Universidade Federal Rural do Semi-Árido
Pró-Reitoria de Pesquisa e Pós-Graduação
https://periodicos.ufersa.edu.br/index.php/caatinga

ISSN 0100-316X (impresso)
ISSN 1983-2125 (online)
http://dx.doi.org/10.1590/1983-21252020v33n313rc

CHARACTERIZATION OF BACTERIAL ISOLATES FOR SUSTAINABLE RICE BLAST CONTROL[1]

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ABSTRACT - Rice blast (*Magnaporthe oryzae*) limits rice (*Oryza sativa*) grain yields worldwide. The objective of this investigation was to morphologically, biochemically, and molecularly characterize six bacterial isolates, BRM 32109, BRM 32110, BRM 32111, BRM 32112, BRM 32113, and BRM 32114, and to determine their potential as antagonists to *M. oryzae*. Morphological characterization was based on colony formation and color, Gram staining, and fluorescent pigment production. Biochemical studies were based on cellulase, chitinase, phosphatase, indoleacetic acid, and siderophore production, as well as biofilm formation. The molecular identification used specific primers for PCR amplification of the 16S rRNA region, followed by sequencing. The antagonism studies involved three experiments, which had randomized designs. Two of them were conducted in laboratory conditions, pairing bacterial colonies and *M. oryzae*, using bacterial filtrates, and the third was conducted in greenhouse conditions. BRM 32111 and BRM 32112 were identified as *Pseudomonas* sp., BRM 32113 as *Burkholderia* sp., BRM 32114 as *Serratia* sp., and BRM 32110 and BRM 32109 as *Bacillus* spp. BRM 32112, BRM 32111, and BRM 32113 inhibited the colony of *M. oryzae* by 68%, 65%, and 48%, respectively. The bacterial suspensions of the BRM 32111, BRM 32112, and BRM 3212 filtrates suppressed leaf blast by 81.0, 79.2, and 66.3%, respectively. BRM 32111 and BRM 32112 were determined to be antagonists of *M. oryzae* and were found to solubilize phosphate, produce siderophores and cellulose, form biofilms, and suppress leaf blast. These isolates should be further investigated as potential biological control agents for leaf blast control.

Keywords: *Oryza sativa*. Biological control. Antagonism. *Pyricularia oryzae*.

CARACTERIZAÇÃO DE ISOLADOS BACTERIANOS PARA O CONTROLE SUSTENTÁVEL DA BRUSONE DO ARROZ

RESUMO - A brusone (*Magnaporthe oryzae*) limita a produção do arroz (*Oryza sativa*), no Brasil e no mundo. Os objetivos foram caracterizar seis isolados bacterianos morfológica, bioquímica e molecularmente, e determinar seu potencial como antagonistas a *M. oryzae*. Utilizaram se os isolados bacterianos BRM 32109, BRM 32110, BRM 32111, BRM 32112, BRM 32113 e BRM 32114. Avaliou-se o formato e a cor das colônias, coloração de Gram e produção de pigmentos fluorescentes. Identificou-se a produção de celulase, quitinase, fosfatase, ácido indolacético, sideróforo e formação de biofilme. A identificação molecular foi realizada utilizando-se oligonucleotídeos iniciadores específicos para amplificação por PCR da região 16S rRNA, seguidos de sequenciamento. Os dois ensaios envolvendo antagonismo foram realizados em laboratório, em delineamento inteiramente casualizados, um com cultivo pareado entre isolado de bactérias e *M. oryzae*, e outro com filtrados bacterianos. Um terceiro ensaio em casa de vegetação para avaliar a supressão da brusone. Identificou-se *Pseudomonas* sp. (BRM 32111 e BRM 32112), *Burkholderia* sp. (BRM 32113), *Serratia* sp. (BRM 32114) e *Bacillus* spp. (BRM 32110 e BRM 32109). Os isolados BRM 32112, BRM 32111 e BRM 32113 inibiram o crescimento da colônia de *M. oryzae* em 68, 65 e 48%, respectivamente. As suspensões bacterianas BRM 32111, BRM 32112 e seu filtrado suprimiram a brusone foliar em 81,0, 79,2 e 66,3%, respectivamente. BRM 32111 e BRM 32112 são antagonistas ao fungo *M. oryzae*, solubilizam fosfato, produzem sideróforos, celulase e biofilme e suprimiram a brusone foliar, mostrando-se potenciais agentes biológicos para o controle da brusone foliar.

Palavras-chave: *Oryza sativa*. Controle biológico. Antagonismo. *Pyricularia oryzae*.

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[1]Received for publication in 12/18/2019; accepted in 05/28/2020.
Paper extracted from the master dissertation of the first author.
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INTRODUCTION

Rice (*Oryza sativa* L.), a primary staple food for half of the global population and the source of approximately 20% of many peoples' daily caloric intake, is mainly composed of carbohydrates, calcium, iron, thiamine, and vitamin E (RATHNA et al., 2019). According to the Food and Agricultural Organization of the United Nations (FAO) and Ricepedia (2020), global rice demands are expected to rise from 496 million tons in 2020 to 555 millions tons in 2035 (http://ricepedia.org/challenges/food-security). While a great deal of scientific research has previously focused on improving and protecting rice production, it continues to be affected by numerous biotic stresses throughout its growth and development. Among these, rice blast caused by *Magnaporthe oryzae* B. C. Couch (anamorph– *Pyricularia oryzae* Cavara.) is a major yield-limiting pathogen, that can result in losses of 100% in susceptible rice cultivars (TALBOT, 2003, PRABHU; FARIA; ZIMMERMANN, 1989). Yield losses could be reduced by preventive measures, such as integrating genetic resistance, cultural practices, and chemical controls. It is common practice for example, that pesticides are excessively applied close to harvest time to avoid the loss of rice panicles, but these practices are unsustainable, not cost effective, and have negative impacts for humans and the environment (ASIAH et al., 2019). Overrall, chemical controls are often utilized in an abusive manner affecting the quality of the product and causing harm to the environment. The global crop protection market showed a growth rate of 3.8% from 2001 to 2016, and while from 2014-2016 the trend line declined globally, in Latin America it remained constant (NISHIMOTO, 2019).

In recent years, the search for alternative control measures has been intensified, and this has included biocontrols as a significant part of integrated rice blast management. The rhizosphere is found at the soil-root interface, where several microorganisms proliferate, such as plant growth promoted bacteria (PGPR) (MOREIRA; SIQUEIRA, 2002). PGPR interact with plants, resulting in the promotion of growth as a direct effect, while inderctly they elicit biochemical and molecular defense responses within the plant. The same PGPRs can also be antagonists to plant pathogens as they can secrete compounds that can affect pathogen growth and reproduction (FILIPPI et al., 2011).

There have been many previous investigations of rice plant endophitics and rhizosphere microorganisms that have antagonistic activities against rice pathogens (WALITANG et al., 2019, WIDIANTINI; HERDIANSYAH; YULIA, 2017). Most of these investigations have been conducted under laboratory conditions. This could be a limitation with respect to developing stable biological agents for application in field conditions. As a result of this, currently there are few, if any, biological control options for rice diseases (HASSAN; HOSSEIN; HOSSEIN, 2019).

The main objectives of this investigation were the morphological, biochemical, and molecular characterizations of six bacterial isolates and the identification of potential antagonists, both *in vitro* and *in vivo*, for *Magnaporthe oryzae*.

MATERIALS AND METHODS

The isolates utilized in the present study are preserved on sterilized filter paper discs in the Embrapa Rice and Bean Multifunction Microorganism and Fungi Collection. The *M. oryzae* isolate BRM 10900 was obtained from leaf lesions of the cultivar BRS Primavera. Six bacterial isolates, designed by the collection codes (BRM 32109, BRM 32110, BRM 32111, BRM 32112, BRM 32113, and BRM 32114) were obtained from the rhizospheres and phyllospheres of healthy rice plants (in a commercial field) that were selected by Filippi et al. (2011) and Pereira Filho (2013). The bacterial isolates were cultivated in Petri dishes containing culture medium 523 (sucrose 10 g, hydrolyzed casein 8 g, yeast extract 4 g, K_2HPO_4 2 g, $MgSO_4.7H_2O$ 300 mg, agar-agar 20 g, and distilled H_2O, 1000 mL) (KADO; HESKETT, 1970) in triplicate, and incubated for 24 h at 28 °C. The morphological, biochemical, genetic, and antagonistic characteristics of each bacterial strain were then assessed.

Morphological characterization

The colony characteristics surface, form, margins, and coloration of the gram staining, were observed using a compound microscope at 100 × magnification, following the methods described by Benson (2002).

Production of fluorescent pigment

The bacterial isolates were each cultivated in Petri dishes containing Kings B medium (protease 20 g, K_2HPO_4. $3H_2O$, 2.5 g, $MgSO_4.7H_2O$, 6 mg, glycerol 15 mL, agar-agar 15 g, distilled H_2O, 1000 mL), for 24 h at 28 °C. They were then examined under ultraviolet light (λ = 365 nm) for the presence or absence of fluorescent pigments (MARIANO et al., 2004), when compared with a no pigment producer isolate.

Biochemical characterization

Cellulase: According to Strauss et al. (2001), the tripticasein soybean agar (TSA) culture medium was supplemented with 10 g L^{-1} of cellulose. The cultures for each bacterial strain were incubated for five days at 28–30 ºC, and later saturated with 0.03%

red Congo solution for 10 min, and then washed with NaCl to reveal transparent halos around the colonies, indicating yes or no cellulose production.

Indole acetic acid (IAA): According to Bric, Bostock and Silverstone (1991) and Cattelan (1999), TSA culture medium was diluted to 1/10 and enriched with 5 mM de L-tryptophan (1021 $g{\cdot}L^{-1}$). After 24 h, the colonies for each bacterial strain were covered with a sterilized nitrocellulose membrane, and incubated at 28 °C, for 24 h. The membrane was then transferred to another plate, and saturated with Salkowski´s solution (GORDON; WEBER, 1951), and incubated at room temperature for up to 2 h, and then the changes (yes or no) in the membrane colors were observed.

Phosphate production: According to Sylvester -Bradley et al. (1982), the TSA medium was diluted to 1/10 with the addition of a phosphorus source ($CaHPO_4$). A fine precipitate was obtained by mixing a solution of 50 mL K_2HPO_4 (0.57 M) with 100 mL of $CaCl_2$ (0.90 M), and adding it to 850 mL of 1/10 TSA. The solution and culture medium were autoclaved separately, the medium´s pH was adjusted to 7.0, and the colonies were incubated at 28 -30 °C for 2 days. The isolates were qualitatively detected as phosphate solubilizer when a halo formed around the bacterial colonies.

Siderophore production: Isolates for each bacterial strain were cultivated in 50 mL Erlenmeyer flasks containing 10 mL of the liquid culture medium tryptic soy (TSL1/10) and incubated for 24 h at 28 °C, with constant agitation. Next, cell suspensions were centrifuged at 12 000 rpm for 10 min. One milliliter of supernatant was transferred to a culture tube and 1 ml of the indicator solution chrome azurol S (CAS) was added. The production of siderophores was indicated when the solution color changed to blue (CATTELAN, 1999).

Chitinase production: According to Hsu and Lockwood (1975), a culture medium that contains chitin as the only carbon source (chitin, 4 g; K_2HPO_4, 0.7 g; KH_2PO_4, 0.3 g; MgSO4.5H2O, 0.5 g; $FeSO_4.7H_20$, 0.01 g; $ZnSO_4$, 0.001 g; $MnCl_2$, 0.001 g; agar, 20.0 g and 1000 mL of ionized water) was utilized to qualitatively detect halos that formed around the colonies, to indicate chitinase production.

Biofilm formation: According to Mathur et al. (2006), after cultivating the bacterial colonies on culture medium 523, they were transferred to Petri dishes containing Congo Red Agar (CRA). Plates were then incubated at 26 ºC for 24-48 h. Then, the black colonies with a dry crystalline consistency were qualitatively identified as the biofilm producers.

Genetic characterization

DNA extraction and amplification of 16S rRNA region: Bacterial colonies of each isolate were grown on solid 523 medium for 48 h. DNA was extracted according to Li and Bouer (1995), quantified by spectrophotometer (Nano Drop® ND-2000 UV-Vis, at 260 nm wavelength) to determine the impurity. The total reaction volume was of 25 µL (1 µL of DNA 25 µM, 1 µL of forward primer 25µM, 1 µL of reverse primer 25 µM, 5µL of Master Mix, 1 µL of Q-solution, 1 µL of sterile water MilliQ). The forward primer was F984 (AACGCGAAGAACCTTAC), and the reverse primer was R1492 (TACGG(C/T) TACCTTGTTACGACTT) (HEUER et al., 1997). Later, the reaction products (10 µL) were then run using electrophoresis on 1.5% agarose gels (p/v), and the bands were observed in a photo documenter (Universal Hood II, Bio-Rad). Images were captured using the Quantity One (Bio-Rad) software.

Sequencing A BigDye® Terminator Cycle Sequencing kit (Life Technologies, USA, used according to the manufacture guidelines) in combination with the model ABI 3500 Genetic Analyzer atomized sequencing system, were utilized. For the sequencing reactions, 20 ng of the purified PCR product with cyclase, and the reaction products were transferred to a sequencing plate with 96 cavities to be purified. The samples were denatured for 5 min at 95 °C, and then stored at 4 ºC in the dark, until the samples were injected into an ABI 3500 Genetic Analyzer atomized system. The DNA sequences were analyzed utilizing the BLASTn (Basic Local Alignment Search Tool - version 2.215 of BLAST 2.0) program available on the NCBI site (http://www.ncbi.nlm.nih.gov/blast/), developed by the National Center for Biotechnology Information (ALTSCHUL et al., 1990). The bases of low-quality sequences were removed using the Bio Edit Sequence Alignment Editor.

Data analysis: Principal component analysis (PCA) was performed to study the correlation among the biochemical characteristics (production of IAA, phosphatases, siderophores, chitinase, solubilization of phosphate, and formation of biofilms) and the diversity of bacteria isolates utilized in the present study. All variables were normalized using the presence or absence of proper vectors in the correlation matrix, as well as the variance of the percentage explained by each component. The PCA was calculated using the PAST 3 software (HAMMER; HARPER; RYAN, 2001).

Antagonism characterization

Dual plate: The bacteria were cultivated in Petri dishes containing culture medium 523 at 28 °C for 24 h. Then a suspension adjusted to 540 nm, corresponding to 10^8 $ufc{\cdot}mL^{-1}$ was prepared, according to Dennis and Webster (1971). *M. oryzae* (BRM 10900) was cultivated in Petri dishes containing PDA for seven days at 28 °C. The assay conducted included seven treatments (T1-BRM 32111, T2-BRM 32113, T3-BRM 32114, T4-BRM

32110, T5-BRM 32109, and T6-BRM 32112) and one control, using a completely randomized design and three replications. A 5 mm disc of *M. oryzae* mycelium was transferred to the central part of a Petri dish containing PDA. Twenty microliters of the bacterial suspension were distributed at four equidistant points. The plates were then incubated at 28 °C, under continuous fluorescent light, and those containing only mycelial discs of *M. oryzae* were used as the controls. Ten days after incubation, the radial growth of the pathogen colony in each treatment was measured and compared with the control.

Filtrate thermostability: The bacterial suspension was prepared in water and adjusted to 540 nm (10^8 ufc·mL^{-1}), according to Dennis and Webster (1971).

Bacterial cell filtrate: One microliter of each bacterial cell suspension was then transferred to Erlenmeyer flasks containing 250 ml of Simmons culture medium (SIMMONS, 1926), with the following modifications: citrate was substituted by 0.1 g L^{-1} of glucose as the only carbon source, and incubated in an orbital agitator at 150 rpm, in the dark for seven days at 25 °C.

Filtration: the liquid part was filtered twice, first with filter paper (0.45 µm), utilizing a Buchner funnel 240 mm, under vacuum. The second, using a cellulose membrane (0.22 µm). The resulting liquid was sterilized in an autoclave and incorporated in PDA culture medium at a proportion of 25% (v/v), based on the method described by Isaias et al. (2014).

The assay was conducted using a completely randomized block design and five repetitions, including the following treatments: T1-sterilized cell suspension of BRM 32111; T2-sterilized cell suspension of BRM 32112; T3- BRM 32111 filtrate; T4- BRM 32112 filtrate; T5-sterilized BRM 32111 filtrate; T6-sterilized BRM 32112 filtrate; T7-*M. oryzae* (control).

Greenhouse experiment

The cultivar BRS Primavera was sown in plastic trays (15 × 30 × 10 cm) containing 3 kg of soil, fertilized with 5.0 g of NPK (5-30-15). Twenty days after planting, 3 g of ammonium sulfate was applied as a top dressing. The conidial suspension of *M. oryzae* was made according to Filippi et al. (2011). The conidial suspension was adjusted to 3 × 10^5 mL^{-1}.

Filtrated co-cultures of bacterial isolates and *M. oryzae*

The isolates BRM 32111 and BRM 32112 were selected based on the results from the *in vitro* assays. One milliliter of bacterial suspension (10^8 ufc·ml^{-1}) was transferred to Simmons liquid culture medium containing 3 × 10^5 conidia mL^{-1}of *M. oryzae* and kept in a shaker for 10 days. After centrifuging the resulting solutions, the supernatant was filtered using Whatman, nº 4 under vacuum.

Rice plants, at the V3 growth stage, were sprayed until the point of dripping, with a suspension containing conidia (3 × 10^5 mL^{-1} at final concentration) of *M. oryzae* mixed with: T1-control (water), T2-cell suspension of BRM 32111, T3- cell suspension of BRM 32112, T4-filtrate of BRM 32112, T5-filtrate BRM 32111, T6- filtrate of BRM 32111 + *M. oryzae* conidia, T7- filtrate of BRM 32112 + *M. oryzae*. After spray inoculations, the plants were incubated at temperatures varying from 28 to 30 ºC at high humidity.

RESULTS AND DISCUSSION

Morphological and biochemical characterizations of the bacterial isolates

The morphological characteristics of all six isolates are presented in Table 1. BRM 32112 was the only isolate to fluoresce. BRM 32109 and BRM 32110 were Gram positive, and BRM 32111, 32112, 32113, and 32114 were Gram negative. The biochemical characteristic analyses showed that all six isolates produced cellulose and phosphatases, and that none produced chitinase. Only BRM 32110 did not produce siderophores, BRM 32113 and BRM 32114 produced AIA, and BRM 32111 and BRM 32112 produced biofilms.

Table 1. Morphological and biochemical characterization of bacterial isolates

Characteristics	Isolates BRM					
	32113	32111	32114	32112	32110	32109
Morphological						
Color	Rose	yellow	cream/rose*	white	white	white
Opaqueness	translucent	translucent	translucent	translucent	opaque	opaque
Form	Circular	circular	Circular**	circular	circular	circular
Margin	smooth	smooth	smooth	smooth	irregular	irregular
Elevation	convex	undulated	elevated	convex	undulated	flat

*Initially the colonies were in cream color and later changed to pink after 5 days.
**Circular with elevated margins.

The principal component analysis (PCA) (Figure 1) showed that only the isolates BRM 32113 and 32114 were positively correlated with IAA production. All six isolates were positively correlated with celullase and phosphatase production. Only BRM 32111 and BRM 32112 were positively correlated with fluorescence and biofilm production, while BRM 32110 was negatively correlated with siderophore production.

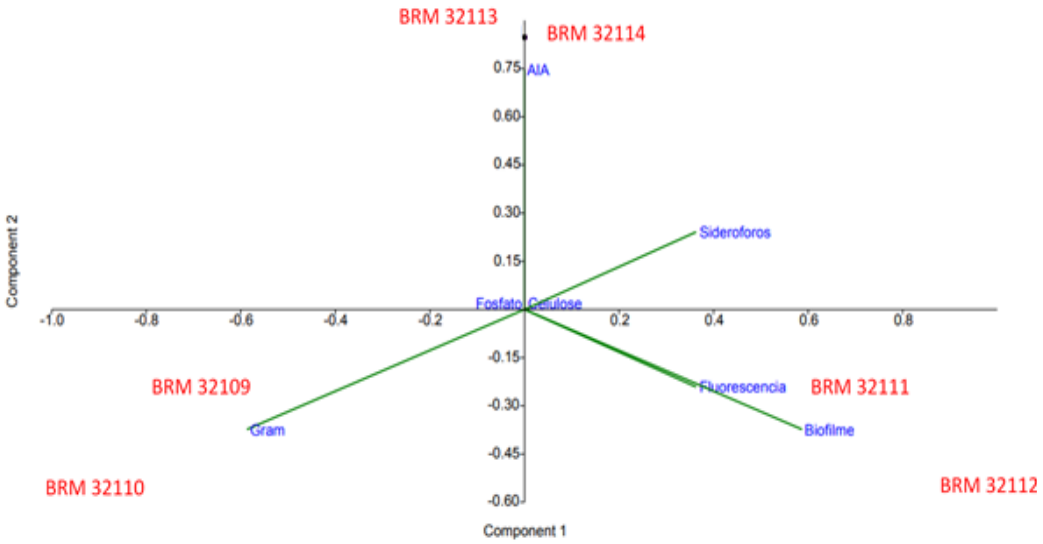

Figure 1. Principal component analysis (PCA) of bacterial isolates based on Euclidean coefficient of similarity among biochemistry.

Identification of isolates using PCR

The molecular characterizations of the bacterial isolates by partial sequencing of their 16S rRNA (fragments of 600-700 base pairs) revealed the following matches: BRM 32111 was a *P. fluorescens* Migula, BRM 32113 was a *Burkholderia* sp., BRM 32114 was a *Serratia* sp., BRM 32112 was a *Pseudomonas* sp., and BRM 32109 and BRM 32110 were *Bacillus* sp (Table 2).

Table 2. Molecular characterization of bacterial isolates by partial sequencing of the 16S rRNA region (fragments of approximately 600-700 base pairs).

Isolate	NCBI*	GeneBank accession number
BRM 32111	*Pseudomonas fluorescens*	MT188711
BRM 32113	*Burkholderia* sp	MT188713
BRM 32114	*Serratia* sp.	KX 378747
BRM 32112	*Pseudomonas* sp.	MT188712
BRM 32109	*Bacillus* sp.	MT188714
BRM 32110	*Bacillus* sp.	KX378746

*National Center for Biotechnology Information.

In vitro antagonism between bacterial cell suspensions and M. oryzae

All isolates statistically inhibited the mycelial growth of *M. oryzae.* The best results were observed with the BRM 32111, BRM 32112, and BRM 32113 isolates, as they inhibited colony growth by 68%, 65%, and 48%, respectively, when compared to the control treatment. (Figure 2 A).

In vitro antagonism between filtrates of bacterial isolates and M. oryzae

Treatments T1, filtrate from BRM 32112 and T2, and filtrate from BRM 32111 inhibited *M. oryzae* colony growth the most, when compared to the control. Filtrates obtained from co-cultivations of *M. oryzae* and BRM 32112 (T3) and BRM 32111 (T4) did not inhibited *M. oryzae* colony growth presented the best inhibition (Figure 2B).

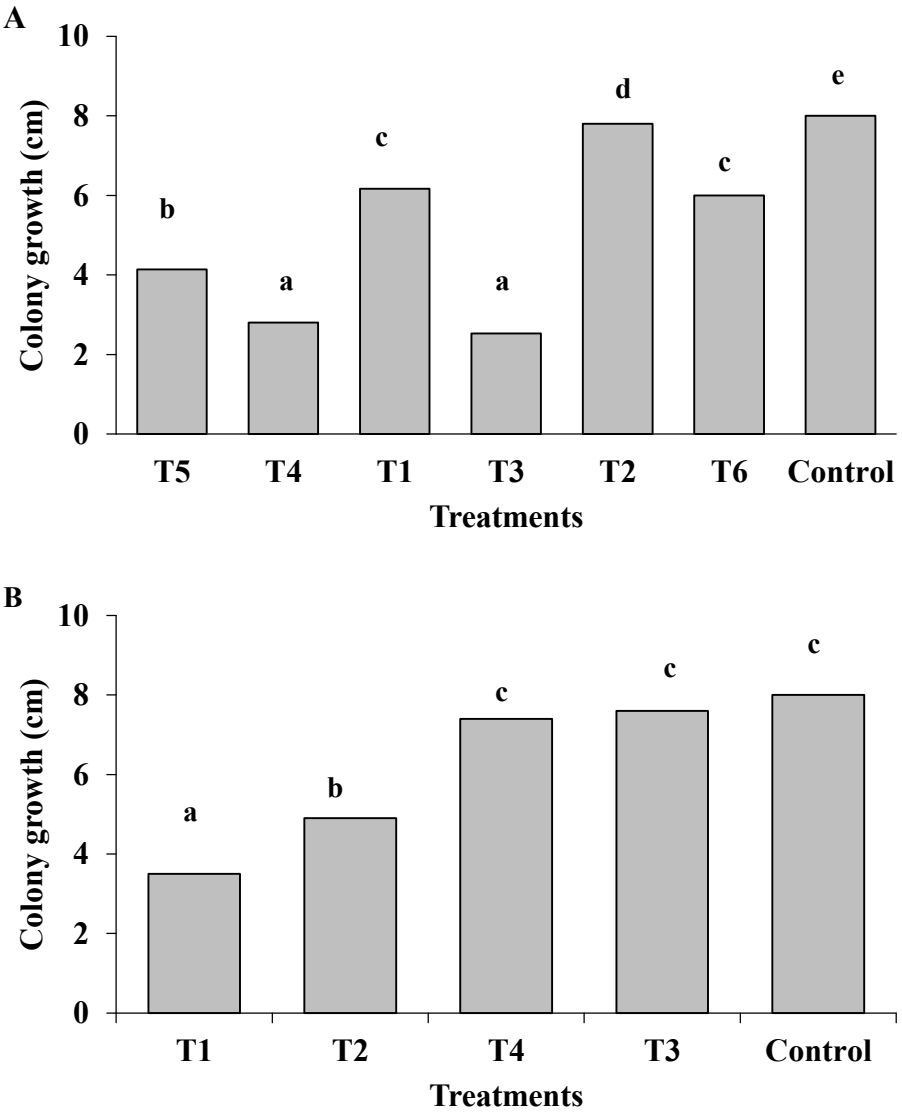

Figure 2. Inhibition growth of *Magnaporthe oryzae* colony by 6 bacterial cell suspensions and 4 filtrates. **A**: Antagonism between *M. oryzae* and BRM 32110 (T1), BRM 32109 (T2), BRM 32112 (T3), BRM 32111 (T4), BRM 32113 (T5), and BRM 32114 (T6); control (no bacterial suspension). **B**: *M. oryzae* growth in medium supplemented with: BRM 32112 filtrated (T1), BRM 32111 filtrated (T2), filtrated of co-culture of *M. oryzae* and BRM 32112 (T3), and filtrated of co-culture of *M. oryzae* and BRM 32111 (T4) and water (control). Bars with the same letter do not differ statistically by Duncan test ($p < 0.05$).

The efficiency of bacterial isolates and respective filtrates on leaf blast suppression

The treatments T2, T3, T4, , T5 and T7 statistically suppressed disease severity when compared to T1 and T6 (Figure 3).

Strategies to maintain and enhance agricultural productivity include high input practices, such as fertilizers and defensives. With climate change, the major influencing factor will be the availability and management of soil water and extreme temperature events, such as heatwaves and droughts, which decrease the annual recharging of soil and groundwater levels (YADAV et al., 2007). In this sense, microbial diversity in the rhizosphere, rhizoplane, and phyllosphere could assecure the sustainability of agricultural production systems.

In the present study, BRM 32111 and BRM 32112 were identified as *Pseudomonas* sp., BRM 32113 as *Burkholderia* sp., BRM 32114 as *Serratia* sp. and BRM 32110 and BRM 32109 as *Bacillus* spp. (Table 2).

Our results present six bacterial isolates classified according to colony characteristics and Gram stain (Table 1). Cabeen and Jacobs-Wagner (2005) emphasized the importance of describing and classifying bacterial species based on cultural characteristics complemented with biochemical and molecular characterizations (Table 1). The sequencing of the 16S rRNA region has been widely utilized, by several authors, to identify bacterial

genera (LOCATELLI et al., 2002; WAHYUDI et al., 2011; SOUZA et al., 2013). The genetic plasticity of bacteria that permits DNA transfer through plasmids, bacteriophages, and transposons complicates the identity of bacterial species (FEDRIZZI, 2006), and for this reason, the methods for studying soil bacterial diversity can be divided into biochemical and molecular techniques.

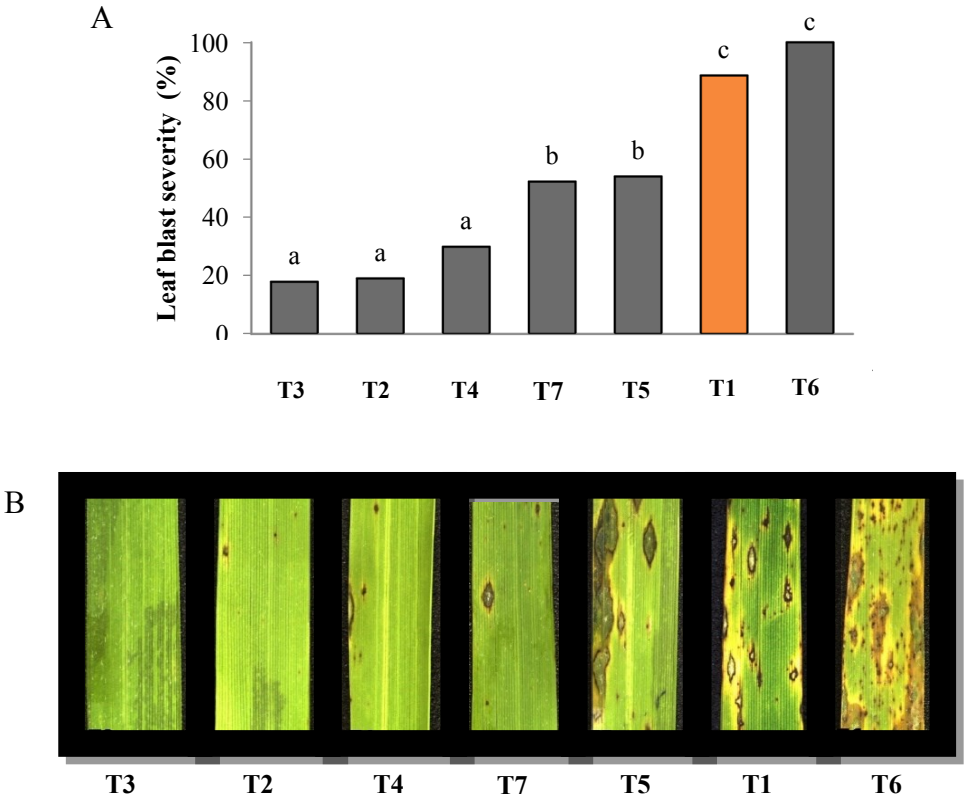

Figure 3. Leaf blast suppression by BRM3211 and BRM 32111 cell suspensions and filtrates. **A**: Leaf blast severity after spray inoculation of *M. oryzae* (*Mo*) suspension mixed with: T1: control (*water*); T2: BRM 32111 cell suspension; T3: BRM 32112 cell suspension, T4: filtrate of BRM 32112, T5: filtrate BRM 32111, T6: filtrate of co-culture of BRM 32111 + *Mo*; T7: filtrate of co-culture of BRM 32112 + *Mo*. **B**: Rice leaf blast typical symptoms, rinsing from pin-like lesions (T3) to typical susceptible lesion (T6). Bars with the same letter did not differ according to Duncan test ($p < 0.05$).

The BRM 32113 and BRM 32114 isolates were identified as indole acetic acid (IAA) producers, confirming the previous results obtained, showing that BRM 32113 and BRM 32114 (Figure 1) can promote plant growth. When active in the root systems, they can increase the size and number of ramifications of adventitious roots, and the contact area with the soil, making considerable quantities of nutrients available to the plant. In turn, rhizobacteria benefit from higher levels of root exudates. The application of biological inducers, with the capacity to promote the initial growth of seedlings, constitutes a viable alternative for a no-tillage production system (SPERANDIO et al., 2017). Rice seeds treated with BRM 32113 and BRM 32114 resulted in rice shoots with a biomass increase of 70% (FILIPPI et al., 2011; NASCENTE et al., 2017; SPERANDIO et al., 2017).

Of the six isolates investigated, BRM 32113, BRM 32111, BRM 32112, BRM 32110, and BRM 32114 were all identified as siderophore producers (Table 1). The soil microorganisms usually compete for nutrients of low availability. One mechanism that confers an advantage in this competition is the production of siderophores that are capable of forming Fe^{3+} complexes (HALFELD-VIEIRA et al., 2015). It is important to emphasize that, when the Fe levels are low in the soil, siderophores can be produced (CATTELAN, 1999). Siderophore producing isolates are fundamentally important for the Cerrado biome. According to Fageria, Moreira and Coelho (2001), soil quality in the West-Central region of Cerrado is naturally acidic and requires the addition of micronutrients such as iron (Fe). The adoption of siderophore producing rhizobacteria into the integrated management of rice diseases, permits the coating of plant roots with Fe and protects against plant pathogens (GRAY; SMITH, 2005;

SOLANKI et al., 2014; NAUREEN et al., 2015). Plant pathogens are sensitive to the actions of siderophores produced by antagonists due to their low affinity for iron (MELO, 1998).

Only BRM 32112 and 32111 formed biofilms (Table 1), which correlates with the results of Rêgo et al. (2014). This is an essential characteristic for successful interactions between plants and rhizobacterium. According to Recouvreux (2004), this structure constitutes a form of protection that permits microorganisms to survive in an adverse environment. Spaepen, Vanderleyden and Okon (2009) showed that quorum sensing is involved in processes such as virulence, symbiosis maturation of biofilms, mobility, and survival of bacteria. They concluded that pH, iron level, and availability of nutrients are essential modulators of biofilm production.

Finally, the principal component analysis (PCA) revealed the correlations between the biochemical and morphological characteristics observed in this investigation, to complement the genetic characterizations (Figure 1). It is essential to emphasize the importance of a broad approach to integrate the different data and information obtained from the bacteria, since this polyphase called "taxonomy", allows us to affirm that one test complements the other. The molecular identification and biochemical tests were all highly correlated.

The BRM 32111 and BRM 32112 isolates were markedly distinct as antagonists of *M. oryzae,* inhibiting pathogen growth *in vitro* (Figure 3). Microorganisms that act by antibiosis, generally, possess a broad spectrum of action. In the inhibition process of fungi, the production of toxic substances is more effective than any other mechanism involved (KUPPER; GIMENES-FERNANDES; GOES, 2003). Similar results were obtained by Amorim and Melo (2002) when they investigated antagonism between different rhizobacteria isolates and *Phytophthora parasitic*, as seven bacterial isolates were found to be capable of inhibiting the mycelial growth of the pathogen. The authors concluded that antagonism must occur by both siderophore production as well as by toxic substances of the pathogen (antibiosis).

In vitro, the selected filtrates (BRM 32112, and BRM 32111) inhibited *M. oryzae* mycelial growth (Figure 2). Besides, these two isolates significantly suppressed leaf blast in tests conducted in the greenhouse. The T2 (suspension BRM 32111 + *M. oryzae*), T3 (suspension BRM 32112 + *M. oryzae*), and T4 (filtrate BRM 32112 + *M. oryzae*) treatments suppressed leaf blast, when compared with the control, by 81.0%, 79.2%, and 66.3%, respectively (Figure 3). For the T1 (control) and T6 (suspension BRM 32111 + Mo (filtrate of co-cultivation) + *M. oryzae*) treatments, the lesions were typically elliptical with brown margins and grey centers, sporulating, and coalescing to occupy more than 50% of the leaf area. Treatments T2 (suspension BRM 32111), T3 (suspension BRM 32112), and T4 (filtrate BRM 32112) resulted in small brown pinhead sized necrotic, non-sporulating lesions. T5 (filtrate BRM 32111) resulted in prominent elliptical lesions that were a few millimeters in diameter with necrotic centers and brown margins, some coalescing on the leaf margins was observed. T7 (filtrate of co-cultivation of BRM 32112 + Mo) resulted in elliptical slightly elongated lesions with brown margins, yellow halos, and grey centers. Some researchers have suggested the use of substances derived from the metabolism of rhizobacteria to control fungal diseases (CATTELAN, 1999; KUPPER; GIMENES-FERNANDES; GOES, 2003; BETTIOL; MORANDI, 2009). The *Pseudomonas* spp. have a high capacity to suppress soil pathogens, are nutritionally versatile, and possess the ability to grow in a wide range of environmental conditions, besides producing high quantities of antibiotics, siderophores, and plant growth hormones. The mechanisms of action of this group include the production of antibiotic compounds, as they act directly to suppress pathogens. In the present study, the partial sequencing of the 16S rRNA genes of the BRM 32111 and BRM 32112 isolates showed a similarity to the *Pseudomonas* genus and they were found to be efficient both in *in vitro* antagonism (Figure 2) as well as *in vivo* suppression of rice leaf blast (Figure 3).

There have been several previous investigations on the inhibitory effects of bacteria from the genus *Pseudomonas*, on the growth of fungi. For example, Jousset et al. (2006) investigated the antifungal activity of 30 *fluorescens* isolates against the main rice pathogens. The authors identified an isolate capable of inhibiting the mycelial growth of all pathogens. They proceeded to extract the compounds produced by this single isolate, and they were also found to inhibit the growth of the same pathogens. After isolating and purifying one of the compounds called 2,4-diacetylfloroglucinol (DAPG), it was also found to inhibit the growth of all rice pathogens.

Bajpai et al. (2018), showed that several types of antifungal compounds that are secreted by *Pseudomonas* sp. have been characterized as secondary metabolites, including 2,4-DAPG, pyoluteorin, phenazines, and pyrrolnitrin. Rhizospheric bacteria depend upon root exudates as carbon and nitrogen sources to produce secondary metabolites. The same secondary metabolites produced by bacterial cells were possibly responsible for inhibiting *M. oryzae* growth and suppressing rice plant colonization.

CONCLUSION

According to Dean et al. (2012), *M. oryzae* fungus was ranked as one of the top 10 fungal pathogens in the world. As more than half of the world's population relies on rice as their primary source of energy, *M. oryzae* can have devastating economic effects on rice cultivation, threatening food security. The approach presented here allows for the search of alternative disease control measures in agriculture to be intensified, such as for example, the use of biocontrol agents. Biological controls, when combined with integrated management techniques, may lead to the developmenmt of sustainable disease control strategies.

Agricultural practices are currently facing many challenges, such as soil impoverishment due to degradation processes such as erosion, salinization, pollution, or biological degradation, which have all become intensified in recent years. Thus, maintaining and restoring soil fertility through a rational fertilization plan should be part of any program of agricultural activity (SILVA FILHO; NARLOCH; SCHARF, 2002). BRM 32111 and BRM 32112 were identified as phosphatase, cellulose, and siderophores producers.

The results show that the bacterial isolates BRM 32111 and BRM 32112 were identified as *Pseudomonas* sp., and could potentially, be highly effective at controlling rice blast. Further research regarding the utilization of these two isolates as components of integrated disease management of upland rice is ongoing. Arriel-Elias et al. (2018, 2019) have developed a sustainable formulation for BRM 32111 bacterial isolate, presenting a viable prototype for commercial scale use under field conditions, to reduce production costs, pollution, and hazards to human health.

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