20 at 590 nm. An aliquot (20 µL) of the six actinobacteria strains were inoculated in spot shape on the center of the surface medium on Petri plates and were incubated at 28 ºC for 10 days. After this period, colonies were flooded with 10 mL of 0.5% Congo red solution for 15 minutes and then washed with 1M NaCl. The occurrence of enzymatic activity was detected observing a clearing zone around the colonies. The detection of lipases activity was determined inoculating as described above all the actinobacteria isolates in Sierra solid culture media [18] supplemented with Tween 80 as carbon source. The Petri plates were incubated at 28 ºC for 10 days and the production of lipases was indicated by opaque halo formed by calcium oleate precipitate around the colonies. Catalase production was accessed by streaking loop full of the actinobacteria biomass on glass slides and the smears were allowed to dry. One drop of 3% H_2O_2 was added to slide and the occurrence of oxygen bubbles indicated the catalase activity [19]. For amylase activity detection, the inoculum prepared as described above was inoculated in Petri plates containing Starch-agar (HIMEDIA®) culture media with 6.6 % of soluble starch [20]. The Petri plates were incubated at 28 ºC for 10 days. After incubation, starch hydrolysis was detected by the addition of Lugol's iodine solution and a clear zone surrounding the colonies observed. The enzymatic index (E.I.) was determined by the following formula: E.I. = D_h/D_c; D_h- diameter of hydrolytic zone; D_c- diameter of colony.

Calcium phosphate and zinc oxide solubilization

To assess the ability to solubilize calcium phosphate or zinc oxide, an aliquot of 2 µL of AGS liquid media, cultivated with all the *Streptomyces* isolates was used to

inoculate the center of Petri dishes containing the solid medium. The medium used to evaluate calcium phosphate solubilization [21] has the following composition: 10 g L^{-1} glucose, NH_4Cl (5 g L^{-1}), NaCl (1 g L^{-1}), $MgSO_4.7H_2O$ (1 g L^{-1}), $Ca_5(PO_4)_3OH$ (1.0 g L^{-1}), agar (15 g L^{-1}), distilled water (1 L) and pH 7.0. The solid medium to evaluate zinc oxide solubilization [22] has the following composition: glucose (10 g L^{-1}), $(NH_4)_2SO_4$ (1 g L^{-1}), KCl (0.2 g L^{-1}), K_2HPO_4 (0.1 g L^{-1}), $MgSO_4.7H_2O$ (0.2 g L^{-1}), ZnO (1.0 g L^{-1}), agar (15 g L^{-1}), distilled water (1 L) and pH 7.0. The plates were incubated at 28 °C for 7 days. The positive solubilization ability was visualized by formation of a translucent halo around the colonies.

Production of related indole compounds

The production of indole acetic acid (IAA) by all actinobacteria strains was determined according to the method of Bano and Musarrat (2003). Discs (8 mm diameter) from colonies of the six actinobacteria isolates previously cultured on AGS media and incubated at 28 ºC for 5 days, were transferred to 5 mL AGS liquid media containing 2 mg mL^{-1} L-tryptophan (tryp +) or not (tryp -). These cultures were incubated at 30 °C in orbital shaking at 125 rpm for 7 days and then collected by centrifugation at 11 000 x *g* for 15 minutes. One milliliter of the supernatant was mixed with 2 mL of Salkowski reagent. The IAA production was indicated by appearance of a pink color and its absorbance was read at 530 nm using a spectrophotometer (Shimadzu UV-visible - 1240). The level of IAA produced by the isolates was estimated against a standard curve obtained with increased concentration of IAA.

Salinity and pH tolerance

Arginine Glycerol Salt Agar (AGS) media (Poter *et* al., 1960) was used to evaluate tolerance to salinity and pH of the six isolates. AGS culture media has the following composition: Arginine (1 g); glycerol (1.25 % v/v); K_2HPO_4 (1 g); NaCl (1 g); $MgSO_4.7H_2O$ (0.5 g); micronutrients solution (1 mL), agar (20 g), distilled water (1000 mL) and pH 7.2. The halotolerance of the actinobacteria was tested in AGS media supplemented with different concentrations (0.1 %, 1 %, 1.5 %, 2 %, 2.5 % e 3 % w/v) of NaCl. The pH tolerance was investigated by variations in initial pH (4.5, 5.0, 5.5, 6.0,

6.5 and 7.0) of the media. The actinobacteria isolates were streaked on each of the six salinity or pH plates values. The plates were incubated at 28 ºC for 7 days. An arbitrary scale was elaborated based on different quantitative growth behavior as: much growth (+++), medium growth (++), low growth (+) and (-) no growth.

DR5::GUS reporter Micro-Tom tomato assay

To evaluate a possible biostimulation mechanisms related to the actinobacteria isolates obtained, we applied the conditioned liquid medium containing the secreted metabolites (as described above) of the isolate AC05 in tomato plantlets (*Solanum Lycopersicum* L.) cv Micro-Tom DR5::GUS, which is a synthetic auxin-responsive gene promoter linked with to the reporter gene *gus* (beta-glucuronidase). Transgenic seeds were surface disinfested, sowed in Vivatto® substrate and inoculated with one milliliter of the AC05 growth liquid culture. Sterilized water or AGS liquid were used as control for the assay. Twenty-one day old plantlets were harvested and histochemical GUS staining was performed as described previously (Jefferson *et al*., 1987) with minor modifications: incubation time of the root segments for 16 hours in a drying oven at 37º C, using the X-Gluc solution (5-bromo-4-chloro-3-indolyl glucuronide). Root segments were observed under stereomicroscopy and light microscopy, where a clear blue color is developed as a product of the GUS activity in plant cells and tissues where auxin accumulates. Representative seedling phenotypes were photographed. Also, number of mitotic sites and lateral root were evaluated in five root segments from AC05 and control treatment. Seeds carrying the *DR5::GUS* gene were kindly provided by Dr. Lázaro Eustáquio Pereira Peres from Universidade de São Paulo, Brazil.

Assessment of maize growth promotion

The actinobacteria isolates (AC01, AC05, AC06, AC09, AC15 and AC31) were grown for 10 days in AGS liquid media and then centrifuged (10 000 x *g* for 30 min). The pellet was discarded and the liquid medium conditioned by the secreted compounds during the growth of the actinobacteria were sterilized by filter membrane 0,22 µm (Millipore®). At the first assay, the effect of the obtained “secreted metabolites” were tested on maize seed germination. To perform it, Germitest® paper was placed into a “gerbox” apparatus and were soaked with a solution prepared using 1 mL of

filtered extract metabolites diluted in 9 mL of distilled water. Maize seeds (*Zea mays* var. UENF 506-11) were sowed on soaked paper and the boxes were placed in a B.O.D. germination chamber at 28 ºC with light regime 12/12 hours of photoperiod (light/dark). After 96 hours, the germination percentage was calculated with the following formula: GR (%) = a/b x 100; GR- mean germination; a- number of germinated seeds; b- number of total seeds. The experimental design was completely randomized with 3 replicates and data transformed in arc sin $\sqrt{GR(\%)}/100$. For the second assay, spores of all actinobacteria strains were introduced in gnobiotic condition to evaluate the plantlets growth promoting effects. Maize seeds (*Zea mays* var. UENF 506-11) were surface disinfested with ethanol 70% (1 minute), Sodium hypochlorite 1% (5 minutes) and 3 times washes in sterilized water (5 minutes each wash). Followed by sowing the seeds in agar-water plates for pre-germination for 4 days in a germination chamber at 28 ºC with light regime 12/12 hours of photoperiod (light/dark). Seedlings with 2.5 cm long radicles were inoculated with spore suspensions of the isolates at optical density of 0,4 at 560 nm. The inoculation was performed by immersion of seeds in 1 mL of suspension for 30 min with a total of six treatments (AC01, AC05, AC06, AC09, AC15 and AC31). Water was used as a control. The seeds were transferred to glass tubes (4.0 cm x 20 cm, diameter and height) containing 40 g of an autoclaved substrate (washed sand: vermiculite; 2:1 w/w). The experiment was conducted in a growth chamber room at 28 ºC with light regime 12/12 hours of photoperiod (light/dark). Plantlets were irrigated daily with sterilized water and after 10 days the plantlets were collected for measurement of the following variables: root and shoot fresh matter, and root and shoot dry matter. The experimental design was completely randomized with 4 replicates.

Statistical analysis

The results were analyzed using the SISVAR (Ferreira, 2000) statistic program and submitted to ANOVA F-test and, when significant, means comparison were performed using Skott & Knott tests at the $P < 0.05$ probability level.

RESULTS

Isolation of actinobacteria associated with matured vermicompost

Using a solid medium with the mineral composition of the JNFb medium (Baldani et al., 1992) and an added low concentration of humic acid (0.025 g L^{-1}) as a sole C-source replacing C-malic acid (5 g L^{-1}) we were able to obtain isolated colonies in serial dilutions of 10^{-3} to 10^{-4}. The colonies were very small (less than 1 mm), dry, light to dark brown, resembling actinomycete. At the end of the isolation procedures, a total of sixteen actinobacteria-like isolates were obtained from 120-d matured vermicompost using sunflower cake (biodiesel byproduct) and sugarcane filter cake (bio-ethanol byproduct) as raw materials. However, based on similarities in colony and cell morphology (Fig. 01), six distinctive isolates designated (AC01, AC05, AC06, AC09, AC 015 and AC31) were selected as representatives for further studies.

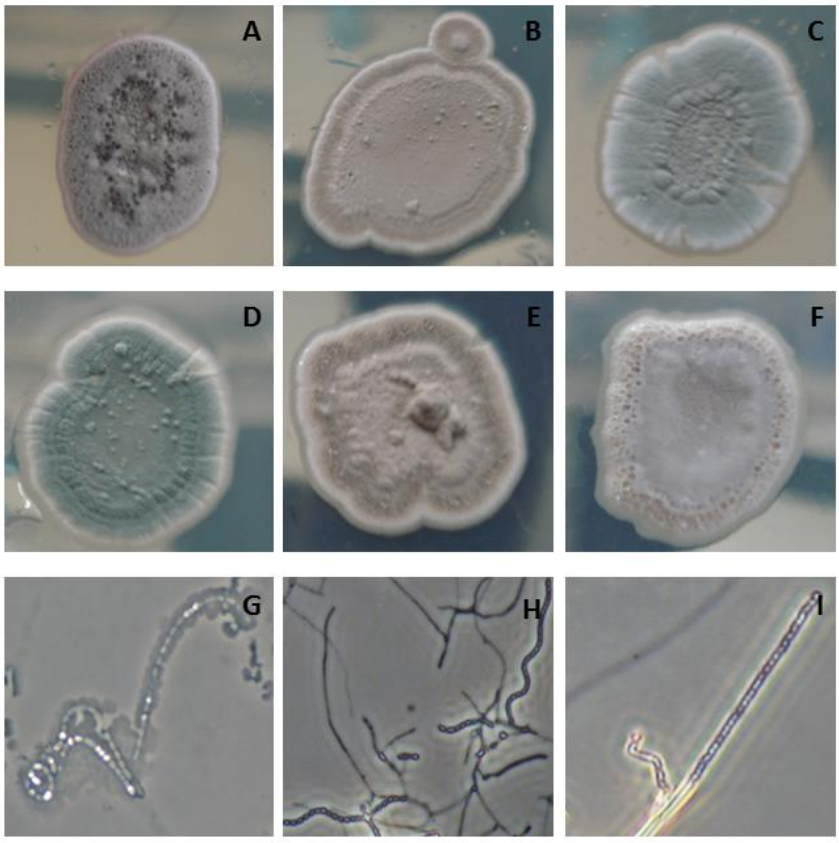

Figure 1. Colony morphology and spore arrangement of representative *Streptomyces* isolates (**A, G** - *S. diastaticus* strain AC01; **B** - *S. hyderabadensis* strain AC05; **C**, **H** - *S. bellus* strain AC06; **D** - *S. bellus* strain AC09; **E**, **I** - *S. diastaticus* strain AC15; **F** - *S. variabilis* strain 31). Bars = 5 µm.

Identification of actinobacteria isolates

Phenotypic characteristics of the six isolates (colony appearance in AGS solid media, spore-mass color, spore mass arrangement and cell morphology under light microscopy) were consistent with their placement in the genus *Streptomyces* genus (Fig. 1; Table 1) [23]. The phylogenetic position of all strains as *Streptomyces* spp. was confirmed based on partial 16S rRNA gene sequence region using BLAST sequence analysis tool, allowing also assign of four species. The 16S rRNA genome sequence of the strain AC01 (848 bases) and AC15 (1277 bases) showed 99% identity with that of *Streptomyces diastaticus*. The strain AC 05 (609 bases) showed 95% identity with *Streptomyces hyderabadensis* strains AC 06 (1308 bases) and AC09 (1277 bases) showed greatest identity with *Streptomyces bellus* (99%) and strain AC31 (1275 bases) as *Streptomyces variabilis* (99%) (Table 01). The 16S rRNA sequences of AC01, AC05, AC06, AC 09, AC15 and AC31 strains were submitted to the NCBI GenBank with accession numbers from KX845573 to KX845578, respectively.

Carbon and nitrogen nutritional requirements

The pattern of 95 carbon source catabolism obtained with the BIOLOG SFP2™ system revealed a versatile metabolic profile of all *Streptomyces* isolates. The number of carbon source used ranged from 55 (*Streptomyces variabilis* strain AC31) to 68 (*S. hyderabadensis* strain AC05 and *Streptomyces bellus* strains AC 06). The common core carbon profile was 51 C-sources (53,7%) with 44 C-sources (46.3%) being discriminative for at least one strain. The *Streptomyces variabilis* strain AC31 was the most distant strain with 15 distinguishable carbon sources (positive for D-Fructose-6-phosphate. N-Acetyl-D-glucosamine and α-D-Glucose; negative for Adenosine-5-monophosphate, Salicin, D-Raffinose, α and β-metil-D-Glycoside, D-Melibiose, L-Fucose, β-metil-D-Galactoside, Succinamic acid, α -metil-D-Mannoside, 2-Deoxi-adenosine and D-Glucose-6-phosphate). D-Mannitol, L-Rhamnose, Glycerol and Acid-D-gluconic were separated AC06 and AC09 (*S. bellus* group) from AC01, AC05 and AC15. Cluster analysis of C-use profile similarities between the six *Streptomyces* strains (Fig. 2) showed 70% similarity level for all isolates with 4 similarities groups. The most distant cluster was represented by the AC 31 strain of *S. variabilis*, followed by a cluster containing AC 06 and AC09 (*S. bellus* group). At 84 % similarity two more

separated groups were formed, one containing AC05 strain (*S. hyderabadensis* cluster) and other grouping AC 01 and AC15 strains (*Streptomyces diastaticus* cluster). The dendrogram obtained from carbon use profile had shown a good agreement with molecular identification based on partial 16S r-RNA sequencing.

The growth in solid medium containing different inorganic and organic N-sources were also evaluated. All of six *Streptomyces* isolates were capable to use the different N-organic sources tested, except AC 31 for arginine and urea (Table 2). Arginine, urea and the media with nitrogen complex composition (yeast extract, peptone, soybean tryptone and tryptone type I) were the best sources for faster growth in comparison with N-inorganic sources (N-NH_4^+). Among the six strains, AC 15 and mainly AC31presents the more distinct N-nutrient requirement. Changes in colony appearance and pigmentation were also noted for the strains according with organic (L-arginine, yeast extract, bacteriological peptone, tryptone, tryptone Type-1) or inorganic (ammonium chloride, ammonium sulphate, urea) nitrogen source (Fig. 3).

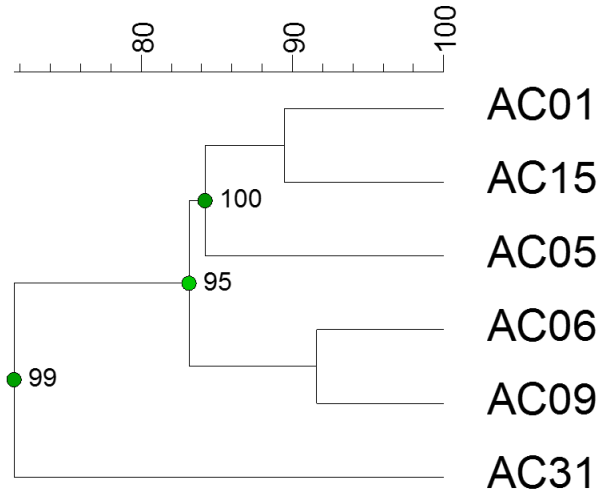

Figure 2. Dendrogram based on the similarity between the biochemical profiles of six *Streptomyces* isolated from matured vermicompost. Tree was generated by the UPGMA algorithm and similarity matrix calculated by the index "Simple Matching" from the use of 95 carbon sources BIOLOG™ system.

Table 1. Phenotypic characteristics and distribution of 16S rDNA sequences from six actinobacteria isolated from matured vermicompost in solid medium with humic acid (25 mg. L^{-1}) as a sole C-source.

Strain identification	Vermicompost raw material origin and dilution isolated	Aerial spore-mass colour	Spore Arrangement	Identification based on 16S rDNA gene†	Max ident (%)‡	bp	Accession number§
AC01	Sunflower cake (-3)	Red	*Spira* - Open loops	*Streptomyces diastaticus*	99	848	NR_112454.1
AC05	Sunflower cake (-4)	Gray	*Rectus flexibilis* - Flexuous	*Streptomyces hyderabadensis*	95	609	NR_116934.1
AC06	Sunflower cake (-4)	Blue	*Rectus flexibilis* - Flexuous	*Streptomyces bellus*	99	1308	NR_041222.1
AC09	Sunflower cake (-4)	Blue	*Rectus flexibilis* - Flexuous	*Streptomyces bellus*	99	1279	NR_041222.1
AC15	Sugarcane Filter cake (-4)	Gray	*Rectus flexibilis* - Straight	*Streptomyces diastaticus*	99	1277	NR_112454.1
AC31	Sunflower cake (-4)	White	*Rectus flexibilis* - Flexuous	*Streptomyces variabilis*	99	1275	NR_043840.1

† Organism with the partial 16S rDNA sequence with greatest identity to that of the respective isolate.
‡ Percentage of sequence identity between the sequence of fruit tree isolate and the related organism.
§Accession number of the related organism sequence.

Table 2: Growth of *Streptomyces* strains in AGS media with different concentrations of NaCl, pH levels and nitrogen sources.

Strain identification	Growth																			
	NaCl (%)						pH						Nitrogen source							
	0,1	1,0	1,5	2,0	2,5	3,0	4,5	5,0	5,5	6,0	6,5	7,0	Arginin	NH_4Cl	YE	$(NH_4)_2SO$	Urea	BP	T	TI
AC01	+++	+++	+++	++	++	++	-	+	+	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
AC05	+	-	+	+	+	+	-	+	+	++	+++	+++	+++	++	+++	++	+++	+++	+++	+++
AC06	+++	++	++	++	++	+	-	-	-	++	+++	+++	+++	++	+++	+	+++	+++	+++	+++
AC09	+++	+	++	++	++	+	-	-	-	++	++	+++	+++	++	++	+	++	+++	+++	+++
AC15	++	++	++	++	++	-	-	-	-	++	++	+++	+++	-	+	++	++	+	+++	+
AC31	+	++	-	-	-	-	-	-	-	+	+	+	-	-	+	-	-	+	+	+

(+++) Much growth; (++) Median growth; (+) Low growth; (-) No growth. YE: Yeast extract; BP: Bacteriologic peptone; T: Tryptone and TI: Tryptone type I.

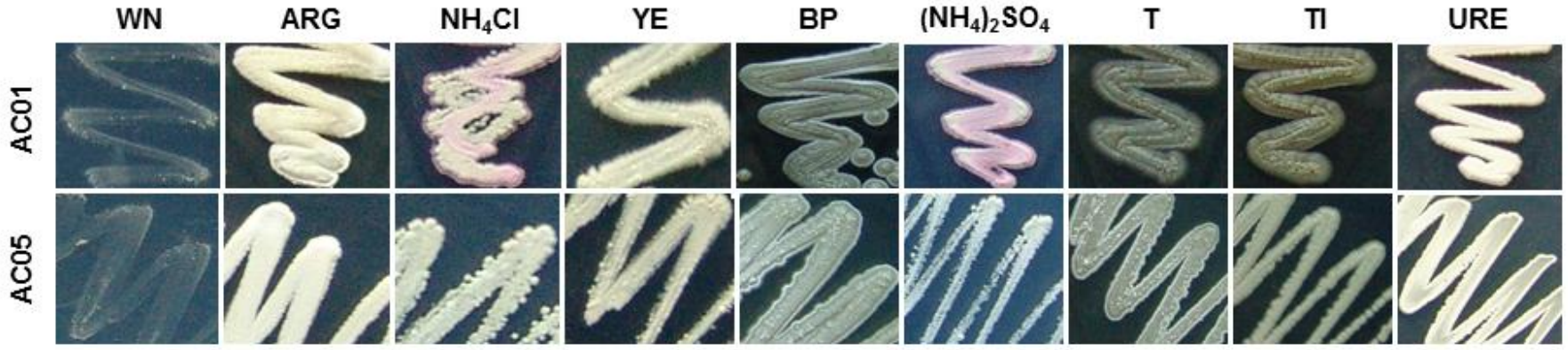

Fig. 3: *Streptomyces diastaticus* strain AC01 e *Streptomyces hyderabadensis* strain AC05 cultured in different nitrogen sources. WN: Without nitrogen; ARG: Arginine; YE: yeast extract; BP: bacteriologic peptone; T: Tryptone; TI: Tryptone type I; URE: Urea.

Other Physiological Features

Related to sodium chloride and initial pH value tolerance in AGS solid medium, most of the isolates were tolerant to 3% NaCl (except AC15 and AC31). The optimal pH level for *Streptomyces* strains were observed at 6,5 to 7,0 and no growth under pH 5,5 was observed (except AC01 and AC05). Overall results suggested that the *Streptomyces diastaticus* strain AC01 had shown the broadest range environment adaptation for salinity and acidity and *S. variabilis* AC31 the narrowest range.

All the six strains had shown catalase, amylase (except AC01) and lipase (except AC31) enzymatic activity. Both xylanolytic and cellulolytic activity were not detected (Table 3). Besides extracellular enzyme production and biosolubilization activity were tested for calcium phosphate and zinc oxide (Table 03). All the strains (except AC 01) were able to convert insoluble P-calcium in available P, and Zn solubilization activity was not noted. Regarding to biostimulation traits it was observed that the six strains were able to produce indole compounds, when tryptophan was supplemented into the AGS liquid media (Table 3). The highest detected value for indole production was noted for *S. hyderabadensis* strain AC05 (34,8 ± 0,02 µg mL^{-1}). Tryptophan-independent indole production was only observed for *Streptomyces diastaticus* strain AC01.

Micro-Tom DR5::GUS reporter response to Streptomyces secreted metabolites

Microscopic of Micro-Tom *DR5::GUS* transgenic tomato plantlets inoculated with AGS liquid medium conditioned by *Streptomyces hyderabadensis* strain AC05 compound secreted compared with control treatment (plants treated only with AGS medium components) was observed (Fig. 4). Plantlets treated with *S. hyderabadensis* strain AC05 secreted compounds had shown β-glucosidase activity in lateral root mitotic sites over the root axis (Fig. 4B, 4I), root tip (Fig. 4C, 4G, 4H) and pericyclic layer of the vascular tissue system (Fig. 4D). Contrary, control plants had much less visible expression of β-glucosidase activity (Fig. 4A, 4E, 4F), with few blue color associated with lateral root mitotic sites and vascular system layer boundaries. Interesting to quote that border cell were released from axial and lateral root in the presence of AC06 metabolites and expressed blue color (Fig. 4G, 4H). Furthermore, root segments of tomato cv Micro-Tom plantlets were evaluated for architectural

changes related to AC06 and had shown significant increased number of mitotic sites and lateral root related to control plantlets (Fig. 5).

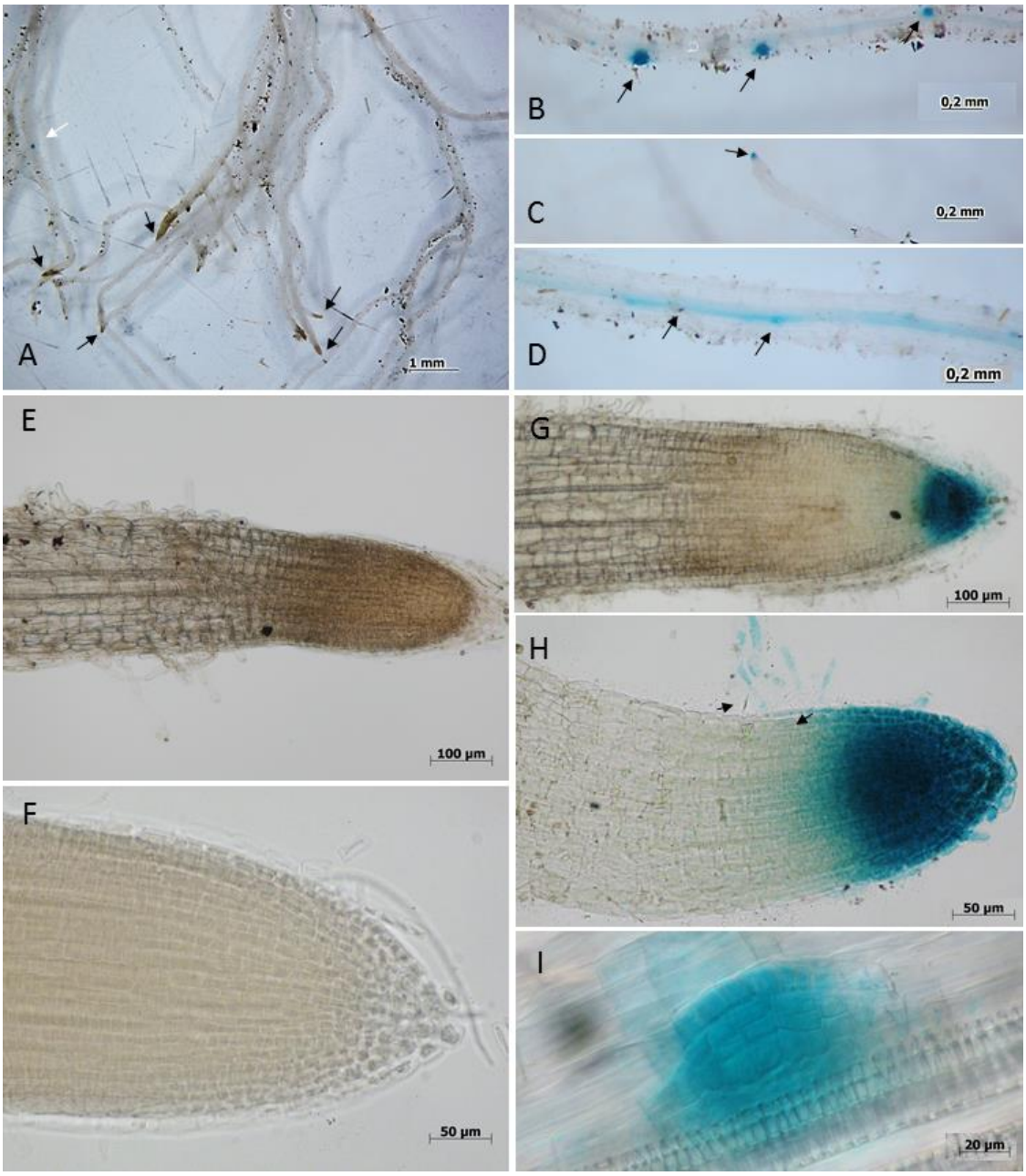

Figure 04: Stereomicroscopy (Figs. A – D) and Light microscopy (Figs. E – I) of Micro-Tom *DR5::GUS* transgenic tomato plantlets (21 days after germination) inoculated with AGS liquid medium conditioned by *Streptomyces hyderabadensis* strain AC05 compound secreted compared with control treatment (plants treated only with AGS medium components). Fig. A: Control plants without visible expression of β-glucosidase activity (arrows); Fig. B: Plants treated with *S. hyderabadensis* strain AC05 secreted compounds showing β-glucosidase activity in mitotic sites over the root axis (Fig B, arrows), root tip (Fig. C, arrow) and pericycle layer of the vascular tissue system; Fig E-F: lateral and axial root segment showing no of β-glucosidase activity in control plants; Fig G-H: Prominent β-glucosidase activity in root tips of lateral and axial root, respectively in plants treated with AC05 secreted compounds. Note border cell released from in both roots; Fig. I: Detail of mitotic site and cell division pattern induced by AC05 secreted compounds.

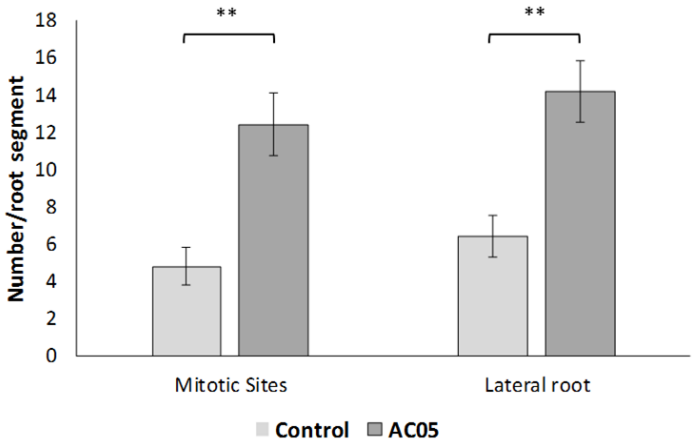

Figure 5. Number of mitotic sites and lateral root per root segment of twenty-one day old *DR5::GUS* reporter transgenic Micro-Tom (MT) tomato seedlings treated AGS liquid medium containing secreted metabolites from *Streptomyces hyderabadensis* strain AC05 compared with control plants treated only with AGS medium components. Data represent means of 5 replicates (root segments) and Student test was performed at $p < 1\%$.

Table 03. Extracellular enzyme production and plant growth promoting characteristics of six actinobacteria isolates obtained from matured vermicompost.

Strain identification	Catalase activity	Cellulase activity	Xylanase activity	Lipase activity	Amylase activity	Solubilization of ZnO	Indol production (µg/ml)		Solubilization of Calcium Phosphate
							Absence Tryptophan	Presence Tryptophan	
AC01	+	-	-	2.20 b†	n.d.	-	1,9 ± 0,01	12,7 ± 0,04	-
AC05	+	-	-	1.52 d	2.12 a	-	n.d.	34,8 ± 0,02	+
AC06	+	-	-	1.85 c	1.10 c	-	n.d.	30,6 ± 0,09	+
AC09	+	-	-	1.67d	1.22 c	-	n.d.	26,9 ± 0,01	+
AC15	+	-	-	2.72 a	1.80 b	-	n.d.	18,0 ± 0,01	+
AC31	+	-	-	n.d.	2.10 a	-	n.d.	16,1 ± 0,02	+

† Means followed by the similar letter do not differ by Tukey test at 5% probability; n.d. = Not detected.

Gnobiotic studies for maize growth promotion response inoculated with the *Streptomyces* strains

Remarkable effect of the secreted metabolites of the six *Streptomyces* over maize seed germination was observed after 96 hours after germination (Table 04). *S. hyderabadensis* strain AC05 metabolites significantly promoted germination related to the control (water or AGS medium), as well related to all strains tested. The second best strain was AC06 (*S. bellus*), followed by a group that has no positive effect on maize germination rates (AC01, AC09 and AC15), and do not differ from control treatments. On the other hand, a significant reduction of germination percentage was observed for seeds treated with metabolic extract produced by *S. variabilis* strain AC31.

Table 4: Percentage of germination of maize seeds treated with metabolic extracts of *Streptomyces* strains.

Treatments	Germination percentage (%)*
Control A**	46.6 c
Control B***	43.3 c
AC01	55.0 c
AC05	89.0 a
AC06	63.3 b
AC09	43.3 c
AC15	43.3 c
AC31	25.0 d
CV (%)	**20.6**

*Data presented as original values of percentage of germination, but data for statistical analysis were transformed for $arc\ \sin\sqrt{(x/100)}$. Mean followed by same letter in the column did not differ by Scott-Knott test at 5% probability.

**Control A mean application of the same volume of distillated water.

***Control B mean application of the same volume of AGS liquid medium.

Maize plantlets biomass accumulation over 10 days after *Streptomyces* spp. inoculation was observed for all the six strains related for non-inoculated plants (Fig. 06) with root and shoot fresh matter respectively ranging from 27.8 to 77.2% and 20.0 to 58.2%. Similar records were obtained for root and shoot dry mass respectively ranging from 26.9 to 111.11 % and 53.7 to 119.9% related to control. Here, the most effective strains for root biomass accumulation were AC06, AC15 and AC31, and for shoot biomass accumulation. *S. hyderabadensis* strain AC05, even better than control

plantlets, had shown less effective plant growth promotion activity compared to the five other strains.

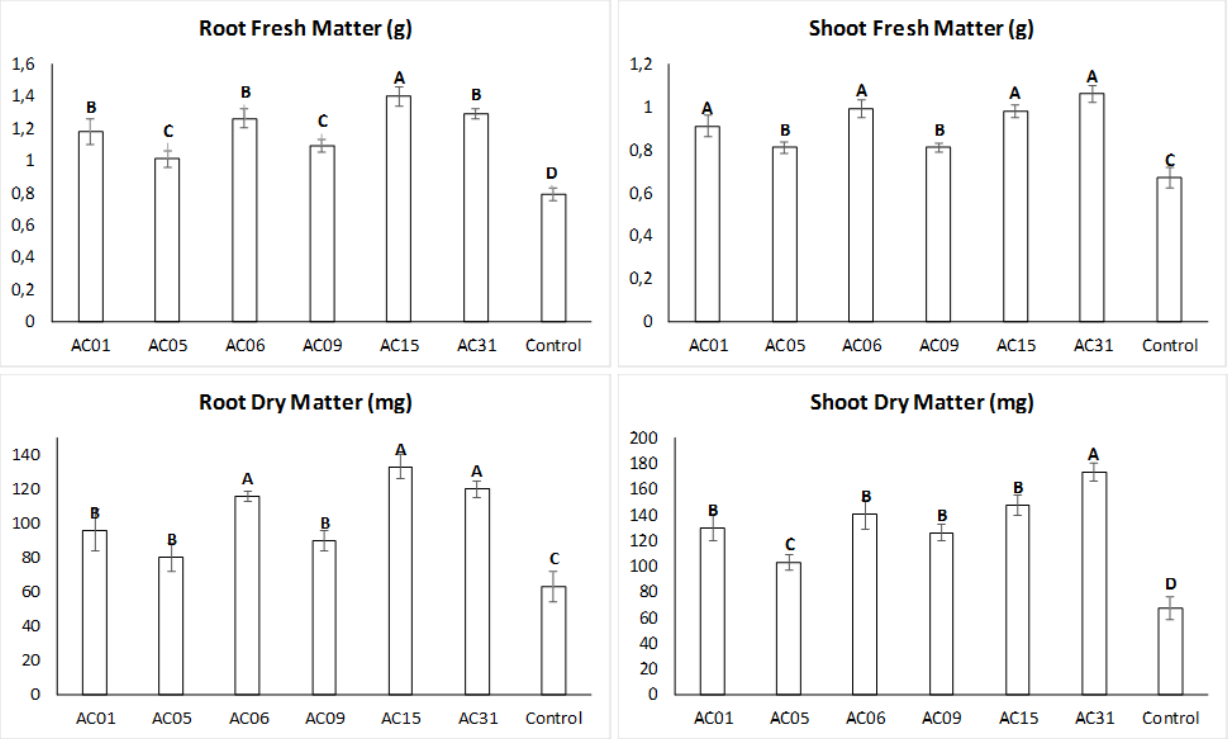

Figure 6: Biomass accumulation of maize plantlets variety UENF 506-11 growing under gnobiotic condition and inoculated with six different *Streptomyces* isolates strains AC01, AC05, AC06, AC09, AC15 and AC31, which representing six inoculated treatments and one non-inoculated control. Plant parameters evaluated are root fresh matter (A), root dry matter (B), shoot fresh matter (C) and shoot dry matter (D). Data represent mean of 3 replicates grouped by Scott-Knott test at 5% probability.

DISCUSSION

In this study, sixteen actinobacteria strains were isolated from vermicompost of sunflower and sugarcane filter cake being six isolates (AC01, AC05, AC06, AC09, AC15 e AC31) selected as representatives for microbial characterization and screening for agro-biotecnological features related to plant growth promotion. There are scarce prospective ecological surveys applied to vermicompost materials compared to environmental sampling from diverse soil-plant system. Considering the extensive use of compost and vermicompost in organic agriculture and its beneficial effects on soil biota and soil structure, as well its ability to promote plant growth and

inhibit plant pathogens, it would be presumable the huge potential of this material as a source of beneficial microorganism for agro-biotechnological.

The predominance of actinobacteria isolates in such high microbial diversity associated material can be explained by the use of humic acid (HA) as a sole carbon source during the isolation procedures. Soil organic matter (SOM) plays a central role in soil structure, fertility and biological activity and HA represent an operational fraction of the stable SOM that is well recognized as microbial recalcitrant material. However, it is also well quoted that some microbial groups have the ability to degrade HA especially actinomycete group, which are the most active in its degradation. It's reasonable to postulate that microorganism able to use stable organic matter as a carbon nutritional source has competitive saprophytic advantages related to other members of the soil microbial community. Thus, survival and competitiveness in soil, one of the most critical aspects of the microbial inoculation biotechnology would be partially overcome considering such feature in microbial screening.

Polyphasic taxonomical approach combining morphological properties and partial 16S rRNA sequence analysis revealed that all six isolates belong to *Streptomyces* genus from actinobacteria phylum. This result is consistent with the fact that many soil *Streptomyces* spp. have been actively involved in the breakdown of soil organic matter and capable of degrading humic acid. Moreover, the predominance of *Streptomyces* genus among actinobacteria isolated from vermicomposts have been reported [24-27]. The actinobacteria occurrence is associated with thermophilic stage of composting process when recalcitrant molecules are degraded, which is an important process for humus production [28].

The closest species affiliation for the six *Streptomyces* strains based on the maximum identity percentage were *S. variabillis*, *S. hyderabadensis*, *S. bellus* and *Streptomyces diastaticus*. All the four species assigned have been originally isolated from soil samples and produces antibiotics (*S. bellus:* althiomycin; *S. variabillis*: variapeptin, citropeptin and ammosamide D; *S. diastaticus*: oligomycin A, oligomycin C, rimocidin). The former species is also recognized as one of the best producer of α-L-arabinofuranosidase activity, related to hemicelulose cell wall degradation. None of these species have been previously isolated from compost or vermicompost or ever have been investigated for plant growth-promoting potential. Great emphasis have been given *Streptomyces* capabilities to produce extracellular enzymes and several

secondary metabolites with antibiotic activity, and comparatively less evidences reports showing direct plant growth promotion mechanisms [14].

Good agreement between partial 16S rDNA sequence analysis and BIOLOG SFP2^{TM} data were observed. The six *Streptomyces* isolates were placed in four groups that correspond to the four identified species. The use of more than 70% of different classes of carbon source by the isolates reveals a wide metabolic versatility and cosmopolite behavior of these actinobacteria. Also, it was noted a preference for defined and complex forms N-organic compared to inorganic N-forms. Taken together, these nutritional requirements support the saprophytic competence and the development of an evolutionary machinery to access complex biomolecules. It was also described changes in morphological properties and colony pigmentation of the *Streptomyces* strain modulated by different N-sources (Fig 3). It would be explained by the fact that secondary metabolism of streptomycetes may being affected by the nitrogen source [29, 30]. Imbalance in carbon and nitrogen availability, for example an excess of ammonium, will demand more carbon structure to incorporate it into the biomass, consequently less carbon is readily available for secondary metabolism, which may interfere in pigmentation [31].

Since under water stress actinobacteria produce survival structures as spores, pH acidity and salinity assume great importance the abiotic factors controlling soil survival. All *Streptomyces* isolates growth in AGS media with pH ranging from 6.0 to 7.0, with only two strains growing until pH 5. The optimum growth pH for the most of *Streptomyces* actinobacteria is about neutral for alkaline, ranging from 6.0 to 8.0 [41] however, acidophilic species has been also described [42], being of special interest for tropical weathered soils. Highest variability for increased NaCl concentration (0.1 to 3%) was observed for the six strains. Several authors have reported the halotolerant ability of streptomycetes [43-45].

Streptomycetes have been considered one of the most prominent source for enzymes with biotechnological interesting due its chemical heterogeneity, cosmopolite distribution and secondary metabolites [14]. Most of *Streptomyces* isolates from this study produced lipase, amylase and catalase. The microbial production of lipases may represent an alternative approach to bioremediation of hydrocarbon-contaminated soils [32] while the decomposition of macro-polymers by amylases may, in edaphic environment, provides carbon sources to sustain natural microbial community or new

opportunities to design microbial consortium for bioremediation process or other biological enrichment process. The production of both lipases and amylases by *Streptomyces* actinobacteria has been previous reported [33-35]. All *Streptomyces* isolates had shown catalase activity that may play a role in detoxification under environmental stress condition or even mitigate abiotic stress by reducing the damage induced by reactive oxygen species (ROS) in the plant tissue through antioxidant enzymes production [36]. Furthermore, during plant growth-promoting bacteria (PGPB) root infection, plant host may exhibit a defense response based on ROS production [37]. Thus, catalase activity is required by PGPB for reducing cytotoxic effects of ROS, contributing to colonization and survival of the bacteria [38, 39]. A catalase producer *Streptomyces rochei* SM3, a catalase induced tolerance to salinity stress in chickpea plants [40].

A possible auxin-mediated biostimulation mechanism were also observed for the *Streptomyces* strains tested. All isolates (except AC01) strain produced indole compounds in AGS media supplemented with tryptophan (12.7 to 34.8 µg/mL), which was consistent with others published data [46, 47]. Indol-3-acetamide is the most frequent tryptophan-dependent pathway reported to produce auxin-like bioactive compounds [48]. Furthermore, this auxin production pathway is related to increased activity of 1-aminociclopropane-1-carboxilate (ACC) deaminase enzyme, which is involved on degradation of 1-carboxilic-1-aminociclopropane acid, precursor of ethylene, produced by plant under stress conditions to reduce the growth rated of the root system [49]. Positive P-biosolubilization was another plant-growth promotion trait reported for the six *Streptomyces* strains investigated. Contrary to Hamdali et al. (2008) that reported P-solubilization activity of the *Streptomyces* strain related to production of a non-identified calcium chelator, an evident halo around the grown colonies in P-Ca plates associated with pH changes were observed, suggesting excretion of organic acids. Combination of the ability of these strains to solubilize rock phosphate or even release P-label from P-organic structures associated to the stable organic matter, since the strains are able to access humic substances, open up opportunities for formulation of novel bio-phosphate fertilizers and partial replacement of highly soluble P-mineral forms.

Although, there is a good relationship between the presence of auxin and Salkowski positive results for indole compounds, the auxin-mediated biostimulation

effect must to be effectively proved. Using secreted metabolites from *S. hyderabadensis* strain AC05 coupled with transgenic MT tomato seedlings that harbor the auxin responsive marker *DR5::GUS*, it was clearly demonstrated an auxin-like effect by compounds produced by the strain. This synthetic marker is used as a tool to visualize auxin responses in plant cells and tissues [53]. Furthermore, AC05-secreted metabolites displayed an increased number of mitotic sites and lateral root that is compatible with phenotypic architectural root changed modulated by auxin responsive genes [61]. Plant growth can be directly improved by the microbial production of metabolic compounds with phytohormones activity at micromolar to nanomolar concentrations [54]. Auxin and auxin-like compounds regulate many aspects of plant growth and development including cell plasticity, tissue elongation, embryogenesis, tip dominance, and emergence of lateral roots. The potential of IAA or auxin-like bacteria producers to enhance plant growth is extensively studied [47, 51, 55]. However, for investigate possible functional signaling pathways in the host plant, the use of defective endogenous auxin may elucidate whether PGPB inoculation directly promote plant growth [56]. There are consistent evidences for auxin-mediated biostimulation activity linked with plant growth promotion by Gram-negative bacteria using *DR5::GUS* plants [57, 58] and very few evidences accumulates for Gram-positive actinobacteria representatives [14]. In our study, we proved that secreted metabolites from *S. hyderabadensis* strain AC05 display auxin activity, since induced β-glucuronidase activity in *DR5::GUS* reporter transgenic MT tomato seedlings coupled with increased lateral root initiation, underlining an effective phytohormonal effect as part of the plant growth promotion mechanisms operating by AC05 strain.

The plant growth-promoting features described for these humic acid-degrade actinomycete strains isolated from vermicompost are potential mechanisms to promote plant growth. Gnotobiotic assay revealed that all *Streptomyces* strains improved the maize seed germination and plantlets growth. Great variability was observed when maize seed were subjected to the metabolic extract of the six strains with positive and negative effect on seed germination. The presence of bioactive compounds in the secreted metabolites like as auxin (demonstrated in the present study) or compounds that limit growth and infectivity of harmful microorganism such as siderophores, anti-fungal, and/or chitinase, partially explain germination seed fitness [59, 60]. In other hand, metabolic extract of *Streptomyces variabilis* (AC31) had shown inhibitory effect of germination. Although, the molecules responsible for the observed effect was not

determined, phosphinothricin produced by *Streptomyces* sp. has been described as seed inhibitor secreted compound for some agronomic crops [52]. The overall effects of PGPB are not derived from one mechanism, but a combination of various mechanisms such as nutrient solubilization, production of metabolic compounds with hormone-like activity and mitigation of abiotic stress operating under influence of environmental conditions [50]. The *Streptomyces* strains described in this study exhibited plant growth promoting multi-traits being represented: phosphate solubilization, production of extracellular enzymes and bioactive compounds. Similar reports for positive *Streptomyces* inoculation response of other plant species reinforce the biofertilizer and biostimulation potential of this actinobacteria group, which have been previouslly widely recognized as antibiotic producer [51]. The positive outcome of this investigation underline the potential of these *Streptomyces* strains to promote plant growth and fitness in soil. Greenhouse and field assay under proper environmental condition would evaluate the ability of these strains to overcome seed dormancy, increase rates of germination and stand uniformity, as well as increase nutrient use efficiency, maintain plant productivity with lower rates of mineral fertilizers.

CONCLUSIONS

Several environmental problems emerge from the excessive use of agrochemical in plant nutrition and protection. Thus, search for effective multifunctional microbial strains as candidates for inoculant formulation is important in order to develop effective alternative technologies to replenish nutrient in agricultural ecosystems. We underlined the importance of vermicompost as prospective material and the presumable importance to select microorganism able to access stable organic matter as C-source to support growth and activity in the soil. The six strains assigned as *Streptomyces* were grouped in four species similarities groups and were able to improve maize and tomato plant growth. Among the plant growth-promoting features described we evidenced that secreted compound exhibit auxin-like activity and displayed root architectural changes resembling auxin biostimulation mechanism. Field studies combining these strains and stable organic matter are the next step for development of commercial biofertilizers based on *Streptomyces*.

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CAPÍTULO 3

INTERAÇÃO ENTRE A BACTÉRIA PROMOTORA DO CRESCIMENTO VEGETAL *Herbaspirillum seropedicae* estirpe HRC54 E ESTREPTOMICETOS ISOLADOS DE VERMICOMPOSTOS EM PLÂNTULAS DE TOMATE.

RESUMO

A utilização de rizobactérias promotoras de crescimento de plantas (RPCP) na formulação de bioinoculantes representa uma tecnologia mais sustentável frente aos impactos ambientais causados pela utilização maciça de fertilizantes minerais e outros agroquímicos. RPCP podem atuar produzindo fitormônios, solubilizando minerais, produzindo enzimas extracelulares ou ainda controlando fitopatógenos. A maioria dos trabalhos geralmente avalia o efeito dessas rizobactérias individualmente e não sua interação com outros microrganismos. Neste sentido, este estudo buscou compatibilizar a bactéria diazotrófica *Herbaspirillum seropedicae* estirpe HRC54 e isolados de estreptomicetos (AC01, AC05, AC06 e AC31) obtidos a partir de vermicompostos maturados e avaliar a inoculação de pelos menos uma combinação compatível em plântulas de tomate. Particularmente, a interação entre HRC54 e o isolado AC06 foi estudada quanto a ocorrência de cooperação metabólica para o amido. Por fim, a atividade auxínica de metabólitos secretados por HRC54 e/ou AC06 foi investigada.Com exceção do isolado de estreptomicetos AC05, todos os demais isolados apresentaram compatibilidade com *H. seropedicae* estirpe HRC54. Plântulas de tomate cv. Santa Clara cultivadas em substrato orgânico incubado por 45 dias com o isolado AC01 apresentaram aumentos significativos na altura (25%), área foliar (216,2%), número de raízes laterais (138%) e massa seca de parte aérea (145,1%). A densidade populacional da bactéria diazotrófica cultivada sob o fornecimento de amido como única fonte de carbono foi significativamente aumentada quando o amido foi previamente incubado pelo isolado AC06. A inoculação simples ou combinada com *H. seropedicae* estirpe HRC54 e *S. bellus* isolado AC06 foi eficiente em promover o aumento significativo da altura, massa seca da raiz e da parte aérea de plântulas de tomate cv. Santa Clara em relação ao controle não inoculado. Compostos metabólicos produzidos por HRC54 e por AC06 desempenham atividade auxínica, induzindo a atividade da β-glucuronidase em tomate Micro-Tom DR5-GUS, além de estimular a indução de raízes laterais.

Palavras-chave: *Streptomyces* spp., *Herbaspirillum seropedicae*, interação microbiana, promoção do crescimento vegetal.

ABSTRACT

The use of plant-growth promoting rhizobacteria (PGPR) in bioinoculant formulations represents a more sustainable technology facing environmental impacts caused by the massive use of mineral fertilizers and other agrochemicals. PGPR may improve plant growth by phytohormone production, mineral solubilization, production of extracellular enzymes or controlling plant pathogens. Most researches generally evaluates individual effect of PGPR and not their interaction with other microorganisms. Thus, this study aimed to compatibilize the diazotrophic bacteria *Herbaspirillum seropedicae* strain HRC54 and Streptomyces isolates (AC01, AC05, AC06 and AC31) obtained from matured vermicompostos. Further, we evaluated the inoculation of at least one compatible combination on tomato plantlets. In particular, the interaction between HRC54 and isolate AC06 was studied focused on potential occurrence of metabolic cooperation starch. Finally, auxin-like activity of metabolites secreted by HRC54 and/or AC06 was investigated. All isolates showed compatibility with *H. seropedicae* strain HRC54 (except AC05 isolate). Tomato plantlets grown on 45-d incubated organic substrate with the isolated AC01 showed significant increases in height (25%), leaf area (216.2%), number of lateral roots (138%) and shoot dry weight (145 ,1%). The population density of *H. seropedicae* strain HRC54 grown on culture medium with starch as sole carbon source was significantly increased when the starch was previously incubated with AC06. The simple inoculation of AC06 or its combination with *H. seropedicae* strain HRC54 was effective in increase significantly height, dry weight of roots and shoots of Santa Clara tomato plantlets. Metabolic compounds produced by HRC54 and AC06 play auxin-like activity, inducing the activity of β-glucuronidase in tomato Micro-Tom DR5-GUS, and further stimulated the induction of lateral roots.

Keywords: *Streptomyces* spp., *Herbaspirillum seropedicae*, microbe interaction, plant growth promotion.

INTRODUÇÃO

Nas últimas décadas, a expansão dos impactos ao meio ambiente e à saúde humana causados pelo uso indiscriminado de agroquímicos tem impulsionado uma reconsideração da lógica do sistema de produção agrícola convencional, a partir de uma visão mais holística de exploração dos recursos naturais. Neste sentido, o uso de rizobactérias promotoras do crescimento plantas (RPCP) na agricultura, na forma de bioinoculantes, tem sido empregado e tem alcançado aceitação mundial [1-3].

Em termos gerais, a indução do crescimento vegetal mediada por RPCP pode ocorrer diretamente, pela facilitação da aquisição de nutrientes (fixação do nitrogênio, solubilização de fósforo e outros nutrientes essenciais) [4, 5] ou por meio da produção de compostos com atividade fitormonal semelhante aquelas de natureza auxínica e giberélica [6, 7]. Por outro lado, RPCP podem atuar também de forma indireta sobre o crescimento vegetal agindo, principalmente, no controle de fitopatógenos [8, 9] e na redução de danos causados por fatores de natureza abiótica, como estresse hídrico e deficiência nutricional [10]. Como consequência, os benefícios ao crescimento vegetal pela inoculação com RPCP incluem incremento na biomassa de raiz e parte aérea, aumentos da taxa de germinação, crescimento radicular, área foliar, teor de clorofila, conteúdo de nitrogênio e proteínas, tolerância ao estresse hídrico e diminuição da senescência de folhas [11]. A indução do crescimento vegetal não está condicionada a ativação de um único mecanismo ou da ação interdependente entre eles, mas conforme é sugerido na “hipótese aditiva” [12], a ativação desses mecanismos pode ocorrer de forma simultânea ou sequencial em diferentes estágios do crescimento vegetal [13]. Diante disso, estudos direcionados para micro-organismos que apresentem propriedades múltiplas para a indução do crescimento vegetal são essencialmente necessários. Tal multifuncionalidade pode ser observada, por exemplo, em bactérias diazotróficas e actinobactérias as quais consistem em dois grupos distintos de procariotos que possuem diversas espécies representantes descritas na literatura como RPCP [14, 15].

Através da fixação biológica do nitrogênio (FBN) atmosférico, bactérias diazotróficas tornam-se responsáveis por uma das principais vias de fornecimento desse elemento ao sistema solo-planta, beneficiando não apenas plantas leguminosas como também espécies gramíneas [16]. A título de exemplo podem ser citadas as espécies diazotróficas do gênero *Herbaspirillum* que são β-Proteobacterias

Gram-negativas capazes de se associar com diversas culturas não-leguminosas, beneficiando o crescimento por meio de muitos dos mecanismos supracitados [17, 18]. A espécie notadamente promotora do crescimento vegetal *Herbaspirillum seropedicae* está entre as mais estudadas do gênero [19-21], a qual apresenta características como FBN, produção de fitormônios, ACC deaminase e sideróforos, e cuja inoculação é capaz de induzir o crescimento de arroz, milho e cana-de-açúcar [22, 23].

Em termos de número e espécies identificadas, o Filo *Actinobacteria* representa uma das maiores unidades taxonômicas, incluindo bactérias Gram-positivas com diversas propriedades fisiológicas e metabólicas como a produção de enzimas extracelulares e uma ampla gama de metabólitos secundários [24]. No que diz respeito a interação entre plantas e bactérias Gram-positivas, aquelas pertencentes ao gênero *Streptomyces* estão entre as mais estudadas [25]. Algumas características relacionadas à indução do crescimento vegetal encontradas em interações positivas entre plantas e estreptomicetos incluem solubilização de fosfato [26], produção de ácido indol-3-acético [27], aumento da tolerância ao estresse salino [28] e controle de vários fitopatógenos [9, 29, 30]. Além disso, uma abordagem diferenciada da capacidade de promoção indireta do crescimento plantas por estreptomicetos consiste na sua utilização como um aditivo biológico capaz de beneficiar o desempenho de outros micro-organismos promotores de crescimento vegetal, configurando um sistema tripartite [31]. Estudos tem demonstrado que a aplicação de estreptomicetos pode atuar como um modulador na simbiose de fungos micorrízicos ou bactérias fixadoras de nitrogênio [32, 33].

A despeito de características que RPCP podem apresentar *in vitro*, a efetividade do desempenho na indução do crescimento está condicionada a sua interação com a espécie vegetal. Nesse sentido, o uso de plantas modelo nos estudos de promoção de crescimento por RPCP é de grande relevância para melhor compreensão deste processo biológico. *Arabidopsis thaliana* (L.) Heynh tem sido frequentemente utilizada como planta modelo para pesquisas relacionadas à inoculação com bactérias diazotróficas [34, 35]. Porém, plantas como tomateiro (*Solanum lycopersicum* L.) e o milho (*Zea mays*) também têm sido sugeridos como plantas modelos para pesquisas básicas e aplicadas em plantas [36, 37]. A cultivar Micro-Tom (MT), por exemplo, é uma miniatura do tomateiro, criada para fins ornamentais e proposta como modelo

genético para estudos também fisiológicos. O mutante *diageotropica* (*dgt*) dessa cultivar é insensível à auxina, sendo caracterizado pelo crescimento horizontal da raiz e parte aérea, dominância apical reduzida e ausência de raízes laterais [37]. O uso do modelo MT-*dgt* é, por tanto, uma importante ferramenta para investigar se uma determinada substância, sintética ou microbiana, possui atividade auxínica [38].

Diante disso, o objetivo desse trabalho foi estudar a compatibilidade entre a bactéria diazotrófica *Herbaspirillum seropedicae* estirpe HRC54 e isolados de estreptomicetos obtidos a partir de vermicompostos maturados e avaliar a inoculação de pelos menos uma combinação compatível em plântulas de tomate.

MATERIAL E MÉTODOS

Bactéria diazotrófica e isolados de estreptomicetos: condições de cultivo

A bactéria diazotrófica *Herbaspirillum seropedicae* estirpe HRC54 utilizada nos ensaios pertence à bacterioteca do Laboratório de Biologia Celular e Tecidual (LBCT) da Universidade Estadual do Norte Fluminense Darcy Ribeiro. A bactéria foi mantida a 4 ºC em meio DYG'S sólido com a seguinte composição: glicose (2 g), ácido málico (2 g), peptona bacteriológica (1,5 g), extrato de levedura (2 g), K_2HPO_4 (0,5 g), $MgSO_4.7H_2O$ (0,5 g), ácido glutâmico (1,5 g), agar (15 g) e pH 6,0. O inóculo foi obtido pelo cultivo em meio DYG'S líquido por 36 horas em agitador orbital (140 rpm, 30°C) [39].

Os quatro isolados de estreptomicetos (*Streptomyces diastaticus* isolado AC01, *S. hyderabadensis* isolado AC05, *S. bellus* isolado AC06 e *S. variabilis* isolado AC31) selecionados para este estudo, também pertencentes à bacterioteca do LBCT, foram mantidos a 4 ºC em meio AGS sólido com a seguinte composição: Arginina (1 g); glicerol (1.25 % v/v); K_2HPO_4 (1 g); NaCl (1 g); $MgSO_4.7H_2O$ (0.5 g); solução de micronutrientes (1 mL), agar (20 g), água destilada (1000 mL) e pH 7.2. O inóculo foi preparado por meio do seu cultivo em AGS [40] líquido por 5 dias em agitador orbital (160 rpm, 28 ºC) ou arroz autoclavado, por 10 dias (28 ºC) [41].

Ensaio de compatibilidade

O ensaio de compatibilização foi feito segundo a metodologia proposta por Cuesta *et al*. (2012) [42] modificada. Alíquotas de 1 mL do inóculo de *H. seropedicae* estirpe HRC54 foram transferidas para tubos de ensaio contendo 15 mL de meio sólido caldo nutriente (NB) (40°C), homogeneizadas, e o volume final vertido em placas de Petri. Uma vez solidificado o meio, quatro discos miceliais (6mm de diâmetro) dos estreptomicetos foram sobrepostos ao meio. As placas foram incubadas em B.O.D. a 28 °C por 7 dias e a compatibilidade verificada pela ausência de halo de inibição.

Potencial de interações para promoção de crescimento de plântulas de tomate

Este ensaio foi realizado em condições gnotobióticas, em delineamento experimental inteiramente casualizado, sendo avaliados 2 isolados de estreptomicetos (AC01 e AC31) e *H. seropedicae* estirpe HRC54, inoculados de forma isolada ou em combinação, com 4 repetições. O substrato comercial Vivatto® foi infestado com suspensão de esporos dos isolados AC01 e AC31 com uma densidade óptica (D.O.) de 0,4 a 560 nm, utilizando 10 mL de suspensão para cada 400 cm^3 do substrato, seguido de incubação por 45 dias em temperatura ambiente (28 ± 2 ºC). A colonização do substrato foi verificada por meio do reisolamento dos estreptomicetos utilizando a técnica de diluição seriada. Sementes desinfestadas de tomate (*S. licopersicum*) cv. Santa Clara, pré-germinadas em Agar-água (0,6%) foram semeadas no substrato colonizado e recobertas com 1 mL do inóculo de *H. seropedicae* estirpe HRC54, com densidade de 10^8 células por mL. O ensaio foi mantido em sala de crescimento a 26 °C com fotoperíodo de 16 horas. A coleta das plântulas foi realizada 15 dias após a semeadura, avaliando-se os seguintes parâmetros: altura, área foliar, número de raízes laterais e massa seca da raiz e parte aérea. Amostras das raízes foram retiradas para quantificação de *H. seropedicae* estirpe HRC54 por meio de diluição seriada, seguida de contagem pelo método do número mais provável (NMP). O plaqueamento das diluições em meio de cultivo sólido AGS também foi realizado para verificar a presença estreptomicetos.

Interação entre *H. seropedicae* estirpe HRC54 e S. bellus isolado AC06, um produtor de amilases

Análises mais restritas da combinação de *H. seropedicae* estirpe HRC54 e *S. bellus* isolado AC06 foram realizadas, a fim de investigar uma possível cooperação metabólica para o amido como fonte de carbono, bem como, a coexistência de mecanismos de promoção de crescimento vegetal na interação.

Flutuação populacional de H. seropedicae estirpe HRC54 em co-cultura com S. bellus isolado AC06

A avaliação de existência de cooperação metabólica foi feita em meio semissólido designado JNFb-amido com a composição mineral do meio JNFb [43] e amido como única fonte de carbono, com a seguinte composição final para 1 litro de meio: amido (5 g), K_2PO_4 (6 mL da solução a 10%), KH_2PO_4 (18 mL da solução a 10%), $MgSO_4.7H_2O$ (2 mL da solução a 10%), NaCl (1 mL da solução a 10%), $CaCl_2.2H_2O$ (2 mL da solução a 1%), FeEDTA (4 mL da solução a 1,64%), azul de bromotimol (2 mL da solução de 0,5% em KOH 0,2 N), solução de micronutrientes (2 mL), vitamina para meio de cultura (2 mL) e pH 5,8. Inicialmente, meio semissólido JNFb-amido contido em frascos de ampicilina foram inoculados (ou não) com 5 mL de uma suspensão de esporos do isolado AC06 (D.O. 0,4 a 560 nm). Frascos de ampicilina contendo meio JNFb com malato [43] também foram inoculados (ou não) com o isolado AC06. Os frascos foram incubados em câmara de crescimento tipo B.O.D. a 30 °C por 5 dias. Após incubação, o meio foi inoculado com 100 µL de suspensões de células lavadas de *H. seropedicae* estirpe HRC54 (D.O. 1,0 a 430 nm). Ao fim da incubação, a população da bactéria diazotrófica foi quantificada por meio de diluição seriada e pelo método de NMP. O ensaio foi delineado de forma inteiramente casualizada, com quatro repetições.

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Avaliação do sistema tripartite H. seropedicae estirpe HRC54, S. bellus isolado AC06 e plântulas de tomate

Para avaliar o potencial da inoculação de *H. seropedicae* estirpe HRC54, *S. bellus* isolado AC06 ou da combinação de ambos micro-organismos, foi realizado um

ensaio inteiramente casualizado, utilizando plântulas de tomate cv. Santa Clara, com 7 repetições. As condições de inoculação são semelhantes àquelas descritas anteriormente. As plântulas foram mantidas em sala de crescimento a 26 °C com fotoperíodo de 16 horas, por 21 dias. Após este período, 3 repetições foram utilizadas para avaliação da capacidade de colonização da rizosfera pelo isolado AC06 em função de *H. seropedicae* estirpe HRC54, seguindo a metodologia proposta por Corral-Lugo e colaboradores [44], com modificações. As raízes das plântulas foram cuidadosamente sacudidas, inseridas em tubos tipo Falcon contendo 20 mL de água destilada esterilizada e agitadas vigorosamente em agitador do tipo vortex. A suspensão gerada foi submetida a diluição seriada. As 4 repetições restantes foram coletadas, avaliando-se altura, massa seca de raiz e da parte aérea.

Atividade auxínica de metabólitos secretados por H. seropedicae estirpe HRC54, S. bellus isolado AC06 em plantas transgênicas de tomate

Para avaliar uma possível existência de mecanismo bioestimulante do crescimento vegetal induzido por *H. seropedicae* estirpe HRC54, *S. bellus* isolado AC06 ou da combinação, foi aplicado o meio líquido condicionado contendo os metabólitos segregados destes micro-organismos. Os metabólitos de *H. seropedicae* estirpe HRC54 foram obtidos por meio de seu cultivo em meio DYG'S líquido acrescido de triptofano (100 $mg.L^{-1}$) [45] por 36 horas. Os metabólitos de *S. bellus* isolado AC06 foram obtidos pelo seu cultivo em meio AGS líquido também acrescido de triptofano (2 $g.L^{-1}$) por 7 dias. As culturas foram centrifugadas (10 000 x *g* por 15 minutos) e filtradas em filtro Milipore® (0,22 µm). Os metabólitos foram aplicados de forma isolada ou combinada em plântulas de tomate (*S. Lycopersicon* L.) cv. Micro-Tom *DR5::GUS*, que é um promotor do gene responsivo para auxina sintética ligada com o gene repórter GUS (β-glucuronidase). As sementes transgênicas foram desinfestadas, semeadas em substrato Vivatto® e inoculadas com um mililitro dos filtrados. Água esterilizada ou meio líquido AGS foram utilizados como controle. Após 21 dias as plântulas foram coletadas e o teste histoquímico com GUS foi realizado [38], com tempo de incubação dos segmentos de raízes durante 16 horas numa estufa de secagem a 37 °C, usando a solução X-Gluc (5-bromo-4-cloro-3-indolil glucoronato). Os segmentos de raízes foram observados sob microscopia de luz e microscopia estereoscópica, em que uma cor azul clara é detectada como um produto

da atividade da β-glucuronidase nas células vegetais e tecidos onde se acumula auxina. Mudas com as características fenotípicas foram fotografadas. Além disso, o número de sítios de mitóticos e raízes laterais foram avaliados. As sementes portadores do gene *DR5::GUS* foram gentilmente cedidas pelo Dr. Lázaro Eustáquio Pereira Peres pela Universidade de São Paulo, Brasil.

RESULTADOS

Avaliação de compatibilidade

Com exceção do isolado de estreptomicetos AC05, todos os demais isolados apresentaram compatibilidade com *H. seropedicae* estirpe HRC54 (Tabela 1).

Tabela 1: Compatibilidade *in vitro* entre isolados de estreptomicetos e a bactéria *Herbaspirillum seropedicae* estirpe HRC54.

Isolado	*Herbaspirillum seropedicae* estirpe HRC54
AC 01	C
AC05	I
AC06	C
AC31	C

C = compatível; I = incompatível

Efeito da inoculação de isolados de estreptomicetos e da bactéria Herbaspirillum seropedicae estirpe HRC54 na promoção de crescimento de plântulas de tomate

Plântulas cultivadas em substrato orgânico incubado por 45 dias com o isolado AC01 apresentaram aumentos significativos na altura (25%), área foliar (216,2%), número de raízes laterais (138%) e massa seca de parte aérea (145,1%) (Tabela 2). No entanto, este aumento não foi amplificado pela coinoculação deste isolado com a bactéria diazotrófica *H. seropedicae* estirpe HRC54. Por outro lado, embora a aplicação de *H. seropedicae* estirpe HRC54 não tenha diferido estatisticamente do

controle não inoculado, diferenças significativas foram observadas entre os tratamentos de coinoculação quando comparados ao tratamento inoculado somente com a bactéria diazotrófica. A coinoculação de *H. seropedicae* estirpe HRC54 com o isolado AC01 aumentou em 55,9% a área foliar das plântulas em relação aquelas inoculadas apenas com a bactéria. Reduções foram observadas nos valores de altura (14%), número de raízes laterais (44,4%) e a massa seca de raiz (56%) e parte aérea (40,3%) das plântulas coinoculadas com *H. seropedicae* estirpe HRC54 com o isolado AC31 quando comparadas aquelas inoculadas unicamente com a bactéria.

Tabela 2: Efeito do isolado AC01 de estreptomiceto e *Herbaspirillum seropedicae* estirpe HRC54 no crescimento de plântulas de tomate cv. Santa Clara.

Tratamento	Altura (cm)		AF (cm^2)		RL		MSPA (g)		MSR (g)	
	---	B	---	B	---	B	---	B	---	B
Controle	9,6 Ab	10,7 Aa	3,7 Ac	5,9 Ab	13 Ac	27 Aa	8,2 Ab	11,9 Aa	2,1 Aa	2,5 Aa
AC01	12,0 Aa	11,1 Aa	11,7 Aa	9,2 Aa	31 Aa	29 Aa	20,1 Aa	15,4 Aa	2,2 Aa	1,5 Ab
AC31	9,2 Ab	9,2 Ab	3,9 Ac	4,4 Ab	16 Ac	15 Ab	8,1 Ab	7,1 Ab	2,2 Aa	1,1 Ab
CV (%)	8,70		20,23		18,96		23,36		26,86	

B = inoculado com *Herbaspirillum seropedicae* estirpe HRC54. Letras iguais não diferem estatisticamente pelo teste de Scott & Knott a 5% de probabilidade. Letras maiúsculas comparam na linha o efeito da inoculação com a bactéria diazotrófica para o mesmo isolado de actinobactéria. Letras minúsculas comparam na coluna o efeito dos isolados de actinobactéria entre si. AF = área foliar; RL = raízes laterais; MSPA = massa seca da parte aérea; MSR = massa seca da raiz.

O número mais provável da população da bactéria diazotrófica *H. seropedicae* estirpe HRC54 reisoladas de raízes de plântulas de tomate não foi aparentemente influenciado pela sua coinoculação com os isolados de estreptomicetos, mantendo-se em torno de 8,0 (Tabela 3).

Tabela 3: Densidade populacional de *Herbaspirillum seropedicae* estirpe HRC54 reisoladas de raízes de tomate cv. Santa Clara

Tratamento	NMP.g^{-1} de raiz (Log_{10})
Controle+B	8,17
AC 01+B	8,01
AC31+B	8,10

+B = coinoculação com *Herbaspirillum seropedicae* estirpe HRC54

Flutuação populacional de *H. seropedicae* estirpe HRC54 em co-cultura com *S. bellus* isolado AC06

Não foram observadas diferenças significativas entre o fornecimento de malato (incubado ou não com o isolado AC06) e o fornecimento de amido previamente incubado com o isolado AC06 sobre o NMP da população de *H. seropedicae* estirpe HRC54 (Fig. 1). Entretanto, a densidade populacional da bactéria diazotrófica cultivada sob o fornecimento de amido como única fonte de carbono foi significativamente aumentada quando o amido foi previamente incubado pelo isolado AC06.

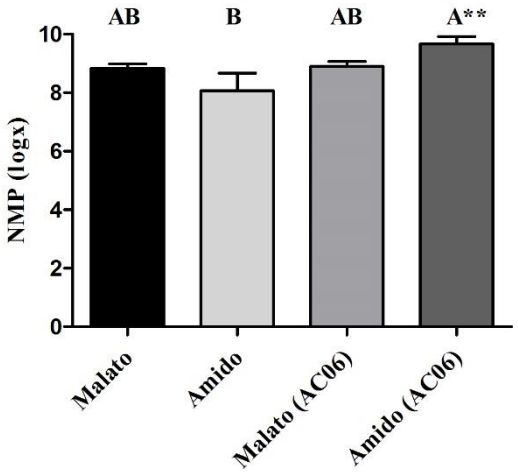

Fig. 1: Número mais provável (NMP) da população de *H. seropedicae* estirpe HRC54 em função da fonte de carbono ou presença de *S. bellus* (AC06). Médias com letras iguais não diferem pelo teste de Tukey (*p≤ 0,05*).

Sistema tripartite *H. seropedicae* estirpe HRC54, *S. bellus* isolado AC06 e plântulas de tomate

Em aspectos gerais, a inoculação combinada de *Herbaspirillum seropedicae* estirpe HRC54 e *Streptomyces bellus* isolado AC06 foi eficiente em promover o aumento significativo da altura (27,9%), massa seca da raiz (257,3%) e massa seca da parte aérea (165,1%) de plântulas de tomate cv. Santa Clara quando comparadas ao controle não inoculado (Fig. 2). Semelhantemente, a inoculação simples com o isolado AC06 proporcionou incrementos significativos dos parâmetros biométricos avaliados, diferindo em 20,3% da altura, 544,9% da massa seca de raiz e 335% da

massa seca da parte aérea das plântulas em relação ao controle. Além disso, AC06 foi eficiente em colonizar a rizosfera de plântulas de tomate. A inoculação com *H. seropedicae* estirpe HRC54, por sua vez, influenciou significativamente a massa seca da parte aérea na ordem de 257,4%.

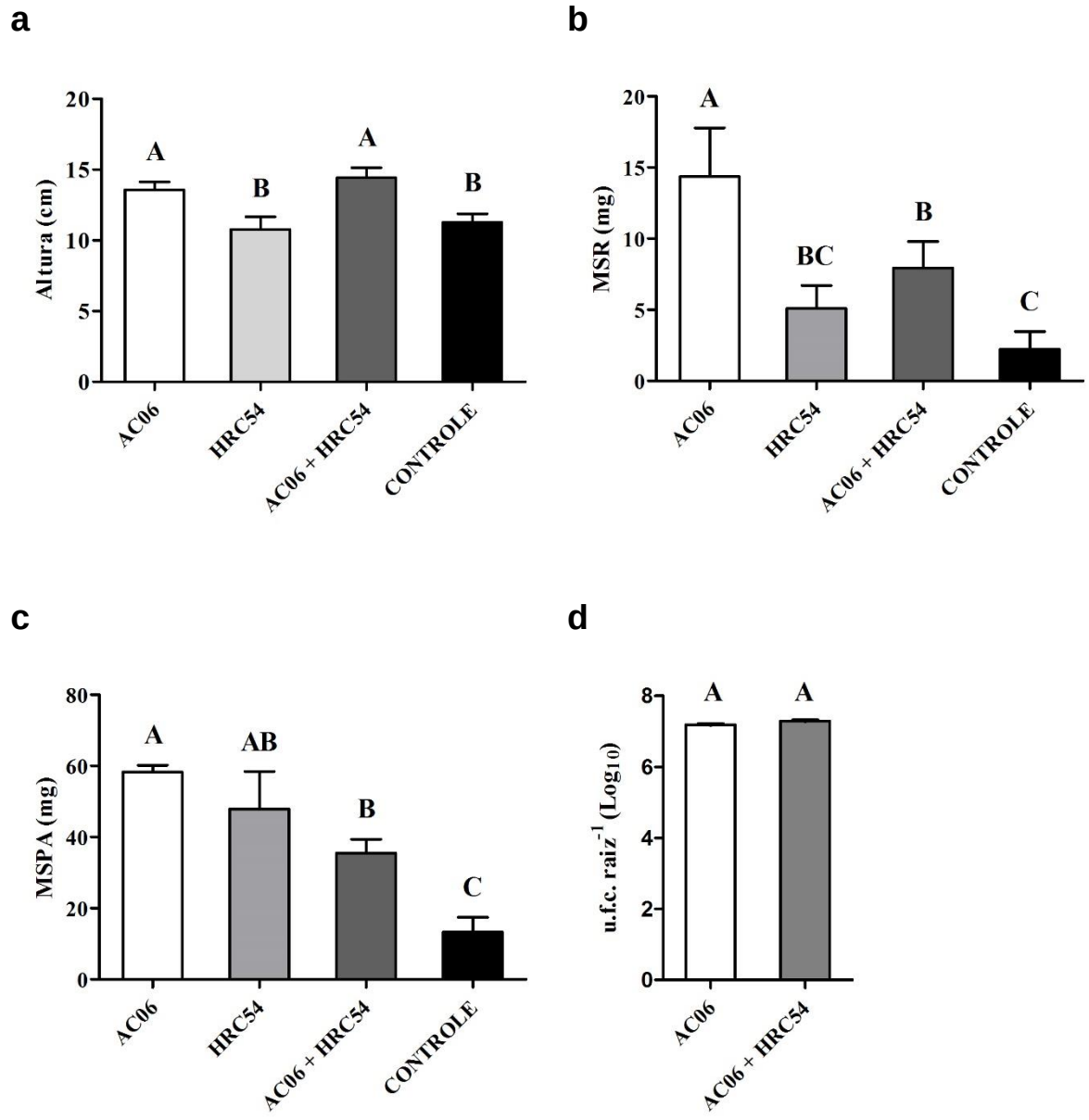

Fig. 2: Inoculação simples ou combinada com *S. bellus* isolado AC06, *H. seropedicae* estirpe HRC54 no crescimento de plântulas de tomate cv. Santa Clara (**a**, **b** e **c**) e colonização rizosférica pelo isolado AC06 na presença e ausência da bactéria. Médias com letras iguais não diferem pelo teste de Tukey (*p≤ 0,05*). MSR = massa seca da raiz; MSPA = massa seca da parte aérea; u.f.c. = unidade formadora de colônia.

Plantas transgênicas de tomate cv. Micro-Tom (DR5::GUS) respondem a metabólitos secretados por H. seropedicae estirpe HRC54 e S. bellus isolado AC06

O ensaio histoquímico de GUS revelou que plantas de tomate cv. Micro-Tom (*DR5::GUS*) foram responsivas ao tratamento com compostos metabólicos secretados por *H. seropedicae* estirpe HRC54, *S. bellus* isolado AC06 ou pela sua combinação, apresentado a coloração azul indicativa da atividade da enzima β-glucuronidase (Fig. 3). Os compostos secretados por ambos os micro-organismos foram capazes de interferir na arquitetura radicular, influenciando positivamente no padrão de fasciculação das plantas (Fig. 3B, C) nas quais a atividade da enzima pode ser observada em sítios mitóticos de raízes laterais (Fig. 3C e O), ápice radicular (Fig. 3G -I e M), pelos radiculares (Fig. 3J) e na camada do periciclo do sistema vascular (Fig. 3E, G e N).

O tratamento com metabólitos secretados por *H. seropedicae* estirpe HRC54 aumentou em 212,5% e 162,5% o número de sítios mitóticos e raízes laterais, respectivamente (Fig. 4). De igual modo, metabólitos secretados por AC06 aumentou significativamente em 120,8% o número de sítios mitóticos e em 162,5% o número de raízes laterais. Parâmetros também foram positivamente influenciados pelo uso combinado dos metabólitos secretados por amos micro-organismos quando comparados ao controle, porém este efeito não foi sinérgico.

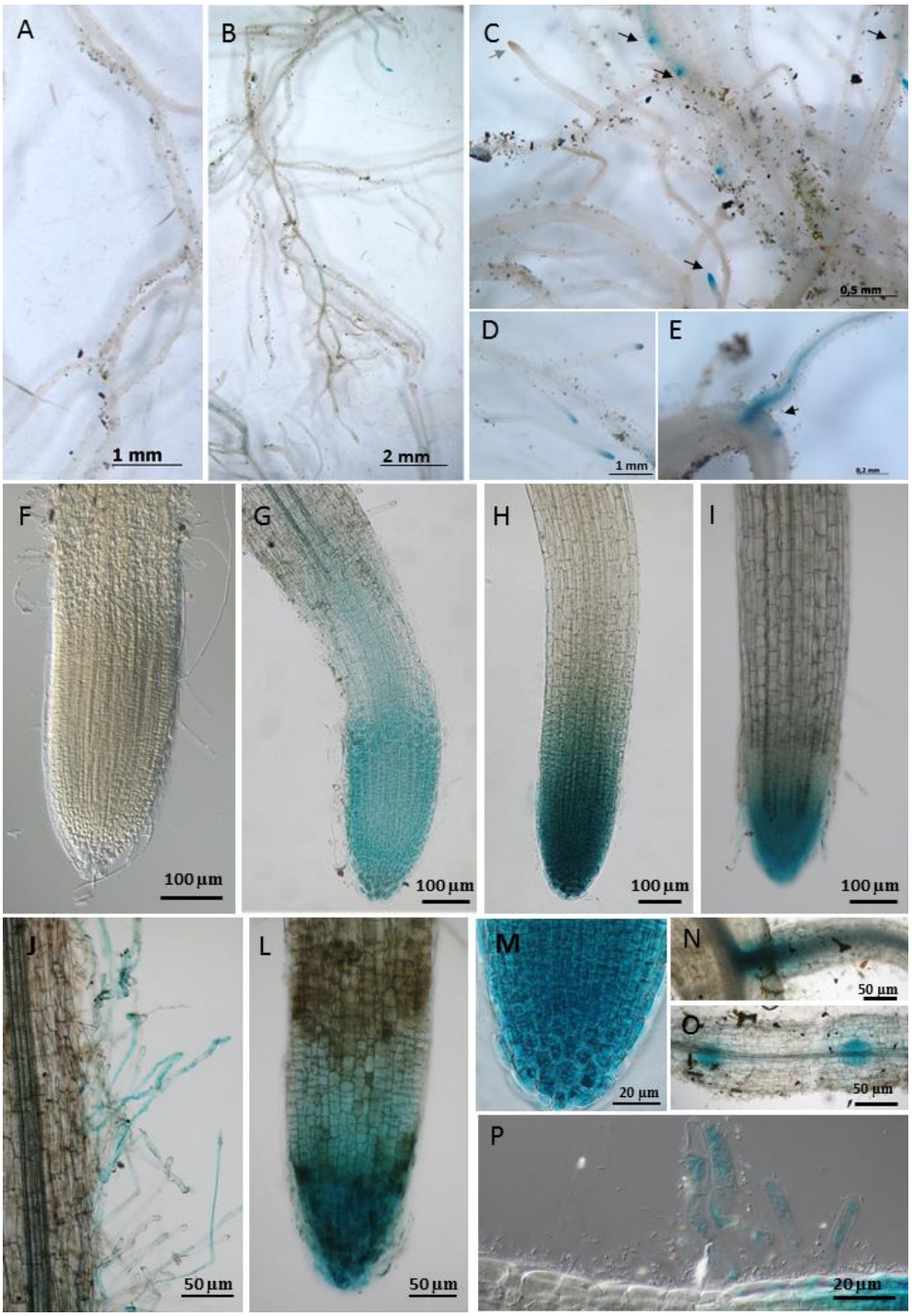

Fig. 3: Estereomicroscopia (Fig. 3. A-E) e Microscopia óptica (Fig. F-P) de raízes de tomate Micro-Tom *DR5:GUS* (21 dias após a germinação) inoculadas *H. seropedicae* estirpe HRC54 (Fig. C, H, L, M, P), *Streptomyces bellus* estirpe AC06 (Fig. B, G, J), e a combinação de HRC 54 e AC06 (Fig. D, E, I, N, O), comparados com plantas controle (Fig. A, F). Fig. A: arquitetura radicular em plantas controle; Fig. B: arquitetura radicular de plantas inoculadas com AC06; Fig. C: arquitetura radicular de plantas inoculadas com HRC54 e atividade da β-glucosidase em tecidos meristemáticos (setas); Fig. D: Perfil de expressão no tratamento HRC54 + AC06, similar ao tratamento HRC54; Fig. E: Expressão da β-glucosidase na região de emissão de raízes laterais e no tecido vascular em HRC54+AC06; Fig. F – I: Expressão da β-glucosidase em resposta a exsudados em plantas controle (Fig. F), plantas tratadas com meio condicionado por AC06 (Fig. G), por HRC 54 (Fig. H) e AC06+ HRC 54 (Fig. I); Fig. J – P: Diferentes sítios anatômicos e padrões de expressão da β-glucosidase em reposta aos secretados de AC06 e HRC54; Fig. J: expressão em pêlos radiculares na zona pilífera; Fig. L: no ápice radicular com estratificação da atividade associada a divisões anticlinais; Fig. M: na coifa e meristema apical; Fig. N: região de emergência e cilindro central em raízes laterais; Fig. O: Sítios mitóticos no eixo radicular, a partir da atividade de células do periciclo; Fig. P: células de fronteira destacadas da coifa.

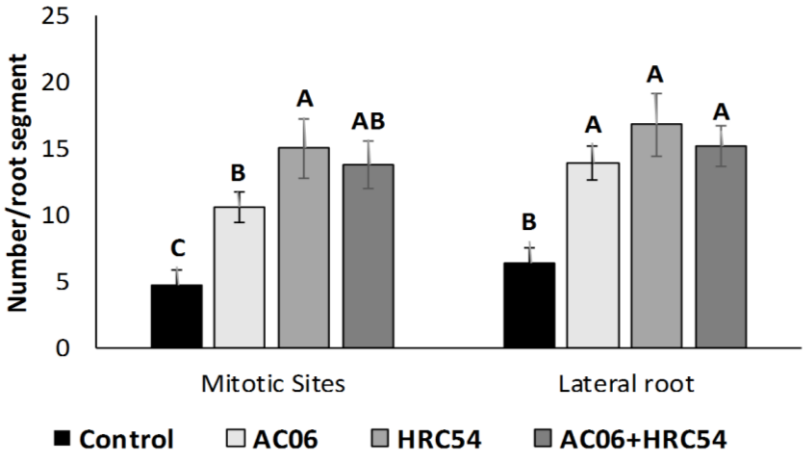

Fig. 4: Efeito do meio líquido condicionado pelo crescimento de *S. bellus* isolado AC06, *Herbaspirillum seropedicae* estirpe HRC54 e a combinação de *Streptomyces bellus* isolado AC06 e *Herbaspirillum seropedicae* estirpe HRC54 em comparação com plantas controle (meio líquido não condicionado pelo crescimento destes microrganismos) sobre a número de sítios mitóticos e o número de raízes laterais de plântulas de tomate Micro-Tom DR5-GUS aos 21 dias após a germinação. As médias de tratamento obtidas a partir de cinco repetições e as diferenças de tratamento foram avaliadas pelo teste Tukey com 5% de probabilidade.

DISCUSSÃO

Bactérias diazotróficas e actinobactérias, particularmente as do gênero *Streptomyces*, representam grupos microbianos extensamente descritos na literatura como agentes de promoção do crescimento vegetal [15, 25, 45, 46]. Entretanto, poucos trabalhos exploram o uso combinado de estreptomicetos com bactérias diazotróficas para a formulação de inoculantes, o que pode ser atribuído a alta performance antagônica dessas actinobactérias. Nossos ensaios *in vitro* demonstraram que três isolados estreptomicetos (*Streptomyces diastaticus* isolado AC01, *Streptomyces bellus* isolado AC06 e *Streptomyces variabilis* isolado AC31) são compatíveis com a bactéria diazotrófica *Herbaspirillum seropedicae* estirpe HRC54 a qual é, notadamente, promotora do crescimento vegetal [17].

Trabalhos têm evidenciado que várias espécies de estreptomicetos são capazes de induzir o crescimento de plantas por meio de diferentes mecanismos incluindo a produção de compostos com atividade fitormonal, solubilização de nutrientes e controle de fitopatógenos [47]. No presente trabalho, a inoculação de plântulas de tomate cv. Santa Clara com *S. diastaticus* isolado AC01 incrementou significativamente a massa seca da parte aérea, o número de raízes laterais e a altura das plântulas. Este resultado é consistente com aqueles descritos por Sousa e colaboradores [48] em que mudas de tomate inoculadas com isolados de estreptomicetos apresentaram altura, diâmetro do caule, massa seca da parte aérea e massa seca das raízes significativamente maiores do que o controle. Não foram observadas diferenças significativas entre os parâmetros biométricos das plântulas inoculadas apenas com os isolados AC01 e AC06 e das plântulas inoculadas com a combinação destes isolados com *H. seropedicae* estirpe HRC54. Porém, plântulas inoculadas com as combinações diferiram entre si. Plântulas inoculadas com a combinação de *H. seropedicae* estirpe HRC54 e o isolado de estreptomiceto AC01 apresentaram maior área foliar quando comparadas ao controle inoculado somente com a bactéria. Em contraste, reduções significativas na altura, número de raízes laterais e biomassa de plântulas inoculadas com a combinação de *H. seropedicae* estirpe HRC54 e o isolado AC31. Recentemente, o potencial da coinoculação de plantas de cana-de-açúcar com bactérias diazotróficas e actinobactérias endofíticas detentoras de características de promoção de crescimento vegetal foi estudado [49]. Os autores observaram que a coinoculação com quatro isolados endofíticos, dentre eles um estreptomiceto, resultou na redução significativa de alguns parâmetros como

massa fresca e seca da raiz ou parte aérea das plantas quando comparada à inoculação simples dos isolados.

Dentre os desafios para se produzir bioinoculantes à base de RPCP encontra-se o desenvolvimento de formulações com características específicas que envolve, por exemplo, o uso de aditivos que aumentem a viabilidade, atividade e desempenho dos micro-organismos [2]. Nesse sentido, o estudo de cooperação metabólica entre RPCP é fundamental para o desenvolvimento de tais formulações. A capacidade de actinobactérias do gênero *Streptomyces* em estabelecer este tipo de cooperação, a qual resulta no acréscimo de reações catabólicas para outros micro-organismos promotores de crescimento, permitindo o acesso a recursos de nutrientes outrora indisponíveis, tem sido estudada [32, 50]. Neste trabalho, a interação entre *H. seropedicae* estirpe HRC54 e *S. bellus* isolado AC06 foi avaliada a fim de investigar uma possível cooperação metabólica tendo o amido como substrato, além da coexistência de mecanismos de promoção de crescimento vegetal.

O cultivo de *H. seropedicae* estirpe HRC54 em meio semissólido JNFb previamente incubado com o isolado AC06 resultou num aumento significativo do NMP da bactéria diazotrófica. Esse resultado permite inferir que, possivelmente, *H. seropedicae* estirpe HRC54 é capaz de acessar as moléculas orgânicas resultantes da degradação do amido pelo isolado AC06 ou que compostos metabólicos gerados por AC06, a partir da degradação do amido, estimulam o crescimento da população de bactérias. Um estudo envolvendo isolados de *Bacillus* sp., produtores de amilases, e bactérias da família *Enterobacteriaceae* demonstrou que a atividade de fixação de nitrogênio por estas bactérias foi significativamente estimulada pela coinoculação com *Bacillus* sp. em meio de cultura contendo amido [51].

A inoculação simples ou combinada com *H. seropedicae* estirpe HRC54 e *S. bellus* isolado AC06 foi eficiente em promover o aumento significativo da altura, massa seca da raiz e da parte aérea de plântulas de tomate cv. Santa Clara em relação ao controle não inoculado. Entretanto, não foi observado um efeito sinérgico entre estes micro-organismos. Observou-se que a massa seca da parte aérea e da raiz de plântulas inoculadas com a combinação foi significativamente menor do que em plântulas inoculadas apenas com o isolado AC06. Conforme discutido anteriormente, este padrão de resposta tem sido observado em outros trabalhos envolvendo a coinoculação de estreptomicetos e bactérias diazotróficas.

Compostos secretados por *S. bellus* isolado AC06 e *H. seropedicae* estirpe HRC54 foram eficientes em influenciar positivamente o padrão de fasciculação das plantas de tomate cv. Micro-Tom DR5-GUS, aumentando significativamente o número de sítios mitóticos e raízes laterais em relação ao controle, sendo os resultados mais expressivos foram encontrados em segmentos de raízes tratadas com metabólitos secretados por *H. seropedicae* estirpe HRC54. Compostos microbianos com atividade fitormonal podem atuar diretamente no crescimento vegetal, influenciando o desenvolvimento de meristemas apicais, estimulando o crescimento de raízes e aumentando o número de pelos radiculares [52]. A utilização de plantas transgênicas de arroz (*Oryza sativa* L.) contendo o gene repórter responsivo a auxina *DR5::GUS* foi anteriormente utilizada para evidenciar a capacidade bioestimulante de substâncias auxínicas produzidas por bactérias diazotróficas Gram-negativas [53] mas, por outro lado, o uso desta técnica para actinobactérias ainda é incipiente. Neste estudo, foi demonstrado que compostos metabólicos produzidos por *Herbaspirillum seropedicae* estirpe HRC54 e por *Streptomyces bellus* isolado AC06 desempenham atividade auxínica, induzindo a atividade da β-glucuronidase em plantas de tomate cv. Micro-Tom DR5-GUS, além de estimular a indução de raízes laterais.

O uso de RPCP na forma de bioinoculantes tem se estabelecido como uma tecnologia mais sustentável para otimização da produção agrícola [54]. Espécies de bactérias diazotróficas do gênero *Herbaspirillum* e actinobactérias do gênero *Streptomyces* não apenas têm sido descritas como promotoras do crescimento vegetal como [17, 19, 55], também, são componentes de bioinoculantes comerciais [56]. Contudo, poucos trabalhos exploram a potencialidade da coinoculação destes representantes microbianos. Neste estudo nós exploramos a possibilidade de usar estreptomicetos isolados de vermicompostos maturados juntamente com a bactéria diazotrófica *Herbaspirillum seropedicae* estirpe HRC54 com vistas à promoção do crescimento de plantas.

CONCLUSÕES

Combinações de *Streptomyces diastaticus* isolado AC01 ou de *Streptomyces bellus* isolado AC06 com *H. seropedicae* estirpe HRC54 foram eficientes em promover o crescimento de plântulas de tomate. Também evidenciamos, pela primeira vez, a compatibilidade metabólica e o efeito auxínico de metabólitos secretados por *Streptomyces bellus* estirpe AC06 e *Herbaspirillum seropedicae* estirpe HRC54 em plantas de tomate cv. Micro-Tom DR5-GUS. Estes resultados apontam para o potencial de combinações de estreptomicetos e bactérias diazotróficas na promoção de crescimento vegetal.

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