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# Screening endophytic actinobacteria with potential antifungal activity against *Bipolaris sorokiniana* and growth promotion of wheat seedlings

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**Actinobacteria secrete substances that limit or inhibit the growth of plant pathogenic fungi and may be used in the biocontrol of these microorganisms. The aim of this study was to characterize physiological and enzymatic activity of endophytic actinobacteria, evaluate their antifungal activity against *Bipolaris sorokiniana* root colonization, and evaluate their efficiency in promoting the growth of wheat seedlings. Antibiosis was analyzed using the double-layer method, the agar well diffusion test, and volatile metabolites. Physiological and enzymatic activity was evaluated through chitinase, glucanase, siderophores, indole-3-acetic acid, nitrogen fixation and phosphate solubilization tests. I*n vivo* assays were evaluated by root colonization, biocontrol test and efficiency to promote the growth of wheat seedlings. From all isolates tested, 69.6% of them presented antifungal activity against at least one *B. sorokiriana* isolate. Among these, 17% of the isolates produced bioactive metabolites in the supernatant when grown in submerging culture. The highest production of bioactive metabolites was at 30°C, between 72 and 96 h of incubation. Three isolates produced volatile compounds, chitinase, glucanase, siderophores and exhibited nitrogen fixation, produced indole-3-acetic acid, efficiently colonized the root system of seedlings of two wheat cultivars. The best isolate [6(2)] showed, under the greenhouse, the capacity to promote an increased biomass and tillers per wheat plant.**

**Key words:** Antagonist actinobacteria, antifungal activity, biocontrol, spot blotch, lytic enzymes.

## INTRODUCTION

The genus, *Bipolaris* includes some significant plant pathogens with worldwide distribution. A common root rot and leaf spot caused by *Bipolaris sorokiniana* are important diseases, causing a large amount of damage, to wheat and barley crop in warm and humid regions of the world (Duveiller et al., 2005). The losses due to foliar blight can vary among the regions. Some studies report that the losses of a wheat plantation may be as high as

100% under very severe conditions of infection (Metha, 1978). In favorable clime conditions, the disease occurs throughout the culture cycle.

Traditionally, chemical controls have been recommended to prevent the losses caused by the pathogen, to treat both seeds and to fight the disease already established in the plantation (Reis et al., 2005). A sustainable agriculture requires a plant-disease control that is more ecological and less dependent on synthetic chemical products. Biological control has been considered as a safe method for controlling soil-borne pathogens (Ling et al., 2010). The biological control maintains the balance in agricultural ecosystems, protecting hosts against significant damage caused by phytopathogens (Júnior et al., 2000).

Actinobacteria have been indicated as potential biocontrol agents against plant pathogens diseases (Igarashi, 2004; Pal and McSpadden Gardener, 2006; El-Tarabily et al., 2010; Costa et al., 2013; Zhang et al., 2014). These microorganisms play a major role in the rhizosphere by secreting a broad range of antimicrobial products that prevent the growth of common root pathogens (Khamna et al., 2009; Oliveira et al., 2010; Sobolevskaya and Kuznetsova, 2010). Besides their potential in biological control, their traits in plant growth promoting is already known (Jog et al., 2012; Nimaichand et al., 2013; Gopalakrishnan et al., 2014). El-Tarabily et al. (2010) observed enhanced plant growth and yield with the application of endophytic *Actinoplanes campanulatus, Micromonospora chalcea* and *Streptomyces spiralis,* individually and in combination, on cucumber seedlings. Costa et al. (2013) evaluating the control of *Pythium aphanidermatum* in cucumber (*Cucumis sativa* L.) under greenhouse conditions, observed that endophytic isolate both of *Streptomyces* genus, were able to reduce damping-off incidence.

Their mechanisms of action involve parasitism of hyphae and lytic enzymes, competition with pathogens, production of antibiotics, siderophores and pesticides (Crawford et al., 1993; Igarashi, 2004; Hasegawa et al., 2006; El-Tarabily and Sivasithamparam, 2006; Khamna et al., 2009; Gangwar et al., 2014).

The metabolic perspective of actinomycetes not only provides an attractive area for research but also offers the possibility of commercialization of the metabolites generated in the process (Sharma, 2014). The worldwide efforts in search of natural products for the protection market have progressed significantly, and Actinobacteria, especially genus *Streptomyces*, appear to be good candidates in finding new approaches to control plant diseases. Several commercial products derived from actinobacteria are available for use in crop protection and growth promotion (Palaniyandi et al., 2013; Hamdali et al., 2008; Minute et al., 2006).

In this scenario, the present study aimed to characterize and evaluate endophytic actinobactéria isolates for their ability to suppress the phytopathogen *B. sorokiniana* based on the production of secondary metabolites and the wheat seedlings root colonization.

## MATERIALS AND METHODS

### Microorganisms

The assays started with twenty-three actinobacteria obtained from tomato roots (*Lycopersicon esculentum*) (Oliveira et al., 2010) and twenty-two *B. sorokiniana* isolates from different Brazilian regions provided by EMBRAPA – Passo Fundo, RS, Brazil. All biological material used in this study belongs to the collection of the Environmental Microbiology Laboratory, DMPI, ICBS, UFRGS, RS, Brazil.

### Antifungal activity

The antifungal activity of 23 actinobacterial isolates was assessed using 22 *B. sorokiniana* isolates. Actinobacteria were inoculated using the spot method in starch casein agar (SCA) medium (10 g starch, 0.3 g casein, 2.0 g $KNO_3$, 2.0 g NaCl, 2.0 g $K_2HPO_4$, 0.05 g $MgSO_4.7H_2O$, 0.02 g $CaCO_3$, 0.01 g $FeSO_4.7H_2O$, 15.0 g agar, distilled water to complete 1 L) and incubated at 28°C for seven days. The double-layer agar method was used; 10 mL of melted potato dextrose agar (PDA) and inoculated with a *B. sorokiniana* suspension ($10^6$ spores/mL), poured on actinobacteria colonies and incubated at 28°C for four days. The essay was carried out in triplicate. The formed antibiosis halos were measured, and the antifungal activity (IA) was determined by the mean differences of the halo diameter and colony diameter.

### Production of antifungal compounds in submerged culture

The actinobacteria that exhibit the wider spectrum activity in the double-layer assay were chosen to optimize growth conditions and metabolites production in submerged culture. The isolates were inoculated in 250 mL conic flasks containing 50 mL starch casein broth (SCB) and incubated at 20, 25, 28, 30 and 40°C for 48 h under agitation (115 rpm). From this culture, an aliquot of 10% (v/v) was transferred to new flasks containing 50 mL of SCB and incubated under the same conditions as described for seven days. Then, 1 mL was retrieved from each flask every 24 h, transferred to microtubes and centrifuged at 13,000 rpm for 10 min. The supernatant was transferred to a new tube and used in the antifungal activity assay.

### Antifungal activity of the supernatant

The antimicrobial activity of the isolates was determined by the agar diffusion method (Bauer et al., 1966). The actinobacteria which showed inhibitory halo equal or greater than 1.0 cm, in the double-layer assay, were grown under submerged conditions at 20, 25, 28 and 30°C. The antimicrobial activity of the supernatant was tested against five *B. sorokiniana* isolates (98004, 98012, 98032, 98040

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and 98041). These isolates were selected based on previous results (Minotto et al., 2014). Wells were punctured, using a sterile cork borer, in PDA plates previously seeded with one of the five *B. sorokiana* chosen. One hundred microliters (100 µL) of the supernatant of each isolate was added to each well. Incubation took place at 4°C for 18 to 20 h for diffusion of the bioactive compound into the medium. After that, incubation of plates took place at 28°C for four days in the dark. Following incubation, inhibition zones were measured. These assays were carried out in triplicate.

**Pairing of cultures and volatile compounds**

Actinobacteria isolates were tested against *B. sorokiniana* using the pairing of cultures in dishes containing PDA and with overlapping dishes (Dennis and Webster, 1971). The pairing of cultures was carried out to analyze the direct antagonistic action of actinobacteria against *B. sorokiniana*. The overlapping dishes technique was used to observe the production of volatile compounds (VCOs) by the actinobacteria, which may influence the growth of phytopathogen (Hutchinson, 1967). Dishes were inoculated for each test and incubated at 28°C in the dark. The pairing of cultures was evaluated after seven days of incubation. The antagonistic activity was determined as the distance between the edges of the antagonist colony and the phytopathogen. VCOs production was recorded after 192 and 336 h of incubation considering the growth of fungal mycelia. Fungal growth inhibition (%) was calculated using the formula (*R1 – R2*/*R1*) x 100, where *R1* is the radial growth of the fungus with no exposure to actinobacteria, and *R2* is the radial growth of the fungus inoculated with the actinobacteria. These experiments were carried out in triplicate.

**Enzymatic and physiological characterization**

The 23 actinobacterial isolates were tested for their capacity to hydrolyze chitinase and β-1,3-glucanase, solubilize phosphate, siderophores production, indole-3-acetic acid (IAA) and fixing nitrogen. All assays were carried out at 25, 28 and 30°C with an exception for IAA production, which was determined only at 28°C. Enzymatic and physiological assays were conducted in triplicate, and the data obtained were analyzed using the analysis of variance and the Tukey's test (α = 0.05) using the software SASM-Agri (Canteri et al., 2001).

**Production of chitinase and β-1,3-glucanase**

Each of the isolates was spot-seeded on a mineral salt agar medium containing 0.5% laminarin (Sigma L9634), or 0.08% colloidal chitin to detect β-1,3-glucanases or chitinases (Renwick et al., 1991), respectively. The colloidal chitin was prepared according to El-Dein et al. (2010). After 14 days of incubation, hydrolysis zone around colonies were measured and chitinolytic and β-1,3-glucanases activity was determined. Enzymatic index (AI) was determined following Rosato et al. (1981).

**Phosphate solubilization**

The phosphate solubilization assay was carried out as described by Nautiyal (1999). Isolates were inoculated on plates containing NBRIP solid medium (National Botanical Research Institute's phosphate growth medium devoid of yeast extract) using the spot method and incubated for 21 days. The evaluation was determined based on the presence (phosphate solubilizers) or absence of halos under the colony growth.

**Production of siderophores**

The production of siderophores was assessed as previously described by Schwyn and Neilands (1987). Isolates were inoculated using the spot method on trypticase soy agar (TSA) ten times diluted and supplemented with chrome-azurol S complex ([CAS/iron (III)/hexadecyl trimethyl ammonium bromide] and incubated for 14 days. The positive reaction shows an orange or yellow halo around the colonies.

**Production of indole-3-acetic acid (IAA)**

Production of IAA follows the methodology described by Gordon and Weber (1951). Isolates were inoculated in TSA supplemented with 10% tryptophan 5 mM and incubated at 28°C for 14 days under agitation of 100 rpm. Then cultures were centrifuged, and 2 mL of the supernatant was transferred to test tubes containing 1 mL of Salkowski reagent (Gordon and Weber, 1951). The mixture was incubated at 28°C for 30 min in the dark. The evaluation was carried out in a spectrometer at 530 nm. AIA concentration ($mg.L^{-1}$) was determined to prepare a calibration curve with different concentrations of synthetic IAA (0, 1, 2, 3, 6, 10 and 16 $\mu g\ L^{-1}$).

**Nitrogen fixation**

The assay for nitrogen-fixing was performed as described by Döbereiner et al. (1995). Actinobacteria were spot-inoculated on test tube containing NFb semisolid medium and the culture was incubated at 25, 28 and 30°C for 14 days. A positive reaction was indicated by presence of veil inside the growth culture medium

***In vitro* colonization of wheat seedling roots by actinobacteria isolates**

Wheat seedlings roots colonization by the actinobacteria was assessed as described by Queiroz et al. (2006) with adaptations. Wheat seeds of BRS Buriti and BRS Camboatá cultivars were surface disinfected and then submitted to microbiolization. The seeds were immersed in separate suspensions containing propagules of actinobacteria and incubated under agitation for 4 h at 25°C. Suspensions were prepared growing microorganisms in SCA medium for 10 days at 28°C. The bacterial propagules concentration was adjusted to $A_{540}$ = 0.50 nm.

After microbiolization, two seeds of each wheat cultivar were transferred to tubes containing agar-water (0.8%) and incubated at 25°C with 12 h photoperiod for seven days. Wheat seedlings roots colonization by actinobacteria was assessed as described by Queiroz et al. (2006). Roots were sliced into 2 to 3 cm fragments long and placed in plates containing SCA and PDA media and plates incubated for seven days at 28°C to confirm the presence of actinobacteria in the rhizosphere. The experimental design was totally randomized with five repetitions, with four actinobacterial isolates, which showed the best results in all assays, and wheat cultivars (BRS Buriti and BRS Camboatá). Seeds immersed only in saline were the negative control.

***In vivo* assay**

The *in vivo* experiment was conducted under natural sunlight and temperature and receiving different conditions of the weather. The

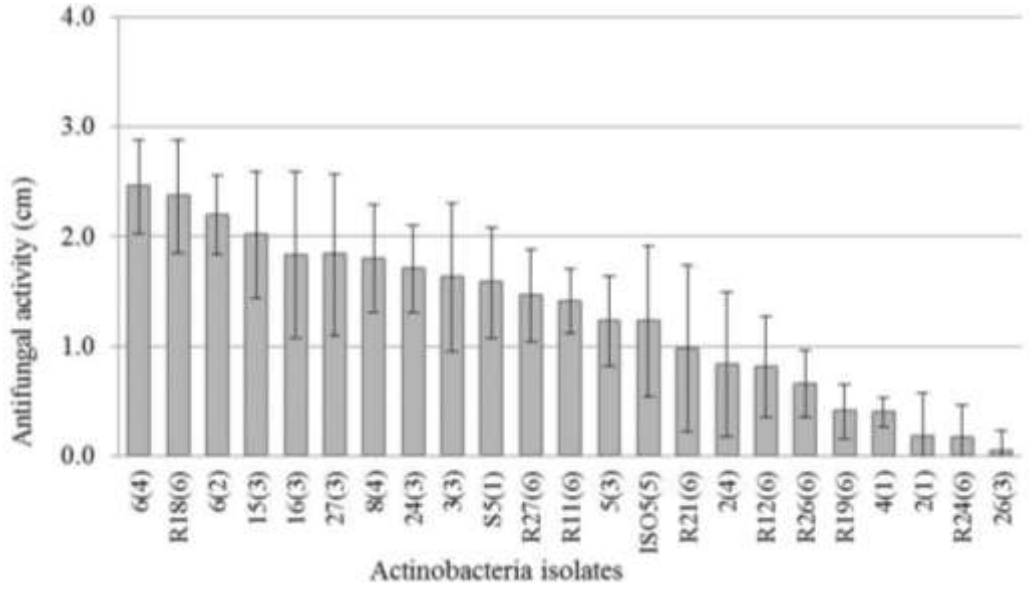


**Figure 1.** Antifungal activity (cm) of 23 endophytic actinobacteria against 22 *Bipolaris sorokiniana* strains isolated from different Brazilian regions, using the double-layer method.

assay was performed in pots containing sterile vermiculite and sand (2:1). In each pot, were placed five wheat seeds of BRS Buriti or BRS Camboatá cultivars. After germination, thinning was performed leaving three seedlings per pot.

For this study, eight treatments were determined: (1) Seeds immersed in SCB / soil without infestations (control); (2) Microbiolized seeds with actinomycetes/soil without infestations (control); (3) Microbiolized seeds with actinomycetes/soil infested with *B. sorokiniana*; (4) Untreated seeds/soil infested with actinomycetes; (5) Untreated seeds/soil infested with *B. sorokiniana*; (6) Microbiolized seeds with actinomycetes/sprayed *B. sorokiniana*; (7) Microbiolized seeds with actinomycetes/soil infested *B. sorokiniana* in time of plantation; (8): untreated seeds/soil infested with *B. sorokiniana* at plantation time;

Treatments 7 and 8 soil was infected with a pathogen suspension ($5 \times 10^5$ spores/mL in the proportion of 0.5 g/mL of the substrate) at the time of planting. In treatments 3 and 5, the soil infestation occurred at the stage GS 15, 23 (Zadoks et al., 1974). In treatment 4, a suspension of $10^8$ CFU/mL antagonists was added to the soil at the planting of the seeds. In treatments 4 and 6, the aerial part of the wheat plants was sprayed with *B. sorokiniana* ($5 \times 10^5$ spores/mL) using a manual spray with a distance of 40 cm from the sheet. The infected plants were at the stage GS 15, 23 (Zadoks et al., 1974). The plants were subjected to the humid chamber for 24 h before and 48 h after spraying.

The experiment took place from June 13 to 2 September 2011. The period was characterized by high rainfall (172.3 mm), 26% above the average of the three previous years for the same period (BDMEP, 2015). The average of temperature was between 10.6 and 19.4°C, relative humidity average between 79 and 84% and the average monthly insolation period was 109.13 h (BDMEP, 2015). The plants were watered as necessary and received a nutritive solution (Voss and Scheeren, 2006) three times a week. The evaluation of leaf necrosis was performed at the fourth true leaf in the 7, 14, 21 and 28 days after inoculation. The assessment based on the rating scale infection response (IR) described by Fetch Jr and Steffenson (1999) was used. Also, the numbers of tillers per plant, fresh mass weight, dry biomass weight and shoot height. The experiment consisted of eight treatments for each of the two cultivars, five replicates per treatment were evaluated with three observational units.

## RESULTS

### Antifungal activity

All actinobacterial isolate inhibited, at least one *B. sorokiniana* isolate. Of the 23 actinobacteria tested, 65.2% (15) presented antifungal activity against *B. sorokiniana* with values greater than 1.0 cm. The isolates 6(4), R18(6), 6(2),15(3) presented the highest inhibition zone against fungi growth (2.45, 2.37, 2.20 and 2.01 cm, respectively) (Figure 1), which represents growth inhibition of 86.4, 77.3, 86.4 and 59.1% of *B. sorokiniana* isolates, respectively.

### Production of antifungal compounds in submerged culture

The results obtained in submerged culture shows that isolates 6(2), 6(4) and 16(3) produced the highest amounts of active metabolites against *B. sorokiniana* at 30°C with 72 h of growth (Figure 2A, B and C, respectively). Active metabolites were produced by isolate R18(6) at temperatures of 20 and 25°C with 168 and 72 h of incubation, respectively (Figure 2D). Although, microorganisms' growth was observed at all tested temperatures, the largest antifungal activity occurred between the 72 and 144 h of growth at 30°C (Figure 2).

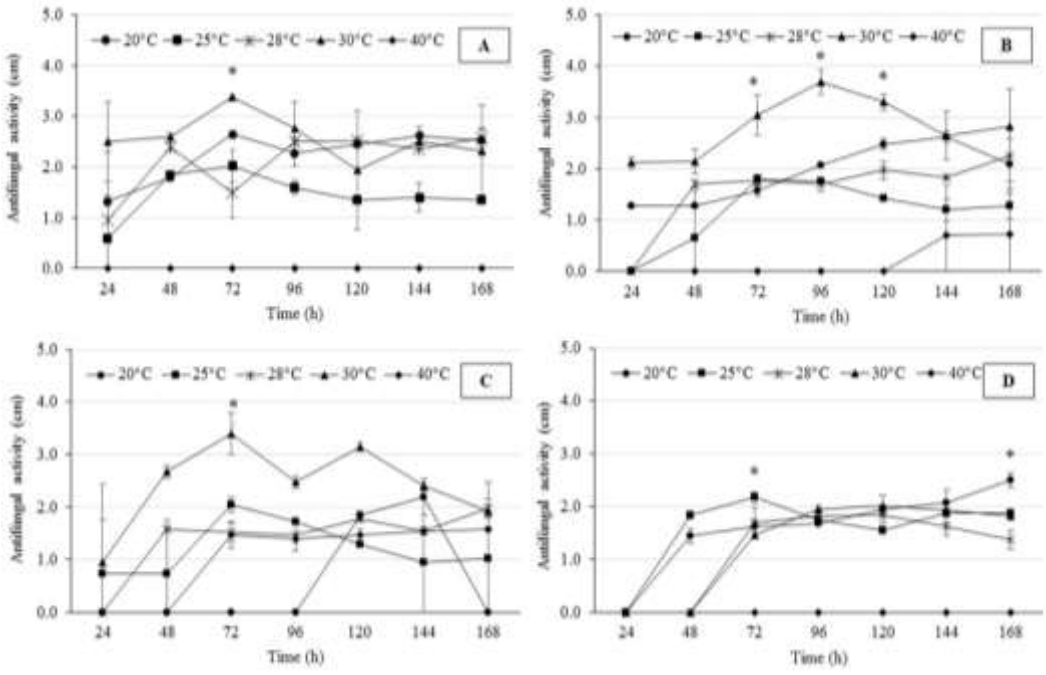


**Figure 2.** Production of antifungal compounds with inhibitory activity (cm) by actinobacteria in submerged culture at different incubation times and temperatures. A: Isolate 6(2); B: Isolate 6(4); C: Isolate 16(3); D: Isolate R18(6). *Statistically significant means in the Tukey's test (α = 0.05).

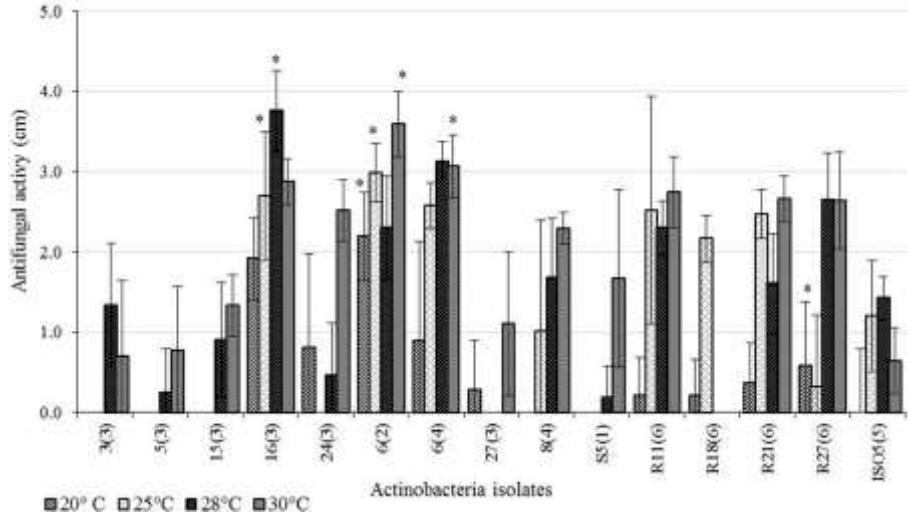


**Figure 3.** Antifungal activity (cm) against *B. sorokinana* isolates of the supernatant from the 15 actinobacteria, grown at different temperatures using the agar diffusion method. *Statistically significant means in the Tukey's test (α = 0.05).

## Antifungal activity of the supernatant

The supernatant produced by 15 actinobacteria isolates showed antifungal activity against *B. sorokiniana* at least in one of the measured temperature (Figure 3). The highest number of isolates (93.3%) showed antifungal activity when grown at 30°C. However, isolate 6(2) showed antifungal activity with a statistical difference as compared to the results of the other isolates and temperatures. Isolate 16(3) showed the higher inhibitory activity as compared to all of them when grown at 28°C (Figure 3).

**Table 1.** Physiological and enzymatic activity (phosphate solubilization, siderophore production, nitrogen fixation, chitinase, β-1,3-glucanase and indole-3-acetic acid (AIA)) of actinobacteria isolates grown at different incubation temperatures.

| Actinobacteria isolates | Phosphate solubilization | | | Siderophore production | | | Nitrogen fixation | | | Chitinase | | | Glucanase | | | AIA (Mean±SD) (µg $L^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 25°C | 28°C | 30°C | 25°C | 28°C | 30°C | 25°C | 28°C | 30°C | 25°C | 28°C | 30°C | 25°C | 28°C | 30°C | 28°C |
| 6(2) | + | + | + | - | +++ | ++ | - | - | + | 2.0 | 2.0 | 2.1 | 2.8 | 2.5 | 2.3 | 33.21±8.75 |
| 3(3) | + | + | + | +++ | + | - | - | + | + | 16.0* | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 32.10±1.28 |
| 5(3) | - | - | + | + | + | +++ | + | + | + | 5.4 | 10.5 | 0.0 | 0.0 | 5.4 | 4.9 | 33.69±3.63 |
| 15(3) | - | + | + | - | ++ | +++ | - | - | + | 13.7 | 11.9 | 16.0* | 0.0 | 5.0 | 4.7 | 32.09±1.50 |
| 16(3) | + | + | + | + | - | - | + | + | + | 6.0 | 2.9 | 6.4 | 4.5* | 3.8 | 3.4 | 32.09±1.50 |
| 24(3) | + | + | + | - | - | - | - | + | + | 5.1 | 3.5 | 6.1 | 0.0 | 3.2 | 3.0 | 32.72±0.64 |
| 27(3) | - | + | + | ++ | - | - | - | - | - | 6.0 | 3.1 | 6.3 | 0.0 | 2.9 | 2.6 | 33.59±4.49 |
| 6(4) | + | + | + | - | ++ | ++ | + | + | + | 0.0 | 3.0 | 3.7 | 0.0 | 2.0 | 2.4 | 32.57±3.74* |
| 8(4) | + | + | + | +++ | +++ | +++ | + | + | + | 11.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 32.79±1.07 |
| S5(1) | + | + | + | - | ++ | +++ | - | - | - | 5.2 | 2.4 | 6.1 | 0.0 | 4.0 | 3.8 | 33.27±2.35 |
| R11(6) | + | + | + | +++ | - | ++ | - | - | + | 8.0 | 0.0 | 2.1 | 0.0 | 0.0 | 0.0 | 32.75±1.28 |
| R18(6) | - | - | - | - | ++ | - | - | + | - | 1.1 | 1.1 | 2.3 | 2.0 | 1.2 | 0.9 | 33.77±1.67 |
| R21(6) | + | + | + | - | +++ | - | + | + | + | 2.5 | 2.0 | 2.5 | 2.1 | 1.0 | 0.7 | 33.14±7.05 |
| R27(6) | + | + | + | ++ | +++ | ++ | - | - | - | 4.5 | 1.6 | 1.8 | 0.0 | 1.4 | 1.3 | 32.50±2.99 |
| ISO5(5) | + | + | + | - | +++ | - | - | + | + | 2.13 | 2.13 | 2.17 | 0.0 | 1.1 | 1.3 | 32.89±1.07 |

(-) Isolate with no activity; (+) Isolate with positive activity and siderophore production H/C ≤ 1; (++) Isolate with positive activity and H/C ≥ 1 ≤ 3; (+++) Isolate with positive activity and H/C > 3. (SD) Standard Deviation; (*) statistically significant means in the Tukey's test (α = 0.05).

## Physiological and enzymatic activity

The enzymatic activity and physiological assays showed that isolates 6(2), 6(4) and 16(3) were able to solubilize phosphate, produce siderophore, fix atmospheric nitrogen and hydrolyze chitin and glucan. At 30°C, 14 isolates were positive for phosphate solubilization on solid medium. The isolate R18(6) did not solubilize phosphate at any of the measured temperature (Table 1). Siderophore production was observed in 93.3% of the isolates. Out of that 10 isolates, 66.6% produced an enzymatic index greater than 3.0. However, isolate 24(3) did not produce siderophores. With the increase in growth temperature from 25 to 30°C, there was an improvement in 40% of isolates in their capacity to fix nitrogen. Isolates 27(3) and S5(1) were unable in fixing nitrogen (Table 1).

Chitinase and β-1,3-glucanase production was observed for 100 and 80% of the isolates, respectively. For chitinase production, 26.6% of the isolates showed an enzymatic index greater than 10. The highest enzymatic index for chitinase was for isolates 3(3) and 15(3) at 25 and 30°C, respectively, and for glucanase was for isolate 16(3) at 25°C (Table 1). All actinobacteria incubated at 28°C for seven days synthesized auxins. IAA production oscillated between 6.7 µg $L^{-1}$ for isolates 3(3) and 5(3) and 30.7 µg $L^{-1}$ for isolate 6(4) (Table 1).

## Pairing of cultures and volatile compounds

The results of the antibiosis assays carried out by direct comparison of two cultures show that isolates 6(2), 6(4) and 16(3) suppressed the development of five *B. sorokiniana* isolates (98004, 98012, 98032 and 98040). Antifungal activity was observed as the formation of an inhibition area ≥ 2.0 cm, between the edge of the

**Table 2.** Inhibition of *Bipolaris sorokiniana* isolates growth (%) by volatile compounds produced by actinobacteria.

| Incubation time (h) | Inhibition of mycelial growth of *B. sorokiniana* (%) by actinobacteria isolates | | |
|---|---|---|---|
| | 6(2) | 6(4) | 16(3) |
| 48 | 5.04 | 0.51 | 4.87 |
| 96 | 5.64 | 4.32 | 13.51 |
| 144 | 5.14 | 10.62 | 13.87 |
| 192 | 6.76 | 11.77 | 14.47 |
| 264 | 7.92 | 13.90 | 16.23 |
| 336 | 11.32 | 14.85 | 16.95 |

**Table 3.** Hypersensitivity reaction (IR) (extension of leaf necrosis) in wheat plants caused by *B. sorokiniana*, subjected to different treatments.

| Treatment | Cultivar BRS Buriti | | | | Cultivar BRS Camboatá | | | |
|---|---|---|---|---|---|---|---|---|
| | 7days | 14 days | 21 days | 28 days | 7days | 14 days | 21 days | 28 days |
| 1 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 1 |
| 2 | 0 | 0 | 0 | 1 | 2 | 0 | 0 | 0 |
| 3 | 1 | 1 | 1 | 1 | 3 | 0 | 0 | 0 |
| 4 | 3 | 5 | 7 | 8 | 4 | 2 | 6 | 6 |
| 5 | 0 | 0 | 0 | 1 | 0 | 5 | 0 | 1 |
| 6 | 2 | 3 | 4 | 4 | 2 | 4 | 5 | 6 |
| 7 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 |
| 8 | 1 | 1 | 1 | 2 | 0 | 0 | 0 | 1 |

IRs 1, 2, and 3 low compatibility; IRs 4 and 5 intermediate compatibility.; IRs 6, 7, 8 and 9 high compatibility. (1) Seeds immersed in AC broth medium/ soil without infestations (control); (2) Microbiolized seeds with actinomycetes / soil without infestations (control); (3) Microbiolized seeds with actinomycetes/soil infested with *B. sorokiniana*; (4) Untreated Seeds/soil infested with actinomycetes; (5) Untreated seeds / soil infested with *B. sorokiniana*; (6) Microbiolized seeds with actinomycetes/Sprayed *B. sorokiniana*; (7) Microbiolized seeds with actinomycetes/soil infested *B. sorokiniana* in time of plantation; (8): untreated seeds/soil infested with *B. sorokiniana* at plantation time.

actinobacteria colony and the edge of the phytopathogen colony. The volatile compounds produced by the isolates showed decreased in the development of the fungal mycelium of *B. sorokiniana* with the increase of exposure time to the metabolites (Table 2). The reduction of *B. sorokiniana* growth was 4.16% in the first evaluation after 48 h of exposure, to 15.66% in the last assessment at 336 h of incubation.

Volatile compounds produced by isolates 6(2), 6(4) and 16(3) promoted the radial decrease in fungal mycelia (11.32, 14.85 and 16.95%, respectively), after 336 h of incubation (Table 2).

### *In vivo* experimental assay

The results obtained in the *in vivo* assay showed that the extent of leaf necrosis in plants of treatments 4 and 6, which had shoots sprayed with *B. sorokiniana* showed small necrotic lesions (IRs 3), in the first assessment (seven days), and extensive leaf lesions with distinct chlorotic margins (IRs 6, 7 and 8) after the third evaluation (28 days), for both cultivars studied (Table 3). The other treatments showed only slight necrotic lesions without chlorosis or very light and diffuse marginal chlorosis (IRs 1, 2 and 3), considered to be indicative of low compatibility (Table 3).

The soil infestation with the isolate 6(2) (treatment 4) provided a significant increase in fresh weight (12.69 and 12.64 g) and dry weight (3.51 and 2.54 g) for both BRS Buriti and BRS Camboatá cultivars, respectively, as compared to the other treatments. This result showed that soil infestation with the antagonist promoted further development of wheat plants as compared to treatments with or without microbiolization (Table 4). Data analysis for plant height variable showed no significant difference between treatments for any of the cultivars (Table 4). Also, the soil infestation with isolate 6(2) significantly increased the mean number of tillers per plant for both cultivars as compared to treatment with or without seeds

**Table 4.** Fresh weight (g), dry weight (g) and average height (cm) of wheat plants submitted to different treatments, after 80 days of plantation.

| Treatment | Cultivar BRS Buriti | | | Cultivar BRS Camboatá | | |
|---|---|---|---|---|---|---|
| | Fresh weight (g) | Dry weight (g) | Height (cm) | Fresh weight (g) | Dry weight (g) | Height (cm) |
| 1 | 9.98 | 2.40 | 48.80 | 10.02 | 2.19 | 37.40 |
| 2 | 10.46 | 2.45 | 45.00 | 9.64 | 2.08 | 38.00 |
| 3 | 8.74 | 2.09 | 45.00 | 9.38 | 1.93 | 37.80 |
| 4 | 12.67* | 3.51* | 48.60 | 12.64* | 2.84* | 40.00 |
| 5 | 9.56 | 2.19 | 47.80 | 10.88 | 2.43 | 41.80 |
| 6 | 9.87 | 2.26 | 47.20 | 8.50 | 1.70 | 39.20 |
| 7 | 9.59 | 2.19 | 48.40 | 10.10 | 2.15 | 38.80 |
| 8 | 8.90 | 2.12 | 47.00 | 8.85 | 1.80 | 38.20 |
| C.V. | 16.17 % | 23.96 % | 8.60 % | 18.92 % | 20.51% | 7.82 % |

(C.V.) Coefficient of variation. (1) Seeds immersed in AC broth medium/soil without infestations (control); (2) Microbiolized seeds with actinomycetes/soil without infestations (control); (3) Microbiolized seeds with actinomycetes/soil infested with *B. sorokiniana*; (4) Untreated Seeds/soil infested with actinomycetes; (5) Untreated seeds / soil infested with *B. sorokiniana*; (6) Microbiolized seeds with actinomycetes/Sprayed *B. sorokiniana*; (7) Microbiolized seeds with actinomycetes / soil infested *B. sorokiniana* in time of plantation; (8): untreated seeds / soil infested with *B. sorokiniana* at plantation time. *Statistically significant means in the Scott-Knott test (α = 0.05).

**Table 5.** Average number of tillers per plant wheat subjected to different treatments during the period of 80 days.

| Treatment | Average number of tillers / plant | | | | | |
|---|---|---|---|---|---|---|
| | Cultivar BRS Buriti | | | Cultivar BRS Camboatá | | |
| | 7 days | 21 days | 28 days | 7 days | 21 days | 28 days |
| 1 | 1.13 | 1.20 | 2.07 | 1.53 | 2.03 | 2.60 |
| 2 | 0.73 | 2.07 | 2.20 | 1.53 | 2.13 | 2.27 |
| 3 | 0.73 | 0.73 | 1.80 | 1.20 | 1.67 | 3.13 |
| 4 | 2.33* | 2.40* | 2.80* | 2.07* | 2.47 | 3.07 |
| 5 | 0.73 | 1.40 | 2.07 | 1.73 | 1.93 | 2.67 |
| 6 | 0.60 | 1.33 | 2.60 | 0.67 | 2.33 | 3.53 |
| 7 | 0.47 | 1.13 | 1.47 | 1.20 | 1.93 | 2.27 |
| 8 | 0.87 | 1.20 | 1.93 | 1.07 | 2.13 | 2.93 |

(C.V.) Coefficient of variation. (1) Seeds immersed in AC broth medium/ soil without infestations (control); (2) Microbiolized seeds with actinomycetes/soil without infestations (control); (3) Microbiolized seeds with actinomycetes/soil infested with *B. sorokiniana*; (4) Untreated Seeds/soil infested with actinomycetes; (5) Untreated seeds/soil infested with *B. sorokiniana*; (6) Microbiolized seeds with actinomycetes/Sprayed *B. sorokiniana*; (7) Microbiolized seeds with actinomycetes / soil infested *B. sorokiniana* in time of plantation; (8): untreated seeds / soil infested with *B. sorokiniana* at plantation time. * Statistically significant means in the Scott-Knott test (α = 0.05).

microbiolized (Table 5). The number of tillers, subject to treatment 4, remained high throughout the evaluation period for cultivar BRS Buriti. For BRS Camboata cultivar, a significant difference was observed only in the first assessment (Table 5).

## DISCUSSION

Actinobacteria populations are essential components of the endophytic and rhizospheric microbial community of several higher plant species. The production of antibiotics or other toxic metabolites by these microorganisms has been widely reported as a biocontrol tool against plant diseases (El-Tarabily et al., 2000; Cao et al., 2005; Castillo et al., 2006; Qin et al., 2011; Costa et al, 2013). Additionally, endophytic *Streptomyces* may improve the agricultural production, reducing the impact of root and crown rot (Coombs et al., 2004). Of the endophytic actinobacteria investigated in the present study, 69.6%

presented significant inhibitory activity against *B. sorokiniana* in the double-layer assay. Of these, isolates 6(2), 6(4) and 16(3) were able to maintain high antifungal activity when grown in submerged culture, as well as in the pairing of cultures in solid media. These results are higher than the findings reported in the literature for the inhibition of 6.5% of the fungi *Alternaria solani, B. sorokiniana, Fusarium oxysporum fsp. lycopersiccum, Gerlachia oryzae, Sclerotinia sclerotiorumaa, Verticillium alboatrum* and *Rhizoctonia* sp. with endophytic actinobacteria (Oliveira et al., 2010). In an antagonism screening, Costa et al. (2013) evaluated if the endophytic *Streptomyces* strains were able to inhibit the plant pathogenic fungi growth. The results show that *S. sclerotiorum, P. aphanidermatum, R. solani, Fusarium* sp. and *P. parasitica* were inhibited by 47.5, 55.0, 62.5, 77.5 and 90% of the isolates, respectively. Li et al. (2014) isolated an endophytic *Streptomyces* strain CNS-42 isolated from *Alisma orientale* and showed a potent effect against *F. oxysporum* f. sp. *cucumerinum* and a broad antimicrobial activity against bacteria, yeasts and other pathogenic fungi. The *in vivo* biocontrol assays showed a significant reduction in disease severity and plant shoot fresh weight and height increased greatly in plantlets treated with strain.

In a previous study, the largest inhibition zones in the growth of *C. sativus* by *Chaetomium globosum* was 6.3 mm in the pairing of cultures (Aggarwal et al., 2004). The authors discovered that the filtered cultures of this antagonist reduced the pathogen's growth by 19.6 to 100%. In the present work, the supernatant of actinobacteria inhibited the growth of *B. sorokiniana*, forming inhibition zones up to 3.7 cm in diameter. Temperature determines the optimal conditions of metabolite production in submerged cultures. It is known that the optimal growth temperature ranges are wide while optimal temperature for secondary metabolites production lies in a narrow interval, of 5 to 10°C (Iwai and Omura, 1982). In the production of streptomycin by *Streptomyces griseus*, an increase of 1°C leads to a drop of 80% in antibiotic production (Dunn, 1985). However, in our case, isolate 6(2) produced antifungal compounds against *B. sorokiniana* throughout the incubation period (168 h). This flexibility in antifungal activity has also been demonstrated by the presence of inhibition halos at temperatures of 20, 25, 28 and 30°C.

The conditions of secondary metabolites production depend on the actinobacterial isolate. Iwai and Omura (1982) study has shown that the mean production times of metabolites with antimicrobial action was between 120 and 240 h of incubation. In the present study, the highest production of bioactive compounds was at 30°C between 72 and 96 h of incubation, with inhibition zones between 3.4 and 4.1 cm in diameter. Salamoni et al. (2012) working with *Streptomyces* 1S observed the highest production of antimicrobial compounds between 48 and 120 h of incubation at 28°C. Bervanakis (2008) observed that the optimal production of secondary metabolites was after 240 h of incubation at 27°C.

The volatile compounds produced by *Streptomyces philanthi* RM-1-138 isolated from the rhizosphere soil of chili pepper suppressed the growth of the *Rhizoctonia solani, Pyricularia grisea, Bipolaris oryzae* and *Fusarium fujikuroi* (52.85 to 100%) (Boukaew et al., 2013). Different results observed in this study, where the reduction of radial growth of the pathogen was 11.32, 14.85 and 16.95% shown by isolate 6(2), 6(4) and 16(3) after 336 h of incubation, respectively. The evolution of volatile organic compounds by soil microorganisms has been associated with the promotion of plant growth (Ryu et al., 2003) and the induction of systemic resistance in cultures (Farag et al., 2006), growth inhibition (Fiddaman and Rossall, 1994) and germination of spores of pathogenic fungi (Mckee en Robinson, 1988).

Isolates 6(2), 6(4) and 16(3) were selected from a set of 23 actinobacteria because they presented high antifungal activity in all tests they were subjected to. Also, these actinobacterial isolates exhibited other properties often associated with biocontrol agents, such as the ability to hydrolyze chitin and glucan, to produce siderophores and IAA, solubilize phosphate, fix nitrogen and colonize the rhizosphere of wheat seedlings. Furthermore, these isolates are efficient producers of several hydrolases enzymes, (Minotto et al. 2014). Studies have shown that the action of *Streptomyces* includes the inhibition of pathogens by the production of antifungal compounds (El-Tarabily and Sivasithamparam 2006), the competition for iron in siderophore production, and the production of degradation enzymes, like chitinase and glucanase (El-Tarabily et al., 2000).

Among the 15 actinobacteria, 26.6% presented high enzyme index for the two enzymes chitinase and glucanase (EI >10.0 and EI >4.0, respectively) (Table 1). A previous study has shown that EI higher than 1.0 indicates the secretion of enzymes with biotechnological potential (Fungaro et al., 2002). Also, *Streptomyces* isolates prescribed for the control of white rot in coal and for the growth promotion of plants have been shown to produce significant amounts of chitinase and of β-1,3-glucanase (Gopalakrishnan et al., 2013). Additionally, the production of chitinase and of glucanase was the main mechanism associated with the biocontrol potential of *Streptomyces viridodiasticus* against *Sclerotinia minor* (El-Tarabily et al., 2000). Similar observation was also reported in control of *Phytophthora fragariae* (Valois et al., 1996), *Fusarium oxysporum* (Singh et al., 1999) and other phytopathogens (Cretoiu et al., 2013).

Microbial enzymes, especially chitinases, are highly important in biocontrol strategies due to their ability (a) to degrade chitin (the main component of the cell wall of most fungi), (b) to inhibit the germination of fungal spores and the elongation of the germinative tube, and (c) to lyse hyphae (Kishore et al., 2005).

In this study, 100% of the isolates produced indole-3-

acetic acid (ranging from 6.7 to 30.7 $mg.L^{-1}$ IAA), siderophores (93.33%), phosphate solubilization (93.33%) and fix nitrogen (86.6%) (Table 1). This result was higher than that reported by Oliveira et al. (2010) who observed IAA production by 72.1% of actinobacteria tested, 86.8% solubilized phosphates and 16.2% produced siderophores. Among the 15 actinomycetes isolates tested by Jog et al. (2012), 78% produced auxins (ranging from 2.6 to 19.22 $mg.L^{-1}$ IAA equivalents), 60% produced siderophores (hydroxamate type) and only five solubilized phosphate on buffered tricalcium phosphate agar.

Although, several bacterial siderophores differ in their ability to sequester iron, as a rule these structures deprive fungi of this essential element due to their higher affinity for it (Loper and Henkels, 1999). Some bacteria which are plant growth promoting may go beyond, and attract iron from heterologous siderophores which were produced by co-inhabiting microorganisms (Lodewyckx et al., 2002). In this sense, the microorganisms selected in the present study may play a beneficial role in plant development, since growth promotion effects are also associated with the production of IAA (Khamna et al., 2009) and phosphate solubilization (Hamdali et al., 2008).

Despite its importance, phosphate solubilization has been reported in a small number of microorganisms (Hameeda et al., 2008). However, the absence of detection does not mean that the microorganism does not have this property. Rather, it may just indicate that the conditions used to detect it are not suitable. Li et al. (2015) observed that the solubilization of $Ca_3(PO_4)_2$ or $MnO_2$ was not detected between the *Trichoderma* inoculated cultures and the controls, but analyses of P in the calcium phytate medium revealed measurable concentrations of soluble P, significantly different from the control concentrations. It is suggested that phytase released by *Trichoderma* played a major role in solubilizing organic P (phytate) (Li et al., 2015). It was observed that increasing the incubation temperature of 25 to 30°C increased the isolates number with the ability to produce siderophores, phosphates solubilization and nitrogen fixing in 20, 28.5 and 40%, respectively. This results corroborate those found by Rinu and Pandey (2010) who observed phosphate solubilization by different *Aspergillus* species at a maximum of 28 or 21°C and the biomass production was favored at 21 or 14°C. Conversely, *A. nidulans* and *A. sydowii* exhibited maximum phosphate solubilization at 14°C and biomass production at 21°C.

In the rhizosphere soil, root exudates are the natural source of tryptophan for rhizospheric microorganisms, which may increase the biosynthesis of auxin on this site (Khamna et al., 2009). In the present study, isolates 6(4), 6(2) and 16(3) colonized the root system of seedlings of the two wheat cultivars used. This result suggests the possibility that high levels of tryptophan are present in wheat root exudates, which may enable the biosynthesis of large amounts of IAA, as the efficient colonization of roots.

Isolate 6(2) was selected for testing *in vivo* because it presented significant antifungal activity in a wide temperature range, especially at low temperatures (20°C) as well as provide a positive activity for all enzymatic activity tests, physiological and CVOs assay. The microbiolization and application of bacterial suspension to the substrate did not prevent infection of wheat seedlings by *B. sorokiniana*, as well as the extension leaf necrosis (Table 3). However, soil infestation with the actinobacteria (Treatment 4) promoted further development of wheat seedlings, with a significant increase in fresh weight, dry weight and an average number of tillers per plant for both cultivars.

Similar results were reported by Jog et al. (2012), when wheat plants were inoculated with actinomycetes isolates; they observed a high number of root branches, the number of branches, and significant biomass as compared to un-inoculated control. However, a significant change in root length was not observed by these authors. The results obtained for soil infestation with the antagonist were higher than those obtained from seeds microbiolization. This result probably was because the low concentration of bacterial inoculum adhered to the seed as compared to treatment with the soil infestation, where the inoculum concentration was much higher.

The nutrients, mostly available from plants, are broadly termed Rhizodeposition and enzymes are essential for the properly utilization of nutrients available in the rhizosphere. Many factors contribute to constructing a nutrient pool containing polymers, sugars, peptides and amino acids, organic phosphates, among others in the rhizosphere (Jog et al., 2012).

Several studies investigated the potential of secondary metabolites of actinobacteria to be used in control of diseases caused by phytopathogens and plant growth promoting. In this study, actinobacterial isolates were tested for the production of extracellular metabolites often associated with biocontrol strategies, with excellent *in vitro* results for three of these actinobacteria. These isolates were capable of utilizing nutrients such as indole-3-acetic acid, inorganic phosphates, iron, chitin, glucan and fix nitrogen, also have antifungal activity, VCOs production and rhizosphere colonization. The data shows that activities were extremely beneficial for wheat plants. Isolate 6(2) was able to promote an increased biomass and tillers per plant. Gopalakrishnan et al. (2014), in a rice field study observed that six isolates of actinomycetes significantly enhanced tiller numbers, panicle numbers, filled grain numbers and weight, stover yield, grain yield, total dry matter, root length, volume and dry weight over the un-inoculated control.

The isolates from this work may be seen as potential agents in the control of *B. sorokiniana*. However, further studies should be carried out to obtain more conclusive

results on the effectiveness of these actinobacteria against spot blotch *in vivo*.

## Conflict of interest

The authors have not declared any conflict of interest

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