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Combined Effects of Biological and Chemical Treatment on Rice Seed Physiological and Sanitary Quality

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Abstract

This study aimed to evaluate the efficiency of integrated biological and chemical control of pathogens in rice seeds and their effects on seed quality. The experiment was conducted in a 2 × 5 factorial completely randomized design. Fungicide-treated (carboxin/thiram) and untreated rice seeds were inoculated with distilled water (control), *Azospirillum brasilense*, *Bacillus subtilis*, *Pseudomonas fluorescens*, or *Trichoderma harzianum*. Seed vigor and viability, shoot and root length, and seedling dry weight were determined. The blotter test was carried out to assess seed health. Fungicide treatment improved seed vigor and viability and reduced the incidence of fungi. Biological treatment did not enhance the physiological quality of seeds but was able to control fungi. *A. brasilense*, *B. subtilis*, *P. fluorescens*, and *T. harzianum* controlled *Phoma sorghina*; *B. subtilis*, *P. fluorescens*, and *T. harzianum* were effective against *Aspergillus flavus*; *P. fluorescens* and *T. harzianum* controlled *Pyricularia oryzae*; and *T. harzianum* was effective against *Gerlachia oryzae*.

Keywords: bioinoculant, seed pathology, vigor

1. Introduction

Rice (*Oryza sativa*) is a staple food of great economic and social importance in Brazil and several other countries. According to data from the United States Department of Agriculture (USDA), world rice production in 2018/2019 will be of 501.57 million tonnes, an increase of approximately 6.2 million tonnes compared to the 2017/2018. The largest rice producing, importing and consuming country is China. India is already the world's largest exporter of the grain. Forecast of world supply and demand for 2018/2019, production of 501.57 million tons, export of 47.37 million tons and inventory versus consumption ratio of 35.00 million tons (Companhia Nacional de Abastecimento, 2019). In 2018/2019, the yield of rice in Brazil is estimated to reach 5,994 kg ha^{-1} (Companhia Nacional de Abastecimento, 2018).

High rice yields depend, among other factors, on seed health. Seed treatment with chemical agents is an important strategy to control phytopathogens and prevent disease transmission to other plants and areas (Corrêa et al., 2008). An alternative to chemical control is the use of biological agents, which include non-pathogenic microorganisms capable of increasing host resistance or limiting pathogen activity (Choudhary & Johri, 2009; Busby, Ridout, & Newcombe, 2016). Biological agents are able to combat not only seed-borne pathogens but also those present in the soil. The main microorganisms used for biological seed treatment are bacteria (such as *Agrobacterium radiobacter*, *Bacillus* spp., and *Pseudomonas* spp.) and fungi (such as *Aspergillus* spp., *Chaetomium* spp., *Gliocladium* spp., and *Trichoderma* spp.) (Lucca Filho & Farias, 2012).

Some rhizobacteria promote plant growth and control diseases. The beneficial effects of root-colonizing microorganisms decrease production costs and minimize the need for pesticides, consequently reducing the environmental impacts associated with their use (Harthmann, Mógor, Wordell Filho, Luz, & Biasi, 2009). For instance, studies have shown that *Bacillus* spp. decreases the incidence and severity of pathogen attack by inducing resistance through cytochemical changes in plant tissues (Mertz, Henning, & Zimmer, 2009). Seed inoculation with *Bacillus subtilis* enhances plant growth in common bean (Custódio, Araújo, Ribeiro, Souza Filho, & Machado Neto, 2013). Inoculation with *Azospirillum brasilense* increases plant dry matter, nitrogen accumulation, and grain production (Reis Junior, C. C. T. Machado, A. T. Machado, & Sodek, 2008). *Pseudomonas fluorescens* and other species of the genus promote plant growth and pathogen control by inducing hormone production in plants, producing siderophores and antibiotics, and competing for space and nutrients with pathogenic microorganisms (Corrêa, Bettiol, & Sutton, 2010).

Trichoderma is a genus of free-living and symbiotic fungi. They can survive in the soil, rhizosphere, and within plants. *Trichoderma*-based products are used for seed, substrate, and foliar treatment; to prevent damping-off of seedlings; and to reduce the severity of soil-borne diseases, such as those caused by *Pythium*, *Rhizoctonia*, *Sclerotium*, *Sclerotinia*, *Fusarium*, and *Phytophthora* (Pomella & Ribeiro, 2009).

Despite the various benefits of biological agents, there is no conclusive evidence that chemical treatment affects biological agents or that their combined use can promote beneficial effects. Therefore, we aimed to investigate the efficacy of integrated chemical and biological control of seed-borne pathogens in rice and evaluate their effects on the physiological quality of seeds.

2. Methods

The experiment was carried out at the Seed Technology Laboratory of the Department of Agronomy of the State University of Maringá, Umuarama, Paraná, Brazil. A 2 × 5 factorial completely randomized design was carried out with four repetitions. A commercial seed lot of cultivar SCS 112 was used. We evaluated the physiological quality of fungicide-treated and untreated seeds inoculated with distilled water (control), *A. brasilense*, *B. subtilis*, *P. fluorescens*, or *Trichoderma harzianum*. The fungicide combination carboxin/thiram (Vitavax-Thiram 200 SC®) was used at 300 mL 100 kg^{-1} seed. *A. brasilense* (Az Total®), *B. subtilis* (Accelerate BS®), and *P. fluorescens* (Accelerate PF®) were used at 100 mL 100 kg^{-1} seed. *T. harzianum* (Ecotrich WP®) was used at 3×10^{12} colony forming units 100 kg^{-1} seed, diluted in 300 mL of distilled water. For each treatment, seeds were placed in a plastic bag and received the addition of the corresponding amount of fungicide and/or biological agent. The bag was then shaken vigorously to ensure a homogeneous distribution of the agents.

Seed health was evaluated by a standard filter paper method (blotter test) using four repetitions of 100 seeds per treatment (Brazil, 2009a). Seeds were placed, 1 cm apart from each other, on top of three sheets of germination paper, moistened with a volume of distilled water equal to 2.5 times the weight of a dry sheet, and placed in a germination box. Each repetition was composed of four boxes containing 25 seeds each. Boxes were kept at 20±2 °C in a BOD incubator equipped with white fluorescent lamps under a photoperiod of 12:12 h light/darkness for 8 days. After incubation, seeds were examined individually for any sign of fungal fruiting bodies using a stereomicroscope (4-10× magnification). Fungal species were identified by comparing fruiting bodies with reference slides of identified fungal structures. Results are expressed as percentage of incidence of fungi in seeds.

The germination test was carried out with four repetitions of 50 seeds per treatment. Seeds were placed on top of two sheets of germination paper, covered with a third sheet, moistened with a volume of distilled water equal to 2.5 times the weight of a dry sheet, rolled, and placed in a BOD incubator at 25±2 °C under a photoperiod of 12:12 h light/darkness. Seeds were examined on days 5 and 10 of incubation to determine, respectively, seed vigor and viability (Brazil, 2009b).

Shoot length was determined with four repetitions of 10 seeds sown in line on the upper third of a previously moistened paper substrate, as described for the germination test. Substrates were rolled and placed vertically in a BOD incubator at 25 °C under a photoperiod of 12:12 h light/darkness. After 14 days, the shoot length, length of the primary root, and number of seedlings were determined. Subsequently, remaining seed reserve tissue was removed, and seedlings were packed in paper bags and dried in a forced-air oven at 65 °C for 48 h. Seedling dry weight was determined by dividing the weight of the sample by the number of normal seedlings (Nakagawa, 1999).

Data were tested for normality by the Shapiro-Wilk test and found to be normally distributed. Analysis of variance was performed, and means were compared by Tukey's test using Sisvar version 5.3 (Ferreira, 2011).

3. Results

Seed vigor was positively influenced by fungicide treatment (Table 1). Biological treatment did not increase seed vigor in comparison with control, regardless of association with fungicide treatment. *A. brasilense* and *P. fluorescens*, without fungicide treatment, reduced seed vigor in relation to control, *B. subtilis* and *T. harzianum*.

Seed viability was majorly affected by fungicide treatment, increasing this parameter with the use of the chemical product.

Table 1. Vigor and viability of fungicide-treated and untreated rice seeds inoculated with biological agents

Biological agent	Seed vigor (%)	
	Fungicide-treated	Untreated
Control (distilled water)	91.5 Aa	74.0 Bab
Azospirillum brasilense	88.0 Aa	63.0 Bbc
Bacillus subtilis	84.5 Aa	76.0 Aa
Pseudomonas fluorescens	89.0 Aa	57.0 Bc
Trichoderma harzianum	81.5 Aa	74.5 Aab
	Viability (%)	
	Fungicide-treated	Untreated
	91.7 A	82.4 B

Note. Means followed by the same lowercase letter in each column or uppercase letter in each row are not significantly different (Tukey's test, $p < 0.05$).

P. fluorescens and *T. harzianum* reduced shoot and root length in relation to *B. subtilis* treatment, but no treatment changed this parameters in relation to control (Table 2).

Table 2. Shoot and root lengths of rice seedlings treated with biological agents and fungicides

Biological agent	Shoot length (cm)	Root length (cm)
Control (distilled water)	9.28 ab	15.11 ab
Azospirillum brasilense	9.40 ab	15.63 ab
Bacillus subtilis	10.26 a	17.90 a
Pseudomonas fluorescens	8.44 b	14.31 b
Trichoderma harzianum	8.43 b	13.86 b

Note. Means followed by the same letter are not significantly different (Tukey's test, $p < 0.05$).

Seedling dry mass was determined; however, no significant differences were observed between treatments (data not shown).

For the analysis of fungi incidence on seeds (Table 3) note that a single seed can be infected by more than one species of fungus. Application of fungicide was efficient in controlling *Aspergillus flavus*, *Pyricularia oryzae, Gerlachia oryzae* and *Phoma sorghina*. Moreover, fungicide-treated seeds had the highest percentage of seeds without signs of fungal infection. There was no additional effect in fungi control when fungicide and biological treatment were associated.

B. subitilis, *P. flourescens* and *T. harzianum*, without application of fungicide, were able to control *A. flavus* and *P. oryzae* (Table 3). Additionally, *G. oryzae* was best controlled by *T. harzianum*, followed by *A. brasilense*, than by *P. fluorescens* and *B. subitilis* afterall. All inoculants were equally effective against *P. sorghina*.

Biological agents, without application of fungicide, showed different performances in combating fungi in rice seeds (Table 3). *B. subtilis*, *P. fluorescens*, and *T. harzianum* were more effective against *A. flavus* than *A. brasilense*. *T. harzianum* was the most effective in reducing the incidence of *G. oryzae*. Inoculation with *T. harzianum* resulted in the highest percentage of seeds without signs of fungal infection, being the only biological treatment with similar results as fungicide treatment for this evaluation.

Table 3. Incidence (%) of fungi in fungicide-treated and untreated rice seeds inoculated with biological agents

Biological agent	*Aspergillus flavus*		*Pyricularia oryzae*	
	Fungicide-treated seeds	Untreated seeds	Fungicide-treated seeds	Untreated seeds
Control (distilled water)	5.5 Ba	16.5 Aa	0.0 Ba	13.0 Aa
Azospirillum brasilense	1.5 Ba	14.5 Aa	0.0 Ba	12.0 Aa
Bacillus subtilis	1.0 Aa	3.5 Ab	0.0 Ba	6.0 Aab
Pseudomonas fluorescens	0.0 Aa	2.0 Ab	0.0 Aa	2.0 Ab
Trichoderma harzianum	0.0 Aa	2.0 Ab	0.0 Aa	1.0 Ab
Biological agent	*Gerlachia oryzae*		*Phoma sorghina*	
	Fungicide-treated seeds	Untreated seeds	Fungicide-treated seeds	Untreated seeds
Control (distilled water)	5.0 Ba	66.0 Aa	1.0 Ba	45.5 Aa
Azospirillum brasilense	7.0 Ba	25.0 Ac	12.0 Aa	13.5 Ab
Bacillus subtilis	2.0 Ba	42.5 Ab	1.0 Aa	6.0 Ab
Pseudomonas fluorescens	6.0 Ba	33.0 Abc	0.0 Aa	8.5 Ab
Trichoderma harzianum	0.0 Aa	2.0 Ad	0.5 Aa	6.5 Ab
Biological agent	Seeds without visual signs of fungal infection			
	Fungicide-treated seeds		Untreated seeds	
Control (distilled water)	90.0 Aa		17.0 Bc	
Azospirillum brasilense	87.0 Aa		35.0 Bbc	
Bacillus subtilis	99.5 Aa		42.0 Bb	
Pseudomonas fluorescens	89.0 Aa		54.5 Bb	
Trichoderma harzianum	85.0 Aa		88.5 Aa	

Note. Means followed by the same lowercase letter in each column or uppercase letter in each row are not significantly different (Tukey's test, $p < 0.05$).

4. Discussion

Several studies reported the beneficial effect of fungicide treatment on seed quality (Pereira, Oliveira, Rosa, Oliveira, & Costa Neto, 2009; Pereira et al., 2011; Silva, Lucca Filho, Zimmer, & Bonini Filho, 2011; Hossen, Corrêa Junior, Guimarães, Nunes, & Galon, 2014). Ramos, Marcos Filho, and Galli (2008) working with supersweet corn, observed that fungicide treatment improved seed vigor under accelerated aging conditions. Pereira et al. (2011) found that treatment of soybean seeds with fungicide increased seedling emergence by 44.3%, confirming the importance of controlling naturally occurring pathogens in seeds even under optimal germination conditions. Similar beneficial effects of chemical treatment were observed on rice seed vigor and viability (Table 1).

Initial seed quality determines the response of seeds to biological and chemical treatment; that is, seeds with high physiological quality do not benefit as much from treatments as do medium-quality seeds (Carvalho & Nakagawa, 2012). This fact probably explains the little effect of biological inoculation on rice seeds viability and seedling growth in the current study.

Another observation of the present study was the reduction of seed vigor after the use of some biological treatments. These results differ from those found in literature. There are evidences showing that seeds biological treatment improves (Brotman et al., 2013) and accelerates seed germination, increases seedling vigor and ameliorates water, osmotic, salinity, chilling and heat stresses (Mastouri, Björkman, & Harman, 2010). The interaction of the biological control agent with seeds/seedlings is complex and depends on factors such as the gene constitution of the plant (Simon et al., 2001; Smith, Tola, de Boer, & O'Gara, 1999), the production of organic acids by it is essential for establishing plant/bacterial interaction (as reviewed by Bloemberg & Lugtenberg, 2001), for example, and the presence of other microorganisms in the environment (Bloemberg & Lugtenberg, 2001). The germination test is carried out on paper substrate, changing the interaction conditions that the seed and the biological treatment would find in the soil. Therefore, in the mentioned test the absence of several factors which interfere in establishing the interaction, could contribute to alter the energy balance and consequently could contribute to reduce seed vigor.

However, biological treatment on seeds can be responsible for several other advantageous effects as it will be discussed further. Some characteristics of *B. subtilis* make it a particularly effective seed inoculant, such as its sporulation ability, tolerance to desiccation, and improved survival in polymer formulations (Choudhary & Johri, 2009). These properties probably explain the high shoot and root lengths observed in rice plants inoculated with *B. subtilis* (Table 2). Bacteria of the genus *Bacillus* are known for their versatile defense mechanisms and antagonistic activities against plant pathogens, which are required for their survival and maintenance in specific ecological niches (Lanna Filho, Ferro, & Pinho, 2010). A study showed that inoculation of rice, common bean, chickpea, soybean, and cotton seeds with *B. subtilis* improves seed vigor, seedling emergence, and seedling dry weight (Custódio et al., 2013). In the same study, corn and cotton plants inoculated with the bacterium were found to have higher phosphorus concentrations in leaves than control plants, and inoculated corn plants were shown to absorb significantly higher amounts of phosphorus even in phosphorus-deficient soil (Araujo, 2008). *B. subtilis* is also beneficial to peanut seeds, improving plant vigor and harvest yield (Abd-Allah & Didamony, 2007).

Additionally, other microorganisms were found to exert positive effects on plants. *A. brasilense*, for instance, improved plant nutrition, water and mineral absorption, tolerance to drought and salinity stress, and root growth by increasing the resistance of plants to pathogen attack (Hungary, 2011). According to Gava and Menezes (2012), when *T. harzianum* is able to colonize the endosperm, it protects the seed and radicle from infection. Interestingly, *Trichoderma* colonization was found to occur preferentially at specific sites of the rhizoplane, mainly at regions of secondary root emergence and at points of contact between soil and roots, where abrasion occurs. Although a certain degree of root damage by soil is considered normal during development of the root system, it makes plants more susceptible to infection. *Trichoderma* inoculation may be a solution to this problem.

Inoculation with *B. subtilis*, *P. fluorescens*, or *T. harzianum* was able to control *A. flavus* in rice seeds (Table 3). The same effect was observed by Reddy, Raghavender, Reddy, and Salleh (2010), who reported growth inhibitions of 72, 74, and 65% by *B. subtilis*, *P. fluorescens*, and *T. harzianum*, respectively. Yang, Zhang, Chen, Liu, and Lu (2017) found growth inhibitions above 80% in the control of *A. flavus* by *P. fluorescens*.

Pseudomonas and *Bacillus* isolates were reported to control the rice blast fungus *P. oryzae* (Suryadi, Susilowati, Riana, & Mubarik, 2013). Inoculation with *P. fluorescens* and *Bacillus* isolates via seed treatment was effective in controlling the brown spot fungus *B. oryzae* and the leaf scald fungus *G. oryzae* in rice (Moura et al., 2014). Another study found that rice seeds inoculated with *B. subtilis* DFs422 and infected with *G. oryzae* showed low-severity symptoms of the disease for the first 21 days and became resistant to the fungus after this period (Ludwig, Moura, Santos, & Ribeiro, 2009).

Biological agents differ in their mechanisms of action. Some exert beneficial effects during seed germination, others during seedling growth, and others may be effective during all stages of the plant life cycle. It is interesting to note that *A. brasilense* has been gaining popularity in recent years as a nitrogen-fixing rhizobacterium (Fibach-Paldi, Burdman, & Okon, 2012), but its effects are not limited to plant growth promotion. In a pioneering work, Russo et al. (2008) demonstrated that *A. brasilense* is an excellent biological control agent against *Rhizoctonia* spp. and, since then, efforts have been made to elucidate its mechanisms of action. The bacterium was shown to produce and secrete phenylacetic acid, which has antimicrobial action against phytopathogenic fungi and bacteria (Somers, Ptacek, Gysegom, Srinivasan, & Vanderleyden, 2005), and siderophores, which are iron-chelating compounds shown to reduce the incidence of *Colletotrichum acutatum* in strawberry (Tortora, Díaz-Ricci, & Pedraza, 2011).

B. subtilis produces a variety of antibiotic substances, including iturine and fengycin, which are inhibitory to *Fusarium*, *Penicillium*, *Aspergillus*, *Colletotrichum*, and *Rhizoctonia solani* (Nagórska, Bikowski, & Obuchowski, 2007); surfactin, which has synergistic effects with fengycin against *A. flavus* (Farzaneh, Shi, Ahmadzadeh, Hu, & Ghassempour, 2016); and bacillomycin, a compound with fungicidal activity (Gong et al., 2014). *P. fluorescens* produces 2,4-diacetylphloroglucinol, a prominent antimicrobial that inhibits the growth of several phytopathogenic bacteria, oomycetes, and fungi (Couillerot et al., 2011). *T. harzianum* secrets hydrolytic enzymes, produces fungistatic compounds (Contreras-Cornejo, Macías-Rodriguez, Del-Val, & Larsen, 2016), and parasitizes other fungi (Silva et al., 2017). All these data support our findings regarding fungi control on rice seeds using biological control (Table 3).

Different pathogens induce different responses in biological control agents. For instance, *R. solani* alters the expression of genes associated with secondary metabolite detoxification and metabolism in *P. fluorescens*, whereas *Pythium aphanidermatum* does not (Hennessy, Glaring, Olsson, & Stougaard, 2017).

Overall, these findings suggest that inoculation of rice seeds with more than one microorganism might be an effective strategy for the control of pathogens (Babalola, 2010). Coinoculation of tomato leaves with

Trichoderma spp. and *B. subtilis*, *Trichoderma* spp. and *P. fluorescens*, or the three microorganisms combined was more effective against the pathogen *Ralstonia* spp. than inoculation with a single microorganism or chemical control (Yendyo, Ramesh, & Pandey, 2018). Similar effects are expected for coinoculation of seeds.

In this study, biological inoculation was found to be more effective in promoting seed vigor and viability and protecting rice seeds from fungi when combined with fungicide treatment (Tables 1 and 3). In the absence of fungicide treatment, microbial inoculation was more effective than the control (Table 3). These results indicate that chemical treatment had a much greater effect on seeds than biological treatment, thereby precluding observation of the beneficial effects of biological agents. Fungicide application might have decreased the positive effects of *T. harzianum* inoculation and might even have affected plant responses to *A. brasilense*, *B. subtilis*, and *P. fluorescens*. A previous study showed that fungicides can have deleterious effects not only on fungi but also on bacteria and are not compatible with *A. brasilense* inoculated via seed treatment (Munareto et al., 2018).

Fungicide treatment increased rice seed vigor, viability, and resistance to fungi. Microbial inoculation did not improve the physiological quality of seeds. *A. brasilense*, *B. subtilis*, *P. fluorescens*, and *T. harzianum* inoculated via seed treatment were effective in controlling *P. sorghina*, *B. subtilis*, *P. fluorescens*, and *T. harzianum* were effective against *A. flavus*, *P. fluorescens* and *T. harzianum* successfully controlled *P. oryzae* and *T. harzianum* was effective against *G. oryzae*.

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