Brazilian Journal of Biology®

ISSN 1519-6984 (Print)
ISSN 1678-4375 (Online)

Original Article

Biocontrol potential of actinobacteria against *Pantoea ananatis*, the causal agent of maize white spot disease

Potencial de biocontrole de actinobactérias contra *Pantoea ananatis*, agente causal da doença mancha branca do milho

J. C. M. Dornelas[a], P. H. F. Carmo[a], U. G. P. Lana[b], M. A. G. Lana[c], C. A. O. Paiva[b] and I. E. Marriel[b,d*]

[a]Universidade Federal de Minas Gerais – UFMG, Instituto de Ciências Biológicas, Departamento de Microbiologia, Laboratório de Micologia, Belo Horizonte, MG, Brasil

[b]Embrapa Milho e Sorgo, Laboratório de Microbiologia e Biologia Molecular, Sete Lagoas, MG, Brasil

[c]Ministério da Agricultura, Pecuária e Abastecimento – MAPA, Laboratório Nacional Agropecuário – LANAGRO, Pedro Leopoldo, MG, Brasil

[d]Universidade Federal de São João Del Rei – UFSJ, Departamento de Ciências Agrárias, Sete Lagoas, MG, Brasil

Abstract

Pantoea ananatis is the causal agent of maize white spot, a foliar disease responsible for significant maize yield reduction worldwide, especially in Brazil. In general, the maize foliar diseases control involves the adoption of resistant genotypes and pesticides application. However, the use of agrochemicals can significantly cause increase production costs, damage to human health and negative environmental impacts. In this sense, the use of biological control agents has been considered among the most promising eco-friendly technologies for sustainable agriculture. Actinobacteria, particularly of *Streptomyces* genus, has been widely recognized as agroindustrially important microorganism due to its potential in producing diverse range of secondary metabolites, including antibiotics and enzymes. Thus, the aim of this work is to characterize and to evaluate the potential of soil actinobacteria for *P. ananatis* control. We observed that 59 actinobacteria strains (85%) exhibited proteolytic or chitinolytic activity. Only the strains *Streptomyces pseudovenezuelae* ACSL 470, that also exhibited high proteolytic activity, *S. novaecaesareae* ACSL 432 *and S. laculatispora* ACP 35 demonstrated high or moderate antagonist activity *in vitro* against *P. ananatis*. Temporal analysis of metabolites produced by these strains growth in different liquid media indicated greater antibacterial activity at 72 h. In this condition, chromatographic and mass spectrometry analysis revealed that *S. pseudovenezuelae* ACSL 470 strain produced neomycin, an aminoglycoside antibiotic that displayed high bactericidal activity *in vitro* against *P. ananatis.* This is the first report of actinobacteria acting as potential microbial antagonists for *P. ananatis* control. Further studies are needed to determine the control efficacy of maize white spot disease by *Streptomyces* strains or their metabolites in greenhouse and field conditions.

Keywords: actinomycetes, biological control, antagonism, foliar maize disease.

Resumo

Pantoea ananatis é o agente causal da mancha branca do milho, doença foliar responsável pela redução significativa da produtividade do milho em todo o mundo, especialmente no Brasil. Em geral, o controle de doenças foliares do milho envolve a adoção de genótipos resistentes e a aplicação de agrotóxicos. No entanto, o uso de agroquímicos pode causar aumento significativo dos custos de produção, danos à saúde humana e impactos ambientais negativos. Nesse sentido, o uso de agentes de controle biológico tem sido considerado uma das tecnologias ecologicamente corretas mais promissoras para a agricultura sustentável. Actinobactérias, particularmente do gênero *Streptomyces*, têm sido amplamente reconhecidas como microrganismos de importância agroindustrial devido ao seu potencial de produção de diversos metabólitos secundários, incluindo antibióticos e enzimas. Assim, o objetivo deste trabalho é caracterizar e avaliar o potencial de actinobactérias do solo para o controle de *P. ananatis*. Observamos que 59 cepas de actinobactérias (85%) apresentaram atividade proteolítica ou quitinolítica. Apenas as cepas *Streptomyces pseudovenezuelae* ACSL 470, que também exibiu alta atividade proteolítica, *S. novaecaesareae* ACSL 432 e *S. laculatispora* ACP 35 demonstraram alta ou moderada atividade antagonista *in vitro* contra *P. ananatis*. A análise temporal do crescimento dos metabólitos produzidos por essas cepas em diferentes meios líquidos indicou maior atividade antibacteriana em 72 h. Nesta condição, análises cromatográficas e de espectrometria de massa revelaram que a cepa *S. pseudovenezuelae* ACSL 470 produziu neomicina, um antibiótico aminoglicosídeo que apresentou alta atividade bactericida *in vitro* contra *P. ananatis*. Este é o primeiro relato de actinobactérias atuando como potenciais antagonistas microbianos para o controle de *P. ananatis*. Mais estudos são necessários para determinar a eficácia do controle da doença da mancha branca do milho por cepas de *Streptomyces* ou seus metabólitos em condições de casa de vegetação e campo.

Palavras-chave: actinomicetos, controle biológico, antagonismo, doença foliar do milho.

*e-mail: ivanildo.marriel@embrapa.br
Received: September 19, 2022 – Accepted: April 14, 2023

1. Introduction

Maize (*Zea mays* L.) is one of the most economically important cereals worldwide, extensively used for food, feed and in high-tech industries (Langner et al., 2019). Although Brazil occupies the third position on world maize production, this crop is susceptible to various foliar diseases (Paccola, 2002; FAO, 2020). Furthermore, due to changes in cultivation strategies, climate, and the extensive use of susceptible hybrids, the occurrence and incidence of maize diseases has increased considerably (Faria et al., 2016; Mueller et al., 2020).

Maize white spot, caused by the bacterium *Pantoea ananatis*, emerged as one of the most important foliar maize disease widely distributed in America (Kistner et al., 2021), Africa (Goszczynska et al., 2007), Europe (Krawczyk et al., 2010) and Asia (Cui et al., 2022), and is considered a potential threat to maize production in regions where high humidity and low night time temperatures are prevalent during the growing season. Symptoms are initially expressed as aqueous lesions in the basal leaves, rapidly spreading to the plant with a chlorotic appearance at more advanced stages of the disease, causing drastically reducing cycle and photosynthetic area of the affected plants (Derera et al., 2007). In Brazil, yield loss was as high as 60% due to the reduction in grain size and weight (Escanferla et al., 2018; Sun et al., 2020).

The adoption of maize resistant hybrids and pesticides application have been employed for maize white spot disease management. The use of disease-resistant genotypes has been the most efficient strategy to reduce yield losses and collaborate with environmental preservation, although it presents limitations and increases the cost of production (Mueller et al., 2020; Kistner et al., 2021). In the other side, the indiscriminate use of pesticides can lead to negative environmental impacts, damage to human health and the selection of resistant pathogens (Bastos et al., 2019; Lopes-Ferreira et al., 2022). Then, there is an urgent demand for ecologically compatible and efficient strategies to suppress pathogens in both conventional and organic agriculture.

In this scenario, the use of biological control by microorganisms as natural agents can be a viable, promising and ecologically alternative for plant diseases control. In recent decades, the selection of microorganisms with potential use in plant disease biocontrol has become one of the main targets of agricultural research (Chanthasena and Nantapong, 2016; Kaur et al., 2019). The adoption of biocontrol agents can increase productivity, improve the phytosanitary status of plants, stimulate more sustainable food production, decrease the application of pesticides and reduce the costs involved in the process (Zou et al., 2021). Several studies have identified efficient antagonist microorganisms for biological control (Dornelas et al., 2017; Melo et al., 2021).

Actinobacteria, especially of *Streptomyces* genus, have been widely recognized as agroindustrially important microorganism due to its potential in producing diverse range of secondary metabolites, including antibiotics and enzymes, with potential use in biocontrol of phytopathogenic microorganisms (Kaur et al., 2019). Antibiotics and enzymes may to inhibit protein synthesis and several enzymes necessary for bacterial and fungal growth. Furthermore, they act directly on structures such as cell membrane and ribosomes, leading to cell damage, cell shrinkage, cytosolic loss and microorganism death (Alekhya and Gopalakrishnan, 2014; Gopalakrishnan et al., 2020; Sharma and Thakur, 2020). Thus, the aim of this work is to evaluate *in vitro* the potential of Brazilian soil actinobacteria strains and the mechanisms involved in the control of *P. ananatis*, the causal agent of maize white spot disease.

2. Material and Methods

2.1. Microorganisms and inoculum preparation

In this study, we used sixty-nine actinobacteria strains from the genera *Streptomyces*, *Amycolatopsis* or *Kitasatospora* (Dornelas et al., 2017) and two phytopathogenic *Pantoea ananatis* strains (CMPC 40 and CMPC 105) belonging to the Multifunctional Microorganisms Collection from Embrapa Milho e Sorgo, Brazil. The actinobacteria strains were grown in agar glycerol-asparagine (AGA) medium supplemented with cycloheximide (30 µg mL^{-1}). After inoculation, the plates were incubated for 14 days at 28 °C (Pridham and Lyons Junior, 1961). *Pantoea ananatis* CMPC 40 and CMPC 105 strains were grown in Potato Dextrose Agar (PDA) medium at 30 °C for 24 h. The pure cultures of actinobacteria and *P. ananatis* were diluted with 0.85% (w/v) NaCl and adjusted to 1.5 x 10^8 cells mL^{-1} (Costa et al., 2018).

2.2. Evaluation of proteolytic and chitinolytic activity

We evaluated proteolytic and chitinolytic activity of all actinobacteria strains. Chitinase production was verified in chitin-yeast-salt extract (CYS) (Kavitha and Vijayalakshmi, 2011) and proteases in agar-gelatin-milk (AGL) (Sarmento et al., 2021). The formation of a clear halo surrounding the colony indicates proteolytic or chitinolytic activity.

2.3. Evaluation of the antibacterial activity of actinobacteria against Pantoea ananatis

In order to select actinobacterial strains with antibacterial activity against *P. ananatis*, we performed a primary selection in a solid medium. Aliquots containing 10^5 cells.mL^{-1} of the sixty-nine actinobacteria strains were individually inoculated, in the form of spots, in Petri dishes containing AGA medium and incubated for 14 days at 28 °C. After incubation, the plates were treated with and without ultraviolet (UV) radiation for 15 min for inactivation of actinobacteria vegetative cells. Then, the colonies were covered with 5 mL of Tryptone Soya Broth (TSB) medium containing *P. ananatis* cells (Williams et al., 1983), followed by incubation at 28 °C for 48 h. After this period, the inhibition halos indicative of antimicrobial activity against *P. ananatis* were measured. Antimicrobial activity was considered absent (halo = 0 mm), low (halo: 7 – 10 mm), moderate (halo: 11 – 14 mm) and high (halo > 14 mm) (Silva et al., 2020) (Figure 1). The actinobacteria with moderate and high antibacterial activity against *P. ananatis* CMPC 40 strain were selected for secondary antibacterial activity assay.

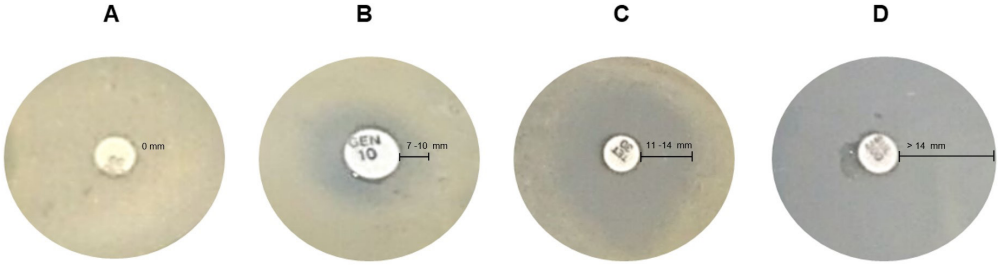

Figure 1. Diagram representing the (A) absent, (B) low, (C) moderate, and (D) high antimicrobial activity of commercial antibacterials against *Pantoea ananatis*. Antimicrobial activity is based on the absence, presence, and the measurement of halos indicative of microbial growth inhibition.

The actinobacteria selected were initially grown in Yeast Malt (ISP-2) medium under constant agitation at 28 °C for 48 h. Then, aliquots of each isolate (10^5 cells.mL^{-1}) were transferred to tubes containing ISP-2 or M1 minimal medium (M1) (Shirling and Gottlieb, 1966). The actinobacteria strains growth in both culture medium was carried out under constant agitation for 120 h at 28 °C. Every 24 h, 5 mL of the samples were centrifuged at 4,000 rpm for 15 minutes and the supernatant was stored at -80 °C.

Paper discs of 6 mm of diameter with 20 µL of the supernatants (metabolites) were transferred to plates individually covered with *P. ananatis* strains, CMPC 40 and CMPC 105. The plates were incubated at 30 °C for 48 h and inhibition halos was evaluated (Bauer et al., 1966; Rodrigues et al., 2019). After determining the culture medium and time of growth in which there was greater antibacterial activity, the samples containing the metabolites produced by actinobacteria strains were lyophilized and resuspended in 5 mL of sterile deionized water.

2.4. Analysis of antibacterial agents produced by actinobacteria strains by ultra-high performance liquid chromatography coupled to mass spectrometry

Based on the results of primary and secondary selection for antibacterial activity against *P. ananatis,* we evaluated the antibiotic production by the actinobacteria strains *Streptomyces pseudovenezuelae* ACSL 470, *S. novaecaesareae* ACSL 432 and *S. laculatispora* ACP 35. Initially, 1 mL of the concentrated supernatant containing the metabolites from each sample was transferred to conical tubes containing 9 mL of 5% (w/v) aqueous trichloroacetic acid (TCA) solution. The mixture was stirred for 5 min and 2 mL of the mixture was transferred to microtubes and centrifuged at 14,000 rpm for 12 min at 4 °C. The extracts were filtered through a nylon membrane with a 0.22 µm pore and a diameter of 13 mm in glass vials for an automatic injector and analyzed by ultra-high performance liquid chromatography coupled to mass spectrometry (UHPLC-MS/MS) with a source of electrospray ionization (ESI in positive mode). The UHPLC-MS/MS system consisted of a binary pump with an automatic injector coupled to a triple quadrupole mass spectrometer with ESI source, RRLC 1200 (Agilent Techonologies, EUA) and API 5000 system (Applied Biosystems, EUA). Data acquisition was performed using Analyst® software version 1.5.1 (Agilent Techonologies, EUA). The analyzes of the extracts were carried out according to the following chromatographic conditions: Zorbax Eclipse XDB C18 column, 50 x 4.6 mm, 1.8 µm; injection volume of 10 µL; column temperature of 35 °C; mobile phase A – 0.2% (v/v) of heptafluorobutyric acid in deionized water; mobile phase B – acetronitrile; flow of 0.6 mL/min and gradient system (initial time – 90% A; 2.0 min – 50% A; 2.5 min – 50% A; 3.0 min – 90% A and 6 min – 90% A). The conditions used in mass spectrometry were: electrospray + ionization mode, 650 °C source temperature, 5500 V ion spray voltage (IS), collision gas (CAD) 6, curtain gas (CUR) 20, ion source gas 1 (GS1) 50 and ion source gas 2 (GS2) 50.

The analyzed metabolytes were from the group of aminoglycosides (spectinomycin, streptomycin, dihydroestreptomycin, amikacin, hygromycin, apramycin, gentamicin, neomycin, tobramycin, kanamycin) and tetracyclines (oxytetracycline, tetracycline, clortetracycline and fluoxoxin and fluoxycline). Positive control (culture medium fortified with a standard solution of the analytes in a concentration varying between 15.0 and 500.0 µg/L) and a negative control (culture medium only) were included.

2.5. Susceptibility of Pantoea ananatis to commercial antibacterials agents

Since the production of metabolites with antimicrobial action has been analyzed, we evaluated the susceptibility of two *P. ananatis* strains (CMPC 40 and CMPC 105) to 25 commercial antibacterial agents (M02-A12, CLSI, 2012). The disks containing antibiotics (CECON, Brazil) had their quality controlled with the following standardized bacterial strains: *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853) and *Enterococcus faecalis* (ATCC 33186) (Martinez et al., 1996).

Pantoea ananatis strains were grown in PDA at 30 °C for 24 h. The inoculum was spread over the entire surface of the Petri dishes containing PDA medium. The disks containing individual antibacterials (norfloxacin, ciprofloxacin, nalidixic acid, levofloxacin, tetracycline, chloramphenicol, cefoxitin, sulfazotrim, amikacin, kanamycin, neomycin, tobramycin, gentamycin, clindamycin, sulphonamide, ampicillin, cefazolin, vancomycin, oxacillin, cefotaxime, erythromycin, penicillin G, nitrofurantoin, ceftriaxone and cephalothin) were placed equidistant and the plates incubated at 30 °C for 48 h. The diameter of the inhibition halos was measured for determination of the susceptibility of *P. ananatis* strains against commercial antibacterial agents (Mamede et al., 2022).

2.6. Statistical analysis

All tests were arranged in completely randomized design with three replications per sample. The antibacterial activity and proteolytic and chitinolytic activity assays were analyzed according to a 2 x 69 factorial scheme. The temporal production of metabolites with antibacterial action in liquid medium was analyzed according to a 2 x 3 x 5 factorial scheme. The antibiogram assays were analyzed according to a 2 x 25 factorial scheme. The results of the antibacterial and enzymatic activity assays were analyzed individually and, when significant differences occurred by the F test ($p \leq 0,05$), the data were subjected to analysis of variance and the means compared by the Scott-Knott test, at 5% probability. For the temporal evaluation, the analysis was performed by the Tukey's test, at 5% probability. Statistical analysis was performed using the SISVAR 5.3 program (Ferreira, 2010). All assays were performed at least twice and the results were reproducible.

3. Results

3.1. Proteolytic and chitinolytic activity of actinobacteria

Enzymatic activity assays of 69 actinobacteria strains, belonged to *Streptomyces*, *Amycolatopsis* and *Kitasatospora* genera, revealed that 59 (85%) strains exhibited proteolytic or chitinolytic activity. *Streptomyces longwoodensis* ACSL 18A exhibited the highest proteolytic or chitinolytic activity when compared to the other strains. *Streptomyces hygroscopicus* ACSL 6 exhibited higher chitinase activity, but lower protease activity. In contrast, *S. pseudovenezuelae* ACSL 470 exhibited higher protease activity, but lower chitinase activity. Taken together, the strains *S. longwoodensis* ACSL 18A, *S. hygroscopicus* ACSL 6 and *S. pseudovenezuelae* ACSL 470 showed the best results for proteolytic or chitinolytic activity (Table 1).

Table 1. Chitinase and protease enzymatic index (EI) of soil actinobacteria strains.

Strain	Enzimatic index*	
	Chitinase	Protease
Streptomyces seymenliensis ACSL 1A	5.60 b	4.26 f
S. massasporeus ACSL 1B	3.35 e	2.67 h
S. chartreusis ACSL 2	5.00 c	3.29 g
S. hygroscopicus ACSL 6	6.55 a	5.70 c
S. galbus ACSL 7	3.10 f	1.25 l
S. sporocinereus ACSL 8	3.60 e	2.15 i
Kitasatospora atroaurantiaca ACSL 12	4.74 c	4.12 f
S. higroscopicus ACSL 13	5.15 c	5.45 c
S. purpeofuscus ACSL 16A	0.00 h	0.00 n
S. galbus ACSL 16B	4.41 d	2.25 i
S. longwoodensis ACSL 18A	6.50 a	6.40 a
S. phaeochromogenes ACSL 18B	2.90 f	1.95 j
S. yunnanensis ACSL 22	5.10 c	4.30 f
S. indiaensis ACSL 23	4.30 d	3.06 g
Amycolatopsis rifamycinica ACSL 25	3.92 e	5.01 d
S. lydicus ACSL 27A	0.00 h	1.92 j
S. corchorusii ACSL 27B	4.44 d	3.27 g
S. sampsonii ACSL 50	2.88 f	2.73 h
K. paracochleata ACSL 53	2.85 f	2.91 h
S. sasae ACSL 54	4.35 d	5.09 d
S. coacervatus ACSL 64A	5.10 c	3.29 g
S. griseoruber ACSL 64B	3.50 e	0.00 n
S. phaeopurpureus ACSL 67	3.65 e	2.60 h
K. phosalacinea ACSL 77	4.55 d	6.05 b
S. phaeochromogenes ACSL 80	0.00 h	0.00 n
K. paracochleata ACSL 82	3.55 e	5.57 c
S. longwoodensis ACSL 83	2.75 f	2.18 i
S. phaeochromogenes ACSL 85	5.31 b	4.45 e
S. yunnanensis ACSL 91	3.78 e	4.15 f
A. echigonensis ACSL 93	4.20 d	3.39 g
S. thioluteus ACSL 115	0.00 h	0.00 n
S. chartreusis ACSL 404	4.05 d	4.92 d
S. novaecaesareae ACSL 432	0.00 h	0.00 n

*Mean values followed by the same letter in each column do not differ by the Scott-Knott test at 5% probability.

Table 1. Continued...

Strain	Enzimatic index*	
	Chitinase	Protease
S. sioyaensis ACSL 448	4.45 d	3.20 g
S. yunnanensis ACSL 449	3.45 e	2.62 h
A. bullii ACSL 450	4.25 d	0.00 n
S. galbus ACSL 453	4.57 d	4.00 f
A. pretoriensis ACSL 457	5.50 b	3.22 g
S. pseudovenezuelae ACSL 470	4.22 d	6.40 a
S. psammoticus ACSL 485	3.85 e	0.00 n
A. kentuckyensis ACSL 490	3.95 e	2.54 h
A. lexingtonensis ACSL 495	5.70 b	4.82 d
S. deserti ACSL 509	3.40 e	1.55 k
S. phaeochromogenes ACSL 517	2.72 f	3.40 g
S. olivochromogenes ACPM 5	3.20 f	4.55 e
S. scabiei ACPM 29	3.70 e	2.00 j
S. phaeopurpureus ACPM 31	0.00 h	0.00 n
S. rishiriensis ACPM 38	4.50 d	3.60 g
S. Sioyaensis ACPM 66	3.80 e	3.15 g
S. endophyticus ACPM 346	1.55 g	0.00 n
S. galbus ACPM 363	4.10 d	3.10 g
K. viridis ACPM 364	3.91 e	0.00 n
S. lannensis ACPM 641	4.15 d	5.14 d
S. ossamyceticus ACJ 1	4.15 d	3.42 g
S. bangadleshensis ACJ 17	0.00 h	0.00 n
S. capoamus ACJ 26	4.40 d	2.80 h
S. galbus ACJ 29	2.90 f	3.45 g
S. psammoticus ACJ 36	3.73 e	0.00 n
S. psammoticus ACJ 43	0.00 h	1.11 m
S. curacoi ACJ 45	1.80 g	0.00 n
S. chiangmaiensis ACJ 48	4.90 c	4.87 d
A. rhabdoformis ACJ 49	4.00 e	2.34 i
S. griseoruber ACJ 51	1.57 g	2.60 h
S. yaanensis ACJ 52	0.00 h	2.14 i
S. cyslabdanicus ACJ 53	4.20 d	1.95 j
S. galbus ACJ 66	4.00 e	3.45 g
S. yunnanensis ACJ 76	1.78 g	1.65 k
S. laculatispora ACP 35	0.00 h	0.00 n
S. variabilis ACCB 1	3.55 e	4.56 e

*Mean values followed by the same letter in each column do not differ by the Scott-Knott test at 5% probability.

3.2. Primary selection of actinobacteria strains in solid medium against Pantoea ananatis

We performed the primary selection of all sixty-nine actinobacteria strains in solid medium against phytopathogenic *P. ananatis* CMPC 40 strain. The actinobacteria strains were also exposure to UV radiation to eliminated vegetative cell and to verify only the effect of their metabolites against *P. ananatis*.

Only nine actinobacteria strains (13%) showed antimicrobial activity against *P. ananatis* CMPC 40 strain (Table 2). Of then, three isolates (4.4%), belong to *Streptomyces* genus, showed moderate or high antimicrobial activity when exposed or not to UV radiation. The maintenance of antimicrobial activity even after exposure to UV radiation suggests the production of stable metabolites by *Streptomyces* against *P. ananatis*. Thus, the strains *S. pseudovenezuelae* ACSL 470, *S. novaecaesareae* ACSL 432 and *S. laculatispora* ACP35 were selected for analysis of temporal changes in metabolites production in two different liquid media against two phytopathogenic *P. ananatis* strains.

3.3. Temporal evaluation of antibacterial activity of metabolites produced in liquid mediums by actinobacteria strains against Pantoea ananatis strains

We also evaluated the temporal production of metabolites by actinobacteria *S. pseudovenezuelae* ACSL 470, *S. novaecaesareae* ACSL 432 and *S. laculatispora* ACP 35 grown for until 120 h in ISP-2 and M1 liquid media. The metabolites produced in at 72 h in both media exhibited greater inhibition of two phytopathogenic *P. ananatis* strains, CMPC 40 and CMPC 105 (Table 3).

Table 2. Antibacterial activity of actinobacteria strains grown in solid medium, exposed and no exposure to UV radiation, against *Pantoea ananatis* CMPC 40 strain.

Actinobacteria strain	Diameter of inhibition halo (mm) of *P. ananatis**		Antibacterial activity
	No exposure to UV	Exposure to UV	
Streptomyces pseudovenezuelae ACSL 470	36.00 a	24.00 a	High
S. novaecaesareae ACSL 432	19.00 b	14.00 b	High
S. laculatispora ACP35	13.00 c	12.00 c	Moderate
S. sporocinereus ACSL 8	10.00 d	0.00 d	Low/Absent
S. indiaensis ACSL 23	10.00 d	0.00 d	Low/Absent
Amycolatopsis bullii ACSL 450	9.00 e	0.00 d	Low/Absent
S. massasporeus ACSL 1B	9.00 e	0.00 d	Low/Absent
S. yaanensis ACJ 52	9.00 e	0.00 d	Low/Absent
S. phaeochromogenes ACSL 517	8.00 e	0.00 d	Low/Absent

*Means followed by the same letter in each column do not differ by the Scott-Knott test at 5% probability.

Table 3. Antibacterial activity of metabolites produced by *Streptomyces* strains grown in liquid medium against two *Pantoea ananatis* strains (CMPC 40 and CMPC 105).

Actinobacteria strain	Liquid medium	Grow time (h)	Diameter of inhibition halo (mm) of *P. ananatis* strains*	
			CMPC 40	CMPC 105
Streptomyces pseudovenezuelae ASCL 470	ISP-2	24	0.00 d	0.00 d
		48	6.00 c	6.00 c
		72	10.00 a	11.0 a
		96	8.00 b	8.00 b
		120	8.00 b	7.00 b
	M1	24	0.00 d	0.00 d
		48	6.00 c	7.00 b
		72	10.00 a	10.00 a
		96	7.00 b	7.00 b
		120	7.00 b	7.00 b
S. novaecaesareae ASCL 432	ISP-2	24	0.00 d	0.00 d
		48	6.00 c	6.00 c
		72	10.00 a	10.00 a
		96	7.00 b	8.00 b
		120	7.00 b	7.00 b
	M1	24	0.00 d	0.00 d
		48	6.00 c	6.00 c
		72	9.00 a	9.00 a
		96	7.00 b	7.00 b
		120	7.00 b	6.00 c
S. laculatispora ACP 35	ISP-2	24	0.00 d	0.00 d
		48	7.00 c	7.00 b
		72	9.00 a	10.00 a
		96	8.00 b	7.00 b
		120	7.00 c	7.00 b
	M1	24	0.00 d	0.00 d
		48	6.00 b	6.00 b
		72	8.00 a	9.00 a
		96	6.00 b	6.00 b
		120	6.00 b	6.00 b

*Means followed by the same letter in strain and liquid medium do not differ by the Tukey test at 5% probability.

3.4. Prospecting and identification of antibiotics produced by actinobacteria

Based on temporal antibacterial activity of three *Streptomyces* strains (*S. pseudovenezuelae* ACSL 470, *S. novaecaesareae* ACSL 432 and *S. laculatispora* ACP 35) that showed high inhibition *in vitro* against *P. ananatis*, we selected their metabolites produced at 72 h in two different media for the identification of antibacterial substances. The chromatographic analysis coupled with mass spectrometry revealed, in the metabolites produced by *S. pseudovenezuelae* ACSL 470 in ISP-2 medium, one peak at 3.77 min and one peak with mass/charge (m/z) of 615.3, typical of neomycin, an aminoglycoside antibiotic (Figure 2).

3.5. Antibiogram

The *P. ananatis* CMPC 40 and CMPC 105 strains showed high sensitivity for 48% (12) of commercial antibacterial agents tested, including neomycin. Norfloxacin and ciprofloxacin showed, on average, the highest antibacterial activity against both *P. ananatis* strains. The *P. ananatis* strain CMPC 40 showed greater sensitivity to antibacterials. Clindamycin, sulphonamide, ampicillin, cefazolin, vancomycin, oxacillin, cefotaxime, erythromycin, penicillin G, nitrofurantoin and ceftriaxone and cephalothin did not exhibit any antibacterial activity against *P. ananatis* strains (Table 4).

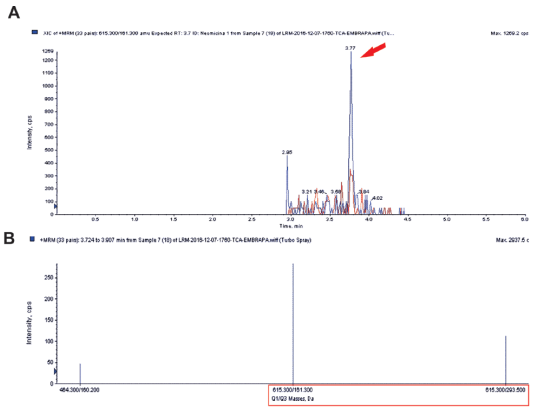

Figure 2. Identification of the antibiotic neomycin produced by *Streptomyces pseudovenezuelae* ACSL 470 in ISP-2 medium after 72 h. (A) Chromatogram with the neomycin pattern (blue line) and the identification of neomycin (red line; 3.77 min) produced by *S. pseudovenezuelae* ACSL 470 (red arrow). (B) Mass/charge transitions (m/z) characteristic of neomycin produced by *S. pseudovenezuelae* ACSL 470 (red box).

Table 4. Antimicrobial activity against *Pantoea ananatis* strains (CMPC 40 and CMPC 105) by 25 commercial antibacterial agents.

Antibiotic	Diameter of inhibition halo (mm) of *P. ananatis* strains*		Antimicrobial activity
	CMPC 40	CMPC 105	
Norfloxacin (10 µg)	37.67 a	39.67 a	High
Ciprofloxacin (5 µg)	37.67 a	39.00 a	High
Nalidixic acid (30 µg)	34.67 b	31.00 b	High
Levofloxacin (5 µg)	34.67 b	28.33 c	High
Tetracycline (30 µg)	35.00 b	27.00 c	High
Chloramphenicol (30 µg)	30.33 c	25.33 d	High
Cefoxitin (30 µg)	27.33 d	23.33 d	High
Sulfazotrim (25 µg)	22.33 e	20.67 e	High
Amikacin (30 µg)	17.33 f	20.00 e	High
Kanamycin (30 µg)	16.33 f	17.00 f	High
Neomycin (200 µg)	16.00 f	16.33 f	High
Tobramlneoycin (10 µg)	15.33 f	13.67 g	High/Moderate
Gentamycin (10 µg)	13.33 g	12.67 g	Moderate
Clindamycin (2 µg)	0.00 h	0.00 h	Absent
Sulphonamide (300 µg)	0.00 h	0.00 h	Absent
Ampicilin (10 µg)	0.00 h	0.00 h	Absent
Cefazolin (30 µg)	0.00 h	0.00 h	Absent
Vancomycin (30 µg)	0.00 h	0.00 h	Absent
Oxacilin (1 µg)	0.00 h	0.00 h	Absent
Cefotaxime (30 µg)	0.00 h	0.00 h	Absent
Erythromycin (15 µg)	0.00 h	0.00 h	Absent
Penicilin G (10 U.I)	0.00 h	0.00 h	Absent
Nitrofurantoin (300 µg)	0.00 h	0.00 h	Absent
Ceftriaxone (30 µg)	0.00 h	0.00 h	Absent
Cephalothin (30 µg)	0.00 h	0.00 h	Absent

*Means followed by the same letter in each column do not differ by the Scott-Knott test at 5% probability.

4. Discussion

Actinobacteria are microorganisms with great biotechnological potential due to their ability to produce metabolites, such a antibiotics, capable of inhibiting and controlling phytopathogenic microorganisms growth (Minotto et al., 2014; Dornelas et al., 2017; Kaur et al., 2019). In this work, we evaluated *in vitro* the antagonism of actinobacteria strains, isolated from Brazilian soil, against *P. ananatis,* the causal agent of maize white spot disease that is responsible for significant maize yield reduction worldwide. Only 4.4% of the actinobacteria strains, from *Streptomyces* genus, exhibited high antimicrobial *in vitro* activity against *P. ananatis*. The low frequency of strain with antimicrobial activity against Gram-negative bacteria, such as *P. ananatis*, may be related to the complex structure of the outer membrane of these microorganisms, which reduces the action of the antibacterial agents (Nithya et al., 2012).

Since actinobacteria strains exhibited antibacterial activity against *P. ananatis*, we also evaluated whether exposure to UV radiation interferes with the degradation and instability of antimicrobial substances spread in the culture medium. We observed that some actinobacteria strains were able to inhibit the growth of *P. ananatis* when not exposed to UV radiation. When the same isolates were exposed to radiation, only three strains were able to inhibit *P. ananatis*, indicating that the UV radiation may have influenced the stability of their metabolites (Cordeiro et al., 2021). Actinobacteria, especially of the genus *Streptomyces,* are capable of producing several molecules with antimicrobial properties, being responsible for producing 80% of the antimicrobials currently commercialized (Sharma and Thakur, 2020). *In vivo* and *in vitro* bioassays demonstrated that *Streptomyces* spp. showed high effect against some bacterial phytopathogens, including *Erwinia* (*Pantoea*) *carotovora*, the causal agent of potato and cabbage soft rot (Qiu et al., 2011; Salem and Abd El-Shafea, 2018).

As fact, it has been well established that antibiotics producing by *Streptomyces* strains can be used for combating certain bacterial diseases of many economically important plants (Vidaver, 2002). Antibiotics can provide a protective barrier on the surface of plants to suppress the growth of the pathogens before infection (McManus and Stockwell, 2001). Some synthetic antibacterials derived from *Streptomyces* have been used to maize white spot disease control. For example, oxytetracycline reduced the number of white spot lesions in maize plants grown in the field by approximately 80-90%, as well inhibited 100% of *P. ananatis* growth *in vitro* (Costa et al., 2011; Gonçalves et al., 2013). Furthermore, the combined use of oxytetracycline and streptomycin demonstrated 60% effectiveness in the management of maize white spot disease in the phenological stages V8 (eight fully developed leaves) and pre-flowering (Manerba et al., 2013).

In the present study, *Streptomyces pseudovenezuelae* ACSL 470, *S. novaecaesareae* ACSL 432 and *S. laculatispora* ACP 35 were responsible for the production of antimicrobial agents against *P. ananatis*. Among them, only *S. pseudovenezuelae* ACSL 470 exhibited proteolytic and chitinolytic activity. These enzymes can play an important role in the degradation of different compounds present in different microorganisms, assisting in nutrition and, consequently, exhibiting antibacterial, antifungal, insecticidal or nematicidal activity (Edreva, 2005; Alekhya and Gopalakrishnan, 2014). The isolation, screening and characterization of secondary metabolite-producing strains of actinobacteria has become an area of interest for research worldwide (Chaudhary et al., 2013). In addition, the search for *Streptomyces* genus actinobacteria in poorly studied habitats raises the prospect of discovering natural products that can be developed with the help of biotechnology (Ogunmwonyi et al., 2010). *In vitro* tests with actinomycetes have shown antagonism against several phytopathogens (Sahilah et al., 2010; Minotto et al., 2014; Yang et al., 2019), revealing the potencial use of these microorganisms for biological control.

In our study, the temporal evaluation of antibacterial activity demonstrated that *S. pseudovenezuelae* ACSL 470, *S. novaecaesareae* ACSL 432 and *S. laculatispora* ACP 35 were able to produce metabolites with activity against *P. ananatis*, reinforcing the potential of *Streptomyces* sp. antimicrobial activity (Daskalaki et al., 2018). The best result for the production of bioactive substances obtained with the ISP-2 medium when compared to M1 medium may have been influenced by glucose concentration (Sánchez and Demain, 2002; Cunha et al., 2009).

In the prospection and identification of antibiotics produced by *S. pseudovenezuelae* ACSL 470, *S. novaecaesareae* ACSL 432 and *S. laculatispora* ACP 35, we verified the presence of neomycin in the ISP-2 fermentative medium from the metabolism of *S. pseudovenezuelae* ACSL 470. As fact, the metabolism of biosynthesis is highly influenced by the availability of nutrients in the medium used, which may be associated with the absence of antimicrobial substances produced in the M1 medium (Charousová et al., 2019). In our study, the antibiogram assay confirmed the high susceptibility of two phytopathogenic *P. ananatis* strains to neomycin. Similar results based on experiments against plant bacterial pathogens showed that other *Pantoea* species, *P. carotovora,* was also sensitive *in vitro* to neomycin (Ma et al., 2011). Neomycin is an aminoglycoside antibiotic that has activity against most gram-negative aerobes, and inhibits protein synthesis by binding, with high affinity, to the A-site on the 16S ribosomal RNA of the 30S ribosome (Kotra et al., 2000). As a result, the antibiotic promotes error prone protein synthesis, allowing for incorrect amino acids to assemble into a polypeptide that is subsequently released to cause damage to the cell membrane and elsewhere (Krause et al., 2016). Post-inoculation spraying with neomycin from the liquid culture of the actinomycete *S. fradiae* HTP has antibacterial activity *in vitro* and *in vivo* against *Pantoea carotovora*, *Ralstonia solanacearum* and *Xanthomonas oryzae*. Surprisingly, neomycin from the liquid culture reducing disease caused by these phytopathogens ranged from 69 to 78% in greenhouse condition (Tao et al., 2011).

In conclusion, *S. pseudovenezuelae* ACSL 470 strain exhibited high antibacterial action against *P. ananatis* and was able to produce hydrolytic enzymes and metabolites contained neomycin, that show high activity against the target microorganism. Therefore, we strongly believe that the use of *S. pseudovenezuelae* ACSL 470 and/or their metabolites for the biocontrol of *P. ananatis* is highly promising.

This is the first report of actinobacteria acting as potential microbial antagonists for *P. ananatis* control. However, it is important to evaluate this effectiveness *in vivo*, carrying out tests in controlled and field conditions.

Acknowledgements

Research supported by Universidade Federal de São João del-Rei (UFSJ), Rede Mineira de Endofíticos and Embrapa Milho e Sorgo (CNPMS).

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