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Native isolates of *Trichoderma harzianum* inducting resistance to *Zymoseptoria tritici* on wheat plants

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Resumen: Aislamientos nativos de *Trichoderma harzianum* inducen resistencia a *Zymoseptoria tritici* en plantas de trigo. La Septoriosis del trigo es endémica en las regiones productoras de Argentina, impactando en su rendimiento. Una alternativa de manejo, es el uso de agentes de biocontrol. Se propuso: determinar la efectividad de los aislamientos de *Trichoderma* obtenidos a partir de suelos de diferentes localidades de la región triguera argentina, sobre el control de la enfermedad y caracterizar la actividad de la serín proteasa (con capacidad antifúngica) del fluido intercelular de trigo generada por los mejores aislamientos. Para determinar la capacidad biocontroladora de los 90 aislamientos de *Trichoderma*, se sembraron semillas recubiertas con cada uno de los antagonistas y posteriormente las plántulas se inocularon con *Zymoseptoria tritici*. Se evaluó la capacidad de cada aislamiento del biocontrolador para restringir el progreso de la septoriosis 21 días después de la inoculación. A las plantas tratadas con las 10 mejores cepas de *T. harzianum*, se les midió el incremento de la actividad proteolítica. Cuatro de los aislamientos (2 y 8, de Los Hornos y 129 y 141 de Manfredi) disminuyeron el porcentaje de cobertura picnidial y se destacaron por el aumento de la actividad proteolítica en un cultivar susceptible. Éste comportamiento se asocia a un mecanismo bioquímico de defensa con un efecto sistémico temprano.

Palabras clave: *Trichoderma harzianum,* biocontrol, trigo, Septoriosis, RSI, serín proteasa.

Summary: *Trichoderma harzianum* inducting resistance to *Zymoseptoria tritici*. Septoria tritici blotch is endemic in the wheat growing cultivated areas of Argentina, which impacts in the crop yield. One management strategy is to use biocontrol agents. The objective of this work was to determine the effectiveness of *Trichoderma* spp. isolated from soils of different growing regions, as antagonist and characterize the *T. harzianum* effect throughout the proteolytic activity of the serine protease in the wheat plant. To determine the antagonistic capacities of *Trichoderma* spp. against *Zimoseptoria tritici*, wheat plants were grown from seeds coated with each of the ninety isolated *Trichoderma*. Antagonism was assessed 21 days after inoculation by the capacity of each *Trichoderma* spp. isolate to restrict the progress of the disease. To measure the increasing proteolytic activity, ten isolated *T. harzianum* characterized by their inhibitory effect over the pathogen, were chosen. Four isolates of the antagonist (strain 2 and 8, from Los Hornos and 129 and 141 from Manfredi localities) simultaneously decreased the pycnidial coverage percentage with a noted increasing of the serine-protease activity in the susceptible wheat cultivar. This behavior is associated to a biochemical mechanism of defense with an early systemic effect.

Key words: *Trichoderma harzianum,* biocontrol, wheat, Septoria tritici blotch, ISR, serine protease.

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Introduction

Septoria leaf blotch or Septoria tritici blotch (STB) is an endemic disease in the wheat-cultivated areas of Argentina, which can severely impact in crop yield. It is caused by the fungus *Zymoseptoria tritici* (Desm.) Quaedvlieg & Crous (fomerly *Mycosphaerella graminicola* and *Septoria tritici*) which damages the leaves and disrupts the grain filling process (Ziv *et al*., 1981; Kraan & Nisi, 1993). Yield losses can reach from 21 to 37% (Kraan & Nisi, 1993) and 20 to 50% (Annone *et al*., 1987), in high-yielding cultivars, and from 16 to 45% depending on the cultivars and fertilization treatments (Simon *et al*., 2002). The typical disease symptoms are chlorosis and fungal colonization of basal leaves. The extent of these symptoms depends on the susceptibility of cultivars to the infection (Sewell & Caldwell, 1960; Annone, 1990). Breeding for resistance is the most economical approach to controlling the disease (Eyal *et al*., 1985). Alternatives as fungicides and various cultural practices that include managing the sowing date, the tillage systems, spacing between sowing rows, soil nitrogen levels and selecting wheat varieties with a distant canopy, have also been used (Eyal, 1981). Genetic resistance of wheat against this pathogen is never complete in most wheat cultivars. Hence, disease control relies heavily on the use of fungicides (Fraaije *et al*., 2005). Triazole and strobilurine or carboxamide and strobilurine combinations are the most widely used chemical classes to control the foliar diseases of wheat. But its constant application shift toward reduced sensitivity of *Z. tritici* to those fungicides. As a consequence, microbial antagonists, either alone or combined with pesticides, have been used to manage wheat diseases (Perelló *et al*., 2006; Cordo *et al*., 2011; Cordo *et al*., 2012). Among the microorganisms, *Trichoderma* spp. has been used to control foliar diseases of different crops. Antagonistic fungi are present in substantial quantity in nearly all agricultural environments, and their use is now being recognized world-over as an alternative in plant disease control (Harman *et al*., 2004; Sutton, 2005). Our previous research in Argentina indicates that some antagonists of phylloplane could be used to control the leaf blotch and other wheat foliar diseases (Cordo *et al*., 2007; Perelló *et al*., 2009; Cordo *et al*., 2011). The diversity of mechanisms available to *Trichoderma* species for pathogen suppression (e.g., competition for nutrients and space, production of antibiotics, mycoparasitism and the control comprising morphological and biochemical changes in the plant) (Viterbo *et al*., 2001) make these fungi attractive as biocontrol agents.

Because of their potential for biocontrol, species of *Trichoderma* have been extensively studied (Mónaco *et al*., 1994; Dal Bello *et al*., 1997; Monte, 2001; Perelló *et al*., 2003; Perelló *et al*., 2009; Stocco *et al*., 2009; Consolo *et al*., 2012; Stocco *et al*., 2012). Most strains of *Trichoderma harzianum, T. aureoviride*, as well as some of *T. koningii* reduce the leaf blotch caused by *Z. tritici* in greenhouses grown wheat (Cordo *et al*., 2007).

Segarra *et al*. (2002) has previously reported the occurrence of proteolytic activity in the intercellular washing fluid (IWF) obtained from the apoplast of wheat plants. It plays a central role in their defense mechanisms because the apoplast is a compartment in which enzymes that are involved in these mechanisms (glucanases, chitinases, and proteases) are accumulated. The present authors reported that, independently of the cultivar from which it was extracted, the extracellular serine protease inhibited the germination of *Z. tritici* conidiospores and its activity also was affected when *Z. tritici* was inoculated on wheat plants. It suggests that proteolytic activity of the leaf apoplast participated in the defense mechanism of wheat plants against *Z. tritici*. The inoculation with *Z. tritici* either increases or decreases the activity of apoplastic serine protease in plants of resistance and susceptible cultivars respectively. The symptoms of leaf botch decreased in susceptible cultivars that were pretreated with some *Trichoderma* isolates before being attacked by *Z. tritici*. In addition, different *Trichoderma* isolates demonstrated a differential capacity for *Z. tritici* biocontrol (Cordo *et al*., 2007).

As a part of ongoing research aimed to demonstrate the efficacy of *Trichoderma harzianum* Rifai to control the *Z. tritici* expression, the objective of this study was to test its ability to protect wheat plants against STB under greenhouse conditions. The specific objectives were (1) to determine the effectiveness of isolates of *Trichoderma* from soils of different Argentinian wheat grown regions on wheat plants at first leaf growth stage; (2) to

characterize the *T. harzianum* effect throughout the proteolytic activity of the serine protease enzyme present in the wheat plant cell apoplast.

Material and Methods

Effectiveness of *Trichoderma* isolates on *Z. tritici* severity of wheat

Isolation of Trichoderma strains

Ninety *Trichoderma* isolates were obtained from soil of wheat rhizosphere sampled in localities of the wheat-growing regions of Argentina. Isolates 1-30 came from Los Hornos (II south), isolates 121-150 from Manfredi (V north), and isolates 151-180 from Lobería (IV) (Fig. 1). Hierarchical sampling method was used for each soil collection. Soil samples were storaged at 4ºC. The *Trichoderma* selective medium (TSM) developed by Elad *et al*., 1981 was used for quantitative isolation of this fungus. Thirty strains were obtained from each locality. *Trichoderma* isolates were storaged on cellulose filter paper following Stocco *et al*. (2010) technique.

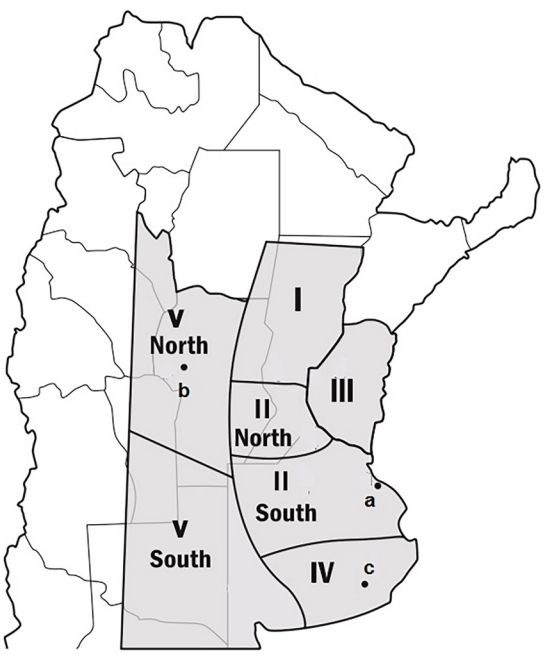

Fig. 1. Map of Argentina wheat area showing sites of isolation of *Trichoderma*: (a) Los Hornos (b) Manfredi (c) Lobería.

Production the Trichoderma spp inoculum

All *Trichoderma* isolates were grown on 2% potato dextrose agar (PDA) medium and incubated 7 days at 26 ± 2 ºC. Conidia of each isolate were harvested by flooding the cultures with sterile distilled water and then rubbing the culture surface with a sterile glass rod. Then, suspensions were through-out two layers of cheesecloth. The concentrations of propagules in suspensions were standardized with the aid of the hemocytometer to 1 x 10^8 conidia ml^{-1} for each *Trichoderma* isolated tested.

For production of *Trichoderma* coated-seed 10 ml of suspension of each isolate was mixed with 90 ml of 0.25% agar in water that served as an adhesive. The mixture was shaken using a magnetic stirring bar until a homogeneous suspension was obtained. Wheat seeds of Buck Guapo cultivar were added to the water-agar-fungal biomass and mixed for pelletizing. Seed uniformly coated with *Trichoderma* formulations were held for air drying overnight in darkness at room temperature and sown 24-48h later.

Production of Zymoseptoria tritici

Two virulent strains of *Z. tritici* (FALP9J008 and FALPLA008) were used for all inocula production. Both strains were isolated in 2008 from wheat field assays of 9 de Julio and Plá localities respectively. Leaves which presented typical lesions with pycnidial were chosen. Fungus was storaged in mineral oil at 4 ºC until it was used for inoculation in the greenhouse at the Centro de Investigaciones de Fitopatología (CIDEFI). Both isolates were recovered on potato dextrose agar (PDA, Difco; Laboratories) until the typical mucous colonies with secondary conidia developed. On day ten, sloops of conidia were replicated in plates onto modified malt agar (30 g malt extract, 5 g mycological peptone, 2 g yeast extract and 1000 ml distilled water) for sporulation. After 7 days, conidia were harvested flooding the plate with 5 ml of sterile distilled water and dislodging the conidia with a bent glass rod. This suspension was filtered through cheesecloth and the concentration of inoculum suspension was adjusted to $1x10^7$ spores per ml for the inoculation on plants. Immediately before plant inoculation, 50 µL L-1 0.05 % (w/v) Tween 20 aqueous solution was added to the conidia solution and mixed.

Greenhouse assays

To determine the antagonistic capacities of *Trichoderma* species against *Z. tritici*, wheat plants were grown from seeds coated with each one of the ninety isolates. The coated seeds germinated in 16 x10 x 5 –cm plastic trays filled with soil fertilized with (NH_4) H_2PO_4 and urea to levels equivalent to 50 kg ha^{-1} P and 100 kg ha^{-1} N. Trials were performed under natural daylight in a greenhouse, from May to July in 2008 and 2010. The average temperature and humidity during these months were 18ºC and 72% RH respectively. The experimental layout was a randomized block designed with 31 treatments and three replications per *Trichoderma* strain. After 10-12 days of sown, wheat seedlings (coming from coating seeds) with the first leaf emerged (GS 1.1 stage following Zadoks *et al*., 1974) were sprayed with a mixture of both strains of the pathogen until run-off using a manually operated sprayer. Those plants that received only pathogen spores suspension were used as controls. After inoculating the pathogen, these plants were covered with plastic bags for 2 days to maintain a high level of humidity. For the evaluation, the first and second leaves of each plant per treatment were selected and the percentages of necrotic area and pycnidial coverage were estimated. This infection was compared to the infection of leaves inoculated only with the pathogen. The necrotic area and pycnidial coverage percentages were recorded 21 days after inoculation. Antagonism was assessed by the relative ability of each isolate to restrict lesions development. Data of each experiment were analyzed by an analysis of variance and means were compared by LSD test ($P \leq 0.05$).

Proteolytic activity characterization

For experiments of proteolytic activity ten isolates of *T. harzianum* characterized for its inhibitor effect over the pathogen *Z. tritici* were chosen. These isolates were obtained in different years and from different field locations. The DNA sequencing of each isolate obtained in this study was deposited in the European Molecular Biology Laboratory (EMBL) and the assigned accession numbers are shown in Table I. The proteolytic activity of the leaf intercellular washing fluid (IWF) was assessed in the following plant treatments: wheat plants without treatment (control); wheat plants inoculated with Z. *tritici* 12 days after sowing; wheat plants coming from seed coated with *T. harzianum* conidia and wheat plants coming from seed coated with *T. harzianum* conidia and inoculated with *Z. tritici* 12 days after sowing. The proteolytic activity obtained from wheat plants inoculated only with *Z. tritici* was defined as 100%.

Preparation of the intercellular washing fluid

The intercellular washing fluid (IWF) was obtained from leaves as described by Segarra *et al*. (2003). In short, 75-100 leaves (nearly 5 g fresh weight) were dipped in a solution containing 50 mM Na-phosphate buffer, pH 7.5, 0.1% (v/v) 2-mercaptoethanol, 0.6 M NaCl, and 0.13% (v/v) Tween 20 at 4 ºC, and exposed to vacuum for three 10s periods separated by 30s. Then, they were dried on filter paper, placed in a glass filter mounted on a tube and centrifuged at 400 g for 20 min. Each gram of leaf yielded nearly 0.25 ml of IWF. This fraction contains the leaf apoplastic serine protease (Pinedo *et al*., 1993; Segarra *et al*., 2003; Cordo *et al*., 2007).

Estimation of protease activity

The proteolytic activity of leaf IWF was assessed in a 400-µL mix containing 25-50 µg of IWF protein, 2 mg of casein and 50 mM Na-phosphate buffer, pH 7.5 (Segarra *et al.,* 2003). After1h of incubation at 37 ºC, the reaction was stopped by adding 50 µL of 50% (v/v) trichloroacetic acid (TCA) and mixed. The mix reaction was stored

Table I. Identification of *Trichoderma isolates* from soils of three localities of Argentina wheat area.

Strains	Year of collection	Field location	Trichoderma sp.	Accession code
2	2008	Los Hornos	*T. harzianum*	HG940467
4	2008	Los Hornos	*T. harzianum*	LN864822
8	2008	Los Hornos	*T. harzianum*	HG940469
10	2008	Los Hornos	*T. harzianum*	HG940470
123	2010	Manfredi	*T. harzianum*	HG940485
129	2010	Manfredi	*T. harzianum*	HG940486
141	2010	Manfredi	*T. harzianum*	HG940489
160	2010	Lobería	*T. harzianum*	HG940490
162	2010	Lobería	*T. harzianum*	HG940491
165	2010	Lobería	*T. harzianum*	HG940492

at 4ºC for 30 min. TCA- soluble material was collected by centrifugation at 2000 x g for 20 min. Aliquots of 200 µL were neutralized with 200 µL of 0.3 M KOH. Then, they were mixed with 400 µL of potassium borate 0.4 M, pH 9.7, and 400 µL of a solution of 0.3 mg mL/L fluorescamine in acetone (Udenfriend *et al*., 1972). The fluorescence generated by Fluorescamine was measured at 390/470 nm (excitation/emission), respectively, using leucine as standard. The proteolytic activity of samples was estimated in units. One unit was defined as the activity necessary to release 30 nmole of leucine h^{-1} (Cordo *et al.,* 2007).

Proteolytic activity of leaf IWF examined by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) in 10% acrylamide slab gels containing 0.1% gelatine was determined as described by Heussen & Dowdle (1980). Samples the IWF corresponding to 150 milligrams of leaf fresh weight were tested. After electrophoresis, the slabs were subsequently washed to eliminate SDS, incubated at 37 °C for 2 h in 50 mM sodium phosphate, pH 7.5, and stained with Coomassie Brilliant Blue. Clear bands revealed proteolytic activity whereas the untouched gelatine appeared as a dark (blue-stained) background. The band intensity was roughly proportional to the activity.

Results

Effectiveness of *Trichoderma* isolates on *Z. tritici* severity of wheat

Greenhouse assays

Significant differences (at the level $p \leq 0.05$) in the necrotic area and pycnidial coverage percentages were found among the plants treated with the *Trichoderma* isolates and inoculated with the pathogen and the control plants (Fig. 2, 3, 4). From Los Hornos locality (Fig. 2) the best four treatments were 2, 4, 8 and 10. From Manfredi (Fig. 3) isolates 123, 129 and 141 also showed significant reduction of the severity parameters evaluated. At least, the best isolates (162 and 165) coming from Lobería locality (Fig. 4) differed significantly from the control because they reduced the necrotic and pycnidial coverage percentage as the results of their biocontrol effect.

Proteolytic activity of the serine protease enzyme in wheat plant cell apoplast

Variation in the activity of the serine protease ranged between -10% (Th 162+ *Z. tritici*) to 1350% (Th 129 + *Z. tritici*), compared to the percentage value of the control (100% for wheat plants

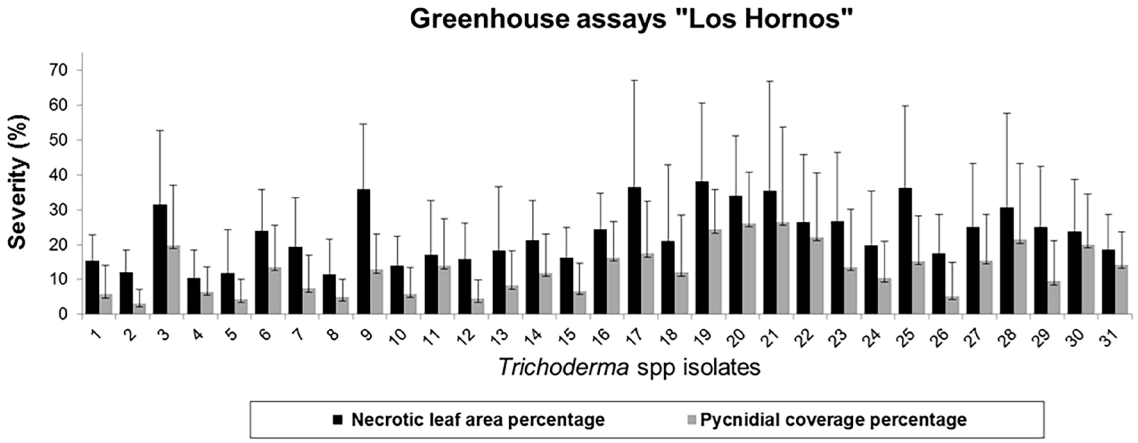

Fig. 2. The necrotic and pycnidial coverage caused by *Z. tritici* in plants pretreated with *Trichoderma* isolates obtained from Los Hornos. Vertical lines on bars represent the standard deviation of each treatment. Isolate 31 corresponds to control.

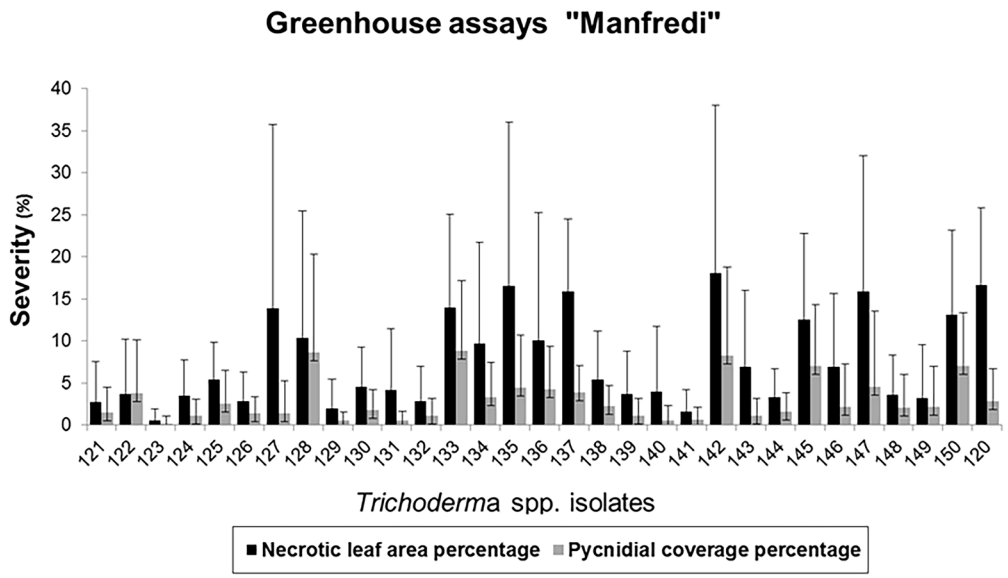

Fig. 3. The necrotic and pycnidial coverage caused by *Z. tritici* in plants pretreated with *Trichoderma* isolates obtained from Manfredi. Vertical lines on bars represent the standard deviation of each treatment. Isolate 120 corresponds to control.

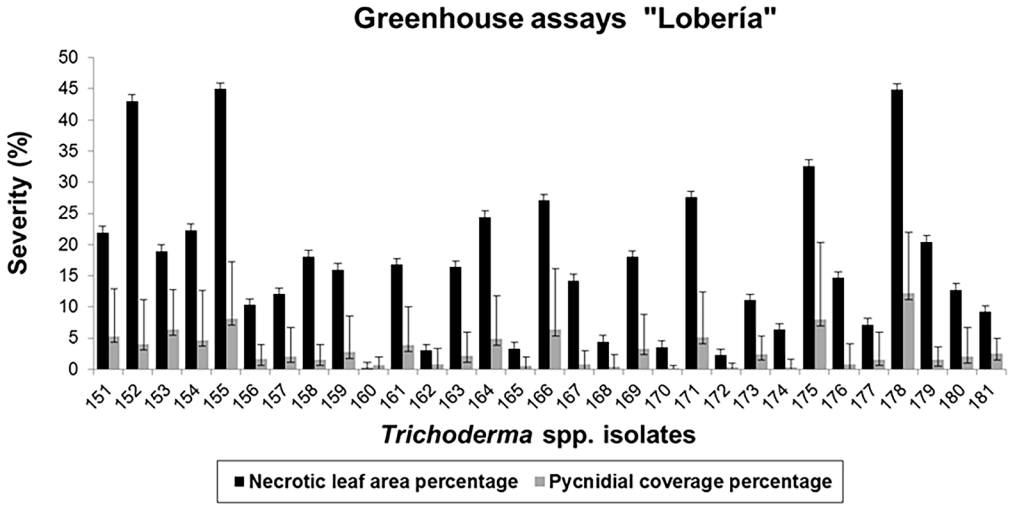

Fig. 4. The necrotic and pycnidial coverage caused by *Z. tritici* in plants pretreated with *Trichoderma* isolates obtained from Lobería. Vertical lines on bars represent the standard deviation of each treatment. Isolate 181 corresponds to control.

inoculated with *Z. tritici*) and plants pretreated with *T. harzianum* and inoculated with *Z. tritici* (Table II). These activities were visible 21 days after sowing. In coincidence, the treatments with *T. harzianum* coming from Los Hornos showed a moderated variation of this activity (Fig. 5 and Table II).

The activity showed three types of behavior: 1- Similarity in proteolytic activity values between plants only pretreated with *T. harzianum* and plants pretreated with *T. harzianum* and inoculated with *Z. tritici* (*T. harzianum* 129, 141 and 160). 2- Values of proteolytic activity increased when pretreated plants with *T. harzianum* and inoculated with *Z. tritici* were compared with plants only pretreated with *T. harzianum* (*T. harzianum* 123). 3- Values of proteolytic activity decreased in plants pretreated with *T. harzianum* 162 and inoculated with *Z. tritici* compared with plants pretreated only with *T. harzianum* (Table II).

The result of the analyses of the IWF with SDS-PAGE copolymerized with gelatine is shown in Fig. 6. The bands present in the gel have different intensity colors that correspond to different levels of activity e.g. treatment 162 + S (*Z. tritici*) shows the minimum level of activity (90%) compared with treatment T+S (2); in contrast, treatment 129 +S shows the maximum level of activity (1450%) compared with treatment T+S (10).

Table II. Effect of *Z. tritici* and *T. harzianum* on leaf apoplast proteolytic activity. (-) the absence of proteolytic activity dates are caused by the loss of IWF.

Plant Treatment	Days after sowing	Proteolytic activity (%)	Proteolytic activity variation(%)
Non treatment	21	100 ±10	
Control	21	100±10	
Th 2	21	-	
Th2 +*Z. tritici*	21	175	75+
Th 4	21	-	
Th4 +*Z. tritici*	21	150	50+
Th 8	21	-	
Th8+*Z. tritici*	21	250	150+
Th 10	21	-	
Th10+*Z. tritici*	21	220	120+
Th 123	21	300	
Th123+*Z. tritici*	21	1000	900+
Th 129	21	800	
Th129+*Z. tritici*	21	1450	1350+
Th141	21	1500	
Th141+*Z. tritici*	21	1200	1100+
Th 160	21	350	
Th160+*Z. tritici*	21	350	250+
Th162	21	280	
Th162+*Z. tritici*	21	90	-10
Th165	21	320	
Th165+*Z. tritici*	21	150	50+

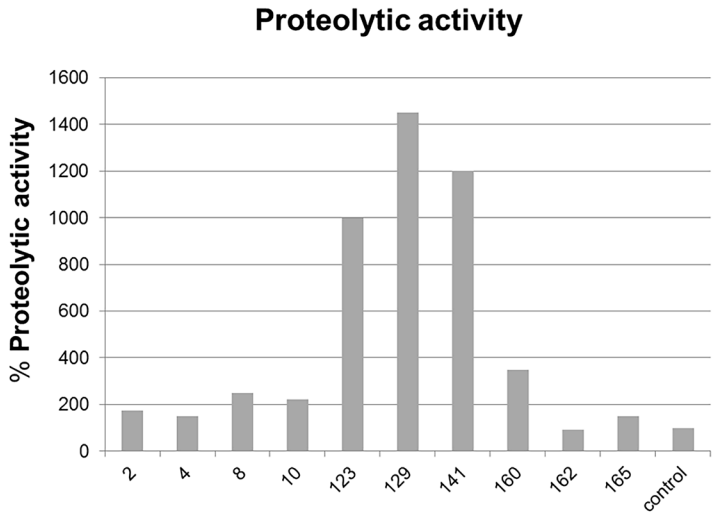

Fig. 5. Proteolytic activity variation of the serine protease produced on wheat after interaction with *T. harzianum* and *Z. tritici*. Numbers in the bottom of the graph correspond to each interaction between *T. harzianum* isolates (2, 4, 8, 10, 123, 129, 141, 160, 162, 165) and *Z. tritici.* Control plants were those inoculated only with *Z. tritici.*

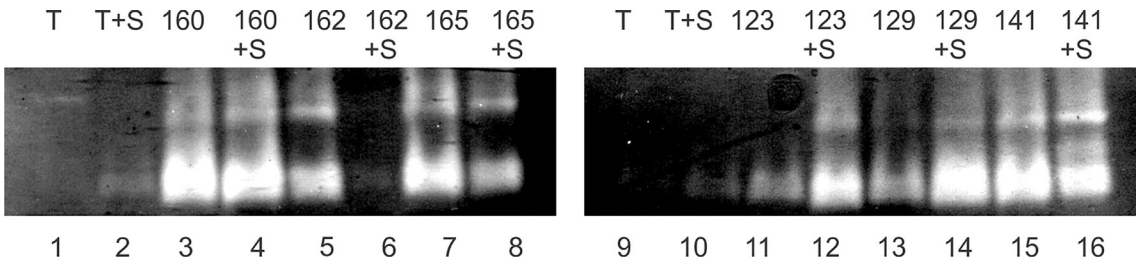

Fig. 6. Proteolytic activity of the IWF analyzed with SDS-PAGE copolymerized with gelatine. In each lane the IWF of the first wheat leaf (G.S.1.1), corresponding to 150 milligrams of leaf fresh weight, was run. Lanes: 1, 9: wheat plants without treatment. 2, 10 wheat plants inoculated with *Z. tritici.* Lanes 3, 5, 7, 11, 13, 15: wheat plants pretreated with isolates 160, 162, 165, 123, 129, 141 of *T. harzianum* respectively. Lanes: 4, 6, 8, 12, 14, 16, wheat plants pretreated with isolates 160, 162, 165, 123, 129, 141 of *T. harzianum* respectively and inoculated with *Z. tritici.* Clear bands indicate proteolytic activity against a dark background of undegraded gelatine. Difference in intensity corresponds to different levels of serine protease activity.

The treatments with isolates 2 and 8, obtained from soil of Los Hornos locality, caused a reduction in the pycnidial coverage of 78 % and 65%, respectively. In addition, these isolates presented high proteolytic activity, estimated between 175% and 250%, respectively (Table II). Similar results were obtained with the isolates coming from soils of Manfredi (123 and 129). These strains decreased the necrotic coverage percentage in values of 90% and 80% respectively, besides decreasing 90 % and 65 % the area covered by pycnidia. Those isolates also showed an increase of the proteolytic activity with values of 1000% and 1450%, respectively (Table II).

Discussion

Trichoderma is a large fungal genus. Most species have cosmopolitan distribution and possess high biotechnological value, so several are currently used as biological control agents. Species of *Trichoderma* protect plants against the attack of soil-borne plant pathogens by competing for nutrients and inhibiting or killing plant pathogenic fungi and Oomycetes, through the production of antibiotics and/or hydrolytic enzymes. In addition to the role of species of *Trichoderma* as biocontrol agents, they have other beneficial effects on plants, including the stimulation of plant defenses and the promotion of plant growth (Hermosa *et al*., 2013).

The results obtained from this work showed that among the 90 *Trichoderma* isolates obtained from wheat cultivated soils, several of them significantly decreased the severity, but only 9 reduced simultaneously more than 50% the necrotic and pycnidial coverage of Septoria tritici blotch (STB). This result demonstrated the importance of using native strains to protect plants. Related to this, Sutton (1995) stated that the ecological adaptation of the microbe to the crop plant would be advantageous for effective and sustained biocontrol in the cropping system. Dal Bello *et al.* (2011) reported, for Argentine conditions, a reduction of disease incidence (65–95%) and severity (50–77%) of the leaf grey mould (produced by *Botrytis cinerea*) on tomato by the application of *Trichoderma harzianum* and *Fusarium semitectum*. Both species have been isolated from cultivated and wild plants of Solanaceae family. Howell (2003) established that the best method for obtaining a potential biocontrol agent might be one where the candidate *Trichoderma* species would be isolated from areas of the plant and soil where it would be expected to function in disease control. Emphasis is placed in the fact that they can grow under conditions of temperature, moisture, and nutrient availability, similar to those found in nature. Besides *Trichoderma* species are strongly influenced by the grown substrate, temperature and the presence of other microorganisms in the soils population that affect the production and activities of enzymes and antibiotics. So the environmental factor that determine the biocontrol activity of *Trichoderma* spp., is limiting its efficacy. For this reason, we used native isolate of *Trichoderma* well adapted to wheat plants as biocontrol agents.

Different authors (Perelló *et al*., 1997, 2006, 2009, Dal Bello *et al.,* 2002, Stocco *et al*., 2009, 2012, Cordo *et al*., 2007, 2011, 2012) applied seed coating technique to incorporate the antagonist. Cordo *et al*. (2007) found that this technique was more effective against leaf blotch than the leaf spraying technique. Besides it is known that the ability of *Trichoderma* species to survive on leaf surfaces decreases rapidly when it is applied as aerial inocula (Perelló *et al.,* 1997, 2003).

When the species of *Trichoderma* colonize and penetrate plant root tissues, they initiate a series of morphological and biochemical changes, that increase resistance to a wide range of plant-pathogenic microorganisms. This response seems to be broadly effective for many plants indicating that its action is not specific of the plant. The scientific reviews indicate that *Trichoderma* species have evolved as opportunistic plant avirulent symbionts (Harman *et al*., 2004). When spores or other propagative structures are added to soil and come into contact with plant roots, they germinate and grow on root surfaces, and at least some infect the outer few root cells. They produce at least three classes of substances that elicit plant defense responses that prevent further infection of roots by plant pathogens. These elicitors include peptides, proteins and low-molecular-weight compounds. The proteolytic enzyme serine-protease would be associated to this mechanism (Harman *et al*., 2004). Segarra *et al*. (2002) and Cordo *et al*. (2007) attribute the reduction of the STB severity to a Systemic Induced Resistance throughout elicitors with enzymatic function as the serine-protease. This process 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. The plant defense reactions can become systemic and protect the entire plant from a range of phylloplane pathogens and diseases, even when *Trichoderma* grows only on the roots (Harman *et al.* 2004).

In this work, four isolates of *Trichoderma harzianum* (strains 2 and 8, from Los Hornos and 129 and 141 from Manfredi localities) simultaneously decreased the pycnidial coverage percentage and induced a remarkable increase in the serine-protease activity for the susceptible wheat cultivar Buck Guapo. These results agree with those of Cordo *et al*. (2007) who demonstrated that the antifungal proteolytic activity, measured 12 days after sowing, increases in plants grown from seed coated with *T. harzianum* (Th 5) isolate and inoculated with *Z. tritici.* This increase conferred resistance to the susceptible wheat studied. Segarra *et al*. (2002) showed that the resistant cultivar (with resistance response to *Z. tritici* infection) was characterized by a gradual and significant increase of proteolytic activity in the IWF, with occurs in parallel, with a limited fungal colonization. Mauch & Staehelin (1989) recognized that an increase in proteolytic activity would be part of a concerted strategy of defense. Pinedo *et al.* (1993) established that this strategy could be possible in wheat leaves, where at least IWF-proteinases are present inside the cells colonized by fungus. Also Cordo *et al.* (2007) suggested that *T. harzianum* stimulated a biochemical systemic induced response against *Z. tritici*.

Our results demonstrated that in asymptomatic leaves, after 12 days of inoculation with *Z. tritici,* the IWF of wheat plants originated from seeds precoated with *T. harzianum,* increased the proteolytic activity. According to Cordo *et al*. (2007) and in coincidence with our results, after 12 days of inoculation *T. harzianum* was not present in the leaves of plants grown from precoated seeds. But, the stimulation of its proteolytic activity was observed. We inferred that this reaction might be considered a systemic induced response, which is one of the biochemical mechanisms of plant defense proposed by Viterbo (2002), Hanson & Howell (2004), Howell (2006) and Hoitink *et al*. (2006).

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