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ORIGINAL ARTICLE

Antifungal activity of *Streptomyces* spp. against *Pyrenophora tritici-repentis* the causal agent of tan spot in wheat

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Abstract

Tan spot, caused by *Pyrenophora tritici-repentis* (*Ptr*), is a worrisome destructive foliar disease of wheat-growing areas around the world. *Streptomyces* spp. have been investigated as biocontrol agents because they beneficially interact with host plants and produce important bioactive substances that can act in the suppression of diseases in plants. In the present study, antifungal activity and plant growth-promoting of *Streptomyces* spp. strains 6(4), R18(6), and their consortium, were evaluated through *in vitro* and greenhouse assays. The Ultra High-Performance Liquid Chromatography-Quadrupole Time-of-Flight Mass Spectrometry (UHPLC-QTOF MS) technique was used to analyze the crude extract of each strain. The results of the *in vitro* tests showed that the 6(4) metabolites caused several abnormalities in the conidial germination of *Ptr.* This strain also produced indole acetic acid (IAA) and siderophores. Strain R18(6) did not alter conidial germination of *Ptr,* and produced IAA and phosphate solubilizers. In the greenhouse, the treatment 'seed inoculation plus foliar spray' with streptomycetes propagules and metabolites contributed to biomass gain, with no statistical difference between the strains ($p < 0.05$). Treatments with 6(4) 'seed inoculation', 'seed inoculation plus foliar spray', and consortium 'seed inoculation' showed the lowest percentage of injured area compared to other treatments ($p < 0.05$). UHPLC-QTOF MS data showed that erucamide is present in the culture of 6(4), but not in the culture of R18(6). Therefore, this substance is one of those involved in *Ptr* hyphal abnormalities, and R18(6) use indirect mechanisms of action to control *Ptr*. We concluded that these *Streptomyces* spp. and their metabolites have a promising potential for biological control of *Ptr* to protect wheat plants from tan spot damage.

Keywords: antifungal compounds, biocontrol agents, *Pyrenophora tritici-repentis, Streptomyces* spp., wheat

Introduction

Wheat (*Triticum aestivum*) is the most important cereal crop in sustaining global food security. However, wheat yields are still lost each year due to diseases (Acevedo *et al*. 2018). A major foliar disease of wheat is tan spot, which is a fungal disease of wheat caused by the necrotrophic fungus *Pyrenophora tritici-repentis* (*Ptr)* (Died.) Drechs. (anamorph, *Drechslera tritici-repentis*) (Died.) Shoem. and is typified by necrotic lesions and regions of chlorosis on infected leaves (Larran *et al.* 2016). Tan spot occurs in many wheat growing areas around the world, including Europe, South America, Canada and Australia. This infection causes a reduction in the photosynthetic area and leads to yield reduction and lower grain quality, causing great

losses to producers (Ciuffetti *et al*. 2014; Savary *et al*. 2019).

Although the use of agrochemicals is necessary to guarantee production, there is a worldwide tendency to reduce the use of these products. Since excessive use of agrochemicals causes several problems and the risk of pathogen resistance development, alternative methods to combat pathogens become important to guarantee yield (Suryanarayanan *et al*. 2018). One of the tools to control plant pathogens with minimal impact on the environment is biocontrol. The use of secondary metabolites of microbial origin is gaining incentive in crop protection and such metabolites may become a supplement or an alternative to chemical control (Larran *et al*. 2016; Rakshit *et al*. 2021).

Streptomyces of the Actinomycetes group stands out as an important producer of antibiotics and other bioactive substances and, continues to provide new antibiotics with a wide variety of chemical structures (Donald *et al*. 2022). These actinobacteria are widely distributed in nature. Some form mutualistic relationships with plants promoting their growth and protecting against pathogens by inducing components of the plant immune system (Olanrewaju and Babalola 2019). Some strains of *Streptomyces* are already registered in commercial products and have their biocontrol capacity reported due to the production of metabolites like siderophores (*S. coelicolor*), chitinase (*S. violaceusniger* YH27A), nigerin, and geldanamycin (*S.violaceusniger* YCED-9) (Som *et al.* 2017; Shrivastava and Kumar 2018). Several researchers have found potential in *Streptomyces* isolates to control fungal diseases, such as those caused by *Fusarium* sp.. This action has been related to the ability of these bacteria to produce metabolites that prevent the growth and development of the fungus. Chromatography has been used to identify these compounds (Chen *et al.* 2018; Qi *et al.* 2022; Zeyad *et al.* 2022). The identification of metabolites is very important for the understanding of mechanisms of action that these compounds exert on the phytopathogen and the host plant.

Thus, this study was aimed to verify the potential of two *Streptomyces* sp. 6(4), R18(6) strains for the control of *P. tritici-repentis* on wheat plants under greenhouse conditions and to investigate by high-resolution liquid chromatography which compounds are involved in this activity.

Materials and Methods

Microorganisms

The *Streptomyces* spp. – 6(4) and R18(6) strains, collected from healthy tomato roots by Oliveira *et al.* (2010), were identified by their morphological characteristics. These strains had their 16S rRNA gene sequenced and were confirmed to be from the genus of *Streptomyces* (Oliveira *et al*. 2010; Borba *et al.* 2022). The strains were cultivated on solid agar plates in casein starch medium – SCA (10 g · l^{-1} Starch; 0.3 g · l^{-1} Casein; 2 g · l^{-1} KNO_3; 2 g · l^{-1} NaCl; 2 g · l^{-1} K_2HPO_4; 0.05 g · l^{-1} $MgSO_4.7H_2O$; 0.02 g · l^{-1} $CaCO_3$; 0.01 g · l^{-1} $FeSO_4.7H_2O$; 15 g · l^{-1} agar), at 28°C for 7 days, and stored in water suspension and 20% glycerol in a freezer at –20°C.

The *P. tritici-repentis* (*Ptr*) isolates (27 and 5-C1/08) were sent by the research corporation Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA) from Passo Fundo – RS, Brazil. They had been isolated from wheat plants with tan spot symptoms. The ITS sequence was amplified with the primer pair ITS1 (5`-TCCGTA GGT GAA CCT GCG G-3`) and ITS4 (5`-TCC TCC GCT TAT TGATAT GC-3`) (White *et al*. 1990) and the PCR products were sequenced. BLAST analysis confirmed the identity of these isolates as *P. tritici-repentis*. The samples were multiplied in a V8-PDA medium [150 ml · l^{-1} V8 Campbell's juice, 10 g · l^{-1} agar, 10 g · l^{-1} PDA (KASVI), 3 g · l^{-1} $CaCO_3$] incubated in a germinator with a photoperiod of 12 h at 25°C for 7 days, and stored in tubes in the form of dehydrated discs in a freezer at –20°C.

The fungal inoculum was produced from sporulating *Ptr*, and grown on a plate with V8-PDA for 5 days in the dark at 25°C, with a lighting schedule of 24 h of light at 25°C and 24 h of darkness at 13°C. The conidia were removed, and the suspension was adjusted to a concentration of 3×10^3 conidia · ml^{-1}.

Production of bioactive metabolites by *Streptomyces* spp.

The *Streptomyces* spp. strains were inoculated in 250 ml Erlenmeyer flasks. Each flask contained 50 ml of starch casein broth. Strain 6(4) was incubated for 96 h and strain R18(6) was incubated for 144 h at 28°C under shaking and aeration conditions (115 rpm). After growth cells were centrifuged at 10,000 rpm for 10 min, the supernatant was transferred to a new tube for further use. For the consortium the two strains were first submitted to a compatibility test on an SCA medium, and neither presented growth inhibition.

Screening for plant growth promoting traits *in vitro*

Phosphate solubilization assays were performed following the protocol of Nautiyal (1999). Plates containing the National Botanical Research Institute's Phosphate (NBRIP) medium were inoculated with

the actinobacterial isolates and incubated at 28°C for 14 days. The assay was performed in triplicate, a positive reaction was determined by the presence of halos under colony growth.

Indole acetic acid (IAA) evaluation was carried out by the method of Gordon and Weber (1951). The *Streptomyces* spp. strains were previously grown on King B broth medium (20 g · l^{-1} peptone, 1.15 g · l^{-1} K_2HPO_4, 1.5 g · l^{-1} $MgSO_4$, 15 g · l^{-1} Glycerin, supplemented with 0.5 g · l^{-1} tryptophan) and incubated at 28°C under agitation at 115 rpm. Every 48 h for 10 days, 2 ml of the culture was transferred to a tube and centrifuged at 13,000 rpm for 5 min. IAA production was determined by transferring 50 µl of the supernatant to a 96-well plate containing 50 µl of Salkowski reagent (2.4 g of $FeCl_3$ and 84.2 ml of H_2SO_4). The 96-well plates were stored in the dark at room temperature for 30 min, and the color intensity was determined by spectrophotometer at λ = 520 nm. The concentration of IAA produced was calculated based on a standard curve of 3-Indoleacetic acid (Neon®) obtained in the range of 1–20 µg · ml^{-1}.

Siderophore assays were carried out by the method of Glickmann and Dessaux (1995). *Streptomyces* spp. strains were inoculated into King B medium for siderophore (4 g · l^{-1} peptone; 0.23 g · l^{-1} K_2HPO_4; 0.3 g · l^{-1} $MgSO_4$; 3 g · l^{-1} glycerol, set at pH 6.8). One milliliter aliquots were withdrawn every 48 h, placed in microcentrifuge tubes, and centrifuged at 13,000 rpm for 5 min. Afterwards, 500 µl of the supernatant was transferred to new tubes containing 500 µl of chromoazurol-S (CAS) dye. To prepare the CAS dye, 60.5 mg of CAS in 50 ml of Mili-Q water was added to 10 ml of $FeCl_3$ solution. The solution was then stirred, and a solution of 72.9 mg of hexadecyltrimethylammonium bromide (CTAB) previously dissolved in 40 ml of water was slowly added, after which the solution was autoclaved for 15 min. A positive reaction was indicated by a change in color from blue to orange or yellow during 30–60 min.

Determination of minimal inhibitory concentration

Minimal inhibitory concentrations were measured by the broth microdilution method, M38-A protocol (CLSI 2002). The compounds (cell-free supernatant, filtered through a 0.22 µm sterile filter (KASVI) and subsequently lyophilized) were dissolved in ultrapure water and diluted by serial dilutions in Roswell Park Memorial Institute (RPMI) broth on a 96-well plate at concentrations of 114, 57, 28.5, 14.25, 7.12, 3.56 mg · ml^{-1} for strain 6(4), and diluted at concentrations of 151.75, 75.87, 37.93, 18.96, 9.48, 4.74 mg · ml^{-1} for strain R18(6). To each well, 100 µl of fungal suspension (8×10^3 conidia/ml) was inoculated and incubated at 25°C for 96 h. Pure RPMI broth was the negative control and fungal suspension (100 µl) plus RPMI (100 µl) was the positive control. The test was performed in triplicate. Fungal growth was assessed by visualization in the optical microscope model Olympus BX41 and the images were analyzed in Mshot Digital Imaging System Software.

Greenhouse experiment

The experiment was performed in a greenhouse of the Departamento de Fitossanidade da Faculdade de Agronomia – Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil from May to June of 2021. The assays were performed in disposable plastic cups with 500 ml of Humusolo® substrate (pH 7.3, N 0.82%, P_2O_5 1%, K_2O 0.25% w/w) and expanded vermiculite (1:1 w/w). In each pot, ten wheat seeds of *Triticum aestivum* cv Ônix, susceptible to leaf spot (OR Sementes®) were sown following the description of each treatment: 1. Application of 5 ml of spore propagules of the *Streptomyces* spp. in sowing; 2. Application of 5 ml of spore propagules of the *Streptomyces* spp. in sowing + spray of the culture supernatant of the *Streptomyces* spp. on the leaves before the pathogen. Untreated control – only spray of *Ptr*, and negative control – seedling wheat with neither antagonist nor pathogen.

Twenty-one days after planting, wheat plants with two expanded leaves were sprayed with suspensions of *P. tritici-repentis* (3×10^3 conidia · ml^{-1}) using a manually operated sprayer. The negative control was not sprayed. The spore propagules of the *Streptomyces* spp. were applied on the seeds in a sowing furrow at the concentration of 1×10^8 spores · ml^{-1}. The culture supernatant of antagonists was applied on the leaves 2 h before the pathogen. After pulverization, the pots were covered with plastic bags for 2 days to maintain a high level of humidity. The seedlings were watered every 2 days, if necessary.

For the evaluation, the second leaf of each plant was selected, for a total of seven leaves for each treatment. The percentage of necrotic area was determined with ImageJ software for 7 days following spraying of the phytopathogen. At the end of the experiment, the seedlings were dried at 50°C for 7 days and the dry weight of the plants was measured.

The experiment was performed in a completely randomized design with seven replications of each treatment. An arcsine transformation was applied to all percentage data before one-way ANOVA ($p < 0.05$), and treatment means were compared using the Scott-Knott test ($p < 0.05$) with software SASM-Agri (Canteri *et al.* 2001).

Liquid chromatography coupled with high resolution mass spectrometry

The presence of compounds with antifungal activity in the previously lyophilized cell-free supernatant was monitored using a Shimadzu Nexera X2 UHPLC system connected to an Impact II QTOF mass spectrometer (Bruker Daltonics). The UHPLC system was equipped with a reversed-phase Luna Omega C18 analytical column. The mobile phase was a mixture of acetonitrile acidified with 0.1% formic acid (eluent A) and water acidified with 0.1% formic acid (eluent B), at a flow rate of 0.35 ml · min^{-1}. The elution gradient was from 5% A (initial condition) to 98% A, in 35 min, returning to the initial condition in 4 min and maintained for another 6 min, giving a total analysis time of 50 min. The QTOF mass spectrometer was operated in positive ionization mode, under the following conditions: capillary at 4000 V, nebulizer at 40 Bar, drying gas at 9 l · min^{-1} and gas temperature at 200°C. The QTOF MS system was operated in wideband collision-induced dissociation (bbCID) acquisition mode, which provided MS and MS/MS spectra at the same time. All MS information was recorded in the m/z range from 50 to 1500 using a sweep rate of 2 Hz. The bbCID mode allows operation with two different collision energies: a low collision energy of 10 eV and a high collision energy of 20 eV (to obtain MS/MS spectra). A list of approximately 150 known *Streptomyces* compounds within the m/z range established for screening in the samples was provided. All suspect data and possible elemental compositions for ions with a deviation of ±5 ppm were evaluated using Data Analysis 4.2 software. The confirmation of compounds was carried out through the free database Mass Bank.

Results

Plant growth regulators

Streptomyces sp. R18(6) was able to solubilize phosphate, and *Streptomyces* sp. 6(4) was able to produce siderophore (Table 1). Both strains produced IAA (Table 1). The strains 6(4) and R18(6), during the 10 days of evaluation, showed the same pattern in IAA production with very close values (13.5 and 12.7 μg · ml^{-1}). The consortium started with a lower production rate but by day 10 (240 h) it produced 16.8 μg · ml^{-1} of IAA.

Table 1. Production of plant growth regulators

Streptomyces sp. strains	Phosphate solubilization	Indole acetic acid [μg · ml^{-1}]	Siderophore
6(4)	–	13.5	+
R18(6)	+	12.7	–
Consortium		16.8	+

(–) – no activity, (+) – positive activity

Determination of minimal inhibitory concentration

After 96 h of incubation, when observing the 96-well plate, from the second well onwards in the presence of the filtered culture of 6(4), germination of conidia was detected and in all wells in the presence of the filtered of R18(6). Investigation revealed that the metabolites caused significant changes in the mycelium morphology at various concentrations in comparison to the control. At the concentration of 114 mg · ml^{-1} of the 6(4) filtered, the conidia of *Ptr* did not germinate. From concentration 57 to 3.56 mg · ml^{-1}, germination took place, but with many changes in the hyphae such as distortions, vesicles, wrinkling of the wall (Fig. 1A) and in the last two concentrations (7.125 mg · ml^{-1} and 3.56 mg · ml^{-1}), the metabolites may have caused disruption of fungal cell walls (Fig. 1B and C). In the control treatment, the conidia germinated without changes (Fig. 1D). In the presence of the filtered culture of the R18(6) isolate, no alterations were detected in the germinated conidia.

Greenhouse experiment

Pereira *et al.* (2022) observed that culture supernatants of these isolates of the *Streptomyces* spp. in the detached leaf assay demonstrated antifungal activity against *P. tritici-repentis*. In the current study we observed the antifungal effect under greenhouse conditions. According to Table 2, the treatments 6(4) 'seed inoculation', 6(4) 'seed inoculation plus foliar spray' and consortium 'seed inoculation' were the ones that showed good performance in reducing the injured area of the leaves, with 4.06, 2.98, and 4.10% of the injured leaf mean area, respectively. No statistical difference between them was observed, but they were statistically different from the untreated seedlings with 14.89% of the injured leaf mean area ($p < 0.05$). The treatments with strain R18(6) also showed good protection of the wheat leaf, resulting in 5.73% and 6.64% of the injured area in the treatments of the 'seed inoculation' and 'seed inoculation plus foliar spray', respectively. These injured areas also were statistically different from the untreated seedlings ($p < 0.05$) (Table 2).

In Figure 2A–F there was a positive effect of *Streptomyces* spp. in controlling tan spot compared

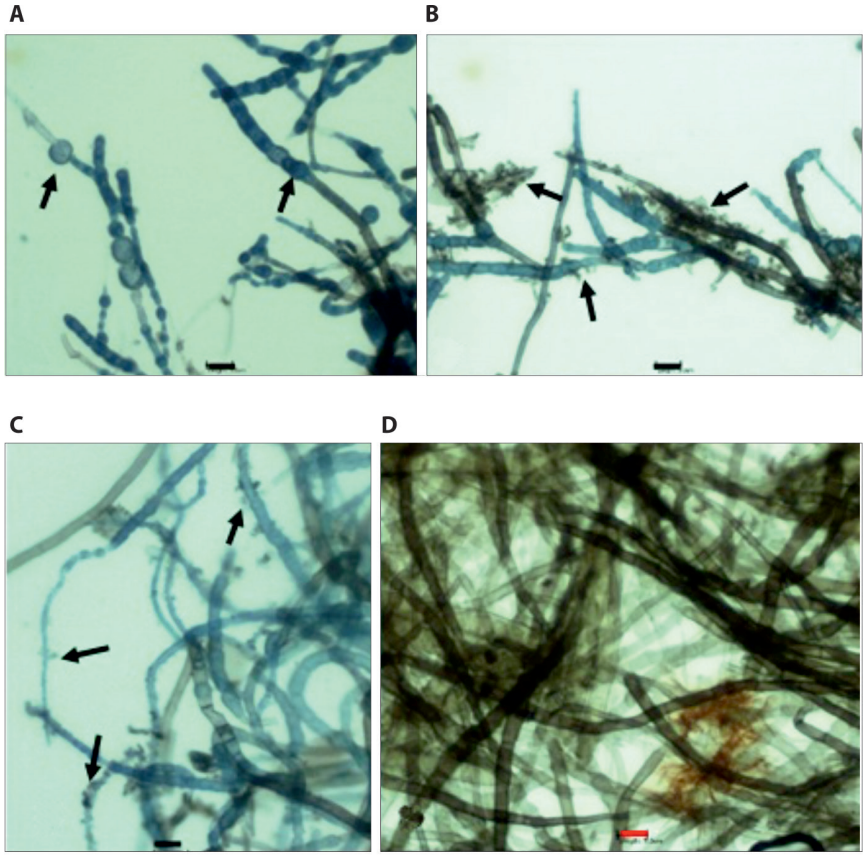

Fig. 1. Light micrographs of minimal inhibitory concentration assay. Abnormal conidia germination in the presence of filtered culture of *Streptomyces* sp. 6(4). A – 57 mg · ml^{-1} – alterations such as vesicles and wrinkling of the hyphae; B – 7.12 mg · ml^{-1} – damage in cell wall; C – 3.56 mg · ml^{-1} – damage in cell wall and wrinkling of the hyphae; D – negative control, without alterations and completely germinated. The black arrows indicate the changes. Scale bar = 5 µm

to untreated plants. In the treated plants there were smaller spots with fewer or no chlorotic borders. In the untreated plants, we observed a greater severity of the tan spot and the chlorotic borders coalescing, debilitating the photosynthetic foliar area (Fig. 2G–H).

In the consortium treatments, the injured area on the leaves was 4.10% and 5.55% from 'seed inoculation' and 'seed inoculation plus foliar spray', respectively (Table 2). Figures 2E and F showed no growth of necrosis and chlorosis in the foliar area until the 7 final

Table 2. Effect of *Streptomyces* spp. and their metabolites in controlling *Pyrenophora tritici-repentis* (*Ptr*) on wheat plants

Treatment	Application method*	Injured leaf mean area [%]**	Leaf mean dry weight [mg]
Streptomyces sp. 6(4)	seed	4.06 (11.1) c	20.64 b
Streptomyces sp. 6(4)	seed and foliar	2.98 (9.5) c	21.94 a
Streptomyces sp. R18(6)	seed	5.73 (13.72) b	19.78 b
Streptomyces sp. R18(6)	seed and foliar	6.64 (14.9) b	21.71 a
Consortium	seed	4.10 (11.5) c	19.58 b
Consortium	seed and foliar	5.55 (13.1) b	23.32 a
Untreated control	spray *Ptr* on leaves	14.89 (22.5) a	19.87 b
Negative control	water	-	23.88 a
CV%		22.66	16

*seed – indicates the application of spore propagules of *Streptomyces* spp. on sowing furrow; seed and foliar – indicates the application of spore propagules of *Streptomyces* spp. on sowing furrow plus foliar spraying with culture supernatant of *Streptomyces* spp. before the pathogen. Untreated control – only foliar spraying of *Ptr;* Negative control: water (healthy plant)

**statistical analysis performed with transformed data, values in parentheses. Means followed by the same letter are not significantly different, the Scott-Knott test was applied at 5% probability level. CV% – coefficient of variation

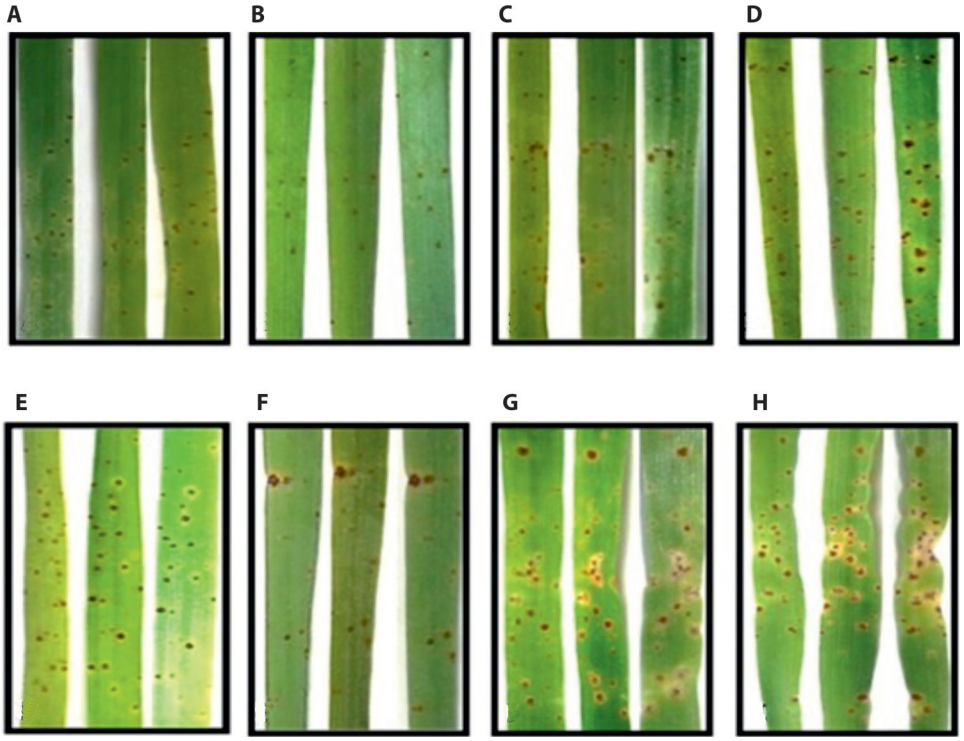

Fig. 2. The low severity of tan spot on leaves of seedlings of wheat treated with the antagonist. The evaluation was performed 7 days after the infection. The three leaves in each frame are the sequence of days captured from the same leaf, the first day, the fourth day, and the seventh day of capture. A – strain 6(4) – sowing furrow; B – strain 6(4) – sowing furrow and foliar spray; C – strain R18(6) – sowing furrow; D – strain R18(6) – sowing furrow and foliar spray; E – consortium – sowing furrow; F – consortium – sowing furrow and foliar spray; G and H – untreated control – foliar spray only of *Pyrenophora tritici-repentis*

days of assessment. Little damage was observed on leaves compared with untreated control (Figs. 2G–H). However, these results were similar to treatment with the single strains ($p < 0.05$), that is, we did not detect a synergism between the strains in the consortium.

We wish to highlight that all treatments with 'seed and foliar' inoculation presented leaf dry weight greater than the untreated seedlings and were similar to the healthy plants. The dry weight of the plants was 21.94, 21.71, and 23.32 mg from 6(4), R18(6), and consortium, respectively, with no statistical difference with healthy plants with 23.88 mg (Table 2). This result indicates that even under biotic stress caused by *Ptr* infection, seedling growth was maintained. Regarding the risk of damage to plant development, these metabolites were not harmful because they do not interfere with plant growth.

Liquid chromatography coupled with high resolution mass spectrometry

This analysis indicated the presence of various compounds in the two samples, with the appearance of several distinct peaks in the spectrum. It was possible

Table 3. Compounds identified from the cell-free supernatant of strain 6(4) through LC MS/MS with error lower than 5 ppm

Name	Meas. [m · z^{-1}]	Ion formula	Mass-to-charge ratio [m · z^{-1}]	Error [ppm]	Score
Erucamide fragment	114.091	$C_6H_{12}NO$	114.0913	2.7	100
Erucamide fragment	121.1008	C_9H_{13}	121.1012	3.2	100
Erucamide fragment	135.1164	$C_{10}H_{15}$	135.1168	3	100
Erucamide fragment	149.132	$C_{11}H_{17}$	149.1325	3.1	100
Erucamide	338.3413	$C_{22}H_{44}NO$	338.3417	1.3	100
Bengamide Z	389.2278	$C_{18}H_{32}N_2O_7$	389.2282	1.1	100

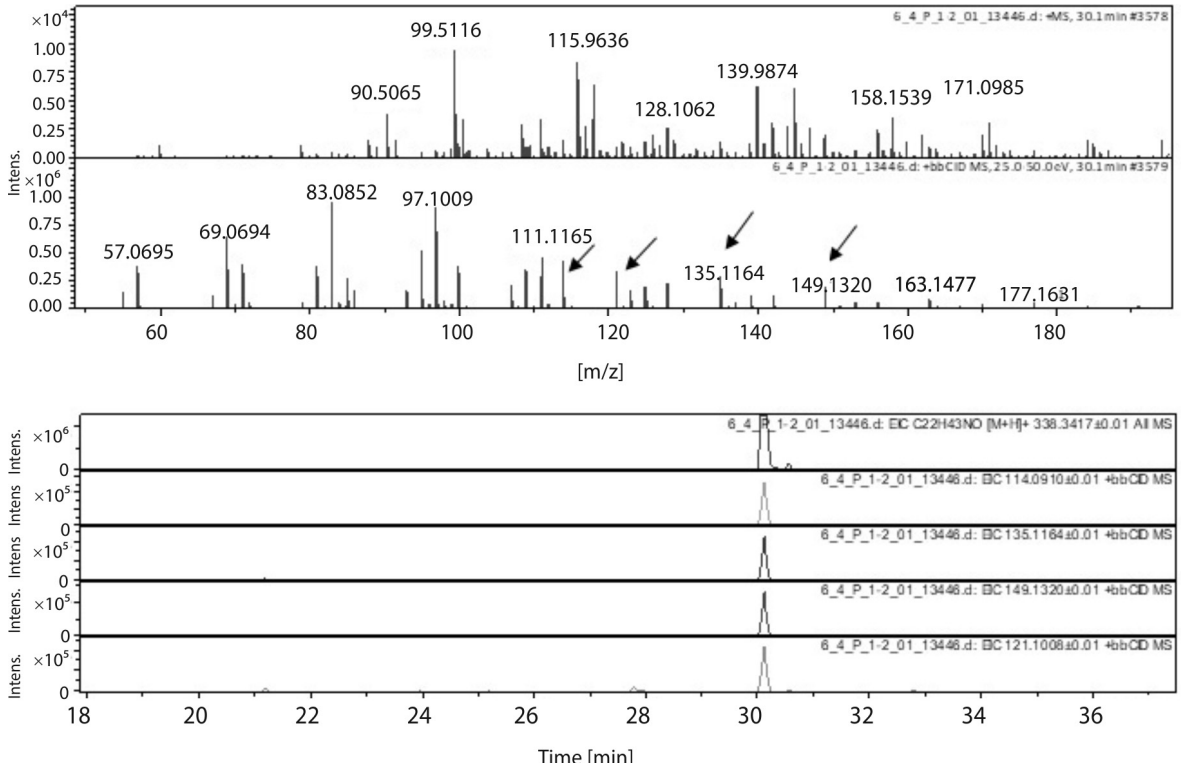

Fig. 3. Mass spectra of sample 6(4) indicate erucamide fragments confirmation, all identified in the same retention time, 30.1 min (last figure). The arrows show corresponding pikes

to confirm the presence of erucamide in the 6(4) supernatant, as shown Table 3 and Figure 3. There is a lot of evidence that bengamide Z is present (Table 3) but it is still has not yet been possible to confirm through the presence of fragments. Erucamide and bengamide were not present in the R18(6) supernatant.

Discussion

Streptomyces spp. are ubiquitous in the soil where they play different ecological roles. They are also producers of several bioactive compounds which demonstrate their potential as microbial biological control agents (LeBlanc 2022). The biocontrol mechanism by *Streptomyces* sp. is a complex process and involves the release of several compounds that will interact with the pathogen and the host plant. In this present work, besides applying the *Streptomyces* spp. propagules at sowing, we also assessed if foliar spraying of cell-free culture would improve the protection of wheat leaves from the infection of the *P. tritici-repentis*.

The results showed that the plants which only received the sowing inoculation with streptomycetes propagules, responded with less severity of tan spot than the untreated plants ($p < 0.05$). The contact of *Streptomyces* spp. on seeds gave plants a better ability to react to infection of the phytopathogen, probably due to the activation of some defense mechanism of the plant. The literature confirms that biological control agents are essential in regulating the resistance of plants against pathogenic organisms and protect plants against biotic stresses (Köhl *et al.* 2019). However, when we also applied the cell-free cultures of streptomycetes on the leaves, the plants had a greater biomass gain than the other treatments ($p < 0.05$). The susceptibility of plants to disease severity is influenced by abiotic and biotic stress factors. We observed through this study that complementation with the pulverization of the metabolites on leaves guaranteed the relief of biotic stress and the plant showed better fitness. Asaturova *et al.* (2022), evaluating seed and plant treatment with *Bacillus* strains against *P. tritici-repentis,* also observed that the treatment of seeds and plants with strain BZR336 was 10% higher than when treating the plants alone. Other authors also observed that cell-free supernatant of *Streptomyces* isolates reduced leaf spots of plants infected with *Botrytis cinerea* and *Alternaria brassicicola,* and contributed to increased dry weight of plants (El-Shatoury *et al.* 2020; Sharma and Manhas 2022).

The results of the present work showed that the cell-free culture, which contains the metabolites from strain 6(4), caused several alterations in the mycelial growth of the *Ptr* at several tested concentrations, e.g., of 57 mg · ml^{-1} until 3.56 mg · ml^{-1}, were capable of causing disruption of the fungal wall and abnormalities in

the hyphae. Therefore, these metabolites have antifungal properties that may have silenced the virulence of *Ptr* on wheat leaves. Effectors are deployed by pathogens, as necrotrophic fungal *Ptr,* to facilitate plant colonization and nutrient acquisition, causing cell death by secreting phytotoxic molecules and degrading plant cell walls (Shao *et al*. 2021). Based on this, the metabolites in treated plants with 6(4) metabolites appear to have altered the communication of fungal effectors with cell plants, preventing the release of toxins that causes necrosis and chlorosis.

The major active compounds from the filtered culture of strain 6(4) identified by UHPLC-QTOF MS was erucamide and bengamide. Erucamide, a fatty acid amide, was found by Qi *et al*. (2022) to be present in the extract from the *Streptomyces* strain. It presented antifungal activity against *Fusarium oxysporum* f. sp. *cubense* tropical race 4 in *in vitro* and *in vivo* experiments, destroying the cell structure and inhibiting the germination and growth of fungal spores. Another recent study by Zeyad *et al*. (2022) found that biopriming with *Streptomyces* isolates modulated the defense response in chickpea against *Fusarium*, and this extract also contained erucamide. The bengamide Z was not fully confirmed but very likely it is present in the sample of 6(4). Bengamide also appeared in LC/MS analyses of the study from Zeyad *et al*. (2022), previously quoted, but the authors did not present any observations about this compound. However, studies from Jamison *et al*. (2019) observed the potent antifungal activity of the bengamide associated with bengazole against *Candida.* The action mechanism was methionine aminopeptidases (MetAPs) inhibition. From this evidence, we infer that erucamide and bengamide produced by strain 6(4) participate in the antifungal activity and modulation of the defense of wheat plants against *Ptr* infection.

Strain R18(6) did not produce the same compounds and did not directly damage the development of *Ptr* as did strain 6(4). Even so, the results showed that in whole plants the R18(6) was also able to reduce the severity of the disease and increase biomass when compared to untreated plants (Table 2). Substances involved in promoting plant growth also play an important role in plant protection. Strain R18(6) produced IAA and solubilized phosphates. IAA is known to play a role in plant morphology as well as stimulating the defense and can contribute to protection against the pathogen. Phosphates are an important factor for vegetable growth (Vurukonda *et al*. 2018). Therefore, these substances must be involved in the mechanism of indirect action of R18(6) against *Ptr*. We cannot attribute antifungal activity just to the substances identified in the spectrum. Unknown compounds may also be involved, because *Streptomyces* do not release only one type of substance. They also produce enzymes and various volatile compounds that are used as mechanisms of biocontrol (Olanrewaju and Babalola 2019).

We also tested the combination of the strains, in a consortium, to verify if there would be a synergism between the two strains to intensify the protection and development of the plants. The results showed that, when inoculated at sowing, the percentage of injured area was statistically equal to treatments with strain 6(4) ($p < 0.05$), while the double treatment was statistically equal to the percentage of R18(6). That is, we did not identify synergism, but further field studies may clarify if there is a coaction of the strains in a consortium, for example, with productivity parameters.

It was clear that *Streptomyces* spp. strains 6(4) and R18(6) produce various metabolites which can play a role in the mechanism of their biological activity, showing the ability to suppress the pathogen. Erucamide, present in the culture of 6(4), may be responsible for damaging *Ptr* hyphae, and at the same time modulate the plant's own defense against *Ptr* infection. The plant growth-promoting compounds and those not yet identified produced by R18(6) took part in the indirect mechanism of biocontrol that decreased the severity of tan spot in wheat seedlings. Therefore, we conclude that these strains, with different mechanisms of action, protect wheat plants from tan spot damage. This study can be the basis for the optimization of further field tests and bioproduct formulation.

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