Antifungal Activity of Bacterial Strains from Maize Silks against *Fusarium Verticillioides*

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Abstract

Fusarium verticillioides is pathogenic to maize and mycotoxin-producer, causing yield losses, feed and food contamination, and risks to human and animal health. Endophytic (ISD04 and IPR45) and epiphytic (CT02 and IM14) bacteria from maize silks were tested in vitro and greenhouse against *F. verticillioides* and for hydrolytic enzyme production (cellulase, pectinase, protease, lipase, and chitinase). The strains were assigned as *Achromobacter xylosoxidans* (ISD04), *Pseudomonas aeruginosa* (IPR45), and *Bacillus velezensis* (CT02 and IM14) by 16S gene sequencing. All strains showed antifungal activity in vitro with inhibition values from 58.5–100%; they changed hyphae morphology and inhibited the conidial germination by up to 100% (IPR45). The four strains produced at least one enzyme with antifungal activity. The microbiolized seeds reduced the fungal development in stored grains and stalk rot severity in the greenhouse by 72.6% (ISD04). These results highlight the potential of these strains as biocontrol agents against *F. verticillioides*

Introduction

The fungus *Fusarium verticillioides* (Sacc.) is one of the major pathogens causing significant economic losses in the maize production globally (Jia 2019; Rosa Junior et al. 2019). The fungus can cause different disease symptoms in almost all plant parts, such as stalk and ear rot (Deepa and Sreenivasa 2017; Gai et al. 2018). Feed and food contaminated with toxic secondary metabolites as fumonisins produced by *F. verticillioides* are harmful to animal and human health (Deepa and Sreenivasa 2017; Blacutt et al. 2018; Zubrod et al. 2019). *F. verticillioides* is transmitted through the seeds, root lesions, wounds on the adult plant, or silks. The latter is the most critical contamination risk factor, leading to grain rupture, ear rot, and fumonisin accumulation in grains (Munkvold et al. 1997).

Synthetic fungicides are among the basic principles of plant disease management and have been used for decades worldwide to control plant pathogenic fungi (Yoon et al. 2013; Haq et al. 2020). However, their systematic and uncontrolled use has undesirable effects on non-target organisms, disrupted the ecological balance, and often led to fungal resistance (Yoon et al. 2013; Kim et al. 2017). Therefore, alternative procedures have been developed for crop protection. Intensive investigations on natural antagonists and their active metabolites with fungicide effects have been undertaken to reduce the use of hazard fungicides, and create new safe products for humans and the environment (Dukare et al. 2019; Jia 2019). The use of antagonistic bacteria for controlling plant pathogenic fungus represents a promising environmental safe and low-cost alternative to reducing synthetic fungicides in agriculture (Rahman et al. 2017). Biocontrol agents act against phytopathogens by different mechanisms, including the synthesis of antifungal metabolites and secretion of hydrolytic enzymes (Dukare et al. 2019). Lipopeptides and enzymes act on the fungal mycelial structure altering its shape, size, and surface and causing breakages (Borah et al. 2016). Enzymes also lead to weakening or degradation of the fungal cell wall and membranes, preventing their growth (Neeraja et al. 2010; Köhl et al. 2019). Different classes of hydrolytic enzymes produced by antagonistic bacteria represent an important mechanism directed against phytopathogens, useful for sustainable plant disease management (Jadhav et al. 2017).

However, the mechanisms involved in the biological control are very complex and encompass competition, antibiosis, parasitism, induction of systemic acquired resistance (SAR), induction of systemic resistance (ISR), and soil suppressiveness (Jadhav et al. 2017; Hornby 1983; Rabbee et al. 2019).

Antifungal lipopeptides produced by *Bacillus* sp., such as iturin, fengycin, and surfactin, have a strong inhibitory effect against pathogenic fungus and harmful rhizosphere microorganisms (Ongena and Jacques 2008; Devi et al. 2019). *Bacillus velezensis* is a plant growth-promoting bacterium with various strains suppressing the growth of microbial pathogens (Rabbee et al. 2019). Genomic analysis showed that this species possesses strain-specific secondary metabolites gene clusters involved in the secondary metabolites synthesis, which play significant roles in pathogen suppression. Bioactive antimicrobial composts produced by *B. velezensis* have full applications in the pharmaceutical industry and medicine (Meena et al. 2019). *B. velezensis* also synthesize secondary metabolites that trigger induced systemic resistance in plants (Chen et al. 2018; Rabbee et al. 2019). Other beneficial metabolites include bacteriocins that inhibit the growth of similar or closely related bacterial strains, and secondary interkingdom interactions (antagonism, mutualism, intra- and interspecies regulation), and at-a-distance influence on bacterial behavior (Tilocca et al. 2020).

Achromobacter xylosoxidans is a plant growth-promoter, and produce the antifungal compost Cyclo (L-Leucyl-L-Prolyl), a cyclic dipeptide that inhibits the growth of phytopathogenic fungi (Kumar et al. 2005; Vyas et al. 2018). A study by Dhaouadi et al. (2018) found that *A. xylosoxidans* reduced significantly 80% of the mycelial growth *Fusarium in vitro*. In the greenhouse conditions, the bacteria reduced considerably in 60% of the disease severity on melon plants inoculated with the pathogen. In another *in vitro* study, the strain AUM54 of *A. xylosoxidans* inhibited by 11% the mycelial growth of *Magnaporthe oryzae*, the causal agent of rice blast disease. In the greenhouse, plants originated from seed treated with the bacterial strain inoculated with the fungus showed a reduction of the disease incidence by 39% (Joe et al. 2012). The strain AUM54 also promotes seed germination, seedling vigor, and enhanced plant growth and yield. AUM54 moved systemically through the roots and stem of plants and significantly increase the activity of defense-related enzymes such as polyphenol oxidase (PPO), peroxidase (POD), phenylalanine ammonia-lyase (PAL), and chitinase (Vaidya et al. 2001; Joe et al. 2012; Veliz et al. 2017). Zhang et al. (2016) demonstrated that the culture filtrate of *Achromobacter xylosoxidans* strain 09X01 caused high mortality of the second-stage juvenile nematodes and reduced egg hatch *in vitro*. In the greenhouse and field trials, the treatments with bacterial suspensions of 09X01 significantly reduced the numbers of white females in roots. The strain 09X01 also increased the wheat yields by 13.2% compared to untreated control (Zhang et al. 2016).

Many strains of *Pseudomonas* sp. has been extensively utilized as a biocontrol agent and plant growth promoters (Manuel et al. 2011). These bacteria exhibit a broad spectrum of activity against a range of phytopathogenic fungi, producing lipopeptides, and proteases, essential for fungal control. Some strains produce phenazine-1-carboxylic acid, phenazine-1-carboxamide, and other exometabolites with antifungal activity (Shtark et al. 2003). The antibiotic 2,4-diacetylphloroglucinol (DAPG) is highly toxic to

many plant pathogenic fungi and contributes to the biological control of plant disease. *Pseudomonas* species secrete fungal cell wall-degrading enzymes, such as glucanases, proteases and lipase (Poritsanos et al. 2006; Dharni et al. 2012; Chalotra et al. 2019). Hydrogen cyanide (HCN), a volatile secondary metabolite produced by *P. aeruginosa*, is recognized as a highly toxic antibiotic against plant pathogens. Siderophores synthesized by *Pseudomonas* sp. also exhibit antimicrobial activity (Keswani et al. 2020).

The isolation of antagonistic bacteria from their natural environment is crucial for the effectiveness of the biological control since they will be better adapted to survive and compete with other microorganisms adapted to the same environment (Figueroa-López et al. 2016; Blacutt et al. 2018). In this study, we tested four bacterial strains from maize silks for antifungal activity against *F. verticillioides*, the causal agent of the stalk and ear rot, and fumonisin producer.

Material And Methods

Microbial strains

The antifungal activity tests against *F. verticillioides* were performed with four bacterial strains from the maize silks from the Coleção de Microrganismos Multifuncionais e Fitopatogênicos of the Embrapa Milho e Sorgo, City of Sete Lagoas, Minas Gerais state, Brazil. The strains were collected in the localities of Sete Lagoas-MG, Sidrolândia-MS and Sertaneja-PR, in the year 2016. The *Fusarium verticillioides* isolate CML2743 used in the antagonism tests was from the Laboratório de Fitopatologia of the Embrapa Milho e Sorgo.

Molecular identification of the bacterial strains

The partial 16S rRNA gene sequence was used for identifying the four bacterial strains. Genomic DNA extraction was performed by the Wizard® Genomic DNA Purification kit (Promega, USA). PCR amplification of the 16S rRNA was carried out with the bacterial universal primers 8F (5'-AGAGTTTGATCCTGGCTCAG-3) and 1492R (5'-GGTTACCTTGTTACGACTT-3) designed by Turner et al. (1999). PCR reactions consisted of 20 ng of bacterial genomic DNA plus 2.0 µL 10X PCR buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl), 0.8 µL of each primer (10 µM), 1.5 µL dNTP (2,5 mM each), 0,6 µL of MgCl2 (50mM), 0,2 µL of Taq DNA polymerase (5 U/µL) (Invitrogen, USA) and the total reaction volume adjusted to 20 µL with ultrapure water. The PCR amplification was performed in a model Veriti® 96-Well Thermal Cyclers (Applied Biosystems, EUA) with the following conditions: one step of 2 min at 95 ºC for DNA denaturation, followed by 30 cycles of 30 s at 94 ºC, 30 s at 59 ºC and 90 s at 72 ºC, and a final extension step at 72 ºC for 10 min. The PCR products were analyzed by 1.0% (wt/vol) agarose gel electrophoresis and documented using the Gel Logic 200 system (KODAK Company, USA). The nucleotide sequences were determined on both strains using the PCR primers in the ABI PRISM 3500xL Genetic Analyzer Sequencer (Applied Biosystem, USA). The sequences alignments were made with the Sequencher 4.1.4 program (Genes Codes Corporation), and the alignment research tool (BLAST) was used to find

similarities with sequences in the NCBI database (http://www.ncbi.nlm.nih.gov/). The edited sequence of each species was deposited in the Gene Bank and received the following accession number: *Bacillus velezensis* strain CT02 (MK461847), *Bacillus velezensis* strain IM14 (MK461831) *Pseudomonas aeruginosa* (MK461572) and *Achromobacter xylosoxidans* (MK461853).

In vitro antifungal activity against *Fusarium verticillioides*

The antagonistic test was carried out by previously growing the four bacterial strains ISD04, IPR45, CT02, and IM14 and *F. verticillioides* CML2743 on potato dextrose agar (PDA) medium. After, a 5 mm diameter from actively growing fungus was transferred to the center of a new plate containing PDA medium, and 10µL of bacterial suspension (10^8 UFC/mL) was applied in four equidistant points, near the periphery of the culture. The plates were incubated at 28 °C under the 12h photoperiod for seven days. The antifungal activity was estimated by measuring the radial growth rate (mm) of the fungus in the confrontation test after the fungus of the control plate reached the entire medium surface. The inhibition ratios were calculated using the following formula: Inhibition ratio (%) = (radial mycelial growth of control - radial mycelial growth with antagonist) / radial mycelial growth of the control × 100.

Antifungal activity of the cell-free supernatant

Pure cultures of the bacterial strains were inoculated in liquid Tryptic Soy Broth (TSB), incubated at 28 °C, and constant agitation rate of 90 rpm for 72 h. Afterward, the culture was centrifuged at 6.000 rpm, and the supernatant filtered through a 0.22 µm pore membrane. Then, streptomycin (20 mg/L) was added to the supernatant and used as a growth medium for *F. verticillioides*. The TSB medium-plus antibiotic inoculated with culture discs of the fungus, and non-inoculated TSB medium with antibiotic was used as controls. The incubation was performed at 28 °C without shaking for ten days to allow *the mycelial growth ofF. verticillioides*. The fungal mycelium was recovered by filtration on Whatman paper filters (n.4) and dried at 60 °C until constant weight. The mycelial growth inhibition rate (%) was determined by the dry weight percentage of the mycelial biomass concerning the control (100%).

Effect of cell-free supernatant on conidia germination and hyphae development of *F. verticillioides*

The bacterial culture filtrates were used to test the inhibitory activity against *F. verticillioides* conidia germination and hyphae growth. Conidia of the fungal pathogen were obtained from a seven-day culture at 25 °C and 12 h photoperiod in the BDA medium. The conidia suspension was filtered through gauze to remove any large fragments of mycelia, and the concentration was adjusted to 1 x 10^4 conidia/mL by counting in a Neubauer chamber. Then, the conidial suspension and each bacterial culture filtrate were mixed in equal proportion and incubated for 24 h at 26 °C under a 12 h photoperiod. The control consisted of TSB medium inoculated with *F. verticillioides*. The effect of each bacterial supernatant on conidia germination and hyphae development was evaluated by observation under an optical microscope. For the conidia germination test, 100 conidia of each treatment were counted, in triplicate, and those germ tubes with twice the size of the conidia were considered germinated (Abou-Jawdah et al.

2002). The percentages of inhibition were calculated by comparison with the fungal culture without bacterial inoculation (control). The experiment was repeated three times.

Hydrolytic enzymes production by the bacterial strains and calculation of the enzyme index

The concentration of bacterial culture grown in TSB for 72 h was adjusted to approximately 10^8 CFU mL^1 and inoculated in the specific medium for each enzyme. After microbial growth for about 48 h, the enzymatic activity was measured by a clear zone surrounding colonies. The enzymatic index (EI) was calculated by EI = diameter (mm) of the discolored halo/diameter (mm) of the colonies.

Cellulase

To determine the cellulase production, the four bacterial strains were grown in minimal M9 culture medium (10 g/L carboxymethylcellulose, 5 g/L yeast extract, 12.8 g/L Na_2HPO_4 .$7H_2O$, 3 g/L KH_2PO_4, 0.5 g/L NaCl, 1 g/L NH_4Cl, 5 g/L $MgSO_4$.$7H_2O$, 0.01 g/L $CaCl_2$.$2H_20$, 15 g/L agar). After microbial growth, 10 mL of Congo Red (1g/L) was distributed on each plate. After 15 min, the plates were washed with 5M NaCl and observed for a yellowish area around the colonies (Teather and Wood 1982).

Pectinase

For pectinase activity, the bacterial strains were grown in the M9 medium plus citrus pectin with pH adjusted to 8.0 and incubated as described above. After the culture growth, ten mL of Lugol were added to the plates and washed with deionized water. A colorless halo around the colonies indicated the pectinase production (Beg et al. 2000).

Protease

The protease production was evaluated in culture medium containing 5 g/L tryptone, 2.5 g/L yeast extract, 2.5 g/L NaCl, 1 g/L of glucose, and 16 g/L agar. After sterilization, 100 mL of boiled skimmed milk were distributed on each plate. Protease activity was expressed by the formation of a colorless halo around the colonies (Beg et al. 2000).

Lipase

For lipase, the four strains were grown in a medium containing 5 g/L peptone, 1 g/L yeast extract, 4 g/L NaCl, 15 g/L agar, 31.25 mL/L olive oil, 0.01 g/L rhodamine B with the pH adjusted to 7.0. After the bacterial growth, the presence of a blue halo around the colonies was visualized by ultraviolet radiation (Savitha et al. 2007).

Chitinase

Chitinase activity was performed, as indicated by Trudel and Asselin (1989), with modifications. A 10µL of the supernatant of bacterial culture was added in a 2mm wells in plates containing agarose gel (1.6%) in sodium acetate buffer (0.1M, pH 5.0) and 0.01% (P/V) of glycol chitin solution. After the incubation at

30 °C for 48 h, the plates were stained with 0.1% calcofluor in 0.5M Tris-HCL buffer, pH 8.9 for 10 min, and washed with distilled water. The formation of a clear halo detected by ultraviolet light (300nm) was indicative of chitinase production.

Growth of *Fusarium verticillioides* in maize seeds microbiolized with the bacterial strains

Maize seeds with no antifungal treatment were disinfested with a 3% sodium hypochlorite solution for 5 min, followed by a wash with sterile distilled water. Then, the seeds were disinfected according to Daniels (1983) by placing the seeds in a 70% (v/v) ethanol solution for 10 min and then transferred to flasks containing sterile deionized water for 4 h. Subsequently, the seeds were transferred to another flask containing sterile deionized water and kept in a water bath at 60 °C for 5 min. Then, the seeds were placed under direct light for 24 h, followed by frozen at -20 °C for 24 h to inhibit seed germination. Afterward, the seeds were infected with a suspension of 1 x 10^6 conidia/mL of *F. verticillioides*. The antagonist bacterial strains were grown in liquid TSB medium for 72h, and the bacterial cell concentrations were adjusted to approximately 10^8 CFU mL^{-1}. The maize seeds were microbiolized by immersion in bacterial suspensions for 24 h at 28 °C with shaking at 130 rpm. The seeds immersed only in TSB served as the negative control. An additional control with seeds disinfected without inoculations was included to assess the effectiveness of the disinfection method. The seeds were germinated in boxes (11 x 11 x 3cm) containing filter paper, moistened with sterile distilled water. Sixty seeds were distributed in three replicates of 20 seeds per treatment and incubated with a photoperiod of 12 h of light at 26 °C for eight days. The evaluation of *F. verticillioides* incidence was carried out by examining the seeds in the Zeiss Stemi 2000 binocular stereomicroscope with a 50X magnification objective.

Effects of seeds microbiolization with bacterial strains in reducing stalk rot disease

The bacterial strains were grown in Lysogeny Broth (LB) medium at 35 °C for 72h and shaking at 300 rpm. Each culture was centrifuged at 2.000 rpm, and the pellet resuspended in a 20% sucrose solution. For microbiolization, seeds of maize hybrids BRS 1010, susceptible to *F. verticillioides* were treated with bacterial culture plus 50% sucrose (w/vol) and incubated at 80 rpm and grown for 30 min at room temperature. Then, the seeds were mixed in starch and dried for 24 h at 30 °C (Figueiredo et al. 2010). The treated seeds were planted in pots containing 5 kg of soil, and 30 days after planting, the fungus was inoculated by stem punctures using sterile toothpick immersed in the conidial suspension (1 x 106 conidia / mL). Five seeds from each treatment were planted in each of three pots. They consisted of 1) seeds treated with each bacterial antagonist inoculated with *F. verticillioides,* 2) seeds without bacterial treatments inoculated with *F. verticillioides*, and 3) seeds without treatment and toothpicks puncture without *F. verticillioides*, As a control, seeds treated with each bacterial strain without *F. verticillioides* inoculation were included in the experiment. After 45 days of infection, the stalk rot severity was determined according to the Symptom Score Scale described by Nicoli et al. (2015).

Statistical analysis

The analysis of variance (ANOVA) was used to check the collected data, followed by the Scott-Knott means comparison test at p <0.05. All experiments were performed in triplicate, and the results were expressed as mean ± standard deviation (SD).

Results

Molecular identification of the bacterial strains

The partial nucleotide sequences of the 16S rRNA gene showed that the four bacterial strains belong to the species *Bacillus velezensis* (IM14 and CT02), *Achromobacter xylosoxidans* (ISD04), and *Pseudomonas aeruginos*a (IPR45). The 16S nucleotide sequences were deposited in the Genbank database, and the accession number are shown in Table 1.

Table 1
Bacterial strains from the maize silks, molecular identification, and Gene Bank accession numbers

Strain	Collection place	Lifestyle	Molecular Identification	Nucleotide length	Genbank Acession number
ISD04	Sidrolândia	Endophytic	*Achromobacter xylosoxidans*	1435bp	MK461853
CT02	Sete Lagoas	Epiphytic	*Bacillus velezensis*	1395bp	MK461847
IPR45	Sertaneja	Endophytic	*Pseudomonas aeruginosa*	1368bp	MK461572
IM14	Sete Lagoas	Epiphytic	*Bacillus velezensis*	1400bp	MK461831

In vitro **antifungal activity against** Fusarium verticillioides

The four bacterial strains were tested for antagonistic activity against the growth of *F. verticillioides* by the confrontation test. The strain ISD04 (*A. xylosoxidans*) showed the highest antagonistic activity (100%) and completely inhibited the fungal development (Fig. 1D). The other three strains CT02 (*B. velezensis*), IPR45 (*P. aeruginosa*), and IM14 (*B. velezensis*) also significantly reduced the *F. verticillioides* growth with inhibition values of 62.5%, 61.5%, and 58.5% respectively. In the three last-mentioned strains, the formation of a clear zone was observed around the colonies (Fig. 1A, 1B, and 1C). Unlike the other three strains, the confrontation test with *A. xylosoxidans* ISD04 did not show a colorless halo around the colony due to the fast bacterial growth overpassing the fungal disc (Fig. 1D).

Effect of cell-free supernatant on conidia germination and hyphae development of F. verticillioides

The results using culture supernatants showed that all strains significantly reduced the mycelial growth of *F. verticillioides* (Fig. 2). The inhibitory effect of the four bacterial antagonists against the fungus was

assessed through the fungus dry weight. The inhibitory values ranged from 47.2 to 74.7%, with no significant differences (Table 2).

The four strains showed different percentages of conidia germination inhibition compared to the control inoculated with *F. verticillioides*, which presented 86.6% of germination, and the cell-free filtrate *Pseudomonas aeruginosa* (IPR45) inhibited 100% the conidial germination. The other strains showed inhibition values varying from 45–56.5% (Table 2).

Table 2
Inhibition of mycelial growth and conidia germination of F. verticillioides **by cell-free supernatant**

Strain	Identification	Mycelial mass (mg)*	Mycelial inhibition (%)	Conidia germination (%)	Conidia germination (%)
Control**	—	367.6 a	—	86.6 a	—
ISD04	*A. xylosoxidans*	194.0 b	47.2	42.6 b	50.8
IM14	*B. velezensis*	147.3 b	59.9	37.6 c	56.5
IPR45	*P. aeruginosa*	99.6 b	72.9	00.0 d	100.0
CT02	*B. velezensis*	93.0 b	74.7	47.6 b	45.0

* Averages followed by the same letter do not differ by the Scott-Knott test at 5% probability

** Control: *F. verticillioides* grown in liquid medium without bacteria

The microscopic evaluation showed that the filtered supernatant of *Bacillus velezensis* (strains IM14 and CT02) impaired the hyphal growth of *F. verticillioides* and caused morphological abnormalities, such as hyphal swelling, reduction in the hyphal compartment length (shortening), and vacuolization (Fig. 3C, 3D and 3E). In addition, the culture filtrate of *P. aeruginosa* (isolate IPR45) completely inhibited the conidial germination and considerably reduced the number of the fungal conidia in suspension (Fig. 3F). Normal hyphal growth was observed in control with TSB medium (Fig. 3A) and *A. xylosoxidans* culture filtrate (Fig. 3B) after 24h incubation.

Hydrolytic enzymes production by the bacterial strains and calculation of the enzyme index

In this study, the four bacterial strains produced at least one of the five enzymes tested (Table 3 and Fig. 4). All strains produced chitinase; three produced protease (IM14, IPR45, and CT02), two produced cellulase and pectinase (IM14 and CT02). Only the isolate IPR45 of *P. aeruginosa* showed lipolytic activity, and the highest chitinolytic activity (Table 3).

Table 3
Enzymatic index (EI) of cellulase, pectinase, protease, lipase, and chitinase of the bacterial cultures *A. xylosoxidans*, *B. velezensis*, *P. aeruginosa*, and *B. velezensis*

Isolate	Identification	Enzimatic Index (EI)*				
		Cellulase	Pectinase	Protease	Lipase	Chitinase
ISD04	*A. xylosoxidans*	0.00	0.00	0.00	0.00	5.40 a
IM14	*B. velezensis*	1.40 a	1.91 b	1.31 a	0.00	1.60 b
IPR45	*P. aeruginosa*	0.00	0.00	1.46 a	2.30	5.70 a
CT02	*B. velezensis*	1.66 b	1.88 b	1.55 a	0.00	1.80 b
*Averages followed by the same letter do not differ by the Scott-Knott test at 5% probability						

Growth of Fusarium verticillioides **in maize seeds microbiolized with the bacterial strains**

The growth of microorganisms was not observed in the control treatment consisting of disinfected seeds, which confirmed the disinfection method (Fig. 5A). Contrary, the fungal growth was remarkable in control inoculated with *F. verticillioides* conidia (Fig. 5B).

The microbiolization of seeds with the four bacterial strains was efficient in reducing the fungal incidence compared with the non-microbiolized seeds inoculated with *F. verticillioides*. The seeds treated with *Pseudomonas aeruginosa*, the growth of the pathogen was completely inhibited (Fig. 5C). The seeds treated separately with the two *Bacillus velezensis* (Fig. 5D and 5E) and *Achromobacter xylosoxidans* (Fig. 5F) only partially reduced the growth of *F. verticillioides*.

Effects of seeds microbiolization with bacterial strains in reducing stalk rot disease

The severity of the stalk rot disease was evaluated by the presence of black or dark brown lesions in maize stalk, a typical diagnostic of *F. verticillioides* infection (Fig. 6). The disease severity in each treatment and the antagonistic effect of the bacterial strains on the percentage of stalk rot reduction are shown in Table 4. Plants treated with A. *xylosoxidans* showed 72.6% in the reduction of the stalk rot prevalence (Fig. 6C), and the effect of the treatment was statistically equal to control without inoculation with *F. verticillioides* (Fig. 6A). The strains of *P. aeruginosa* (IPR45) and *B. velezensis* (CT02) reduced the stalk rot by 50.7% and 32.8%, respectively (Fig. 6D and 6F), compared with the control inoculated with *F. verticillioides* (Fig. 6B). Microbiolization with *B. velezensis* IM14 did not affect disease progression and was not statistically different from the control inoculated with the fungus (Fig. 6E). Microbiolized seeds without *F. verticillioides* inoculation were germinated and developed health plants without stalk rot symptoms (data not shown).

Table 4
Effect of seeds microbiolization with four bacterial strains in reducing stalk rot severity forty-five days after *Fusarium verticillioides* inoculation under greenhouse conditions

Treatment	Disease severity (%)*	Stalk rot reduction (%)
Non-microbiolized (control)	6.02 a	-
Control + *F. verticillioides*	38.06 c	0
A. xylosoxidans (ISD04) + *F.verticillioides*	10.40 a	72.6
P. aeruginosa (IPR45) + *F. verticillioides*	18.75 b	50.7
B. velezensis (IM14) + *F. verticillioides*	50.05 c	0
B. velezensis (CT02) + *F. verticillioides*	25.57 b	32.8

* Averages followed by the same letter do not differ by the Scott-Knott test at 5% probability

Discussion

Fungal diseases are a major threat to crop production. *Fusarium verticillioides* is the primary pathogen of maize and cause ear rot, stalk rot, and a fumonisin producer making the grains improper for human and animal consumption (Rosa Junior et al. 2019). The use of chemical pesticides in the field is a serious threat to environmental and human health (Zubrod et al. 2019). Therefore, biological control is a natural alternative to reducing production costs and environmental disturbances, thereby contributing to sustainable agriculture (Jia 2019). Thus, developing strategies using antagonistic microorganisms represents a safer and cheaper solution to control fungal diseases in crop fields (Dukare et al. 2019). In this work, we aimed to test four bacterial strains isolated from the maize silks to obtain biocontrol agents against the maize phytopathogenic fungus *F. verticillioides*. The fungus *F. verticillioides* growing along or within the stigmatic structures (silks), in association with the pollen tube, gain access to the plant, causing kernel rot and ear rot, and producing fumonisins (Munkvold et al. 1997; Gai et al. 2018). The use of microorganisms from the environment where they will be used guarantees easy adaptation and survival of the antagonists for *in vivo* interactions against the target pathogen and indigenous microorganisms (Figueroa-López et al. 2016). *F. verticillioides* may also enter the plant through the roots. Thus, seeds microbiolized with bacterial antagonists may reduce the levels of plant infection by the fungus. Therefore, adopting these strategies tends to increase biological control efficiency and represent a key point for developing new strategies for plant disease management.

We demonstrated the antagonistic activity of four bacteria isolated from the maize silks against *F. verticillioides* and carried out their identification by DNA sequencing. The four bacterial strains belong to *Bacillus velezensis*, *Pseudomonas aeruginosa*, and *Achromobacter xylosoxidans* (former *Alcaligenes xylosoxidans*). Several studies have shown the potential of these species as biocontrol agents for different phytopathogenic fungi, including *Fusarium* sp. (Borah et al. 2016; Moretti et al. 2008; Passari et

al. 2017). The complete genome sequence and laboratory experiments on purification of the bioactive substances revealed that these three species produce several inhibitory substances (as the antifungal cyclic lipopeptide bacillomycin L, Cyclo (l-Leucyl-l-Prolyl), and several phenazines produced by *Bacillus velezensis*, *Achromobacter xylosoxidans* and *Pseudomonas aeruginosa*, respectively (Stover et al. 2000; Yan et al. 2004; Kumar et al. 2005; Badalamenti and Hunter 2015; Zhang et al. 2020; Gao et al. 2020). Furthermore, many strains in these groups are notable plant growth promoters by producing phytohormones, organic acids, enzymes, and siderophores, or activating mechanisms for mineral phosphate solubilization and nitrogen fixation (Keswani et al. 2020).

In the in vitro confrontation method, all four strains showed high inhibitory activity against *F. verticillioides*. The antagonistic control may occur through different mechanisms of action such as the production of antifungal substances as organic acids, lytic enzymes, hydrogen peroxide, diacetyl, and peptides (Piard and Desmazeaud 1992; Krishan et al. 2018; Souza et al. 2018). Our results showed that cell-free supernatants reduced the germination of conidia and caused morphological changes in the structure of *F. verticillioides* hyphae. As the primary mode of vegetative growth of the fungus, the hyphae play a crucial role in fungal adhesion, invasion, and disease progression in the plant. Morphological damages to the hyphae caused by bacterial metabolites reduce the pathogenicity by preventing the plant colonization by the fungus (Borah et al. 2016).

The inhibitory effect of both cell-free culture supernatants and microbiolized seeds from *P. aeruginosa* reduced the number of conidia of *F. verticillioides*, and the fungus growth affecting the development of the hyphae and altering the conidial cell wall. A previous study by Chan et al. (2003) reported the negative effect of *Bacillus subtilis* culture supernatant on macroconidia germination and hyphal growth of *F. graminearum* due to hyphal swelling and cell disruptions.

The presence of active substances in the filtered supernatant of the strain CT02 of *Bacillus velezensis* reduced the growth of *F. verticillioides* by 74.7%. Since the discovery of *B. velezensis* genes controlling the biosynthesis of secondary metabolites with activity in suppressing the growth of plant pathogens by triggering the systemic resistance induced in plants and plant growth, the species has received considerable attention from the biological control research community (Chen et al. 2018; Rabbee et al. 2019). The complete genome sequencing of the strain B-4 of *Bacillus velezensis* by Zhu et al. (2020) revealed 12 clusters of genes related to the synthesis of antimicrobial metabolites corresponding to more than 19.56% of the genome. Among the primary metabolites with antimicrobial properties of *B. velezensis*, cyclic lipopeptides may be highlighted, such as iturin A, fengycin, and surfactin. Phenicin has toxic activity against filamentous fungi and can induce plant resistance, and surfactin acts as a biosurfactant and induces systemic resistance (Gong et al. 2015; Kim et al. 2017). Iturin, which acts in the cytoplasmatic membrane leading the cell to death, shows intense antifungal activity against yeasts and many fungi (Ongena and Jacques 2008). The use of CT02 and other *Bacillus* species in biocontrol programs represents an advantage concerning the other three strains due to their ability to produce endospores resistant to heat and drying. These features give survival advantages under field conditions and make these microorganisms more suitable for commercial formulations due to their increased shelf

life compared to the lifetime of agrochemicals (Wu et al. 2015). For these reasons, several *Bacillus*-based products are commercially available in the supermarket (Pérez-Garcia et al. 2011).

The excretion of hydrolytic enzymes can weaken or degrading the components of the fungal cell walls and membranes (Neeraja et al. 2010). Proteases can bind to external mannoproteins and open the protein structure, exposing the inner layers of glucan and chitin microfibrils (Choudhary et al. 2014). The enzymes chitinase, glucanase, and protease produced by some bacterial strains can hydrolyze the fungal cell wall but not the plant cell wall, which is the most critical target of fungal pathogens (Neeraja et al. 2010; Khare and Yadav 2017). Other forms of enzymatic actions occur when the endophyte produces cellulase and pectinase, activating the plant defense responses, or when proteases inactivate the enzymes of the pathogen responsible for the destruction of the plant cell wall, which is the determinant factor for plant infection by the fungus (Vaidya et al. 2001; Khare and Yadav 2017; Köhl et al. 2019).

In the test of extracellular enzyme production, only *Pseudomonas aeruginosa* showed lipolytic activity. Chalotra et al. (2019) reported the effect of secreted lipase degrading the fungal cell wall. Together with the other results for *P. aeruginosa*, this suggests the existence of different antifungal mechanisms exerting synergism or cooperative effects to reduce the growth of *F. verticillioides*. Many strains of *P. aeruginosa* have been used in commercial formulations for the biocontrol of diseases caused by *Phytophtora* spp., *Phythium* spp., *Rhizoctonia* spp., and *Fusarium* spp. and other fungi in many crop species such as tomatoes, pepper, taro, and beans (Kumar et al. 2005; Wu et al. 2015; Zohara et al. 2015; Meena et al. 2019). However, studies using *P. aeruginosa* in *F. verticillioides* biological control in maize crops are still scarce.

In the greenhouse, *Achromobacter xylosoxidans* ISD04 reduced the severity of the stalk rot disease, producing a result similar to the control not inoculated with *F. verticillioides*. It was demonstrated that plants treated with A. *xylosoxidans* strain AUM54, followed by inoculation with *Magnaporthe oryzae* (rice blast fungus), showed a significant increase in the activities of defense-related enzymes such as polyphenol oxidase, peroxidase, phenylalanine ammonia-lyase, and chitinase (Joe et al. 2012). The authors (Joe et al. 2012) also reported that plants treated with *A. xylosoxidans* AUM54 showed a reduced incidence of blast disease and significant growth improvement.

Despite the satisfactory results *in vitro*, the performance of *Bacillus velezensis* IM14 in the greenhouse not differed statistically from the control inoculated with *F. verticilioides*. Various studies have shown that antifungal activity in vitro does not necessarily correlate with antagonistic activities in situ (von der Weid et al. 2000; Gopalakrishnan et al. 2011; Köhl et al. 2019; Wang et al. 2019). This finding has been attributed to the fact that the production of antifungal metabolites is determined by more than one mechanism regulated by highly complex interactions among their components, such as the inoculum, the abiotic environment, and the other organisms living in the soil (Watanabe et al. 2001). In this scenario, the antagonist must quickly adapt to a new environment, and its success or failure will depends on its competitive ability for space and nutrients. Thus, the possibility exists that the introduced species may eventually be eliminated from the environment. Chan et al. (2003) presented an alternative solution to

circumvent this problem by applying cell-free culture filtrate to the soil instead of the living cells. This procedure avoids the need for the antagonist to colonize the target plant and eliminates the influence of external factors but decreases the efficiency of the biological control. In addition, this strategy does not eliminate the harmful effects of cell-free culture extracts on the soil microorganisms, and the protective effect of the antagonist will not extend to adult uncolonized plants or the maize kernels. Thus, this critical aspect of biological control remains unanswered. The use of bacterial antifungal metabolites in chemosensitization, instead of the living bacteria, is a promising topic to increase the effectiveness of fungicides by exploring the synergistic action of fungicides and antifungal metabolites and, at the same time, decreasing the risks of negative impact on the environment by reducing the amount of fungicide applied in the field (Kim et al. 2017). Therefore, to solve this limitation, it is undoubtedly necessary to move forward in the biological control approaches by identifying, purifying, and synthesizing the active metabolites and their application to the target. Recently was demonstrated that mycelia of *F. verticillioides* treated with rhamnolipids from *P. aeruginosa* exhibited an irregular shape, surface roughness, breakages, and severe reduction in thickness, leading to suppression of disease symptoms and colonization of maize plants by *F. verticillioides* (Borah et al. 2016). Future studies within these four strains will address this topic.

Conclusions

The data showed the inhibitory effect of four endophytic and epiphytic bacterial strains from maize silks on *Fusarium verticillioides*. All strains evaluated produced at least one of the five hydrolytic enzymes. Supernatants free from *Bacillus velezensis* cells caused morphological abnormalities in the phytopathogen hyphae. *Pseudomonas aeruginosa* inhibited the growth of *F. verticillioides* in microbiolized seeds. In the greenhouse, *Achromobacter xylosoxidans* ISD04, *Pseudomonas aeruginosa* IPR45, and *Bacillus velezensis* CT02 were effective in reducing the maize stalk rot. The four strains are a potential source for control strategies to reduce the *F. verticillioides* incidence in maize.

Declarations

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Conflicts of interest/Competing interests

The authors declare that they have no conflict of interests.

Availability of data and material

Not applicable

Code availability

Not applicable

Authors' contribution

This study was designed by Christiane Abreu de Oliveira Paiva, Gisele de Fátima Dias Diniz, Luciano Viana Cota, Vera Lúcia dos Santos, and Ivanildo Evódio Marriel. Christiane Abreu de Oliveira Paiva and Ivanildo Evódio Marriel, as the main investigators directed and coordinated all aspects of the project. Frederick Mendes Aguiar and Dagma Dionísia da Silva assisted Gisele de Fátima Dias Diniz in the experiments and collecting the samples. Gisele de Fátima Dias Diniz performed the data analysis and, together with José Edson Fontes Figueiredo and Christiane Abreu de Oliveira, did critical review of the results and manuscript. The article was written by Gisele de Fátima Dias Diniz and José Edson Fontes Figueiredo.

Ethics approval

The experiments reported in this study did not involve human participants and/or animals.

Consent to participate

Not applicable

Consent for publication

All authors read and approved the final version of the manuscript for publication.

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Figures

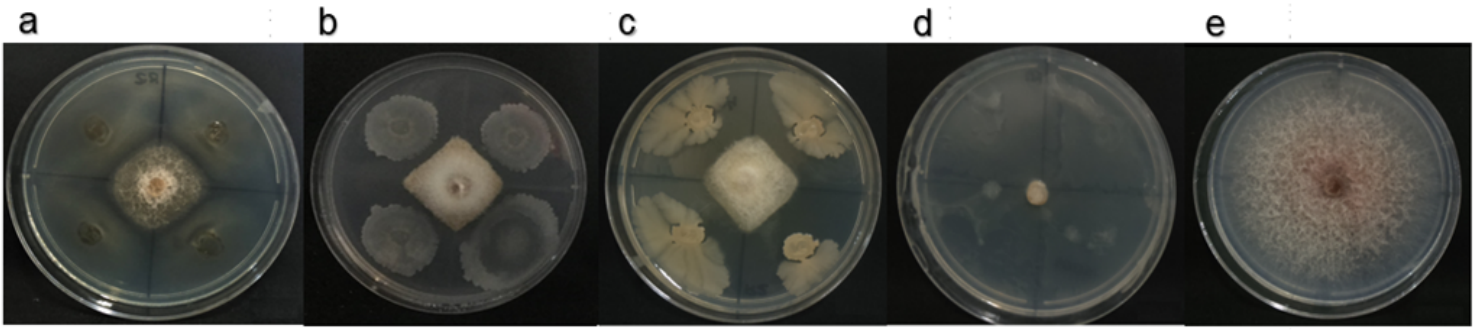

Figure 1

The in vitro antagonistic activity of four bacterial strains against Fusarium verticillioides. (A) Pseudomonas aeruginosa (IPR45), (B) Bacillus velezensis (CT02), (C) B. velezensis (IM14), and (D) Achromobacter xylosoxidans (ISD04). (E) F. verticillioides used as control. Fusarium verticillioides growth inhibition is evidenced by a clear area around the phytopathogen in the center of the plates, except in (D)

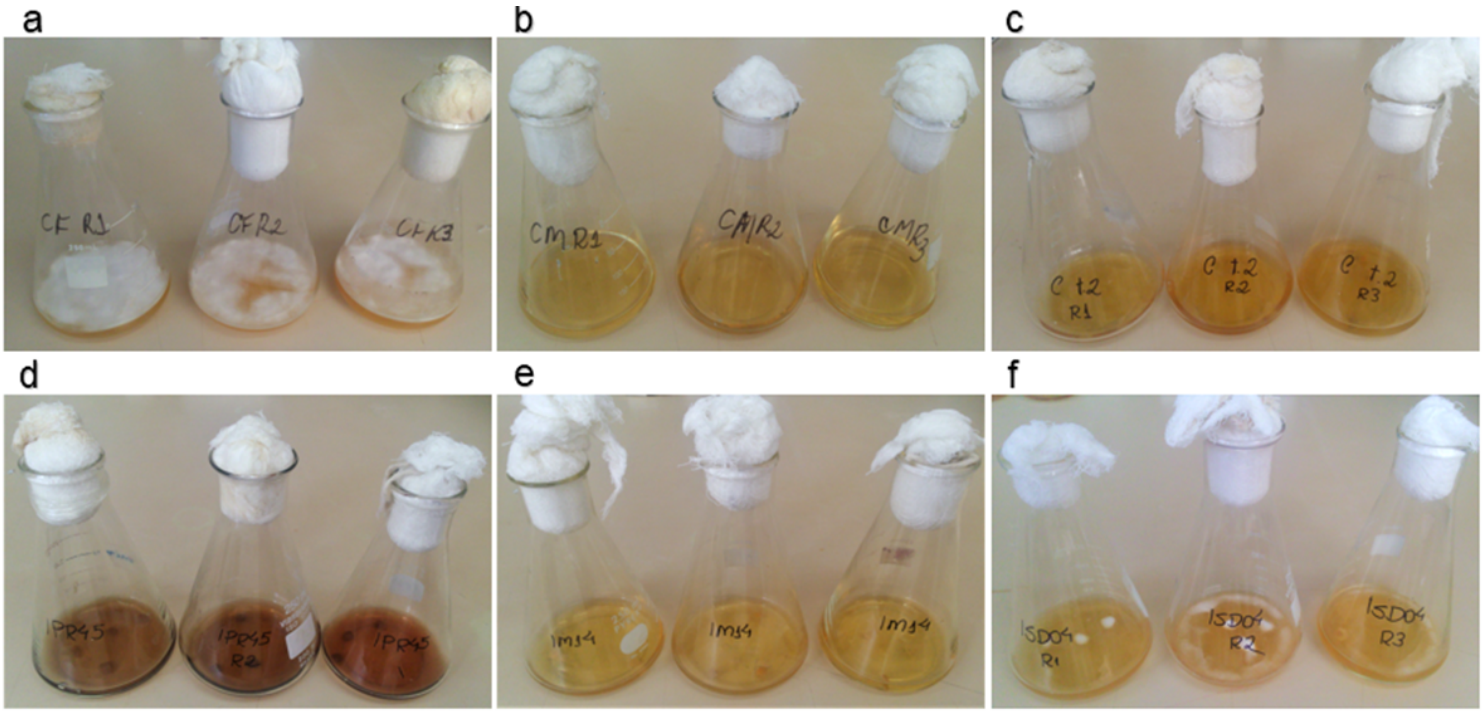

Figure 2

Effects of cell-free bacterial culture supernatants on mycelial growth of Fusarium verticillioides in liquid medium. (A) Control inoculated with F. verticillioides in TSB plus streptomycin, (B) Culture medium without inoculation (control). From (C) to (F), free filtrate of bacterial culture cells inoculated with F. verticillioides discs (C) Bacillus velezensis (CT02), (D) Pseudomonas aeruginosa (IPR45), (E) B. velezensis (IM14), and (F) Achromobacter xylosoxidans (ISD04). The white matters inside the flasks correspond to mycelial growth of Fusarium verticillioides.

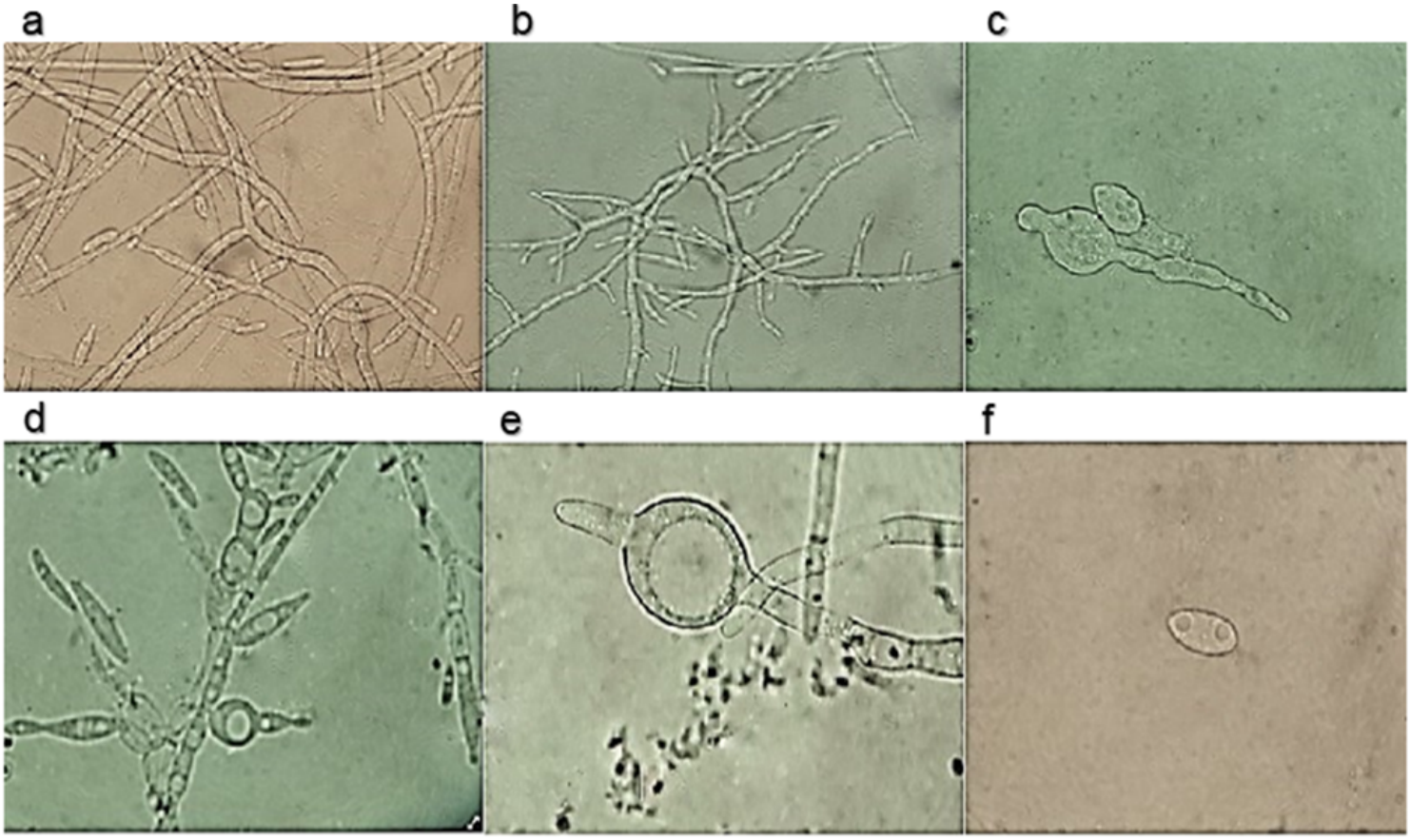

Figure 3

Effect of cell-free filtered culture supernatant on hyphae growth and conidia development. (A) Hyphae growth of Fusarium verticillioides used as the control, (B) Fungal hyphae without abnormalities growing in the presence of cell-free filtrate A. xylosoxidans (isolate ISD04). Morphological abnormalities in the development of fungal conidia in cell-free filtrates of the Bacillus velezensis strains IM14 (C) and CT02 (D and E). (F) ungerminated conidia of F. verticillioides after 24 h of incubation in the Pseudomonas aeruginosa cell-free filtrate

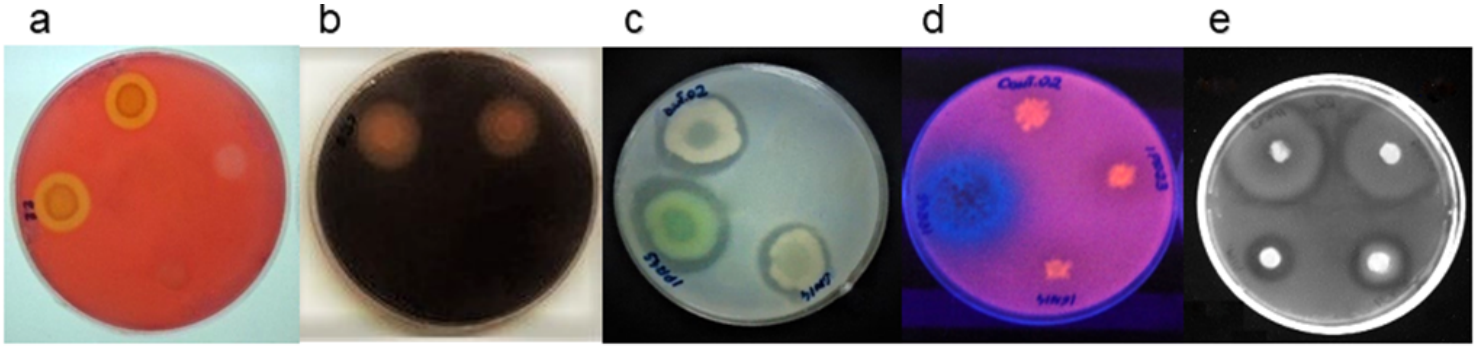

Figure 4

Enzymatic activity test for the four bacteria isolated from the maize silks. Formation of halos indicative of the production of cellulase (A), pectinase (B), protease (C), lipase (D), and chitinase (E). The four strains were inoculated on the same plate

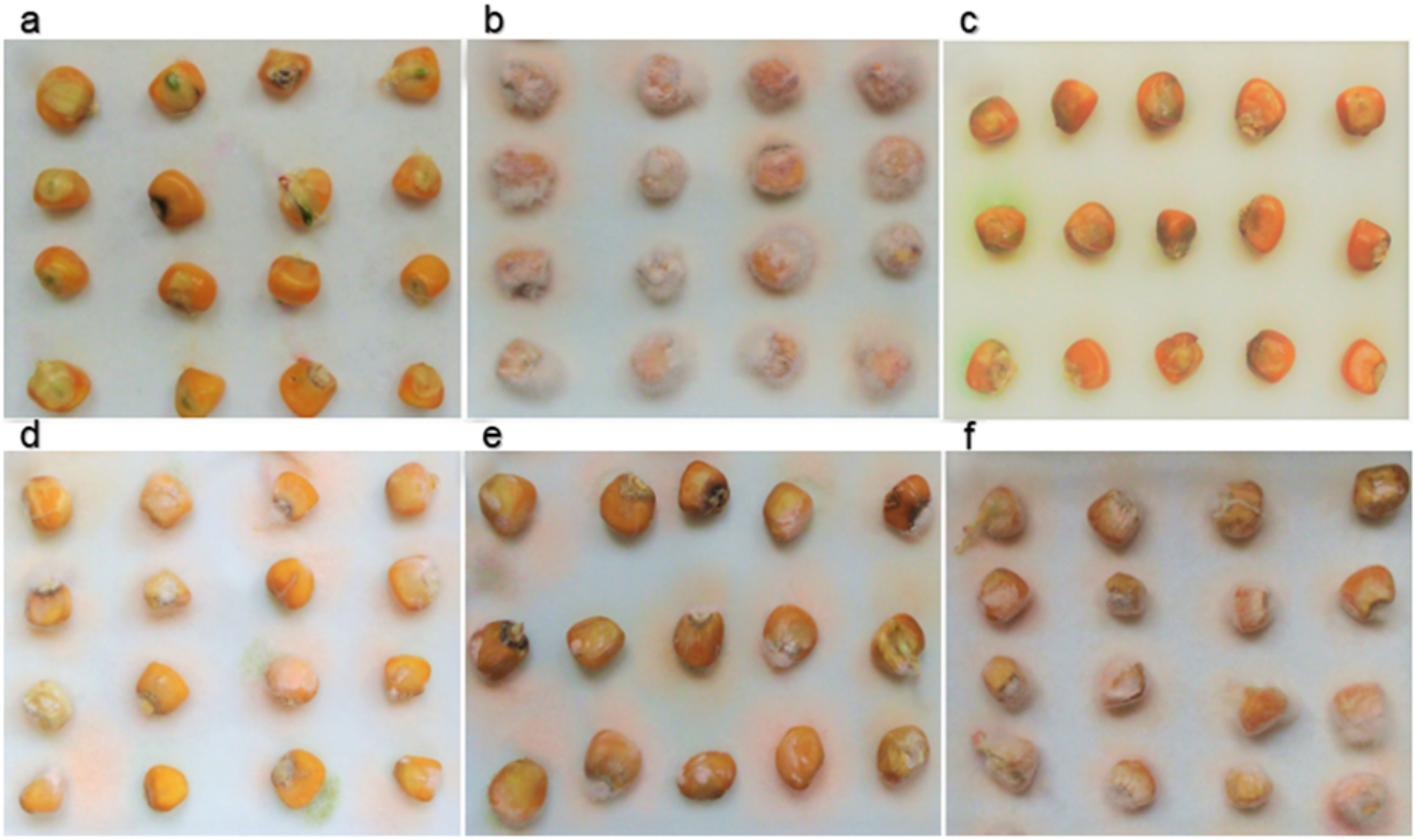

Figure 5

Effects of maize seeds microbiolization on F. verticillioides growth. (A) Absence of fungal growth in disinfected seeds, (B) fungal incidence in seeds inoculated with F. verticillioides, (C) to (F) Seeds microbiolized with Pseudomonas aeruginosa (C), Bacillus velezensis CT02 (D) and IM14 (E), and Achromobacter xylosoxidans ISD04 (F)

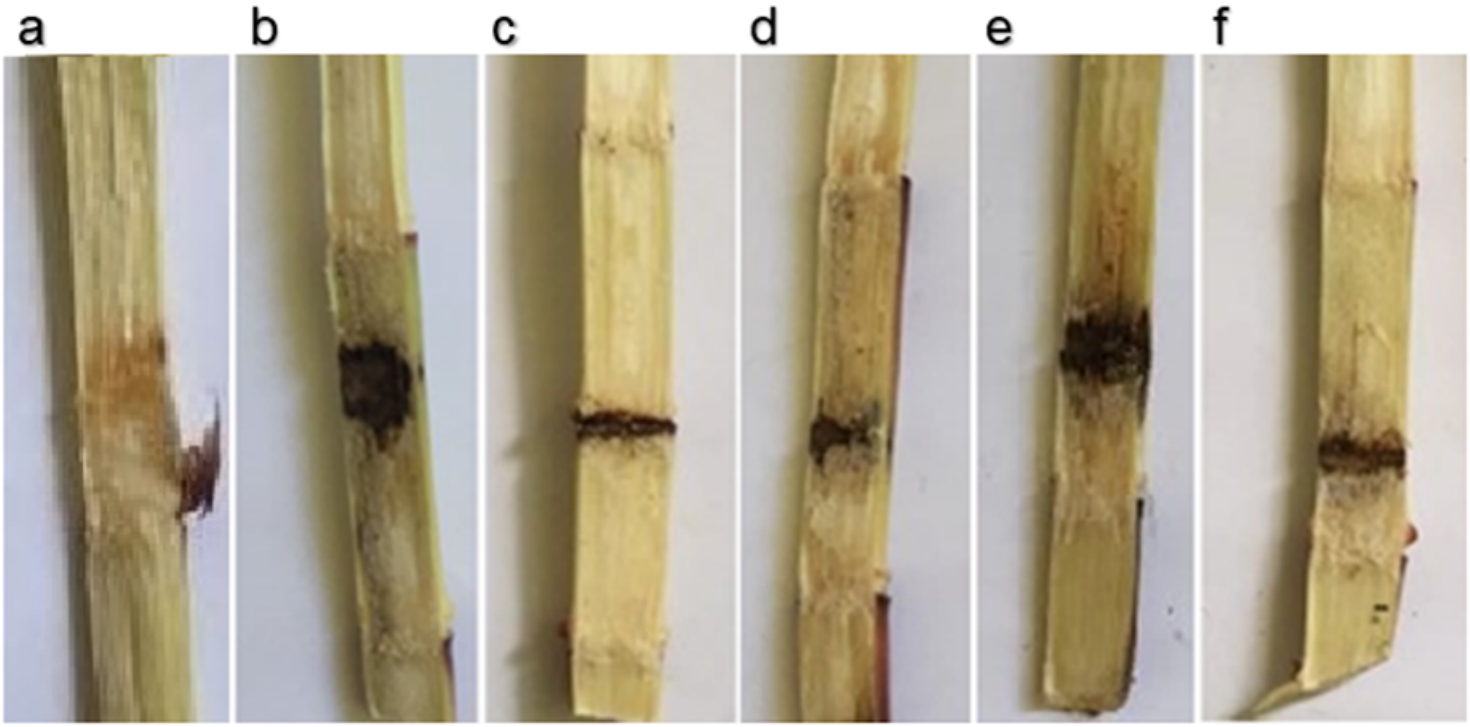

Figure 6

Effect of seed treatment with four bacterial strains on stalk rot caused by Fusarium verticillioides. (A) non-inoculated control (B) inoculation of F. verticillioides in plants originated from non-microbiolized seeds. From (C) to (F), all treatments were inoculated with F. verticillioides (C) Achromobacter xylosoxidans (ISD04), (D) Pseudomonas aeruginosa (IPR45), (E) Bacillus velezensis (IM14), and (F) Bacillus velezensis (CT02)