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FUNGI WITH ENZYMATIC ACTION AGAINST FUNGAL DISEASES AND GROWTH PROMOTING IN WHEAT

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ABSTRACT

Wheat (*Triticum aestivum* L.) is the most important cereal crop in the world. In Brazil, there is a socio-economic interest in increasing wheat production to supply the national demand, since its import still represents almost half of local consumption. Wheat diseases have affected the increase in its production. Some fungi, including *Trichoderma* species and *Aspergillus japonicus*, have shown promise in biological control of the pathogens. The antagonistic activity of these species against plant pathogens has been studied extensively. This research aimed at evaluating the productivity in the field, seedling development, spot blotch, gibberella and rust severity, along with the activity of the enzymes phenylalanine ammonia lyase (PAL), β-1,3-glucanase (GLU), peroxidase (POD), and superoxide dismutase (SOD) in wheat plants under soil inoculation of different fungal pathogens. When used alone, *A. japonicus* allowed some development of the diseases, but still it protected 25 % more than the control. The inoculation of *T. tomentosum* and *T. viride* (combined) led to the lowest severity of fungal diseases of wheat, while the inoculation of *T. tomentosum* (singly) resulted in the greatest development of wheat seedlings. Fungi also increased wheat yield. PAL, POD and SOD activities were higher under soil inoculation of *T. tomentosum*, while the activity of GLU was more expressive under inoculation of *T. tomentosum* and *T. viride* (combined).

Additional key words: Enzymes, peroxidase, severity, superoxide dismutase

RESUMEN

Hongos con acción enzimática contra enfermedades fungosa y promotora del crecimiento en trigo

El trigo (*Triticum aestivum* L.) es el cultivo de cereales más importante del mundo. En Brasil, existe un interés socioeconómico en aumentar la producción para abastecer la demanda nacional, ya que su importación todavía representa casi la mitad del consumo local. Las enfermedades del trigo han afectado el aumento de su producción. Algunos hongos, incluidas las especies de *Trichoderma* y *Aspergillus japonicus*, se han mostrado prometedores en el control biológico de los patógenos. La actividad antagonista de *Trichoderma* spp. y *A. japonicus* contra patógenos de plantas ha sido ampliamente estudiada. Esta investigación tuvo como objetivo evaluar la productividad en campo, el crecimiento de las plántulas y la severidad de la mancha borrosa, la fusariosis y la roya, así como la actividad de las enzimas fenilalanina amoniaco liasa (PAL), β-1,3-glucanasa (GLU), peroxidasa (POD) y superóxido dismutasa (SOD) en plantas de trigo bajo la inoculación de diferentes especies de hongos patógenos en el suelo. Cuando se utilizó de forma individual *A. japonicus* permitió cierto desarrollo de las enfermedades, pero aun así logró proteger un 25 % más que el testigo. La inoculación de *T. tomentosum* y *T. viride* (combinados) condujo a la menor severidad de las enfermedades fúngicas del trigo, mientras que la inoculación de *T. tomentosum* (por separado) dio como resultado el mayor desarrollo de plántulas de trigo. Los hongos también aumentaron el rendimiento de trigo. Las actividades de PAL, POD y SOD fueron mayores en la inoculación de *T. tomentosum* en el suelo, mientras que la actividad de GLU fue más expresiva bajo la inoculación combinada de *T. tomentosum* y *T. viride*.

Palabras clave adicionales: Enzimas, peroxidasa, severidad, superóxido dismutasa

INTRODUCTION

Wheat has been used in food production for humans, manufacture of non-food products and manufacture of products for animals, in the form of grains, component of animal feed or fodder

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(Mori et al., 2007). Wheat farming has a prominent role in crop rotation and/or succession planting in agricultural production units, thus assuring the economic flow and the property sustainability (Penckowski et al., 2010; Silva et al., 2014; Rodrigues et al., 2014).

The pathogenic fungus *Bipolaris sorokiniana* (Sacc.) is the causative agent of spot blotch, which can occur in leaves and ears of wheat and other cereals. This disease leads to a significant reduction in wheat production, with losses up to 20 % (Iftikhar et al., 2012).

The fungus *Fusarium graminearum* infects cereal crops, causing gibberella ear rot (GER). The infection of floral structures reduces grain quality and contaminates the grain with mycotoxins (Foroutan, 2013).

Puccinia triticina produces leaf rust, the most common rust disease of wheat, although the use of biological control may decrease the rust severity (El-Sharkawy et al., 2015).

Based on the current concern with the reduction of environmental impacts caused by some agricultural inputs, the actions of the different microorganisms in biological control need to be understood. Fungi of the genus *Trichoderma* spp. are widely used microbial inocula against various phytopathogenic fungi through foliar and soil application. They have become popular as plant growth inducers (Hermosa et al., 2012).

Some *Trichoderma* species have direct effects on plants, increasing their growth potential and nutrient uptake, fertilizer efficiency, higher seed germination rate and percentage, and stimulating plant defenses against biotic and abiotic damage (Shoresh et al., 2010). Studies have reported that *Trichoderma* increases root development, crop yield, secondary root proliferation, fresh weight and leaf area. *Trichoderma* spp. can release bioactive molecules that induce plant resistance and contribute to increased plant growth and nutrient uptake (Harman, 2006; Sharma et al., 2011).

Among the biotic elicitor factors, endophytic fungi, including species of the genus *Trichoderma* and *Aspergillus* can act directly in the confrontation with pathogens, disputing space or food or indirectly, through the production of compounds or induction of resistance in plants (Dutta et al., 2014; Hosseyni-Moghaddam and Soltani, 2014). Isolates of *A. japonicus* produce different insecticidal compounds and alkaloids that may act in the confrontation with pathogens (Varga et al., 2011).

One way to verify resistance induction is through the enzymes involved in this process. After inoculation of the fungi these can activate such mechanism, thus, the plants can be protected against infection for weeks or even months.

Peroxidases (POD, Enzyme Commission number EC 1.11.1.7) are plant stress-related glycoproteins that catalyze several reactions, including hydrogen peroxide production, cell wall strengthening by acting on lignin, suberin and papilla formation, hydroxyproline-rich glycoprotein binding, lipid peroxidation, and phytoalexin production. Furthermore, PODs are toxic to pathogens and help in wound healing (Oliveira et al., 2016).

β-1,3-glucanases (GLU, EC 3.2.1.39) act through successive hydrolysis from the non-reducing extremity of glucan, thus obtaining glucose and oligosaccharides as final reaction products and, in a longer period, only glucose molecules (Ebrahim et al., 2011).

Phenylalanine ammonia lyase (PAL, EC 4.3.1.5) is an important enzyme of phenyl-propanoid pathway and catalyzes the deamination of L-phenylalanine to form trans-cinnamic acid and ammonia. Trans-cinnamic acid generates several sugar-conjugated compounds in cell walls. Such compounds generate lignin, suberin, coumarins, flavonoids, anthocyanins, phyto-alexins, and salicylic acid (Khan et al., 2015).

Superoxide dismutases (SOD, EC 1.15.1.1) are enzymes involved in scavenging O_2^-, and most of the cellular H_2O_2 is generated from the SOD-catalyzed dismutation of O_2^-. H_2O_2 is a relatively stable free charge oxidant, which can facilitate its passage through the cell membrane, thus favoring a fast elicitation of plant responses. Then, such a molecule can be considered one of the first reactive oxygen species generated during infection (Puthur, 2016).

This study aimed at evaluating the effect of *T. tomentosum*, *T. viride* and *A. japonicus* and their combinations as growth promoters and for controlling the three above mentioned diseases of wheat.

MATERIALS AND METHODS

The experiment was carried out in a

greenhouse belonging to the Cedeteg *Campus*, Unicentro, Guarapuava, PR, Brazil. We tested, as inoculants, *A. japonicus* isolated from leaves of *Brachiaria plantaginea*, *T. tomentosum* from leaves of *Rumex obtusifolius*, and *T. viride* isolated from soil of Atlantic forest of Paraná at 25º25' S, 49º16' W.

Laboratory experiment. The isolation of these endophytic fungi was performed according to Silva et al. (2006). The plants were submitted to the superficial disinfection method, which consisted of washing abundantly in running water, immersed in 70 % ethanol for one minute; immersed in 3 % sodium hypochlorite for three minutes; put back immersed in 70 % ethanol for 30 seconds; rinse twice in distilled and sterilized water; and cut the samples into fragments of 8-12 mm. Then, the fragments of the samples were transferred to Petri dishes containing potato dextrose agar (PDA) medium with pH 6.8, with addition of 100 $\mu g \cdot mL^{-1}$ of chloramphenicol and 100 $\mu g \cdot mL^{-1}$ of streptomycin. Five fragments were placed on each plate and they were kept in an oven at 28 °C ± 1, under photoperiod of 12 hours.

The fungi were identified through the analysis of the products amplified with ITS1 and ITS4 primers specific for internal transcribed (White et al., 1990). The experiments were repeated in 2015 and 2016, and the results presented are the means of these experiments.

The pathogenic inoculum was obtained as follows: For *B. sorokiniana* and *F. graminearum*, the pathogens were cultured in PDA medium and, after multiplication, they were stored in a biochemical oxygen demand (BOD) incubator at 25 ºC and 12 h photoperiod for 15 days. For fungal inoculum standardization, 5 mL sterile saline solution (0.85 %) containing Tween 80 (0.1 %) were added to colonies. Then, colonies were gently scraped using a Drigalski spatula, spores were collected and the suspension was transferred to sterile glass test tubes. The final conidial concentration was adjusted to 10^6 spores per mL in a Neubauer chamber. For *P. titricina*, fresh mixture of aggressive urediospores the pathogen was collected from infected adult wheat plants, then, a dry preparation of urediospores were mixed with talc powder (1:20 v/v) in baby cyclone.

For the biological trial by paired culture, the isolates were first tested in the inhibition assay (antagonistic effect) when in contact with some pathogens in Petri dishes to verify their potential. From this *in vitro* test the fungi were taken for *in vivo* tests. For the study of the antagonistic effect of the fungi was used the technique of coupled culture in Petri dishes (Dennis and Webster, 1971). The percent inhibition of mycelial growth was calculated according to the following formula (Menten et al., 1976):

$Inhibición\ (\%) = \left[\frac{(crtest - crtrat)}{crtest}\right] \cdot 100$, where: crtest = growth radial control; crtrat = radial growth treatment. The experiment was conducted in a completely randomized design, with five repetitions.

Greenhouse experiment. The isolates of endophytic fungi were cultivated in Petri dishes containing PDA medium at 25 ºC (± 2 ºC) and 12 h photoperiod for 7 days in a BOD incubator. For inoculant preparation, 50 g of rice grains, spore suspension (6.3 x 10^8 spores per mL) and 25 mL distilled water were placed into a 250 mL Erlenmeyer flask, followed by storage in BOD incubator at 25 ºC and 12 h photoperiod for 15 days. Then, isolates were introduced into the plant growing media by adding 2 % (w/v) rice colonized by fungi. Treatments were T1=*Aspergillus japonicus*, T2=*A. japonicus* with *Trichoderma tomentosum*, T3=*A. japonicus* with *T. viride*, T4=*T. tomentosum*, T5=*T. tomentosum* with *T. viride*, T6=*T. viride*, T7=autoclaved rice without fungus (control). These treatments were applied in greenhouse and field experiments.

Wheat seeds cultivar Tbio Iguaçu were sown in 11 L pots filled with a growing media of soil and a commercial substrate (1/1:v/v). The substrate contained composted pinus bark, vermiculite, and a base fertilization consisting of C 0.58 %; K 2.9 $cmol_c \cdot kg^{-1}$; Ca 7.8 $cmol_c \cdot kg^{-1}$; Mg 7.5 $cmol_c \cdot kg^{-1}$; cation exchange capacity (CEC) 18.6 $cmol_c \cdot kg^{-1}$ and pH 5.9.

For inoculation, the pathogens were sprayed (5 mL per plant), and pots were maintained in a moist chamber for 48 h.

Seven days after germination, shoot height and root length were measured as described by Garcia et al. (2008). Shoot and root dry matter were obtained after drying in a forced aeration oven at 65 °C till constant weight.

The experiment was conducted in a completely randomized design. Each pot containing five

plants was considered one repetition, and each treatment consisted of five repetitions used for disease evaluations and other five for the shoot and root measurements.

Field experiment. The field experiment was installed in Boa Ventura do São Roque (24°54' S, 51°39' W; 907 m.a.s.l.). The wheat cultivar used at this site was Tbio Iguaçu, in the years 2015 and 2016. Soil contained: C 0.57 %; K 1.4 $cmol_c \cdot kg^{-1}$; Ca 5.7 $cmol_c \cdot kg^{-1}$; Mg 3.1 $cmol_c \cdot kg^{-1}$; CEC 14.1 $cmol_c \cdot kg^{-1}$ and pH 5.3.

The fungi *A. japonicus*, *T. tomentosum* and *T. viride* were cultivated in the same manner as mentioned above, and 250 g of colonized rice was inoculated in the field per square meter, in the plowed soil, with a depth of 5 cm. The inoculation of the pathogens was carried out at thirty days, when the plant was already established and it could survive to perform the severity analysis.

The experiment was carried out in a randomized complete block design, with five repetitions.

The severity of foliar diseases was evaluated according to a diagrammatic scale (James, 1971) every five days. The scale used to evaluate gibberella was the one of Stack and McMullen (1995). Three leaves per plant were established for spot blotch and rust evaluation, totaling ten evaluations in all plants. Since the disease appeared irregularly in cultivars, the evaluations were performed on different days. The area under disease progress curve (AUDPC) was obtained through $\sum\left[\frac{Y_i+(Y_i+1)}{2(\mathrm{Ti}+(1-\mathrm{Ti}))}\right]$, where Yi = disease severity at the time of evaluation i; Ti = plant age at the time of evaluation i (Campbel and Madden, 1990).

To evaluate the agronomic characteristics and productivity, the number of spikes was obtained in samples of one linear meter in the useful area of the plot, and the number of grains in a sample of ten ears collected in the plot. After harvesting and drying the grains, the weight of one thousand grains was obtained; all plots were analyzed and the yield per surface unit was expressed with correction for 13 % moisture. Also, the height of 10 plants of the central rows was measured.

All data were submitted to analysis of variance and Tukey test for experiments on laboratory and greenhouse, and Scott Knott test for the field experiment, using the Sisvar software (Ferreira, 2011).

Biochemical analyses. Leaf tissue samples showing symptoms were collected seven days after pathogen inoculation. The biochemical analysis was performed on this date to evaluate if the fungi inoculated in the vessels were able to activate resistance induction. After sampling, they were immediately frozen in liquid nitrogen and stored at -20 ^{o}C until evaluations. For protein extraction, we followed the method of Moersch Bacher (1988), described by Guzzo and Martins (1996). The total protein content was quantified according to the method of Bradford (1976). For biochemical analysis, one leaf was collected.

PAL activity was assayed through colorimetric quantification (absorbance) of trans-cinnamic acid released from the substrate phenylalanine, according to the method described by Goldson-Barnaby and Scaman (2013).

β-1,3-glucanase (GLU, EC 3.2.1.39) activity was assayed through colorimetric quantification of glucose released from laminarin, using p-hydroxybenzoic acid hydrazide (PAHBAH) (Lever,1972). After cooling to 25 °C, absorbance readings were performed at 410 nm.

Peroxidase (POD, EC 1.11.1.7) assay was done through oxidation of pyrogallol according to Kar and Mishra (1976). The molar extinction coefficient 2.47 $mM^{-1} \cdot cm^{-1}$ was used for calculating the activity of POD.

The specific activity of SOD was assayed through the spectrophotometric method described by Giannopolitis and Reis (1977). Absorbance readings were performed at 560 nm.

The results of the biochemical analyses were compared by the analysis of variance and Tukey test using the "R" statistical computing environment.

RESULTS

As an initial test, we performed the inhibition assay in Petri dishes and it can be observed that when *A. japonicus* and *T. tomentosum* fungi that were isolated from weeds and *T. viride* that was isolated from the soil, were able to inhibit pathogenic fungi, as there was a decrease in the growth of pathogenic fungi (Figure 1). This inhibition was higher than 50 %, and for *T. viride*, the inhibition against pathogens was higher than 70 %. For this reason, these fungi were brought to the *in vivo* test.

The treatments represented by *T. viride* (T6), *T. tomentosum* with *T. viride* (T5) and *T. tomentosum* (T4) led to the highest seedling shoot dry matter and height in comparison with the control group (Table 1). Furthermore, *T. viride* led to the most expressive results of root dry matter and length. All treatments were more efficient than control on seedling development, mainly on shoot and root growth and weight.

In the field experiment the highest productivity was observed with T5 (*T. tomentosum* with *T. viride*), being this one superior in 16.6 % compared to the control, as a year average (Table 2). In relation to the number of ears in the year 2015, the treatments were the same with exception of the control; in the year 2016, the best treatments were T4, T5 and T6, showing the potential of the fungus *Trichoderma*. In the number of grains and the size of wheat plants, the T5 treatment was more expressive, and with respect to plant growth rates (number of ears, number of grains per ear and plant height), all treatments reached higher values than the control. This data set evidences the potential of using fungi inoculation to improve the development of wheat plants and contribute to productivity.

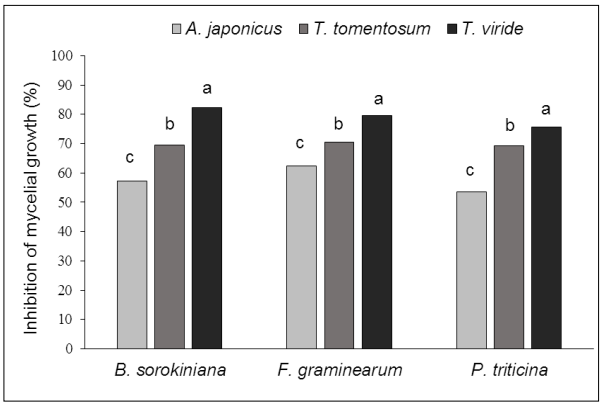

Figure 1. Percent of inhibition of pathogenic fungi in the *in vitro* test with the antagonists *T. tomentosum*, *T. viride* and *A. japonicus*. Columns followed by the same equal letters for each pathogen do not differ from each other according to the Tukey test ($P \leq 0.05$)

Table 1. Effects of fungal inoculum on organ tissue growth of 7-day old wheat seedlings cv. Tbio Iguaçu under greenhouse (Boa Ventura de São Roque, PR, Brazil). Mean of the experiments of 2015 and 2016

Treatment	Shoot dry matter (g)	Root dry matter (g)	Shoot height (cm)	Root length (cm)
1	1,09 b	0,101 b	6,52 bc	7,85 b
2	1,12 b	0,103 b	7,89 a	8,72 ab
3	1,14 b	0,105 ab	7,61 ab	9,85 ab
4	1,21 a	0,112 ab	8,23 a	9,89 ab
5	1,24 a	0,114 ab	8,69 a	8,72 ab
6	1,19 a	0,124 a	8,23 a	10,84 a
7	0,98 ab	0,094 b	6,23 c	8,22 b
CV (%)	10,84	11,97	13,51	16,01

Means followed by the same letter in the column do not differ from each other according to the Tukey test ($P \leq 0.05$). T1=*A. japonicus*, T2=*A. japonicus* with *T. tomentosum*, T3=*A. japonicus* with *T. viride*, T4=*T. tomentosum*, T5=*T. tomentosum* with *T. viride*, T6=*T. viride*, T7=autoclaved rice without fungus

The treatments T5 (*T. tomentosum* with *T. viride*) and T6 (*T. viride*) led to the lowest AUDPC values, thus demonstrating that spot blotch, gibberella and rust severity was approximately 50 % lower than that of the control group (Figure 2). So, the treatments with inoculated fungi were able to minimize the development of fungal diseases.

The T7 treatment (control) was the one that allowed the greater development of the diseases, since it presented the highest AUDPC, followed by the T1 (*A. japonicus*), but still this one protected 25% more than the control; in contrast, the treatments T5 and T6 were the ones that presented the greater protection. These data were observed for the three pathogens tested. It is also shown that T4, T5, and T6 were more efficient when acting against the pathogens *B. sorokiniana* and *F. graminearum* (Figure 2).

Table 2. Agronomic characteristics and yield of wheat cv. Tbio Iguaçu (Boa Ventura de São Roque, PR, Brazil). Field experiment

Treatment	Yield kg·ha^{-1}	Number of ear m^{-2}	Number of grains ear^{-1}	Plant height (cm)
		Crop 2015		
T1	2608 b	347,8 a	25,0 c	87,8 c
T2	2674 b	348,2 a	25,6 b	87,2 c
T3	2684 b	349,6 a	25,6 b	88,0 b
T4	2678 b	348,8 a	25,6 b	88,9 b
T5	2833 a	349,8 a	27,0 a	89,7 a
T6	2719 b	348,6 a	26,0 c	86,9 d
T7	2385 c	339,8 b	23,4 d	85,2 e
CV (%)	1,72	0,32	1,64	0,42
		Crop 2016		
T1	2553 c	346,0 b	24,6 bc	86,5 d
T2	2559 c	346,8 ab	24,6 bc	86,9 d
T3	2661 bc	346,5 ab	25,6 ab	87,7 c
T4	2701 ab	349,0 a	25,8 a	87,7 c
T5	2730 a	350,0 a	26,0 a	91,5 a
T6	2517 c	349,6 a	24,0 cd	89,7 b
T7	2386 d	340,0 c	23,4 d	85,9 e
CV (%)	6.71	1.05	4.15	1.67

Means followed by the same letter in the column do not differ from each other according to the Tukey ($P \leq 0.05$). T1=*A. japonicus*, T2=*A. japonicus* with *T. tomentosum*, T3=*A. japonicus* with *T. viride*, T4=*T. tomentosum*, T5=*T. tomentosum* with *T. viride*, T6=*T. viride*, T7=autoclaved rice without fungus

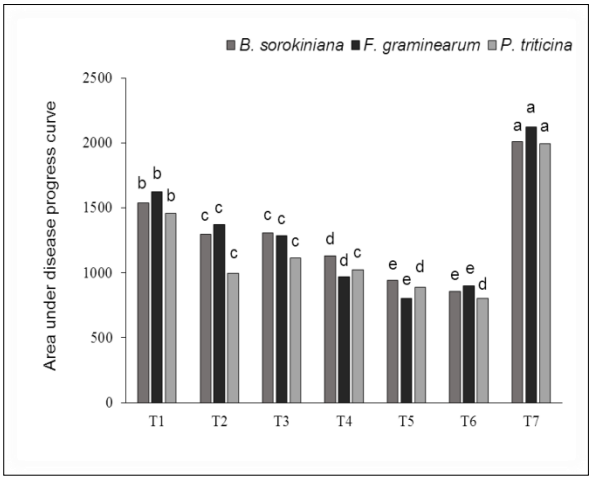

Figure 2. Area under disease progress curve of the wheat plants grown on soil that were inoculated with different fungi and the *B.* sorokiniana, *F. graminearum and P. triticina* pathogens were inoculated. Means followed by the same letter do not differ from each other according to the Tukey test ($P \leq 0.05$). T1=*A. japonicus*, T2=*A. japonicus* with *T. tomentosum*, T3=*A. japonicus* with *T. viride*, T4=*T. tomentosum*, T5=*T. tomentosum* with *T. viride*, T6=*T. viride*, T7=autoclaved rice without fungus. Mean of the experiments of 2015 and 2016

The highest PAL activity in wheat seedling leaves (Figure 3A) was observed by inoculation with *T. tomentosum*, with approximately four-fold higher activity than that of the control group. The treatments T5 (*T. tomentosum* with *T. viride*), T4 (*T. tomentosum*) and T6 (*T. viride*) led to the highest GLU activity (Figure 3B), which where two-fold higher in comparison with the control. Higher POD activity (Figure 3C) was observed under the treatments T4 (*T. tomentosum*) followed by T5 (*T. tomentosum* with *T. viride*). The treatments T4 (*T. tomentosum*) and T5 (*T. tomentosum* with *T. viride*) led to the highest SOD activity in wheat seedling leaves (Figure 3D).

DISCUSSION

In the laboratory experiment (Figure 1), when analyzing the inhibition of pathogens against antagonistic fungi, all antagonistic fungi were efficient in this process. The most expressive results can be observed with the *T. viride* antagonist against all tested pathogens (*B. sorokiniana, F. graminearum* and *P. triticina*). These inhibition indices gave us support for the

greenhouse and field tests, because the results indicate that the antagonistic fungi tested have mechanisms of action that inhibit the growth of pathogenic fungi.

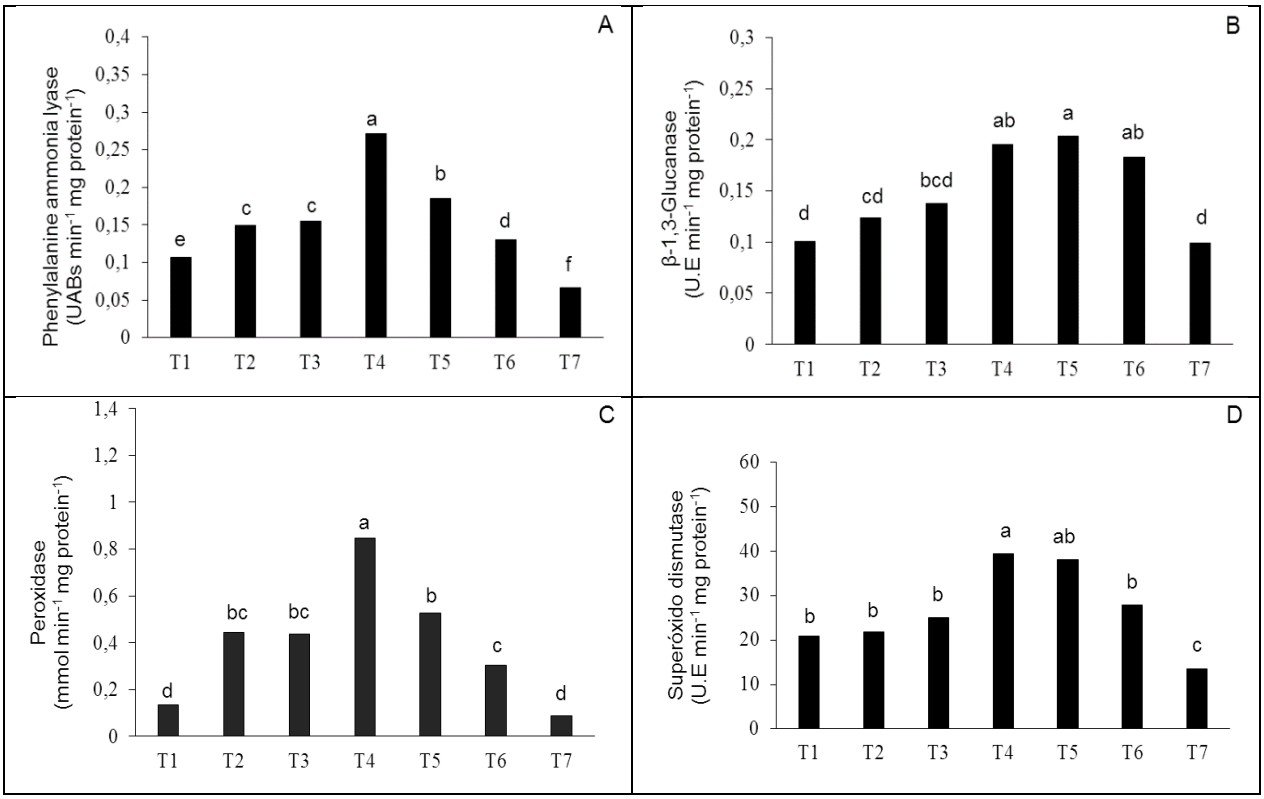

Figure 3. Activity of (A) phenylalanine ammonia lyase (PAL), (B) β-1,3-glucanase (GLU), (C) peroxidase (POD), and (D) superoxide dismutase (SOD) of seven day old wheat plants. Means followed by the same letter do not differ from each other according to the Tukey test ($P \leq 0.05$). T1=*A. japonicus*, T2=*A. japonicus* with *T. tomentosum*, T3=*A. japonicus* with *T. viride*, T4=*T. tomentosum*, T5=*T. tomentosum* with *T. viride*, T6=*T. viride*, T7=autoclaved rice without fungus. Mean of the experiments conducted in 2015 and 2016

In Table 1, in the greenhouse experiment, with treatments based on inocula of antagonistic fungi in the pots, it was possible to observe the development of wheat seedlings, both dry matter and their growth. The least effective treatment was T7 (the control, containing only autoclaved rice without fungus) whereas the treatment with the best result was T6 with *T. viride* fungus. Generally the other treatments with *A. japonicus* and *T. tomentosum* also reached the goal of encouraging the development of the seedlings. Thus, these data show that the antagonistic fungi have activity as growth promoters.

Our results show that the application of the fungi contribute to the increase of the dry matter and height of the shoot, generally improving the development of the shoots. They also indicate that soil inoculation with *Trichoderma* spp. can contribute to the development of wheat seedlings. Similarly, Sharma et al (2012) evaluated the capability of *T. harzianum* for induction of wheat growth through soil, seed and leaf application and observed increase in radicle length, tiller number, grain weight and production under all application methods.

The isolated fungi seem to be endophytic, since they were isolated from the leaves of the plants. And it was found that in general they contributed to the promotion of growth and also to decrease the severity of the diseases, perhaps by the greater activity of the enzymes.

The metabolic cost exists, however, the

positive effect on disease control compensates for the loss of energy spent with the activation of the defense routes. The plant, when defending, loses less by the attack of the disease, because it has less damaged leaves and therefore less photosynthetic area being destroyed; all this compensates the metabolic expense to defend itself, and at the end, still has more production.

In the field experiment, conducted in two years, it was possible to verify that the highest productivity was observed with T5 (*T. tomentosum* with *T. viride*), in both years. Probably, this interaction of antagonistic fungi (T5) over time, that is, while the plants grow they have mechanisms of action that together become potentiated, diminished the action of the pathogens and contributing to the development of the plants. The fungus *Trichoderma* spp. presents different types of actions when applied in plants, as it can act as a growth promoter, contributing to an improvement in the soil organic composition, can produce compounds that benefit the plants and still inhibit the development of pathogens (Vinale et al., 2008).

Such findings also corroborate those of Hasan et al. (2013) and Perello et al. (2003), who carried out a greenhouse experiment and observed that the application of different *Trichoderma* spp. strains significantly reduced disease severity in wheat plants. Furthermore, Perello et al. (2003) reported a significant reduction (16-35%) in tan spot severity after *Trichoderma* spp. application in wheat plants. Our results show that all fungi were able to inhibit the growth of phytopathogens, *in vitro*, in general data around 70 %, and in the field experiments around 30-50 %.

Küçük et al. (2007) obtained better results for wheat growth promotion and protection against *B. sorokiniana* after soil inoculation with *T. harzianum*. This antagonist has also been effective controlling pathogenic species of the genus *Bipolaris* in other cereals, like rice (Pérez et al., 2018).

In Figure 2, where the AUDPC shows the evaluations of the severity of the diseases developed by these pathogens, treatments behaved in a similar way; this may indicate that these antagonists have broad spectrum of action and the treatments T5 and T6 were the most that controlled the development of the disease while the control was the least effective. These data go hand in hand with productivity data (Table 2).

El-Sharkawy et al. (2015) concluded that *T. harzianum* and *Streptomyces viridosporus* bioinducers treatments have best beneficial effects as compared to chemical fungicide, and mentioned that the application of bioinducers is applicable, safe and cost effective method for controlling leaf rust severity in wheat. Their results revealed that the weight of spikes and grains, and amounts of chlorophyll were lower in the infected plants than in healthy ones.

In Figure 3, which shows the enzymatic activity in the plant, the treatments that presented the best results were T4 followed by T5, while the control was the one that less activated the enzymes. The enzyme PAL with the T4 treatment, GLU with T4, T5 and T6, POD with T4, and SOD with T4 and T5 increased their activity in two-fold or higher in comparison with the control

Since these enzymes participate in plant protection metabolism they contribute to the reduction of disease severity (Figure 2) and consequently to the growth and development of plants (Table 2). Antagonistic fungi and the fungi *Trichoderma* sp. are usually associated with the onset of defense mechanism, including expression and enhancement of defense enzymes such as POD, PAL, and accumulation of phenolic compounds, phytoalexins and lignins, thus favoring their protection against pathogens (Waghunde et al., 2016).

Based on our results, the action of the enzymes can be a defense mechanism, protecting the plant from the attacks of phytopathogens and as a consequence there may be a promotion of growth. And the promotion of growth can also be achieved by simple contact of the fungi with the plant.

Foroutan et al. (2013) showed that *T. harzianum* significantly reduced the incidence (7.66 %) and severity (3 %) of disease caused by *Fusarium* 42 days after inoculation and increased the grain weight in greenhouse conditions by 64 % over the *Fusarium* inoculated plants, almost at the level of non inoculated control. For confirmation of the greenhouse tests, the selected antagonists were re-examined in field trials and *T. harzianum* also reduced the disease incidence (10 %) and severity (4.42 %), and increased the yield of wheat under field conditions at more than double

compared with the *Fusarium* inoculated control.

New technologies have emerged for improving agricultural production, but some practices affect the environment. Thus, there is an immediate need to find ecological solutions including a wider application of biological control agents (BCAs). Several microorganisms can be used as BCAs since they induce systemic resistance in plants and reduce diseases in different crops (Hasan, 2013).

Among the various species, the fungus *Trichoderma* produces different substances that play important roles on the biological control of phytopathogenic fungi, such as cell wall degradation, mycoparasitism and competition for space and nutrients. Moreover, they can also induce plant resistance, thus decreasing disease severity (Waghunde et al., 2016).

Similarly, Zafari et al. (2008) evaluated wheat plants presenting leaf rust and observed the effects of biological control using different *Trichoderma* species, which reduced disease severity (25-55 %) and led to increase in sprout dry matter (27-59 %) and root dry matter (23-58 %).

According to Harman (2006), *Trichoderma* fungi have been known since 1920 by their capability of acting as BCAs against plant pathogens. The main control mechanisms of pathogens include parasitism, antibiosis and competition for resources and space. They are also capable of activating localized and systemic resistance, increasing plant growth and nutrient uptake, increasing the activity of some enzymes such as chitinases (EC 3.2.2.14) and GLU, improving microflora composition, and, in the soil, they improve nutrient solubilization and root development.

Similarly, Dutta et al. (2014) reported that the inoculation of endophytic fungi in wheat activates systemic resistance and the gene expression of pathogenesis-related proteins (GLU, chitinase).

Furthermore, Karthikeyan et al. (2006) applied *Trichoderma viride* and *T. harzianum* in coconut roots infected with *Ganoderma lucidum* and observed a significant increase in POD, PPO, PAL, chitinase, and GLU activities and in phenolic compound concentration.

Muthukumar et al. (2011) applied endophytic microorganisms (*T. viride* and *P. fluorescenses*) as BCAs in chilli plants and observed higher PAL, POD and polyphenol oxidase (PPO, EC 1.10.3.1) activities and higher phenolic compound concentration, thus indicating the activation of defense mechanisms, leading to disease reduction and higher productivity. In an attempt to defend against oxidative damage, wheat plants under different stresses, including during host-pathogen interaction, may trigger the activity of enzymes (Ebrahim, 2011).

Our results show that the inoculation of these fungi contributes to the enzymatic activity in the plants because they increased the activity of important enzymes, such as phenylalanine ammonia lyase, β-1,3-glucanase, peroxidase and superoxide dismutase. We also found that *Trichoderma* spp. may improve physiological responses to stress, induce resistance to pathogens, and have mechanisms of mycoparasitism. This antagonist has great potential in agriculture, to be used as plant protector and growth enhancers, besides their application in several industrial processes

CONCLUSIONS

The fungi inoculated in the soil can reduce the spot blotch, gibberella and rust severity since they induce resistance and activate physiological changes in wheat plants. When used alone, *A. japonicus* allowed some development of the diseases, but still it protected 25 % more than the control. The inoculation of *Trichoderma tomentosum* and *Trichoderma viride* (combined) led to the lowest severity of fungal diseases, while the inoculation of *T. tomentosum* (singly) resulted in the greatest development of wheat seedlings. Fungi also increased plant yield. PAL, POD and SOD activities were higher under soil inoculation of *T. tomentosum*, while the activity of GLU and SOD were more expressive under inoculation of *T. tomentosum* and *T. viride* (combined). The isolated fungi seem to be endophytic, since they were isolated from the leaves of the plants. And it was found that, in general, they contribute to the promotion of growth and also to decrease the severity of the diseases, perhaps by the greater activity of the enzymes.

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