Optimization of the Extraction Process and Application of Bacterial Extracts in the Control of Brown Spot and Leaf Blast in Rice Culture

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Abstract

The objective of this work was to optimize the extraction process and application of bacterial extracts of *Bacillus* sp. and *Serratia* sp. in leaf blast control (*Magnaporthe oryzae*) and brown spot (*Bipolaris oryzae*) in rice culture. The work was divided into three stages: 1) Bacterial obtaining extracts through liquid-liquid extraction 2) Antagonistic capacity of bacterial extracts to *M. oryzae* and *B. oryzae* 3) Suppression of brown spot (A1) and leaf blast (A2) in greenhouse. The bacterial isolates in present study were identified as *Bacillus* sp. (BRM32110) and *Serratia marcescens* (BRM32113). The crude extract of both isolates at different extraction times 6, 16, 24, 48 and 72 hours reduced the growth of colonies of *M. oryzae* and *B. oryzae* by up to 92% and 28%, respectively. The extracts that showed highest inhibition of colony growth were those obtained after 6 and 16h of incubation and were selected for subsequent assays. These, for both isolates were able to reduce conidia germination by up to 91% and apressorium formation of *M. oryzae* by up to 93%. In green house, A1 the treatment that stood out was the extract of *Bacillus* sp. (16h) with 6.7% of leaf area affected and in A2 the treatment *S. marcescens* extract (16h) stood out with only 7.6% of leaf area affected with brusone when compared to control. The use of extracts of *Bacillus* sp. and *Serratia marcescens* was efficient in reducing the severity of brown spot and leaf blast in rice crop.

Introduction

The warning about the need to increase food production is recurrent, and rice (*Oryza sativa* L.) stands out for its strategic role in both social and economic development in Brazil and in the world (CONAB 2020). The average consumption in the world is 57 kg person⁻¹ year⁻¹ (Nascente et al. 2019), and in Brazil consumption is on average 45 kg of rice person⁻¹ year⁻¹ (Embrapa 2020). World rice production was approximately 495.23 million tons, and has the highest potential for increased production among other cereals, supplying 20% of calories and 15% of protein consumed in the diet (Sosbai 2012).

The production of this cereal has been showing a drop in profitability, according to the increase in production costs, caused by the excessive number of fungicides and fertilizers, to ensure the production and quality of grains threatened by stresses (Guimarães et al. 2014; Ahmad et al. 2016), as the occurrence of brusone and grain spot. Brusone caused by the fungus *Magnaporthe oryzae* B. Couch ranks first in economic importance (Silva-Lobo et al. 2012; Dean et al. 2005), is difficult to control and can cause up to 100% losses in rice yield (Prabhu et al. 2009). Brown spot (*Bipolaris oryzae*) causes grain yield losses (90%), particularly when the leaf spot stage assumes epiphytic proportions (Sunder et al. 2014).

The cost of pesticides in rice farming has increased by about 180% since the 2007/08 crop (CONAB 2021; Sharma et al. 2019) and fungicides account for up to 45% of production costs for both irrigated and upland systems (DEPEC 2019). In addition, the most commercially used molecules present specific site of action as triazoles and strobilurins, which facilitates the selection of isolates resistant to the most used molecules (Souza 2012; D'Ávila et al. 2021).

On the other hand, the market for biological inputs has been growing in recent years, The global agricultural biologicals market reached a value of US\$ 8.1 Billion in 2020 and is estimated to register a compound annual growth rate of 9.4% from 2020 to 2027. worldwide due to the trend of an increasing demand in integrated pest and disease management (IPM) (Grand View Research 2020).

Despite the fast growing market, the production of a bioproduct for plant health protection faces obstacles such as the development of an efficient and sustainable formulation. The main technical barriers to legalize and use these bioproducts are: lack of stability of the formulated product (low shelf life), low viability of microorganisms after exposure to ultraviolet rays of the sun, difficulty in the application of the product in the field, acceptance of the producers for presenting incompatibility with chemicals used in the management of various crops and species of microorganisms that are close to species pathogenic to humans (Bettiol et al. 2014).

In previous studies conducted by our team, six bacterial isolates were shown to be potential biological agents in rice blast suppression. Among them we highlight those of the genus *Bacillus* and *Serratia*. (Filippi et al. 2011; Martins 2015; Martins et al. 2020). Bacteria of the genus *Bacillus* belong to the phylum Firmicutes, family Bacillaceae, can be found in soil, in association with plants, rivers, and estuaries and produce several metabolites of industrial interest such as enzymes, B-exotoxin, thuricin, petrobactim, bacillibactim (Bach et al. 2011; Sansinenea et al. 2011).

The species of the genus *Serratia* stand out in their capacity to produce siderophores, indol acetic acid, phosphate solubilizers and production of antifungal metabolites, such as pyrrolnitrin, carbapenem, prodigiosin, haterumalide and siderophores (Levenfors et al. 2004; Thompson et al. 2002). These natural products, when compared to synthetic compounds, offer greater structural diversity, synergism between molecules and are considered exceptional sources of new agrochemicals (Tremacoldi and Filho 2006).

We hypothesized that rhizobacteria produce primary and secondary metabolites that are responsible for their biological characteristics and activities, of potential importance, to be used in agriculture, because, they present a wide chemical diversity and can be a new integrant for the integrated management of diseases in rice culture. Therefore, the objective of this work was to optimize the extraction process and evaluate the efficiency of bacterial extracts of *Bacillus* sp. and *Serratia* sp. in the control of leaf blast (*M. oryzae*) and leaf brown spot (*B. oryzae*) in rice culture.

Materials And Methods

Microorganisms

The isolates *Serratia* sp. (BRM 32113), *Bacillus* sp. (BRM 32110) *M. oryzae* (BRM 31295) and *B. oryzae* (BRM46676) belongs to Embrapa Microorganism Collection, preserved by the Castellani and deep freeze methods. Colonies of bacterial strains were transferred to Petri plates containing Nutrient Agar (AN-Extatro de carne, Peptona, Cloreto de sódio, Ágar), and incubated during 48 hours at 28 °C. *M. oryzae* isolate was transferred to Petri plates containing Potato Dextrose Agar (PDA), and incubated for seven

days, at 24 °C, under light regime for seven days. *B. oryzae* isolate were transferred to Petri plates containing PDA, and incubated for seven days, at 24 °C, under dark conditions.

Bacterial isolates identification

The two bacterial isolates had their complete genome sequenced using the Illumina platform, where the Nextera XT kit (shotgun) is used, which contains the adaptor primers required for sequencing. Coverage: 100x (1 million reads). We extracted, for the BRM32110 and BRM32113, the 16S rRNA region from the genomes assembled, using the ContEst16S (Contamination Estimator by 16S) (Lee et al., 2017) in which 16S rRNA gene fragments from the query genome assemblies are screened. The database for both isolates was constructed by species from monophyletic clade of the *S. marcescens* and *Bacillus thuringiensis* (Table 1).

Sequences in the GenBank database were compared to check for molecular identification through the nucleotide BLAST algorithm (Altschul et al. 1990). MUSCLE was used in the alignments (Edgar 2004), implemented in MEGA v.7 (Kumar et al. 2016). Bayesian inference (BI) analysis was carried out, for each isolate reconstruction, based on the Monte Carlo Markov chain method (MCMC). The MrMODELTEST v. 3.04 (Nylander 2004) was adopted to select the nucleotide substitution model for BI analysis. Likelihood values were calculated, and the model was selected according to the Akaike information criterion (AIC). Evolution models selected for each isolates reconstruction were HKY + I for BRM32110 and GTR + I for BRM32113. The BI analysis was carried out in MrBayes v.3.1.1 (Ronquist and Huelsenbeck 2003). The consensus tree was obtained after 10 million generations of a Markov chain were applied to two runs, four chains each, with a 15% burn-in. Likelihood log convergence was analyzed in TRACER v. 1.4.1 software (Rambaut et al. 2018). The tree was visualized and edited in ITol v.4 (Letunic and Bork 2019).

Preparing the bacterial suspensions and obtaining the extracts

The bacterial strains grown in Petri dishes were transferred to 5 different Erlenmeyers, each containing 100mL of the nutrient broth culture medium. The Erlenmeyers were kept under constant 150 rpm shaking at 28 °C for 6, 16, 24, 48, and 72 hours of growth.

After the different growth periods, 50.0 mL of each bacterial suspension was transferred to a separation funnel, in which the same volume (50.0 mL) of ethyl acetate solvent was also added. The mixture was stirred slowly to avoid the formation of internal pressure in the funnel. The system was kept at rest until complete separation of the phases. The aqueous phase was separated from the organic phase and returned to the separation funnel. The aqueous phase was subjected to a further extraction step with the addition of another 50.0 mL of the same solvent. The aqueous layer was collected in an Erlenmeyer, and for one more time the process was repeated until three extraction steps were completed. The phase containing the ethyl acetate was concentrated at 50 °C in a rotary evaporator under reduced pressure.

Thin-layer Chromatography

The samples were analyzed via thin-layer chromatography (TLC) performed on an aluminum foil (4 x 5 cm) coated with a silica gel with fluorescent indicator F254 as the stationary phase. After applying acetone soluble samples on the plates, an ethylacetate:hexane (9:1 v/v) mobile phase was eluted by capillarity. The chemical profile of the samples was visualized by ultraviolet light (254 and 365 nm) and vanillin solution (composed by 135 mL of methanol, 3 mL of vanillin, 135 mL of distillation water and 35 mL of sulfuric acid) as revealing, which forms colored spots, according to its interaction with the natural compounds classes.

Extracts screening for antagonistic activity

The isolates of *M. oryzae* and *B. oryzae* were previously grown in a Petri dish containing BDA (potato dextrose agar) culture medium during seven days of incubation at 24°C. Then, discs of culture medium, 5 mm in diameter, containing mycelia of the fungi were transferred to new Petri plates, 60 mm in diameter, containing BDA culture medium supplemented with 50 µl of the extracts of *Bacillus* sp. and *Serratia* sp solubilized in 1 mL of DMSO (dimethylsulfoxide). The assay was conducted in an entirely randomized design with twelve treatments in four repetitions (tab....). The control treatment consisted of the medium supplemented with DMSO (negative control). The absolute control was considered a BDA plate containing only the mycelium discs of the two pathogens. The plates were incubated under a constant temperature of 24°C for 7 days. At the end of the incubation period, colony diameters were measured using a millimeter ruler. Antagonism was determined by comparing the percentage reduction of the mean colony areas in the different treatments (Filippi et al., 2011).

The best treatment identified in the first assay antagonism assay, it was also quantified the *B. oryzae* conidial production by each colony, per Petri dish. Conida was removed with 3 mL/plate of distilled and sterilized water and a brush. The concentration was estimated with a Neubauer chamber.

Inhibition of conidial germination and appressorium formation of *M. oryzae*

A suspension of *M. oryzae* conidia, prepared at a concentration of 2×10^5 conidia/mL was mixed with the different extracts at different concentrations. Five microliters of each extract were pipetted into an artificial hydrophobic plastic surface to induce germination and appressorium formation. The hydrophobic plastic surfaces containing the droplets were kept in a humid chamber in a growth chamber. Evaluations were performed after 4, 6 and 16 hours, under a 20x optical microscope. Germinated conidia and appressorium formed were quantified. The trial was composed of the treatments described in Table 2.

Phytotoxicity of extracts on rice leaves

Detached leaves of the rice cultivar BRS Primavera with 21 days after planting were placed in a humid chamber and sprayed with the extracts, selected in the test described in previous section. In an entirely randomized design, 4 treatments were evaluated, representing different extraction times and concentrations: T1: BRM 32113 6h of growth and sprayed at 25mg of extract; BRM 32113 16h of

growth, sprayed at 25mg extract; BRM 32110 6h of growth and sprayed at 100mg extract; BRM 16h of growth and sprayed at 50mg extract. The sprayed leaves were incubated at 25 ± 2 ° C under constant common light. Visual assessments of phytotoxicity, characterized by yellowish/whitish spots (chlorotic lesions), or any other changes on the leaf surface compared to the control, were performed daily for seven days, based on Sakthivel et al. (2002). The control of the experiment was represented by spraying leaves with sterilized distilled water.

Assays in greenhouse

Assays E1 and E2: Gray spot and blast leaf suppression

It was composed of 8 treatments and three replicates, totaling 24 trays. Treatments in E1 and E2 were the same, but, E1 were spray inoculated with *B. oryzae*, and E2 with *M. oryzae*. the treatments of both assays are described in Table 3.

Preparation of bacterial suspension: a cell suspension of the *B. pyrocinia* and *Bacillus* sp. isolate was transferred to a 500 ml Erlenmeyer flask, containing 200 ml of nutrient broth culture medium, prepared according to Arriel-Elias et al. (2018). The bottles were under constant stirring at 150 rpm, for 48 h at 28°C. Then, each suspension was adjusted, with a spectrophotometer, to 0.5 absorbance, at a wavelength of 620 nm, corresponding to 1×10^8 CFU.ml⁻¹.

Sowing in greenhouse

Seeds of upland rice cultivar BRS Primavera were disinfected with 70% alcohol and 30% sodium hypochlorite before planting and sown in eight furrows in plastic trays (15 × 30 × 10 cm) in 3 kg of soil, that was fertilized with NPK (5 g of 5-30-15 + Zn and 3 g of ammonium sulphate). Besides, 5 g (NH₄)₂SO₄ + Fe and Bo were applied per tray 19 days after sowing. A daily irrigation regime was adopted, corresponding to approximately 100% of the soil field capacity. Trays planted with rice seeds were maintained at greenhouse until the rice plants get to V3 stage.

***B. oryzae* suspension for spray inoculation (E1)**

B. oryzae isolate, was multiplied in Petri plates containing BDA and incubated at 25 ° C for seven days with continuous dark. Conidium was removed with sterilized distilled water and a brush, and the concentration was adjusted for 1×10^5 con.ml⁻¹.

At the 35st day (V4 stage), rice plants were transferred to cages coated with transparent plastic. Thirty ml of *B. oryzae* conidial was suspended. It was pulverized in each cage contained three trays with rice plants. The pulverization was performed with a pressure pump and a spray gun, at a pressure of 0.001 kg / cm². The plants remained in the greenhouse under controlled environmental conditions, with temperatures ranging from 27 to 28 ° C and relative humidity above 80%, for 8 days.

Leaf gray spot severity was evaluated using a severity scale (1.6%, 3.2%, 6.4%, 12.6, 23.1% and 38.6%), according to Lenz et al. (2010), and determining the percentage of affected leaf area by disease.

M. oryzae suspension for spray inoculation (E2)

M. oryzae isolates (BRM 31295), was multiplied in Petri plates containing agar, oatmeal and incubated at 25 ° C for seven days with continuous light. The aerial mycelium of *M. oryzae* colonies was removed with a sterile glass rod and the plates incubated in a growth chamber for 48 hours, under continuous fluorescent light. Conidium was removed with sterilized distilled water and a brush, and the concentration was adjusted for 3×10^5 con.ml⁻¹.

At the 21st day (V3 stage), rice plants were transferred to cages coated with transparent plastic. Thirty ml of *M. oryzae* conidial was suspended (Filippi et al. 2007). It was pulverized in each cage contained three trays with rice plants. The pulverization was performed with a pressure pump and a spray gun, at a pressure of 0.001 kg / cm². The plants remained in the greenhouse under controlled environmental conditions, with temperatures ranging from 27 to 28 ° C and relative humidity above 80%, for 8 days.

Leaf blast severity was evaluated using a severity scale (0%, 0.5%, 1%, 2%, 4%, 8%, 16%, 32%, 64%, and 82%), according to Notteghem (1981), and determining the percentage of affected leaf area by disease.

Statistical analyses

The averages of each test were calculated, the variances analyzed, and the proportions were compared by the Tukey test at 5% of significance, using the SPSS program, version 2.1.

Results

Bacterial isolates identification

The 16S rRNA region from the genomes assembled, revealed that isolate BRM32113 pertains to the genus *Serratia* and species *marcescens* and isolate BRM32110 belongs to the genus *Bacillus* (Fig 1, A and B).

Thin-Layer Chromatography

The aspects considered were color and height after analysis with UV light and developer. The crude extract of isolates BRM32110 and BRM 32113 at the different extraction times of 6, 16, 24, 48 and 72 hours showed brown, yellow and blue spots on plate eluted with hexane anethylacetate:hexane (9:1 v/v) and revealed with vanillin solution, indicating the presence of terpenoids (yellow-brown) and fatty acids (blue), as described by Wagner & Bladt (1996). Stains with more marked brown coloration were detected at the same height in the extracts obtained after 6 and 16 hours of growth of both isolates.

Antagonistic capacity of extracts

Antagonism to *B. oryzae*

The extracts obtained from *Bacillus sp.* (BRM 32110) and *S. marcenses* (BRM 32113), after 6 and 16 hours of growth, reduced up to 28% of the mycelial growth of *B. oryzae* (Table 4, Fig. 2, A).

Quantification of conidia production of *B. oryzae* colonies grown in culture medium supplemented with extracts of *Bacillus sp.* (BRM 32110) and *S. marcenses* (BRM 32113) reduced conidia germination by up to 62%, statistically different from controls (Fig. 3, A).

Antagonism to *M. oryzae*

The extracts of *Bacillus sp.* (BRM 32110) and *Serratia marcenses* (BRM 32113) strains obtained after 6, 16, 24, 48 and 72 hours of growth reduced the growth of *M. oryzae* colonies. The extracts obtained after 6 and 16 hours, for both *Bacillus sp.* (BRM 32110) and *S. marcenses* (BRM 32113) isolates reduced the mycelial growth of *M. oryzae* colonies by up to 94% (Table 4, Fig. 2, B).

Conidium germination and appressorium formation

After 6 hours of incubation: the extract of the isolate *Bacillus sp.* (BRM32110) (6h) at concentration of 100mg, significantly inhibited in 59.6% the germination of conidia, when compared to controls (Fig. 4-A), and in 85% the appressorium formation. The extract of the isolate *S. marcenses* (BRM32113) at concentrations of 50 and 100mg reduced by 45 and 52%, respectively, the germination of conidia (Fig. 5-A), and at the 25mg concentration reduced by 75% the number of appressorium formed.

After 16 hours of incubation: the extract of the isolate *Bacillus sp.* (BRM32110) (16h) reduced in 45% and 38% the germinated conidia and 81% and 93% the appressorium formation in concentrations of 50 and 100mg (Fig. 4 - B and C). The extract of *S. marcenses* (BRM32113), at concentrations of 100mg and 50mg reduced conidia germination by 82% and 53% and appressorium formation by 91% and 92% respectively (Fig 5 - B and C).

The negative control containing the solvent DMSO delayed conidia germination for both isolates, but after 6 hours of incubation it matched the control containing only water. The first appressorium were observed after 6 hours of incubation (Fig. 4 and 5) in the control treatment.

Phytotoxicity

Extracts individually tested showed negative for phytotoxic effect on detached rice leaves on a visual evaluation (Fig. 6 A and B). Different treatments did not alter the characteristics of leaves in a period of one week of evaluation, mainly in relation to the appearance of yellowish, whitish spots, necrosis or any other alteration of the leaf surface as compared to the control (Fig. 6 B, 1). In *in vivo* tests under greenhouse conditions were also performed with the extracts. The results confirmed that extracts did not generate direct toxicity reactions in rice plants.

Assays A1 (leaf brown) spot and A2 (leaf blast suppression)

Brown spot suppression

In A1, control treatments showed up to 23.5% of leaf area affected with brown spot at eight days after challenge inoculation. Treatments 3 (suspension of *Bacillus sp.* cells (BRM32110), with 7.8% of the leaf area affected, 5 (extract of *Bacillus sp.* BRM 32110, 6h) with 9.8% affected leaf area, 6 (*Bacillus sp.* extract BRM 32110, 16h) with 6.7% affected leaf area and 8 (*S. marcescens* extract BRM32113, 16h) with 6.8% affected leaf area of (Fig. 7 A).

Leaf blast suppression

In A2, the treatments that stood out were 3 (suspension of *Bacillus sp.* (BRM32110) cells), with 10.4% affected leaf area, 4 (suspension of *S. marcescens* BRM32113 cells) with 11.6%, 5 (*Bacillus sp.* BRM 32110 extract 6h) with 8.8%, 6 (*Bacillus sp.* BRM 32110 extract 16h) with 14.4%, 7 (BRM32113 extract 6h) with 10% and 8 (BRM32113 extract 16h) with 7.6%. All treatments differed statistically from controls that showed up to 37% leaf area affected with leaf blast (Fig 7 A).

Discussion

The results obtained in the present study showed that the use of *Bacillus sp.* and *Serratia marcescens* extracts were efficient and have potential for suppression of leaf brusone and brown spot in rice.

The *Bacillus sp.* BRM 32110 and *S. marcescens* BRM32113 species under study, were isolated from the rhizosphere of upland rice by Filippi et al. (2011), who performed the phenotypic, biochemical and molecular characterization, through the amplification of the gene encoding the 16S rRNA region (Martins et al. 2020). Other works of the same research group revealed that these same isolates are growth promoters in rice, soybean and bean crops, antagonists to some phytopathogens such as *M. oryzae* and *B. oryzae* and resistance inducers. (Filippi et al 2011; Sousa et al. 2017; Côrtes et al. 2015; Nascente et al. 2017; Sperandio et al. 2017; Arriel-Elias et al, 2018; Arriel-Elias et al 2019; Silva et al. 2020; Rezende et al. 2021). However, complete genome characterization and chemical characterization of the metabolites of both bacterial isolates had not been performed so far.

Bacteria have a simple genomic structure, comprising one or more chromosomes, and this has allowed the accumulation of a range of knowledge about these microorganisms. In recent years, the complete sequencing of genomes has advanced the knowledge of the phylogeny of these microorganisms (Marques 2012). Previously, phylogenetic identification was based on the sequencing of some regions of their genome, such as the 16S rRNA region.

Sequencing of the ITS (Internal Transcribed Spacer) region separates the 16S rDNA gene and can be amplified with specific primers anchored to this region. This region is highly conserved intraspecifically but variable between different species, which makes distinction at the specific level possible. However, molecular identification of certain bacterial groups by 16S rRNA gene sequencing can lead to inaccurate results. Today a 16S rRNA identity greater than 98.7% is accepted as a minimum threshold to

differentiate species because, in most cases, this similarity corresponds to a DNA-DNA identity greater than 70% (Fungaro 2000; Zanirati 2012).

The bacterial isolate BRM32113 was initially identified by Martins et al. (2020), as *Burkholderia pyrrocinia*, analyzing the sequencing obtained from the amplification of the 16S rRNA gene, and the biochemical and morphological identification. But the analysis of the 16S rRNA gene extract from the complete genome sequencing of isolate BRM 32113 revealed that it is from the genus *Serratia* and species *marcescens*. The sequencing of isolate BRM 32110 also revealed that it is from the genus *Bacillus* and for specie identification we will continuous the whole genome studies (Fig. 1).

The aspects analyzed in the CCD showed the most remarkable presence of brown coloration, indicating the presence of terpenoids, in the extracts obtained at 6 and 16 hours after bacterial growth (Wagner and Bladt 1996). Terpenes are one of the largest groups of natural products and comprise numerous substances with important roles in physiological and pathological processes, they are hydrocarbons formally divided into isoprenic units and present recognized antimicrobial activity (Felipe and Bicas 2017) that act directly in the process of infection of the fungus in the plant, inhibiting the growth of hyphae and the germination of conidia (Achotegui-Castells 2016). Some *Serratia* species produce volatile compounds such as terpenoids, which can influence the growth of other bacteria or fungi (Su et al. 2016). Also found in plants, they can be elicited by beneficial microorganisms and act as growth promoters, for example gibberellins, abscisic acid, carotenoids, and sterols, and also as toxins, inhibitors of phytopathogens and herbivores, such as limonoids, saponins, essential oils, among others (Hussein et al. 2019; Olanhewaju et al. 2017).

The sum of all chemical reactions within a living organism is known as metabolism, classified as primary and secondary. In primary metabolism macromolecules are produced as essential functions for microorganisms, while in secondary metabolism we have the production of specific micromolecules that have adaptive functions to biological activity (Tortora 2012) and that also act as competitive weapons used against other microorganisms, nutrient transport agents, and sex hormones.

We identified that the isolates BRM 21110 and BRM 32113 should grow for 6 and 16 hours in liquid nutrient broth medium for the production of molecules of primary and secondary metabolism, because the extracts obtained at these times were able to reduce the mycelial growth of *M. oryzae* and *B. oryzae* colonies up to 94 and 37% respectively (Fig. 2 A, Table 4). In addition we observed the change in the morphology of the fungal mycelium of both isolates (Fig. 2 B), which directly reflects in the pathogenicity of the fungus when in contact with the plant (Sangappillai and Nadarajah 2020) and also the reduction of up to 62% in the production of conidia of *B. oryzae*.

The process of penetration of these fungi into the plant cuticle is initiated when the conidia come in contact with the surface of rice leaves. It forms the germ tube that extends and differentiates into bulbous melanization, forming the appressorium, which is rich in melanin and contains cell wall degrading enzymes. Melanin is a secondary metabolite that protects conidia from UV damage and is also crucial in generating the turgor force generated by the apressorium to penetrate host tissue (Saha et al.

2020). We also evaluated morphological changes in conidia, germ tubes that did not form appressorium, non-melanized appressorium, and observed that all treatments differed from controls, which showed the formation of elongated hyaline conidia with thin apex and septate, as described in the literature (Prabhu and Filippi 2006).

In the present study we observed that the LD 50 for *M. oryzae*, which was 100mg for the extract of BRM 32110 (6h), 50mg for BRM32110 (16h), 25 for BRM 32113 (6 and 16h), through conidium germination and appressorium formation, of the extracts obtained from both bacterial isolates. These presented a reduction of up to 90% of the appressorium formation, when compared to the control. Therefore, even though all concentrations significantly reduced the aspects analyzed, the statistical and morphological evaluations led us to choose them. These same extracts did not show signs of fiotoxicity to rice leaves (Fig. 6).

The reduction in the mycelial growth of *B. oryzae* and *M. oryzae* colonies, in the germination of conidia and appressorium formation by the metabolites represents a solid perspective for the development of a commercial bioproduct for rice culture because the insertion of a bioproduct in the management of rice culture can contribute to the reduction of doses and number of applications of synthetic chemical fungicides and fertilizers, known to cause problems to the microbiological balance of the soil and the environment. Therefore, aiming at the commercial use of the metabolites, it was necessary to define the LD 50, the ideal concentration and phytotoxicity of the extracts.

The extracts obtained from the isolates BRM 32110 and BRM 32113 showed the same bioactivity of the bacterial cells of these isolates, as described by Filippi et al. 2011; Sousa et al. 2017; Sperandio et al. 2017; Arriel-Elias et al. 2017 and Arriel-Elias et al. 2019, who were successful in controlling diseases in rice culture when using the cells of these microorganisms after 48 hours of growth in culture medium. With 48 hours of culture we will have in the culture medium new cells and old cells, using extracts obtained after 6 and 16 hours of culture we will have a great advantage, which is uniformity by standardizing the growth time, extracting desirable characteristics of these PGPRs.

In the greenhouse trials, to evaluate the suppression of diseases, we found that all treatments containing the extracts and bacterial suspension showed statistical differences from the controls. The solvent used did not harm the suppressive effect of bacteria, as previously proven in the antagonism test. When used alone, it did not show significant differences from the control.

In A1 the best values for leaf brown spot suppression (LGS) were the treatments: 3 (BRM32110), presented 7.8% of affected leaf area, 4 (BRM32113) presented 17.1%, 5 (BRM32110 extract 6h) presented 9.8%, 6 (BRM32110 extract 16h) presented 6.7%, 7 (BRM32113 extract 6h) presented 11.4% and 8 (BRM32113 extract 16h) presented 6.8%. The controls treatments 1 (H₂O), and 2 (DMSO) presented 21 and 23.5% of the leaf area affected by LGS, respectively (Fig. 6, 1. Treatments (3, 4, 5, 6, 7 and 8) presented small lesions and in a shorter amount, keeping most of the leaf area green (Fig. 6, 1 - C, D, E, F,

G and H). In treatment 1 (H₂O) and 2 (DMSO), we observed typical lesions of the disease, considered sporadic lesions, with a grayish center and brown edges that coalesce with time (Fig 7- 1 and 2).

For leaf blast (LB) suppression, in A2, all treatments containing the extracts and bacterial suspension showed statistical differences from the controls and the best values for leaf blast suppression was: 3 (BRM32110), presented 10.4% of affected leaf area, 4 (BRM32113) presented 11.6%, 5 (BRM32110 extract 6h) presented 8.8%, 6 (BRM32110 extract 16h) presented 14.4%, 7 (BRM32113 extract 6h) presented 10% and 8 (BRM32113 extract 16h) presented 7.6%. The controls treatments 1 (H₂O) and 2 (DMSO) presented 32 and 37% of the leaf area affected by LB respectively (Fig. 7 -2). Treatments (3, 4, 5, 6, 7 and 8) presented typical small brown open lesions, pinhead type and in a shorter amount; however, it was in a smaller size and number than the control treatment, and it did not coalesce with time, keeping most of the leaf area green (Fig. 6, 2 - C, D, E, F, G and H). In treatment 1 (H₂O) and 2 (DMSO), we observed typical lesions of the disease, considered sporadic lesions that coalesce with time (Fig 7- 1 and 2).

Similar effects were observed using cell-free extracts of *B. subtilis* and *B. amyloliquefaciens*, in inhibiting the mycelial growth of *Fusarium* up to 50.33%. Similarly, when using *B. pumilus* extracts obtained inhibitory activity for three soil pathogens *Rhizoctonia solani*, *Phytophthora* and *Sclerotium rolfsii* and furthermore identified one of the molecules responsible for the suppression of pathogens, pumilacidin (Mendes et al. 2018; Melo 2005), corroborating our results.

Some authors have achieved similar results to ours when using extracts of *Serratia* sp. strains, as for example, Roberts et al. (2015) obtained the control of *Phytophthora ultimum* using extracts of *Serratia marcescens*, in the treatment of pumpkin seeds and Lenvenfors et al. (2004) when using extracts of *Serratia plymuthica* A 153 observed the inhibition of germination of conidia of species of *Aspergillus* and identified some molecules present in the extracts: pyrrolnitrin, and haterumalide NA, B and NE. These results show the efficiency of bacterial extracts on soil pathogens, and with our work, we achieved efficacy in suppressing leaf diseases in greenhouse.

The most widely used control methods for brown spot and leaf blast, is the integration of genetic resistance and chemical control. But due to the high variability of the pathogen *M. oryzae*, the duration time of genetic resistance in improved rice cultivars is limited, thus increasing the number of fungicide applications which has caused high risk of resistance emergence in phytopathogen populations (Prabhu and Filippi 2006). Oliveira et al. (2015) found *P. oryzae* isolates with the presence of the G143A mutation in the cyt b gene that were 42 time more resistant to pyraclostrobin, due to the high selection pressure exerted by consecutive years of strobilurin applications. D'Ávila et al. (2021) examined the reaction of fungal field isolates, collected over a 26-year period, to the active ingredients of commercially relevant fungicides, and documented the gradual increase in the frequency of fungicide-resistant isolates in rice populations of *P. oryzae* on a long-term basis. The insertion of alternative measures in integrated disease management in rice can decrease the dependence on chemical control in the production system and safeguard the productive potential of genetically improved cultivars.

Although biological control represents a strategy to increase the durability of resistance of rice cultivars to diseases and reduce toxic residues resulting from the indiscriminate use of chemicals (Filippi et al. 2011), there are still no biological products registered for rice cultivation (Agrofit 2021).

To date, no other studies have been conducted using molecules of microbial metabolism of *Bacillus sp.* and *Serratia marcescens* in the control of brown spot and leaf blast in rice crop. The results obtained showed that the use of *Bacillus sp.* and *S. marcescens* bacteria extracts were efficient and have great potential for suppression of brown spot and leaf brusone in rice. Therefore, with this work we have the perspective of inserting a natural product in the agricultural market, aiming to favor producers with one more control tactic that can help the agroecological management to take off in Brazil. With this, more studies will be conducted in order to identify which are the molecules produced by these bacterial isolates and their production in large scale.

We are at the forefront of developing bioproducts or biobased products for rice cultivation, and to help protect natural ecosystems while improving profitability and productivity in rice fields it is necessary to look for more components to integrated management.

Conclusion

The use of *Bacillus sp.* and *Serratia marcescens* extracts was efficient in reducing the severity of brown spot and leaf blast in rice culture. These results are significant because they demonstrate that it is possible to use bacterial extracts in the control of phytopathogens seeking the insertion of natural products in agriculture and is the first report in rice culture.

Declarations

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Author contribution The original idea was suggested by VGPS and MCCF and the concept of this study was developed by MTAE. The experimental work was carried out by MTAE with guidance from GCTFA, GAB and PHDS. MTAE drafted the article. GCTFA and GAB were involved in the critical revision of the article for important intellectual content. All the authors read and approved the final manuscript.

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Competing interests The authors not competing interests.

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Tables

Table 1 Collection details and GenBank accession numbers of isolates included in the present study.

Serratia spp.

Species	Strain	GenBank Accession number (16S rRNA)
<i>Serratia plymuthica</i>	DSM 4540	NR 114579
	S16.1.2	OK284921
<i>Serratia ficaria</i>	NBRC 102596	NR 114155
	DSM 4569	AJ233428
<i>Serratia rubidaea</i>	DSM 4480	AJ233436
	NBRC 103169	NR 114232
<i>Serratia marcescens</i>	ZO1-A	AJ296310
	WO1-A	AJ296309
	BRM32113	In process
<i>Serratia nematodiphila</i>	PSI26	OK423573
	B1	OK560052
<i>Serratia ureilytica</i>	NiVa 51T	AJ854062
	NiVa 51	NR 042356
<i>Bacillus</i> spp.		
<i>Bacillus cereus</i>	wg-3	EU624208
	NVH 391-98	AM747234
<i>Bacillus anthracis</i>	ATCC 14578	AB190217
	LB5YE	MW819989
<i>Bacillus wiedmannii</i>	FSL W8-0169	NR 152692
<i>Bacillus mycoides</i>	ATCC 6462	AB021192
	2861	MT586023
<i>Bacillus weihenstephanensis</i>	DSM 1182	AB021199
<i>Bacillus</i> sp.	BRM32110	In process
<i>Bacillus toyonensis</i>	BCT-7112	NR121761
<i>Bacillus cereus</i> biovar toyoi	NCIB 40112	AJ310100
<i>Bacillus thuringiensis</i>	Bt11	MT292101
<i>Bacillus pseudomycooides</i>	NRRL B-617	AF013121

*Isolates and newly generated sequences are in bold.

Table 2. Treatments description for Conidial germination and appressorium formation assays, combining growth period and extract concentration of BRM32110 and BRM32113

Isolate	Treatments number	Growth period (h)	Concentration
H ₂ O	1	-	-
DMSO	2	-	-
BRM 32113 extration	3	6	100mg
	4	6	50mg
	5	6	25mg
	6	6	10mg
	7	6	5mg
	8	16	100mg
	9	16	50mg
	10	16	25mg
	11	16	10mg
	12	16	5mg
BRM 32110	13	6	100mg
	14	6	50mg
	15	6	25mg
	16	6	10mg
	17	6	5mg
	18	16	100mg
	19	16	50mg
	20	16	25mg
	21	16	10mg
	22	16	5mg

Table 3 Leaf gray spot and leaf blast suppression treatments, extraction time and extract concentration of BRM32110 and BRM32113, assays E1 and E3

Isolate	Treatments number		Extraction time (h)	Concentration
	1	H ₂ O	-	-
	2	DMSO	-	-
BRM32110	3		-	10 ⁸ CFU.mL ⁻¹
BRM32113	4		-	10 ⁸ CFU.mL ⁻¹
BRM32110	5		6	100mg
BRM32110	6		16	50mg
BRM32113	7		6	25mg
BRM32113	8	BRM32113 Extract	16	25mg

Table 4. Reduction of mycelial growth of *B. oryzae* and *M. oryzae* by extracts of BRM32110 and BRM32113, after 7 days of growth in BDA. Means followed by the same letter do not differ statistically from each other by the Tukey's test ($p < 0.05$)

Treatments	Extaction time (h)	Reduction of colony area (%) <i>B. oryzae</i>	Reduction of colony area (%) <i>M. oryzae</i>
Control (H ₂ O)	-	0 c	0 h
Control (DMSO)	-	0 c	0 h
BRM32110 - 6h	6	17.2 b	94,3 a
BRM32110- 16h	16	28.9 a	93,2 a
BRM32110 - 24h	24	0 c	80,0 bc
BRM32110 - 48h	48	0 c	69,1 def
BRM32110 - 72h	72	3.3 c	68,3 def
BRM32113 - 6h	6	17.2 b	89,8 a
BRM32113 - 16h	16	21.5 b	87,3 ab
BRM32113 - 24h	24	3.3 c	76,6 cd
BRM32113- 48h	48	1.4 c	74,4 cde
BRM32113 - 72h	72	0.7 c	68,3 def

Figures

Figure 1

Phylogenetic trees based on the 16S gene recovered from the genomes of *Bacillus* sp. (A) and *Serratia marcescens* (B).

Figure 2

(A) *B. oryzae*'s and (B) *M. oryzae*'s colony growth suppression in Petri plates containing PDA medium enriched with 50 μ l of each extract (BRM32110 and BRM32113) solubilized in 1mL of DMSO (dimethylsulfoxide), seven days after incubation. A: first assay (a) control (PDA medium enriched with water), (b) PDA medium enriched with DMSO; BRM32113 extract collected at: 6h (c), 16h (d), 24h (e), 48h(f), 72h(g); BRM32110 extract collected at: 6h(h), 16h (i), 24h (j), 48h (l), 72h(m). B: second assay (a) control (PDA medium enriched with water), (b) PDA medium enriched with DMSO; BRM32113 extract collected at: 6h (c), 16h (d); BRM32110 extract collected at: 6h(e), 16h (f)

Figure 3

B. oryzae conidial concentration per Petri dish containing PDA culture medium, supplemented with 50 µl of each treatment solubilized in 1mL of DMSO (dimethylsulfoxide) seven days after incubation. Lowercase letters define the effect of treatments by the Tukey test ($p \leq 0.05$). The bars indicate the mean standard error

Figure 4

Percentage of *M. oryzae* conidial germination (A and B) and appressorium (C and D) formation in drops containing conidial suspension and different BRM 32110's extract concentration. The observations time were at 4, 6 and 16 hours after assay incubation. (A) BRM32110 extract obtained with 6h of bacterial cell growth in culture medium, (B) BRM32110 extract obtained with 16h of bacterial cell growth in culture medium, (C) BRM32110 extract 6h, (D) BRM32110 extract 16h. Lowercase letters define the effect of treatments in each incubation hour by the Tukey test ($p \leq 0.05$). The bars indicate the mean standard error

Figure 5

Percentage of *M. oryzae* conidial germination (A and B) and appressorium (C and D) formation in drops containing conidial suspension and different BRM 32113's extract concentration. The observations time were at 4, 6 and 16 hours after assay incubation. (A) BRM32113 extract obtained with 6h of bacterial cell growth in culture medium, (B) BRM32113 extract obtained with 16h of bacterial cell growth in culture medium, (C) BRM32113 extract 6h, (D) BRM32113 extract 16h. Lowercase letters define the effect of treatments in each incubation hour by the Tukey test ($p \leq 0.05$). The bars indicate the mean standard error

Figure 6

Phytotoxicity of adjuvants used in the different formulations in rice leaves, evaluated during seven days. (A) day one, (B) day seven. Treatments: (1) water, (2) DMSO (dimetilsofoxide); BRM32110 extract at 6h (3), BRM32110 extract at 16h (4); BRM32113 extract at 6h (5); BRM32113 extract at 16h (6)

Figure 7

The severity of leaf gray spot (1) and leaf blast (2) according to the scale of notes. Uppercase letters indicate different symptoms in rice leaves of each treatment: (A) water; (B) DMSO; (C) BRM32110; (D) BRM32113; (E) BRM32110 extract 6h (100mg); (F) BRM32110 extract 16 h (50mg); (G)BRM32113 extract 6h (25mg), (H) BRM32113 extract 16h (25mg).Columns (lowercase letters) followed by the same letters were not significantly different from each other according to Tukey's test ($p < 0.05$). The bars indicate the mean standard error