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Biocontrol and seed transmission of *Bipolaris oryzae* and *Gerlachia oryzae* to rice seedlings[1]

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ABSTRACT - *Bipolaris oryzae* and *Gerlachia oryzae*, which cause rice brown spot and leaf scald, respectively, are mainly disseminated by seeds. The aim of this study was to evaluate the potential of seeds microbiolization to reduce transmission of these pathogens to seedlings by using the bacteria DFs185 (*Pseudomonas synxantha*), DFs223 (*P. fluorescens*), DFs306 (unidentified) and DFs418 (*Bacillus* sp.). Seeds naturally infested/infected with both pathogens were immersed in suspension of these bacteria (A_{540} = 0.5) individually or in saline solution (control treatment). After 30 minutes of agitation at 10 °C, 400 seeds were submitted to a sanity test through the blotter method and the isolate DFs223 was the best to reduce the incidence of *B. oryzae* and *G. oryzae* in both seed lots evaluated. Seeds treated like above were sowed in sterilized vermiculite. Seed transmission and growth promotion were recorded after 21 days of incubation in the same conditions. The isolates DFs185 and DFs306 reduced transmission of both pathogens, although the isolate DFs306 was the one wich gave the greatest growth increases. The evaluation of the *in vitro* antibiosis showed that isolates inhibited the mycelial growth of both pathogens, except DFs306. It is possible to affirm that these bacteria have the potential to be used as a seed treatment for seed-borne disease control.

Index terms: biological control, rice brown spot, rice leaf scald, seed treatment, *Oryza sativa* L.

Biocontrole e transmissão de *Bipolaris oryzae* e *Gerlachia oryzae* para plântulas de arroz

RESUMO - *Bipolaris oryzae* e *Gerlachia oryzae* causadores, respectivamente, da mancha parda e da escaldadura do arroz são principalmente disseminados por sementes. Objetivou-se com esse trabalho avaliar o potencial da microbiolização de sementes para a redução da transmissão destes patógenos das sementes para as plântulas usando as bactérias DFs185 (*Pseudomonas synxantha*), DFs223 (*P. fluorescens*), DFs306 (não identificado) e DFs418 (*Bacillus* sp.). Duas amostras de sementes portadoras de *B. oryzae* e *G. oryzae* foram imersas em suspensão dessas bactérias, sendo a testemunha imersa em solução salina (A_{540} = 0,5). Após agitação (30 min./10 °C), 400 sementes foram submetidas ao teste de sanidade pelo método do papel de filtro e o resultado indicou que o isolado DFs223 destacou-se na redução de *B. oryzae* e de *G. oryzae* nos dois lotes de sementes avaliados. Sementes tratadas conforme descrito foram dispostas em vermiculita autoclavada e a transmissão dos patógenos e promoção de crescimento avaliados após 21 dias de incubação em mesmas condições. Os isolados DFs185 e DFs306 reduziram a transmissão de ambos os patógenos, porém o isolado DFs306 foi o que proporcionou maiores incrementos de crescimento. Avaliação de antibiose *in vitro* mostrou que os isolados inibiram o crescimento micelial de ambos os patógenos, exceto DFs306. Pode se afirmar que estas bactérias apresentam potencial para tratar sementes visando ao controle de doenças transmitidas por estas.

Termos para indexação: controle biológico, mancha parda do arroz, escaldadura do arroz, tratamento de sementes, *Oryza sativa*.

Introduction

The transmission of pathogens through seeds is an efficient mechanism by which plant pathogens are spread over long distances, are introduced in new cultivation areas and are spread via plant population as random sources of primary inoculum (Malavolta et al., 2002). Therefore, necrotrophic pathogens use seeds as a vehicle for dissemination, as a shelter and as means of survival.

In Brazil, *Bipolaris oryzae* and *Pyricularia grisea* are

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mentioned as major pathogens associated with rice seeds, followed by *Gerlachia oryzae*, *Cercospora oryzae*, *Phoma* spp., *Alternaria padwickii*, *Fusarium* spp., *Nigrospora oryzae* and *Tilletia barclayana* (Franco et al., 2001).

Damage caused by *B. oryzae* and *G. oryzae*, etiologic agents of brown spot and leaf scald, respectively, is due to the reduction in the number of seeds per panicle and their weight, reflecting on the quality of the grown seeds, causing a decrease in their germination. In addition, there is damage from the early epidemic in the field due to the high percentage of transmission of fungi from seeds to seedlings, which, in the case of *B. oryzae*, may reach 15.1% (Malavolta et al., 2002).

In the search for reducing damage caused by these pathogens, the use of resistant cultivars is recommended, although the cultivars available in the market do not always have the desirable levels of resistance and/or resistance to more than one pathogen (Nunes et al., 2004). Another aspect that limits the possibility of use of resistant cultivars is their reduced usable life time brought about by the emergence of new races of pathogens (Cornélio et al., 2003).

The brazilian market offers various compounds formulated and registered for the control of most diseases present in rice, but only four of them for seed treatment and, of these, only one active ingredient is recommended for the control of *B. oryzae* and *G. oryzae* (Agrofit, 2014). The chemical control is effective but its use increases production costs and its residues are accumulated in the environment besides increases selection pressure, allowing the emergence of pathogen populations resistant to these chemical compounds (Celmer et al., 2007). Faced with this scenario, biological control by microbiolization of seeds appears as a viable alternative. Reports on the potential of different isolates of *Pseudomonas* and *Bacillus* to control rice blast (*P. grisea*) (Krishhnamurthy and Gnanamanickam, 1998) and seedling blight (*Rhizoctonia solani*) (Commare et al., 2002; Wiwattanapatapee et al., 2004; Souza Júnior et al., 2010) have shown encouraging results. Studies on the biological control of brown spot and leaf scald in rice through the use of microorganisms are still scarce, but, Ludwig et al. (2009) reported the potential of bacteria for the control of *B. oryzae* and *G. oryzae* when these pathogens were inoculated on rice leaves. However, nothing is known about the effect of biocontrollers during seed germination and their impact on the transmission of seed borne pathogens.

Thus, the aim of this study was to evaluate the effect of selected bacteria for the biocontrol of *B. oryzae* and *G. oryzae* (Ludwig et al., 2009) on the incidence in seeds and transmission of these pathogens in seed lots naturally infested/infected with these pathogens.

Material and Methods

Effect of the microbiolization of seeds with biocontrollers on the incidence of pathogens

Seeds of cultivars Chumbinho (lot 376) and Formosa (lot 375) with incidence of *B. oryzae* and *G. oryzae* were microbiolized with isolates DFs185 *Pseudomonas synxantha* (Ehrenberg) Hollan; DFs223 *P. fluorescens* Migula; DFs418, *Bacillus* sp. Cohn; and DFs306 (unidentified). Seeds were immersed in a suspension of each bacterium with 24 hours of growth in medium 523 (10 g sucrose; 4.0 g yeast extract, 8.0 g casein hydrolysate; 0.3 g $MgSO_4$; 2.0 g K_2HPO_4; 15.0 g agar) prepared with saline solution (0.85% NaCl) and adjusted to A_{540}=0.5. The control was immersed in saline solution.

After a stirring period of 30 minutes at 10 °C, 400 seeds were placed in gerbox® boxes, according to the blotter test method (Brasil, 2009), incubated at 23 ± 2 °C under 12 hours light/12 hours dark conditions.

The incidence of pathogens was evaluated after seven days, individually examining the seeds in a stereoscopic microscope and an optical microscope to confirm the characteristics of conidia and conidiophores. The percentage of incidence was calculated compared to the control, considered 100%.

Effect of biocontrollers on the transmission of pathogens from seeds to seedlings

Seeds of the same lots from the previous trial were microbiolized as described in the previous section, and then planted in plastic cups with a capacity 50 mL containing sterilized vermiculite, placing one seed per cup. The assay was carried out inside transparent plastic boxes (41 cm x 30 cm x 30 cm) maintained in a moist chamber, where 25 cups were placed and incubated at 23 ± 2 ºC, each box constituting one experimental plot. The statistical design was a randomized block with four replications of each treatment.

After 21 days of incubation in the above-mentioned conditions, we evaluated the number of seedlings (percentage of emerged seedlings). Subsequently, the severity of symptoms caused by the pathogens was evaluated. Each seedling received a score regarding the intensity of symptoms, where: 0 = seedling with no symptoms; 1 = slightly necrotic seedling; 2 = moderately necrotic seedling; 3 = highly necrotic seedling. We calculated the average severity of each plot relative to the total of emerged seedlings.

In addition, seeds that did not germinate (dead seeds) were removed from the substrate and sterilized in sodium hypochlorite at 1% for 1 minute. After that, they were washed in running water and subjected to a moist chamber aiming to determine the incidence of *B. oryzae* and *G. oryzae*, the average incidence of each pathogens was calculated regarding the total of non-germinated seeds in each plot.

Subsequently, of the developed seedlings, the root system was cuted off from the shoot at the neck, and the length of both parts was measured. To calculate the responses of each treatment, the value of the control for each variable was converted to 100%.

Antibiosis in vitro against B. oryzae and G. oryzae

The pathogens used in this test were isolated from the seeds infested with them, observed in the above test.

The four biocontrollers isolates (DFs185, DFs223, DFs306 and DFs418) were cultured in liquid medium 523 (10.0 g sucrose; 4.0 g yeast extract; 8.0 g casein hydrolysate; 0.3 g $MgSO_4$; K_2HPO_4) for a period of 72 h at 28 °C. Subsequently, 1 mL of each culture was centrifuged for 15 minutes at 9860 rpm (10,000 *g*). The supernatant was removed and subjected to ultrasound bath (Ultrasonic Cleaner 1440D) for a period of 20 min. for the disruption of bacterial cells still present in the cultures, thereby obtaining the metabolic liquid from each bacterial isolate. Separately, 10 mL of PDA medium (Acumedia Potato-dextrose-agar) were transferred to Petri dishes. After solidification of the medium, disks were removed using a punch of 5 mm diameter, forming four equidistant holes in the edges of the plates. The liquid formed by metabolites of each isolate was added to the holes (20 mL). Next, a 5 mm disk of *G. oryzae* or *B. oryzae* was placed in the center of each plate. As a control, a disk of mycelium was placed on a plate containing PDA without liquid metabolite. The plates were incubated at 23 ± 2 °C for up to 14 days. The evaluation occurred when the mycelial growth of the control reached the edge of the plates, observing the occurrence of a mycelial growth inhibition halo, considered an indicator of antibiosis. In this test, four replicates for each bacterial isolate were performed.

Statistical analyzes

The data obtained from the developed seedlings in the test of pathogen transmission through seeds (severity of symptoms, germination rate, length of root and shoot) were subjected to analysis of variance and means grouped by the Scott-Knott test at 5% probability.

Results and Discussion

The *G. oryzae* and *B. oryzae* incidence observed in blotter test method for untreated seeds (control) was respectively 36.75 and 11.25% for lot 375; and 24.25% and 4.75 for lot 376. All treatments, when assessed by blotter test, reduced the incidence of pathogens (in media *B. oryzae* by 18.8% and *G. oryzae* by 20.5%), except in the isolate DFs418 in lot 375 for *B. oryzae* and DFs306 and DFs418 in lot 376 for *G. oryzae* (Figure 1). Larger decreases were observed in lot 376 for the fungus *B. oryzae* in the treatment with isolate DFs185 (Figure 1B - 78.9%). When considering the average of the two lots, the isolate DFs185 was the most effective for the control of *B. oryzae* and isolate DFs223 for *G. oryzae*, both providing control percentages of 40%. These isolates in the overall average (pathogens and lots) also showed a similar behavior, resulting in a reduction of 35 and 38% respectively.

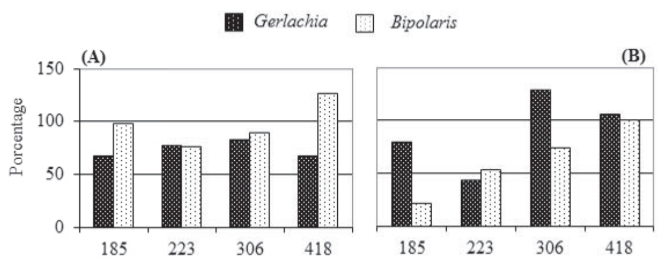

Figure 1. Relative percentage of *Gerlachia oryzae* and *Bipolaris oryzae* incidence in seed lots 375 (A) and 376 (B) naturally infested/infected and microbiolized with different biocontrollers bacteria isolates, as determined by the blotter test. Control considered as 100%.

The high incidence of pathogens resulted in lower emergence in sterile vermiculite, noting 44% of developed seedlings in the control of both lots evaluated. In non-germinated seeds in the control, a pathogen incidence of approximately 68 and 71% in lots 375 and 376, respectively, was observed, and *G. oryzae* represented 68% of these in both seed lots.

The effect of the biocontroller bacteria isolates, when evaluated in autoclaved vermiculite, was similar to that observed in blotter test. In general, all isolates reduced transmission of pathogens from seeds to seedlings, particularly in lot 376 (Figure 2). When considering the results for both seed lots, the most efficient biocontroller isolates in reducing this transmission, measured by the intensity of symptoms emerging from the seedlings, were DFs185 and DFs306, since these were placed in a distinct group from the control by Scott-Knott in both lots evaluated. On the other hand, the incidence of fungi on non-germinated seeds show that the biocontroller isolate DFs223 seemed most effective in reducing the incidence of *B. oryzae* and *G. oryzae* (24.3% and 27.5% respectively).

Growth promoting effects were observed for all bacterial treatments on the length of the roots, which, on average, resulted in an increase of 22 percentage points (Figure 3).

Again, the most intense effects were observed in lot 376. The most effective isolate as a whole, when considering all variables and both lots, was DFs306, resulting in an average increase of 16%.

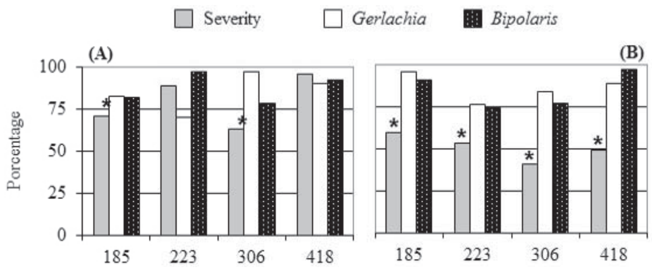

Figure 2. Relative percentage of transmission determined by the severity of symptons in plants and incidence of *Bipolaris oryzae* and *Gerlachia oryzae* in non-germinated seeds in seed lots 375 (A) and 376 (B) naturally infested/infected and microbiolized with biocontrollers bacteria isolates, determined in autoclaved vermiculite after 21 days of incubation at 23 ± 2 ºC. Control considered as 100%.

*Significant values different from the control by the Scott-Knott test ($\alpha = 0.05$).

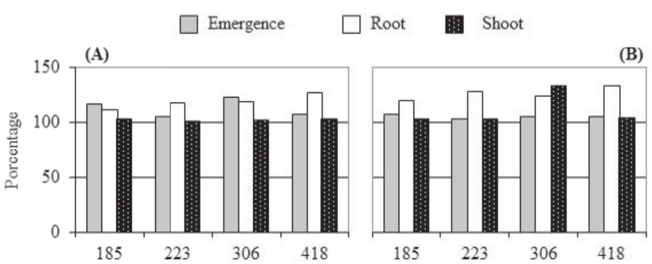

Figure 3. Growth promotion expressed as a percentage compared with the control: emergence rate, length of leaves and roots of rice seedlings originating from seeds of lots 375 (A) and 376 (B) naturally infested/infected by *Bipolaris oryzae* and *Gerlachia oryzae* and microbiolized with biocontrollers bacteria isolates, determined in autoclaved vermiculite after 21 days of incubation at 23 ± 2 °C. Control considered as 100%.

It could be verified that all the isolates have a potential to control pathogens by antibiosis, that is, they all produced at least one compound capable of inhibiting the pathogenic fungi tested, except for DFs306, which did not inhibit mycelial growth of both pathogens (Table 1).

The biocontrol of diseases transmitted by seeds to the seedlings as observed for *B. oryzae* and *G. oryzae* by the bacteria evaluated have already been reported. The microbiolization of seeds with biocontroller has also been used to reduce transmission of pathogenic fungi such as *Fusarium oxysporum* f. sp. *ciceris* by treatment with *Bacillus subtilis* and *Trichoderma harzianum*, used individually or in combination (Herváz et al., 1998). Correa et al. (2008) showed the efficiency of this strategy when they microbiolized bean seeds with biocontrollers bacteria of *Xanthomonas axonopodis* pv. *phaseoli* to reduce the transmission of *Colletotrichum lindemuthianum* of naturally infested/infected seeds to seedlings. On the other hand, the transmission of pathogenic bacteria *Acidovorax avenae* subsp. *citruli* from the plant to the seeds, as well as from seeds to seedlings was strongly reduced by microbiolization of watermelon seeds with an *A. avenae* subsp. *avenae* or *P. fluorescens* isolate (Fessehaie and Walcott, 2005).

Table 1. Mycelial growth of pathogenic fungi provided by biocontrollers isolates determined by *in vitro* antibiosis using liquid metabolite obtained after 72 hours of cultivation at 28 °C of each bacterium individually.

	DFs185	DFs223	DFs306	DFs418
Bipolaris oryzae	+	+	-	+
Gerlachia oryzae	+	+	-	+

(+) presence of inhibition of mycelial growth; (-) absence of mycelial growth inhibition.

The growth promotion exhibited by the antagonists, especially as for the root length (Figure 3), is known in several plants species (Ahemad and Kibret, 2014), including rice (Lucas et al, 2014; Souza et al., 2013). The biostimulator effect in relation to seed physiological potential has been also reported. In this sense, the bacteria studied in this work, in the absence of pathogens and when used to microbiolize seed lots with low quality, showed positive effects, highlighting the isolate DFs185, which provided increased seed germination and seedling emergence (Soares et al., 2012).

The increment of the different variables, especially fast germination and intensive development of the root system, may result, in the first case, in an escape of main soil pathogens (Canteri et al., 1999), and, in the second case, in access to a greater volume of soil, providing better nutritional conditions and increasing tolerance to adverse weather conditions in the field (El-Abyad et al., 1993). Therefore, these bacteria are able to combine positive effects on the growth and health of seedlings, resulting in a highly interesting possibility of use.

In this study, isolates DFs185 and DFs306 stood out in the production of healthy rice seedlings (Figure 3), for both seeds lots evaluated *in vivo*, although DFs306 did not produce compounds capable of inhibiting the mycelial growth of the pathogens when confronted *in vitro*. In this sense, it is worth noting the possibility of DFs306 acting by resistance induction, since this bacterium was not able to inhibit the mycelial growth of *B. oryzae* and *G. oryzae*. On the other hand, the possibility of isolate DFs185 also acting by resistance induction is not ruled out, even knowing its *in vitro* antibiosis capacity. The possible occurrence of induction by both bacteria is based on the fact that when they were used in other studies, it was observed control of *Meloidogyne graminicola* (Ludwig et al., 2013) and *R. solani* (Ludwig and Moura, 2007), therefore exhibiting nonspecific control, besides, they caused increased activity of enzymes related to pathogenesis and induction of resistance (Ludwig and Moura, 2009).

On the other hand, the isolate DFs223 stood out as a whole, reducing both pathogens, both in blotter test and in autoclaved vermiculite (non-germinated seeds), although it did not provide the greatest reductions in all evaluations. Additionally, it provided *in vitro* inhibition halos. This bacterium, as well as DFs185, belongs to a well-studied group of biological control agents, which makes it possible to suggest that these isolates act by antibiosis for the control of these pathogens. This possibility is supported by the fact that these isolates, in addition to presenting antifungal activity against the fungi used in this study, also act by antibiosis against other rice pathogens, such as *Alternaria lunata*, *Curvularia lunata*, *R. solani* (Ludwig and Moura, 2009) and *M. graminicola* (Ludwig et al., 2013).

Another interesting aspect of the biocontrollers used in this study is that they have the ability to colonize the root system of plants of various cultivars of rice, thereby maintaining a high population level during the crop cycle (Ludwig and Moura, 2009). This feature allows the control to be effective, resulting in beneficial effects (Okubara et al., 2004) such as those presented in this study during germination and crop establishment, as well as throughout the crop development (Ludwig et al., 2009).

Finally, isolates DFs185, DFs223 and DFs306 present characteristics that allow their use in leaf scald and brown spot biological control programs. However, further studies are required in the search for the elucidation of the mechanisms of biological control/growth promotion, as well as studies of combinations of isolates with different mechanisms that allow a greater spectrum of activity and/or control amplitude.

Conclusions

All bacteria studied (DFs185, DFs223, DFs306 and DFs418) promote early seedling growth even in the presence of pathogens. Among these bacteria, DFs185 and DFs306 reduce transmission of *B. oryzae* and *G. oryzae* from seeds to seedlings.

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