

## Biocontrol strategies to reduce the impact of *Septoria tritici* blotch in wheat

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*Trichoderma* strains are used as biofungicides for some plant diseases. The aim of this research was to evaluate the effectiveness of *Trichoderma harzianum* isolates, applied alone and in combination with fungicides, to control *Zymoseptoria tritici*, the causal agent of *Septoria tritici* blotch of wheat, and their impact on wheat yield and its components. To this end, field experiments were performed in 2010 and 2011 and 10 different treatments were applied. The disease severity was assessed by visual estimation of the leaf area affected by *Z. tritici* at the first node, anthesis and early dough growth stages. The best results for reducing the area under the disease progress curve (AUDPC) were "coated seed only with *T. harzianum*" and "coated seed plus two foliar application of *T. harzianum*". Regarding the increase in yield gain and the improvement of yield components, the fungicide treatment applied at seedling, and tillering in 2011 provided significant increase. Respect the treatments with the application of *T. harzianum* the best was only one application as coated seed of the biocontroler alone showing yield responses similar to the ones obtained with the fungicide treatments. We recommended the coated seed alone because the protective effect lasts until the early dough stage of ripening. This application produced a comparable yield to that obtained with three applications of *T. harzianum* at different phenological stages of wheat with as well as with more than one application of commercial fungicide. We found a strong relationship between the number of kernels per spike and the wheat yield in 2011 as a consequence of the best environmental conditions for the disease's occurrence. Contrary, it was shown a non-significant association between thousand kernel weight (TKW) and the wheat yield.

**Key words:** biological control; *septoria tritici* blotch; *Trichoderma harzianum*; wheat yield; fungicide

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Las cepas de *Trichoderma* se usan como biofungicidas para el control de algunas enfermedades de las plantas. El objetivo de esta investigación fue evaluar la efectividad de dos cepas de *Trichoderma harzianum*, aplicadas solas y en combinación con fungicidas, para controlar *Zymoseptoria tritici*, el agente causal de la mancha de la hoja del trigo, y su impacto en el rendimiento del trigo y sus componentes. Con este fin, se realizaron experimentos de campo en 2010 y 2011 y se aplicaron 10 tratamientos diferentes. La severidad de la enfermedad se evaluó mediante la estimación visual del área de la hoja afectada por *Z. tritici* en el estadio de primer nudo, en antesis y en grano pastoso. Los mejores resultados para la reducción del área bajo la curva de progreso de la enfermedad (ABCPE) fueron "semillas recubiertas solamente con *T. harzianum*" y "semillas recubiertas más dos aplicaciones foliares de *T. harzianum*". Para un mayor rendimiento y la mejora de los componentes de rendimiento, el mejor tratamiento fue: solo una aplicación de *T. harzianum* (como "semillas recubiertas"). Recomendamos este tratamiento porque el efecto protector dura hasta la etapa temprana de maduración del grano. Esta aplicación produjo un rendimiento comparable al obtenido con tres aplicaciones de *T. harzianum* en diferentes estados fenológicos del trigo y, además de con más de una aplicación de fungicida comercial. Existió una relación significativa entre el número de granos por espiga y el rendimiento de trigo en 2011, como consecuencia de las mejores condiciones ambientales para el desarrollo de la enfermedad. Por el contrario, se mostró una asociación no significativa con el peso de mil granos.

**Palabras clave:** control biológico; *septoria tritici* blotch; *Trichoderma harzianum*; rendimiento del trigo; fungicidas

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## INTRODUCTION

“*Septoria tritici* blotch” (STB), caused by *Zymoseptoria tritici* (Desm.) Quaedvlig & Crous, is an important disease causing significant yield losses in many wheat-producing areas of the world (Thompson & Gaunt, 1986; Rodrigo et al., 2015). Early infections can reduce the number of grains per spike, whereas late infections can reduce the thousand kernel weight (Simón et al., 2002). In Argentina, yield losses of 20-50% were reported (Annone et al., 1991; 1993), whereas Simón et al. (1996) reported a reduction in thousand kernel weight of infected plants of 3-13%. In Argentina, the most frequent genetic background of wheat cultivars varies from moderately susceptible to moderately resistant to STB (Simón et al., 2013). Thus, a frequent approach to control STB is breeding for resistance (Simón et al., 2016), but in the wheat growing area, STB is controlled mainly by the use of fungicides.

The fungicides usually used consist of mixtures of triazoles (which inhibit ergosterol biosynthesis) and strobilurins (which inhibit mitochondrial respiration) in the same formula because the latter could lead to substantial increases in yield in the order of 10-20% above that produced by conventional fungicides. These increases would be associated with the maintenance of canopy photosynthesis during the grain filling period but also with the prolongation of the duration of the green leaf area (Gooding et al., 2000).

In an effort to minimize the use of chemicals, several researchers have investigated the use of antagonistic microbes, both alone and as supplements. In Argentina, for example, the biocontrol of *Z. tritici* via foliar application on wheat leaves has been well documented (Perelló et al., 1997; Perelló et al., 2006; Cordo et al., 2007; Perelló et al., 2008; Stocco et al., 2012).

Members of the fungal genus *Trichoderma* are able to act as biocontrol agents (Harman, 2006; Harman et al., 2004; Perelló et al., 2008). They protect plants against the attack of plant pathogens by competing for nutrients and inhibiting or killing plant pathogenic fungi and oomycetes through the production of antibiotics and/or hydrolytic enzymes. The contribution of *Trichoderma* spp. to plant growth and defense mechanisms were reviewed (Hermosa et al., 2013) by describing the complex plant defense signaling network, that allows the recognition of this fungus as non-hostile microbes.

In Argentina, some products based on *Trichoderma* are manufactured under trademarks as Rizoderma® by Rizobacter and Biagro TL® by Biagro S.A. and used as fertilizers or growth promoters. Moreover, many *Trichoderma* strains are included as active biofungicide matter in registered biological substances or commercial formulations against plant pathogenic fungi (Monte, 2001). Among them, *Trichoderma harzianum* Rifai is a soil-borne free-living fungus that colonizes the roots of many plants as an opportunistic, non-virulent plant symbiont (Harman et al., 2001), which has been used in biotechnological applications both as a biofungicide and as a growth promoter, providing important benefits to agriculture under field conditions (Lorito et al., 2004). So far, all published works on the biocontrol of *Z. tritici* by *T. harzianum* in wheat have studied only the reduction of disease severity. Thus, to gain further insights into the biocontrol of *Z. tritici* by *T.*

*harzianum*, the aim of this study was to evaluate the effect of *T. harzianum*, applied alone and in combination with fungicides, on grain yield and its components in wheat plants affected by *Z. tritici*.

## MATERIALS AND METHODS

### Isolation of *Z. tritici* strains from infected leaves

Two virulent strains of *Z. tritici* (FALP9J008 and FALPLA008), from the cities of 9 de Julio and Plá (Buenos Aires province, Argentina) respectively, were obtained from wheat leaves with typical lesions with pycnidia. Each strain was grown on potato-dextrose agar (PDA; Difco Laboratories) at 20±2°C. Pycnidia developed spores that were released under the form of a cirrus. One single spore was isolated and cultured on water agar. On day 6 after isolation the small colony developed from the single spore was replicated in Petri dishes onto modified malt agar (30 g malt extract, 5 g mycological peptone, 2 g yeast extract and 1000 mL distilled water) for sporulation. On day 9, the mucous mass of conidia for each strain was harvested by flooding the plate with 5 mL of sterile distilled water and dislodging the conidia with a bent glass rod. The resulting suspension was filtered through cheesecloth and the concentration of the conidial suspension was adjusted to 5 x 10<sup>6</sup> spores mL<sup>-1</sup> as were reported by Cordo et al, (2007) and Perello et al. (2008). The inocula from the two strains were mixed at equal volumes to obtain the inoculum volume used in the experiment adjusted for each growth stage. The resulting suspension was mixed with 50 µL<sup>-1</sup> (0.05% v/v) of Tween 20 in aqueous solution as a surfactant.

### Identification of *Trichoderma* species

Two isolates of *Trichoderma* spp. (FALH5cc and FALH118c, isolated from soil samples of cultivated wheat and from soil samples of cultivated tomatoes, respectively) were chosen from the collection of Centro de Investigaciones de Fitopatología of the Facultad de Ciencias Agrarias y Forestales of the Universidad Nacional de La Plata, Argentina. These strains have been previously evaluated in other experiments with different cultures in INBIOTEC-CONICET-FIBA (Cordo et al., 2007; Mónaco et al., 2008).

The DNA of each *Trichoderma* strain was obtained following a modified version of the cetyltrimethylammonium bromide (CTAB) method (Murray & Thompson, 1980). The internal transcribed spacers (ITS) of the ribosomal DNA (rDNA) region from each *Trichoderma* isolate were PCR amplified. The PCR products were separated by electrophoresis in a gel containing 1% agarose (Genbiotech SRL, Buenos Aires, Argentina) in 1x TAE buffer (40 mM tris-HCl, pH 8, and 2 mM EDTA). Gels were run for 1 h at 120 v, stained with gel red (Biotium, Hayward, USA) and photographed with a fotodyne system (Hartland, WI, USA). The PCR amplification products were purified and sequenced by Macrogen (Korea), using the primer ITS4.

Each sequence was uploaded to Genbank and compared with those of known origin by using the blast search program (Altschul et al., 1997). Based on its sequences the strains FALH5cc and FALH118c (Cordo et al., 2007; Mónaco et al., 2008) were PCR amplified with specific primers and sequenced. Both ITS

sequences were in the European molecular biology laboratory (EMBL) database (Miyazaki et al. 2009). The strain FALH5cc, (accession number LN 869401) and the strain FALH118c, (accession number LN 869400), had 99% of similarity with a strain (Accession N° T6776) of *T. harzianum*, and both strains had 99% of similarity with a strain of *Hypocrea lixii* (Cib 1136), that is the teleomorph stage of them.

#### Production of *Trichoderma* sp. Inoculum

The two *T. harzianum* isolates mentioned above were used for inoculum production. The isolates were cultured on PDA in Erlenmeyer flasks and incubated for 7-15 days at  $20\pm 2^\circ\text{C}$  in a growth chamber under a photoperiod of 12 h with fluorescent plus near ultraviolet lights. The conidia of each isolate were harvested by flooding the cultures with sterile distilled water and then rubbing the culture surfaces with a sterile glass rod. After filtering the suspensions through two layers of cheesecloth, the concentrations of propagules in the suspensions were standardized with the aid of a hemocytometer to  $1 \times 10^8$  conidia per mL for each *Trichoderma* isolate tested. Suspensions were amended with one drop of Tween 20 at 0.05 % (v/v) and shaking with a magnetic stirrer bar until a homogeneous suspension was obtained. Wheat seeds (50 g) of the cultivar Buck 55CL2 (moderately susceptible) (reported by Buck catalog 2010) were added to 100 mL of the water agar-fungal biomass mixture to coat the seeds. Seeds were dried overnight at room temperature and stored in hermetically closed glass flasks at  $5^\circ\text{C}$  in a refrigerator for 24-48 h until used.

#### Field experiment

The fungicide used for the combined treatment was the formulated product Amistar Xtra® (20% w/v of Azoxystrobin and 8% w/v of Cyproconazole) at a rate of  $300 \text{ cm}^3 \text{ ha}^{-1}$  in a volume of 150 L of water. To evaluate the sensitivity of *Trichoderma* to this fungicide, it was filtered with a Millipore membrane of  $0.45 \mu\text{m}$  and added to the PDA medium at  $50^\circ\text{C}$  after autoclaving. Aliquots of 19000 ppm (Leroux et al., 2007) of the formulated product were incorporated on each PDA plate. One disc of 6 mm in diameter of each isolate of *T. harzianum* coming from a 6-day-old culture on PDA was placed in the center of the plate on the surface of the culture medium. Ten replicates of each *T. harzianum* isolate used for each condition (with and without fungicide) were performed. The plates were incubated at  $22^\circ\text{C}$  and 12 h of light/dark cycles and observed after 7 day of incubation. The colony size was measured with a caliber and was expressed in cm. Fungicide tolerance for each isolated was determined by calculating the relative colony size with and without the fungicide.

#### Evaluation of *T. harzianum*, applied alone and in combination with the fungicide, on STB severity

Field experiments were carried out at the Estación Experimental J. Hirschhorn, Facultad de Ciencias Agrarias y Forestales, Universidad Nacional de La Plata, Buenos Aires, Argentina ( $34^\circ 52' \text{ S}$ ,  $57^\circ 58' \text{ W}$ ) for two years (2010-2011). The soil was a Phaeozem luvico (USDA –FAO 1975) with a silt loam texture and slight internal drainage deficiency. The analysis of the soil samples (top 0.20 m) indicated the following values by

weight: organic matter 3%, N= 0.17%, P = 7 mg  $\text{kg}^{-1}$ , and pH = 5.9.

Weather conditions (mean monthly values of temperature ( $^\circ\text{C}$ ), precipitation (mm), relative humidity (RH), and global radiation ( $\text{W m}^{-2} \text{ d}^{-1}$ ) were recorded at a meteorological station situated 100 m from the experiments.

Plots consisted of  $4.2 \text{ m}^2$  (1.4 m wide by 3 m long). The total area of each replication, which consisted of 21 plots, was  $84.2 \text{ m}^2$ . The entire experiment was fertilized with N (as urea  $100 \text{ kg ha}^{-1}$ ) and P ( $50 \text{ kg PO}_4 (\text{NH}_4)_2 \text{ ha}^{-1}$ ) at the time of sowing.

The experimental design was a complete randomized block with 10 treatments (T1-T10) and the control (Table 1) and three replications with a row-to-row distance of 15 cm in both years. The bread wheat (*Triticum aestivum* L.) cultivar Buck 55 CL2 was sown at a sowing density of 320 and 300 plants  $\text{m}^{-2}$  for 2010 and 2011, respectively, depending of the date of sown.

Two *T. harzianum* isolates were applied as follows: i) as seed coating ( $1 \times 10^8$  conidia  $\text{mL}^{-1}$  of each *T. harzianum* isolate (Perello et al., 2008), ii) as spraying the spore suspension alone ( $1 \times 10^8$  conidia  $\text{mL}^{-1}$  of each *T. harzianum* isolate (Perelló et al., 2008) or iii) as spraying the spore suspension combined with half the doses of the fungicide Amistar Xtra®, equivalent to  $300 \text{ cm}^3 \text{ ha}^{-1}$ . The goal of the fungicide applications was to compare the efficacy of the *T. harzianum* treatment with that of the fungicide. The fungal inoculum and fungicide were applied three times between August and October at the seedling (GS12), (Zadoks et al., 1974), tillering (GS31) and heading growth stages (GS54). When the fungicide was applied alone, it was sprayed at a rate of  $600 \text{ cm}^3$  in 150 L of water per  $\text{ha}^{-1}$  at each growth stage for the same plants and lastly at heading as the only moment of application. Oat strips were sown between plots to avoid the spread of the inoculum between treatments. Oat strips were also sown between replications.

Strategies used: = T1, T2 and T3 biocontrol only; T4 to T6: biocontrol and fungicide combined; T7 to T10: fungicide only (negative control).

To ensure the vertical dispersion of the disease, the pathogen was inoculated three times. Both for the inoculation with *Z. tritici* and for the application of *T. harzianum* strains, the final volume for each growth stage was changed according to the size of plants. The final volume at seedling was 1.5 L, at tillering 2.75 L, and at heading 5 L for each treatment. Plants were sprayed with the inoculum suspension until runoff.

After inoculations, plants were kept moist by spraying water several times a day (every 3 h from 9 am to 18 pm), for 3 days with a sprayer for applying pesticides. The pathogen was inoculated in all the treatments, including the controls.

In both years, the disease severity was assessed by visual estimation of the percentage of the leaf area affected by *Z. tritici* on twelve plants from each plot at the following growth stages: on the upper two leaves from the second and sixth rows when each cultivar reached the first node (GS31), anthesis (GS62) and early dough (GS82). The area under the disease progress curve (AUDPC) for each treatment was calculated to summarize the progress of the disease (Shanner & Finney, 1977).

Table 1: Treatments applied on the wheat cultivar Buck 55CL2 to control STB either with *Trichoderma harzianum* strains alone or combining *T. harzianum* and a fungicide. The pathogen *Z. tritici* was artificially inoculated in all the treatments.

Treatments	Growth Stages	
Positive Control	GS12, 31, 54	wheat plants inoculated only with <i>Z. tritici</i>
T1*	GS12	wheat seeds coated with strains of <i>T. harzianum</i>
T2	GS12, 31	wheat seeds coated with strains of <i>T. harzianum</i> + <i>T. harzianum</i> spraying onto the leaves at tillering
T3	GS12, 31,54	wheat seeds coated with strains of <i>T. harzianum</i> + <i>T. harzianum</i> spraying onto the leaves at tillering and heading.
T4	GS12	wheat seeds coated with <i>T. harzianum</i> strains + foliar fungicide (half commercial dose) at seedling + <i>T. harzianum</i> spraying onto the leaves.
T5	GS12, 31	wheat seeds coated with <i>T. harzianum</i> strains + foliar fungicide (half commercial dose) at tillering + <i>T. harzianum</i> spraying onto the leaves.
T6	GS12, 31, 54	wheat seeds coated with <i>T. harzianum</i> strains + foliar fungicide (half commercial dose) at tillering and heading + <i>Trichoderma</i> spraying onto the leaves at tillering and heading.
T7	GS12	foliar fungicide (commercial dose) at seedling. Negative control.
T8	GS12, 31	foliar fungicide (commercial dose) at seedling and tillering. Negative control.
T9	GS12, 31, 54	foliar fungicide (commercial dose) at seedling, tillering and heading. Negative control.
T10	GS54	foliar fungicide (commercial dose) at heading. Negative control.

### Evaluation of yield components

The yield components calculated for each plot were: number of spikes per square meter (SPM<sup>2</sup>), number of kernels per spike (KPS), and thousand kernel weight (TKW). To this end, three 1-m sections from the three central rows of each plot were hand-harvested and threshed and the number of spikes per square meter counted. KPS was then determined in 30 spikes, by threshing the heads and counting the kernels. The kernels counted in the 30 spikes were weighed to calculate TKW. Grain yield (in kg ha<sup>-1</sup>) was calculated using the yield components.

The percentage of yield gain due to the effect of the different treatments with *T. harzianum* respect to the control (wheat inoculated only with *Z. tritici*) was calculated as a comparison between the yield in the control (i.e. in the absence of fungicide application but in presence of disease) and the yield obtained in each of the treatments (T1-T10). The yield gain in the control was set as 0.

### Statistical analysis

To assess the effect of the different treatments on the evolution on the AUDPC values and on yield and yield components, an ANOVA was performed for data of both years separately, according to the variables year, treatments, and *T. harzianum* isolates. As there was not different behavior between the *Trichoderma* strains used in this assay, the values of the AUDPC and yield for both strains were averaged. Tukey's test (P<0.05) was applied for the variables and interactions that differed significantly.

Linear regression analysis was used to describe the relationship between AUDPC values and grain yield of wheat plants affected by STB. The equation was  $Y = a X$

+ b, where Y is the predicted yield, b is the expected yield in the absence of disease (both expressed in kg ha<sup>-1</sup>), X corresponds to the disease score, and a is the regression coefficient that expresses the linear yield loss in kg ha<sup>-1</sup>.

## RESULTS

### Effect of *T. harzianum*, applied alone and in combination with the fungicide, on STB severity

In vitro test revealed that none of the *T. harzianum* isolates were affected by the fungicide dose used in the combined treatment. The mycelia of both biocontrol agents reached 9 cm of mean diameter at 7 days of growth.

In 2010, the average temperature for the entire period after inoculation was 15.7°C, the total rainfall was 257.2 mm, the average RH was 71%, and the average radiation was 4347 W m<sup>-2</sup> d<sup>-1</sup>, whereas in 2011, the average temperature was 15.9°C, the total rainfall was 278.2 mm, the average RH was 68 %, and the average radiation was 5852 W m<sup>-2</sup> d<sup>-1</sup>.

The temperature and radiation in 2011 during August and September were higher than in 2010 (Table 2). This increase could favour the expression of tillers. For this reason SPM<sup>2</sup> could be influenced with the increase in number of plants, leaves and the future spikes.

Because of the greater precipitations in 2011, the environmental conditions in this year could be more favorable for the development of the disease. The total rain accumulated between tillering and heading (October 4 and November 21) in 2011 was 141.2 mm, whereas in the same period in 2010, was only 47.8 mm. The 73 mm more of rainfall across 48 days could explain the better development of the *Septoria tritici* blotch in 2011 because

important stages of plant differentiations (from the first node visible to boot stages) took place, increasing the final number of infected leaves.

The AUDPC values were compared with the positive control (wheat plants only inoculated with *Z. tritici*) to obtain the reduction of the severity for the effect of the treatments. The negative controls (T7, T8, T9 and T10) added results to define the effectiveness of the various *T. harzianum* treatments, comparing each *T.harzianum* treatment with each negative control relating them according to their plant growth stages.

There was a significant effect of the year × treatment interaction on the AUDPC variable ( $F = 3.081, P \leq 0.0021$ ) (data not shown). Treatments T2 and T4 for 2010 and T1 and T3 for 2011, were significantly different to the positive control. Treatments T7, T8, T9 and T10 also were significantly different to the same control, although T5 and T6 were not significantly different for both years. All treatments based on fungicide application caused a significant decrease in the amount of disease (T7 to T10) with the lower values of AUDPC for T8, T9 and T10 for both years of testing (Figure 1).

Table 2. Weather variables affecting wheat in the field experiment during August –December 2010-2011 in Estación Experimental J. Hirschhorn, La Plata, Buenos Aires province, Argentina. Ref.: Mean T°C = mean temperature in Celsius degrees; S. mean T°C = soil mean temperature in Celsius degrees; Sun Radiation = in Wats .m-2; Pp = Precipitation in mm; ETP= Evapotranspiration in mm; RH = Relative humidity in percentage (%).

Months	Mean T°C		S. mean T°C		Radiation		Pp		ETP		RH	
	2010	2011	2010	2011	2010	2011	2010	2011	2010	2011	2010	2011
August	9.8	9.7	9.5	10.3	2513	2260	26.8	56	54.1	50.7	76	76
September	13.1	13.7	12.8	13.6	3171	3665	111	21.4	72	94.8	78	64
October	14.8	15.2	14.7	17	4621	4239	40.4	60.4	107	100	73	73
November	18	20.3	17.6	21.4	5302	5586	28	81.8	127	143	70	64
December	22.6	20.4	21.6	21.4	6128	5852	51	58.6	176	152	58	68

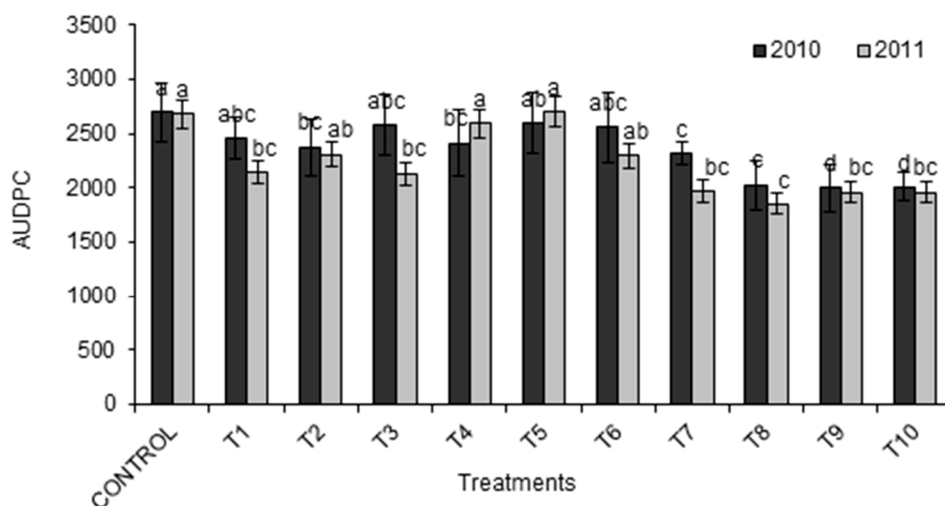


Figure 1. Means of the area under the disease progress curve (AUDPC) (two upper leaves) of the “Septoria tritici blotch” (STB) caused by *Zymoseptoria tritici* in 10 treatments with *Trichoderma harzianum*/fungicide and the positive control (CONTROL) (Table 1) for the wheat cultivar Buck 55 CL2 in field experiments carried out in 2010 and 2011. Strategies used: = T1, T2 and T3 biocontrol only; T4 to T6: biocontrol and fungicide combined; T7 to T10: fungicide only (negative control). Treatments with similar letters are not statistically different. Bars indicated standard errors of the means.

### Effect of *T. harzianum*-based treatments on yield components

The yield values obtained in 2010 and 2011 did not statistically differ from the positive control, except T9 for 2010, which included three fungicide applications. Although the yield values do not differ statistically between treatments, they are higher than those of the control for both years (Table 3).

Table 3. Means of wheat yield ( $\text{kg}\cdot\text{ha}^{-1}$ ) in 10 treatments with *Trichoderma harzianum*/fungicide and the positive control (CONTROL) for the wheat cultivar Buck 55 CL2, infected by *Zymoseptoria tritici* in field experiments carried out in 2010 and 2011. Strategies used: = T1, T2 and T3 biocontrol only; T4 to T6: biocontrol and fungicide combined; T7 to T10: fungicide only (negative control). \*treatments with similar letters are not statistically different.

Treatments	Grain Yield ( $\text{Kg}\cdot\text{ha}^{-1}$ )	
	2010	2011
Control	4287.8ab	2987a
T1	5425.2abc*	4086.1ab
T2	5573.3bc	4366.3ab
T3	5749.6bc	4455.5ab
T4	5380.9abc	4007.2ab
T5	4934.8abc	4354ab
T6	4714.8abc	4417.0ab
T7	4876.4abc	4351.0ab
T8	5209.8abc	5363.3b
T9	5986.3c	3960.3ab
T10	3970.7a	4073.0ab

The regression analysis between the yield and AUDPC was significant only for 2010 ( $F = 4.4054$ ;  $P < 0.0397$ ) with a small value of  $R^2 = 0.0644$ . This suggests that in absence of diseases (AUDPC=0) the expected value of yield would be  $7235 \text{ kg ha}^{-1}$ . On the other hand for an increase in AUDPC of 100 units, the expected yield will decrease  $804 \text{ kg ha}^{-1}$  (Figure 2A).

Concerning the different yield components: (Table 4) for SPM<sup>-2</sup> significant differences were observed between the positive control and treatments T1, T2, T3, T8 and T9 in 2010, and between the positive control and the treatment T2 in 2011. For TKW no differences were observed between the positive control and the treatments T1 to T6 having *T. harzianum* in their composition and treatments T7-T10 with only fungicide application (Table 4) except T9 which was better than the control in both years. Only for KPS in both years, the positive control (only inoculated with *Z. tritici*) differed from treatment T9 (only with fungicide application). Moreover, for KPS the best treatment without fungicide application was T3, with values similar to those observed with treatments with half doses of the fungicide (Table 3).

The Simple Regression model obtained between yield and yield components affected by *Z. tritici* showed that, in 2010, which had non-favourable conditions for STB, only SPM<sup>-2</sup> ( $R^2 = 0.429$ ) was able to explain its association with yield (Figure 2B), whereas, in 2011, which had favourable conditions for STB occurrence, there was a participation of KPS ( $R^2 = 0.264$ ) (Figure 2C) and a non-significant association with TKW ( $R^2 = 0.04$ ). The best yield gains using *T. harzianum* were obtained with T3, (three applications of *T. harzianum* with 25 % of gain for 2010 and 32.9 % for 2011).

Treatment T6, combining *T. harzianum* and half dose of foliar fungicide, resulted in yield gains of 14.3% for 2010 and 29.6% for 2011. On the other hand, foliar applications of fungicide alone (treatments T7-T10) at different phenological stages, resulted in yield gains of 12.5% for 2010 and 31.6% for 2011.

## DISCUSSION

The use of biological agents to reduce the severity of diseases like STB is a complementary strategy within integrated pest management (Hermosa et al., 2013; Lorito et al., 2010). In the present study, the application of *T. harzianum* (two strains combined) at wheat seeds (seed coating), tillering and heading growth stages (T3), provided similar yield increases comparable to three applications of a commercial foliar fungicide at the same phenological stages (T9).

Our results show that the application of *Trichoderma* spp. onto seeds is an effective and least expensive method for the control of diseases severity of *Septoria tritici* blotch in crops compared to fungicides. Because *Trichoderma* is unable to survive on the leaf surfaces, especially under the summer weather conditions, and its population decreases rapidly when it is applied as an aerial inoculum (Perelló et al., 1997; Perelló et al., 2003), several researchers have chosen to incorporate the antagonist species by means of the seed coating technique (Perelló et al., 2006; Perelló et al., 2009; Dal Bello et al., 2002; Cordo et al., 2007; Stocco et al., 2009; Stocco et al., 2012). Regarding this technique, Perelló et al., (2008), established that *Trichoderma* spp. is effective only at the early stages of the diseases. However, our results, especially in the second year, demonstrated that the protection obtained with treatment T1 (only application by seed coating) was achieved until the dough growth stage (data non show). In addition, we have previously demonstrated that seed coating with *T. harzianum* is efficient to protect wheat against the pathogen of STB (Cordo et al., 2007, 2011, 2012) and that leaves of plants grown from these seeds did not contain the fungus. The protective efficacy of wheat seed coating is therefore due to an indirect effect, in which there is no contact between the pathogen and the biocontrol agent. Previous reports (Segarra et al., 2002; Cordo et al., 2007) have mentioned that *T. harzianum* strains induce biochemical plant defense responses similar to those published (Hoitink, 2006; Howell, 2006).

Segarra et al. (2003) demonstrated this biochemical proteolytic action, throughout one described leaf germin-like protease inhibitor.

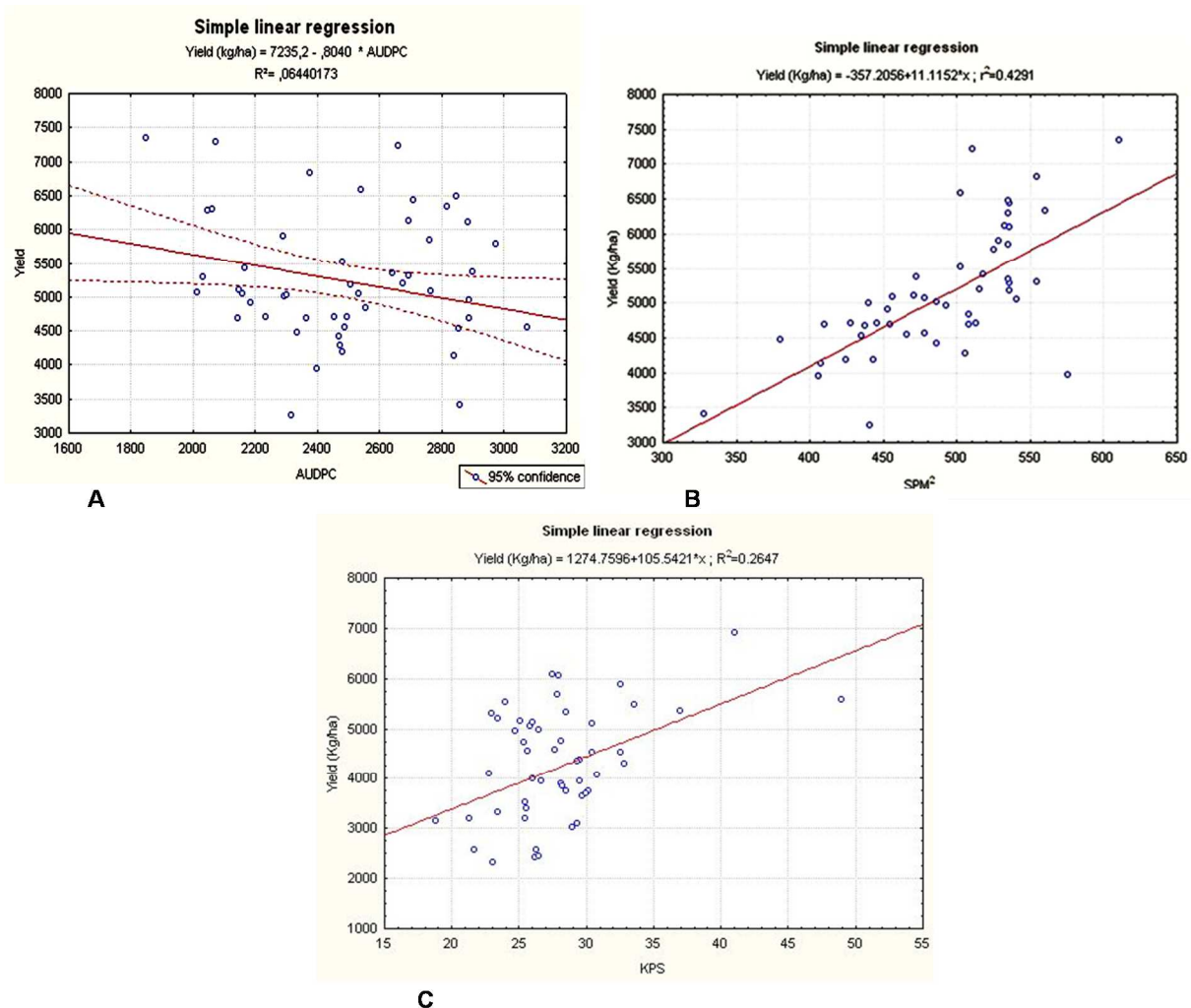


Figure 2. A Relationship between the Yield of the wheat cultivar Buck 55 CL2 and the AUDPC as consequence of STB diseases, 2010 year. B: Relationship between the Yield of the wheat cultivar Buck 55 CL2 and the number of spikes per square meter (SPM-2) as consequence of STB, 2010 year. C: Relationship between the Yield of the wheat cultivar Buck 55 CL2 and the number of kernels per spike (KPS) as consequence of STB, 2011 year. Strategies used: = T1, T2 and T3 biocontrol only, T4 to T6: biocontrol and fungicide combined; T7 to T10: fungicide only (negative control).

The decrease in the AUDPC with treatments T1 and T3 (seed coating and two aerial applications of *T. harzianum* at tillering and heading as reinforcement) as was demonstrated in Figure 1 in 2011 compared with 2010 could be explained by the predominance of rainfall and the decrease in radiation during the period, from the second node visible to heading stages, when yield and its components are defined. The humidity and the cloudy days with low radiation as consequences of rainfall (October-November 2011) favoured the development of the disease (Cordo et al., 2017a; Cordo et al., 2017b) and protected *T. harzianum* spores from drying and becoming inactive. Moreover, treatments T2 and T4 in 2010 and T3 in 2011, with aerial applications of *T. harzianum* as reinforcement, favoured the decrease in the AUDPC. It would be due to an antagonistic mechanism based on

the inhibition of conidia germination of *Z. tritici* by *T. harzianum* on wheat leaves (Perelló et al., 1997). We hypothesized that the reduction of STB symptoms here observed can be attributed to a resistance that could be originated through elicitation triggered by the *T. harzianum* strains, such as the production of the proteolytic enzyme serine protease, that could activate the mechanisms of wheat plant defense (Segarra et al., 2002). In a previous study, we demonstrated the restriction of STB progress in the susceptible cultivar PRO INTA Molinero pretreated with *Trichoderma* spp. isolates, before the infection with *Z. tritici* (Cordo et al., 2007). This protection can be transitory, but strongly potentiates the expression of defense-related proteins when plants are challenged by pathogens at sites distant from the location of the *Trichoderma* strain.



Treatments	Yield components					
	SPM <sup>-2</sup>		TKW		KPS	
	2010	2011	2010	2011	2010	2011
Positive Control	440 a*	360 a	37.3 ab	36.7 a	26.3 b	22.5 a
T1	510 bc	427.5 ab	38.9 a	37.7 a	27.4 ab	25.4 abc
T2	530.5 c	468.9 b	37.3 ab	38.5 a	28.2 ab	24.3 ab
T3	512.5 bc	421 ab	37.3 ab	35.4 a	30.2 ab	28.9 abc
T4	481.2 abc	449.1 ab	38.2 ab	37.3 a	29.3 ab	27.8 abc
T5	459 ab	455.3 ab	37.9 ab	35 a	28.6 ab	27.8 abc
T6	455.3 ab	439.1 ab	38.9 a	35.1 a	27.1 ab	28.5 abc
T7	472.3 abc	369.9 ab	36.5 abc	40.9 a	28.3 ab	28.3 abc
T8	506.3 bc	427.8 ab	35.6 bc	36.6 a	28.9 ab	34.1 bc
T9	528.5 c	415.4 ab	36.8 abc	27.4 a	30.5 a	35.2 c
T10	508.3 bc	397.7 ab	34.5 abc	35.1 a	29.4 ab	30.3 abc

Table 4. Mean values for yield components with 10 treatments with *Trichoderma harzianum*/fungicide and the positive control for the wheat cultivar Buck 55 CL2 infected by *Zymoseptoria tritici* in field experiments performed in 2010 and 2011. \* Treatments with similar letters are not statistically different. Yield components: SPM<sup>-2</sup> spike per square meter; TKW thousand kernel weight; KPS kernel per spike.

In the present study, the highest values of SPM<sup>-2</sup> in 2010 was the result of the highest number of plants per m<sup>2</sup> and the effect of humidity which conditioned tillering. Early protection (seed coating and early application at tillering) may affect the number of spikes, spikelet and flowers, as well as the weight of grains. There was a long period with decreased severity (from the beginning of heading until close to anthesis) that defined a greater number of spikes per plant and therefore per m<sup>2</sup> resulting in an effect similar to that of a multiple application of fungicide at three growth stages.

Treatments with *T. harzianum* protected the plants until the start of flowering, which would favor greater accumulation of photosynthates and the consequent increase in biomass.

Our last results with the application of the antagonist alone were similar to those reported previously by other researchers (Simón et al., 2002; Couretot et al., 2011) using a mix of triazoles and strobilurins. These authors reported an increase in the number of SPM<sup>-2</sup> and the number of grains per spike<sup>-1</sup> as a consequence of an early and delayed protection from the effect of the fungicides on wheat. The good weather conditions during the critical period of 2011, favorable for the occurrence of diseases, could have been one of the reasons because yield of the control was low as a consequence of the high infection, contrasting with the high values of yield and yield gain (%) for treatments T1, T2, and T3 with *T. harzianum* application alone. These results would explain the association between KPS and yield in the Simple linear Regression model with an R<sup>2</sup> = 0.26. The presence of *T. harzianum* on the leaves could stimulate the delay in the duration of the green leaf area increasing yield, similar that was observed with the application of a fungicide composed by strobilurins (Gooding et al., 2000).

In our study, the treatments with *Trichoderma* and/ or *Trichoderma* combined with fungicide or fungicide alone,

generated a healthy green surface from which the grains take advantage of the soluble carbohydrates, that compensate the filling of them (Simón et al., 2002).

Finally, the low association (6%) between yield and AUDPC might be possible explained not only for the amount of diseases but also for the environment and the genes present in the host and the pathogen. Maybe, the proper genes expression of the cultivar guided to a slow rate of the diseases with a reduction in yield (Shaner and Finney, 1977).

## CONCLUSIONS

We concluded that for the first time in Argentina, was demonstrated the beneficial effect of seed coating and seed coating plus two aerial applications of *T. harzianum* for a tendency to increase (even though it was not significant) in grain yield in wheat and the improvement of the yield components. We recommend only one application on wheat seeds (seed coating) because, the protective effect was prolonged until the early dough stage of grain filling, resulting in a low cost of application during the growing cycle of the crop.

We propose that these two strains of *T. harzianum* should become part of the list of already existing microorganisms to be used to obtain biological products.

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