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ORIGINAL PAPER

Selection and characterization of Argentine isolates of *Trichoderma harzianum* for effective biocontrol of Septoria leaf blotch of wheat

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Abstract Species of the genus *Trichoderma* are economically important as biocontrol agents, serving as a potential alternative to chemical control. The applicability of *Trichoderma* isolates to different ecozones will depend on the behavior of the strains selected from each zone. The present study was undertaken to isolate biocontrol populations of *Trichoderma* spp. from the Argentine wheat regions and to select and characterize the best strains of *Trichoderma harzianum* by means of molecular techniques. A total of 84 out of the 240 strains of *Trichoderma* were able to reduce the disease severity of the leaf blotch of wheat. Thirty-seven strains were selected for the reduction equal to or greater than 50 % of the severity, compared with the control. The percentage values of reduction of the pycnidial coverage ranged between 45 and 80 %. The same last strains were confirmed as *T. harzianum* by polymerase chain reaction amplification of internal transcribed spacers, followed by sequencing. Inter-simple sequence repeat was used to examine the genetic variability among isolates. This resulted in a total of 132 bands. Further numerical analysis revealed 19 haplotypes, grouped in three clusters (I, II, III). Shared strains, with different geographical origins and isolated in different years, were observed within each cluster. The origin of the isolates and the genetic group were partially related. All isolates from Paraná were in cluster I, all isolates from Lobería were in cluster II, and all isolates from Pergamino and Santa Fe were in cluster III. Our results suggest that the 37 native strains of *T. harzianum* are important in biocontrol programs and could be advantageous for the preparation of biopesticides adapted to the agroecological conditions of wheat culture.

Keywords Biological control · *Trichoderma harzianum* · Septoria leaf blotch · ISSR

Introduction

The genus *Trichoderma* (Ascomycota, Hypocreales) consists of soil-borne free-living non-pathogenic fungi, which can colonize the roots of numerous plants. They are recognized for their important benefits to agriculture such as their ability to protect crops against diseases (i.e. they are biological control agents) and to increase crop yield under field conditions (Harman et al. 2004). In addition, these fungi have been exploited in biotechnological applications (Lorito et al. 2010) and serve as a potential alternative to chemical control (Kumar and Sharma 2011).

Most *Trichoderma* strains used as biological control agents in agriculture belong to four species: *T. harzianum* (Ospina-Giraldo et al. 1999), *T. atroviride*, *T. virens* and *T. asperellum* (Lorito et al. 2010; Monte 2001; Montero-Barrientos et al. 2011). In Argentina, the use of *Trichoderma* isolates as biocontrollers to reduce foliar disease has

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been extensively studied (Mónaco et al. 1994, 2004; Perelló et al. 2003, 2009; Stocco et al. 2009, 2012).

Septoria leaf blotch (SLB) is a worldwide wheat disease. In Argentina, this disease is endemic to the Southeast region of the cultivating areas of Buenos Aires province and causes a severe impact on the crop yield of this cereal. The causal agent of SLB is the fungus *Zymoseptoria tritici* (Desm.) Quaedvlieg and Crous (fomerly *Mycosphaerella graminicola* and *Septoria tritici*), which damages the leaves and disrupts the grain filling process (Kraan and Nisi 1993; Ziv et al. 1981). In Argentina, yield losses can reach 21–37 % (Kraan and Nisi 1993) and 20–50 % (Annone et al. 1991) in high-yielding cultivars, and 16–45 % depending on the cultivar and fertilization treatment (Simón et al. 2002).

Cordo et al. (2007) showed that most strains of *T. harzianum* and *T. aureoviride*, as well as some strains of *T. koningii,* reduce SLB under greenhouse conditions. These authors found that the symptoms of leaf botch decrease in susceptible cultivars pretreated with some *Trichoderma* isolates before being attacked by *Z. tritici*. In addition, they found that different *Trichoderma* isolates have a differential capacity for *Z. tritici* biocontrol.

The applicability of *Trichoderma* isolates to different ecozones depends on the behavior of the strains selected from each zone (Grondona et al. 1997).

There is an increased interest in finding novel native strains with potential biological activity against SLB. The best strains might be selected from areas of the plant and soil where they are expected to function in disease control and where they grow under ecological conditions that are similar to those found in nature (Howell 2003).

The isolation, identification and genetic characterization of native *Trichoderma* strains could be useful to verify their natural genetic variability, to divide the strains in similarity groups, and to differentiate the original strains from diverse regions (Muthumeenakshi et al. 1998). Several studies assessing genetic diversity (Random Amplified Polymorphic DNA (RAPD), Dodd et al. 2004; Muthumeenakshi and Mills 1995 and Universally Primed PCR (UP-PCR), Cumagun et al. 2000) have been used for the characterization and grouping *Trichoderma* isolates. Other techniques as Inter-Simple Sequence Repeats (ISSR) have been effective to characterize the genetic variability of *Trichoderma* isolates (Consolo et al. 2012). The use of these techniques to assess genetic diversity will help to eliminate duplicated strains in programs on microbial selection (Samson 1995) and during mass production before the risk of contamination.

The present study was undertaken to isolate biocontrollers populations of *Trichoderma* spp from the Argentine wheat regions and to select and characterize the best strains of *T. harzianum* by means of molecular techniques.

Materials and methods

Isolation of *Trichoderma* strains

Soil samples were collected from the wheat cultivated area of Argentina during 2008–2011. Samples were obtained using the hierarchical method sampling from eight representative locations. Samples were stored until their use at 4 °C.

The *Trichoderma* selective medium (Elad et al. 1981) was used for quantitative isolation of this fungus. From colonies determined as *Trichoderma,* according to Samuels et al. (2013), monoconidial isolates were obtained by transferring a germinated conidium on potato dextrose agar (PDA; Difco Laboratories). *Trichoderma* isolates were stored on cellulose filter paper following Stocco et al. (2010).

Production of *Trichoderma* spp. inoculum

All *Trichoderma* isolates were grown on 2 % PDA and incubated for 7 days at 26 ± 2 °C. Conidia from each isolate were harvested by flooding the cultures with sterile distilled water and then rubbing the culture surface with a sterile glass rod. The suspensions were filtered through-out two layers of cheesecloth. The concentrations of propagules in suspensions were standardized with the aid of a hemocytometer to 1×10^8 conidia mL^{-1} for each *Trichoderma* isolate tested.

To produce *Trichoderma* coated-seeds, 10 mL of suspension of each isolate was mixed with 90 mL of 0.25 % agar in water, which served as an adhesive (Stocco et al. 2012). The mixture was shaken using a magnetic stirring bar until a homogeneous suspension was obtained. Wheat seeds of the Buck Guapo cultivar were added to the water-agar-fungal biomass and mixed for pelletizing. Seeds uniformly coated with *Trichoderma* formulations were air-dried overnight in darkness at room temperature and sown 24–48 h later.

Production of *Zymoseptoria tritici*

Two virulent cultures of *Z. tritici* (FALP9J008 and FALPLA008, isolated from the localities of 9 de Julio and Plá respectively) were used for the production of all the inocula. Both cultures were isolated from naturally infected wheat leaves which presented typical lesions with pycnidia, in 2008. The fungus was stored in mineral oil at 4 °C until it was used for inoculation in the greenhouse at the Centro de Investigaciones de Fitopatología (CIDEFI), La Plata, Argentina. Both isolates were recovered on PDA until the typical mucous colonies with secondary conidia were

developed. At 10 days of growth, 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 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 inoculum suspension was adjusted to 1×10^7 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 conidial suspension and mixed (Cordo et al. 2007).

Greenhouse assays

To determine the antagonistic ability of *Trichoderma* spp. against *Z. tritici*, wheat plants were grown from seeds coated with each of 240 *Trichoderma* isolates. The coated seeds were sown in 16 × 10 × 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. The average temperature and relative humidity under control conditions were 18 °C and 72 % respectively. The experimental layout was a randomized block design with 31 treatments and three replications per *Trichoderma* strain. After 10–12 days of sowing, wheat seedlings from coated seeds with the first leaf emerged (stage GS 1.1, Zadoks et al. 1974) were sprayed with a mixture of both strains of the pathogen until run-off, using a manually operated sprayer. The plants used as a control grown from seeds free of *Trichoderma* spp. and receiving spores suspension of the pathogen. After inoculating, all plants were covered with plastic bags for 2 days to maintain a high level of humidity. Disease severity was recorded 21 days after inoculation. For the evaluation, the first and second leaves of each treatment were selected and the percentage of necrotic area and pycnidial coverage were estimated. This infection was compared to the infection of leaves of the control. Antagonism was assessed by the relative ability of each isolate to restrict the development of lesions. Data of each experiment were analyzed by an analysis of variance and means were compared by the LSD test ($P \leq 0.05$) (Infostat). *Trichoderma* strains were selected following their capacity to reduce the necrotic surface and/or pycnidial coverage on wheat plants by more than 50 %.

DNA extraction

Each *Trichoderma* isolate was grown for 5 days at room temperature in 50 mL of liquid malt extract medium on an orbital shaker. Mycelia were harvested under vacuum and lyophilized. Then, 100 mg of powdered mycelia was used for DNA extraction following a modified version of the cetyltrimethylammonium bromide (CTAB) method (Murray and Thompson 1980). DNA quality was determined by electrophoresis on 0.9 % agarose gel.

PCR amplification of fungal-specific genes

PCR amplification of internal transcribed spacers (ITS) of the ribosomal DNA (rDNA) region from each *Trichoderma* isolate was performed with primers ITS1 (5′-TCCGTAG GTGAACCTGCGG-3) and ITS4 (5́-TCCTCCGCTTATT GATATGC-3) (White et al. 1990). Each amplified fragment includes the ITS1, the 5.8S rDNA gene and the ITS2 regions. PCR was performed in a mixture containing 10 ng of genomic DNA, 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each of the four dNTPs, 0.5 mM of each primer, and 1 unit of Taq polymerase (Promega). Thermal cycling conditions involved an initial denaturation step at 94 °C for 2.5 min, followed by 30 cycles of 94 °C for 15 s, 48 °C for 1 min and 72 °C for 1.5 min, and a final extension at 72 °C for 10 min. 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 GelRed (Biotium, Hayward, USA) and photographed with a Fotodyne system (Hartland, WI, USA).

DNA sequencing and data analysis

The PCR amplification products were purified and sequenced by Macrogen (Korea), using primer ITS4. Each sequence was entered into GenBank and compared with those of known origin by using the BLAST search program (Altschul et al. 1997). All the sequences obtained in this work were deposited in the European Molecular Biology Laboratory (EMBL) database and sequence accession numbers for sequences are shown in Table 1.

ISSR fingerprints

Six ISSR primers were selected to analyze the genetic diversity among *T. harzianum* strains: CT(AC)8-(KA5); (AG)8TA–(AA3); ACA(CAA)5 (IA5); (GA)8ACC (FA3); (CAA)5ACG (DA3) and TCA(GT)8 (GA5) (Stenglein and Balatti 2006; Moreno et al. 2008; Consolo et al. 2012). These primers were selected based on their ability to systematically amplify the same fragment(s) from a given isolate. PCRs for ISSR primers were performed in a BIOER equipment (Technology Co., Hangzhou, China) in a 25 μL final volume, containing 10 ng of genomic DNA,

Table 1 Identification of *Trichoderma* isolates from soils of eight localities of the Argentine wheat area

Strains	Year of collection	Field location	*Trichoderma* sp.	Accession code	Similarity
1	2008	Los Hornos	*T. harzianum*	HG940466	84
2	2008	Los Hornos	*T. harzianum*	HG940467	96
5	2008	Los Hornos	*T. harzianum*	HG940468	99
8	2008	Los Hornos	*T. harzianum*	HG940469	99
10	2008	Los Hornos	*T. harzianum*	HG940470	99
12	2008	Los Hornos	*T. harzianum*	HG940471	99
26	2008	Los Hornos	*T. harzianum*	HG940472	99
54	2008	Bragado	*T. harzianum*	HG940473	94
69	2009	Bordenave	*T. harzianum*	HG940474	97
73	2009	Bordenave	*T. harzianum*	HG940475	99
77	2009	Bordenave	*T. harzianum*	HG940476	95
80	2009	Bordenave	*T. harzianum*	HG940477	99
83	2009	Bordenave	*T. harzianum*	HG940478	99
92	2009	Paraná	*T. harzianum*	HG940479	99
93	2009	Paraná	*T. harzianum*	HG940480	97
97	2009	Paraná	*T. harzianum*	HG940481	99
107	2009	Paraná	*T. harzianum*	HG940482	99
108	2009	Paraná	*T. harzianum*	HG940483	98
114	2009	Paraná	*T. harzianum*	HG940484	99
123	2010	Manfredi	*T. harzianum*	HG940485	99
129	2010	Manfredi	*T. harzianum*	HG940486	99
131	2010	Manfredi	*T. harzianum*	HG940487	98
140	2010	Manfredi	*T. harzianum*	HG940488	88
141	2010	Manfredi	*T. harzianum*	HG940489	99
160	2010	Lobería	*T. harzianum*	HG940490	99
162	2010	Lobería	*T. harzianum*	HG940491	99
165	2010	Lobería	*T. harzianum*	HG940492	99
170	2010	Lobería	*T. harzianum*	HG940493	96
172	2010	Lobería	*T. harzianum*	HG940494	98
177	2010	Lobería	*T. harzianum*	HG940495	99
181	2011	Pergamino	*T. harzianum*	HG940496	99
182	2011	Pergamino	*T. harzianum*	HG940497	98
183	2011	Pergamino	*T. harzianum*	HG940498	99
206	2011	Pergamino	*T. harzianum*	HG940499	99
210	2011	Pergamino	*T. harzianum*	HG940500	99
215	2011	Santa Fe	*T. harzianum*	HG940501	99
229	2011	Santa Fe	*T. harzianum*	HG940502	99

20 mM Tris–HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each of the four dNTPs, 2.5 mM of each primer, and 1 unit of Taq polymerase (Promega). Thermal cycling conditions involved an initial denaturation step at 94 °C for 7 min, followed by 33 cycles at 94 °C for 60 s, 48 °C for 75 s, and 72 °C for 4 min. A final extension step of 72 °C for 7 min was added. Then, 15 μL of PCR products were separated by electrophoresis in a gel containing 1.5 % agarose (Genbiotech), in 1X TAE buffer (40 mM Tris–HCl, pH 8, and 1 mM EDTA). Each reaction was performed at least twice. Gels were run for 3–4 h at 100 V, stained with GelRed, and photographed with a Fotodyne system (Hartland, WI, USA).

ISSR analysis

ISSR fingerprints were first scored visually and sorted into groups based on similarities between amplified DNA profiles. These preliminary groups of isolates were then analyzed together on the same gel to confirm fingerprint identity or similarity and to quantify the number of shared fragments among isolates. The software Phoretix 1D

(Totallab) was used to analyze the presence or absence of amplified fragments of each band profile and to determine the genetic relationship among isolates. Isolates sharing more than 95 % of their bands were considered a single haplotype. A similarity matrix was calculated using the DICE coefficient (SD). Cluster analysis based on the unweighted pair group method of averages (UPGMA) (Sneath and Sokal 1973) was performed using NTSYS software, version 2.1 (Rohlf 1998) and a dendrogram was constructed. The cophenetic correlation coefficient was used as a measure of goodness of fit for the cluster analysis. The robustness of the clusters in the dendrogram was assessed by bootstrap analysis using Winboot software (Yap and Nelson 1996), and 1000 repeated samplings with replacement were conducted.

Results

Isolation of *Trichoderma* strains

A total of 240 *Trichoderma* spp. isolates were obtained from the wheat rhizosphere soil sampled from eight localities (thirty from each locality): Los Hornos isolates 1–30, Bragado isolates 31–60 (II South), Bordenave isolates 61–90 (V South), Paraná isolates 91–120 (III), Manfredi isolates 121–150 (V North), Lobería isolates 151–180 (IV), Pergamino isolates 181–210 (II North) and Santa Fe isolates 211–240 (I) (Fig. 1).

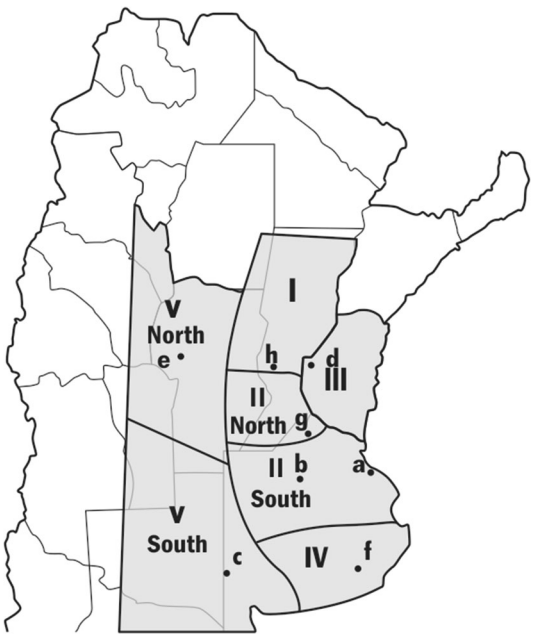

Fig. 1 Map of Argentina wheat area showing sites of isolation of *Trichoderma:* (*a*) Los Hornos (*b*) Bragado (*c*) Bordenave (*d*) Paraná (*e*) Manfredi (*f*) Lobería (*g*) Pergamino (*h*) Santa Fe

Greenhouse assay

Of the 240 strains of *Trichoderma* that were evaluated for biocontrol of leaf blotch caused by *Z. tritici*, 84 were able to reduce the disease significantly, between 1 and 95 %, for both parameters of severity (pycnidial coverage and necrotic area). All the locations sampled were represented by at least one strain of *Trichoderma* biocontrol good fitness behavior. Thirty-seven strains were selected for the reduction of severity, which were equal to or greater than 50 % of the disease development, compared with the control (plants inoculated only with *Z. tritici*) (Fig. 2). The percentage values of pycnidial coverage ranged between 45 and 80 %. Strains 123 (Manfredi) and 170 (Lobería) showed disease reduction values greater than 90 % (Fig. 2).

Identification of *T. harzianum* isolates and ISSR fingerprints

Thirty-seven monoconidial isolates of *Trichoderma* were confirmed as *T. harzianum* after sequencing (Table 1). All six ISSR markers were successfully amplified in all cases and yielded 132 bands ranging in size from 200 to 3000 bp, with an average of 16 polymorphic bands per primer, showing 79.5 % polymorphism and a polymorphism information content (PIC) value ranging from 0.143 to 0.342, with an average of 0.265 (Table 2). Since the maximum value of PIC for dominant markers is 0.5, the PIC value observed in this study shows an intermediate variability among the isolates. A representative banding pattern using the primer $(AG)_8TA$ is shown in Fig. 3. UPGMA distributed the 37 genotypes into 19 haplotypes of *T. harzianum*, which represents that 51 % of the isolates showed a unique banding pattern. The isolates were grouped into three clusters with a similarity between groups of 50 %. Group I included 16 isolates: three from Los Hornos (2, 10, 26), four from Bordenave (69, 73, 80, 83), six from Paraná (92, 93, 97, 107, 108, 114) and three from Manfredi (123, 129 and 140). Group II included 13 isolates: four from Los Hornos (1, 5, 8, 12), one from Bragado (54), six from Manfredi (131, 141, 160, 162, 165, 170), and two from Lobería (172 and 177). Group III included seven isolates: one from Bordenave (77), four from Pergamino (181, 182, 183, 206) and two from Santa Fe (215 and 229) (Table 1; Fig. 4). The cophenetic correlation coefficient was 0.952 and bootstrap values were greater than 90 %, indicating that the generated dendrogram is a good representation of the genetic relationship between isolates.

Discussion

The genus *Trichoderma* has been widely studied and used for biological control for a broad range of phytopathogens (Harman 2000; Monte 2001; Quiroz-Sarmiento et al. 2008).

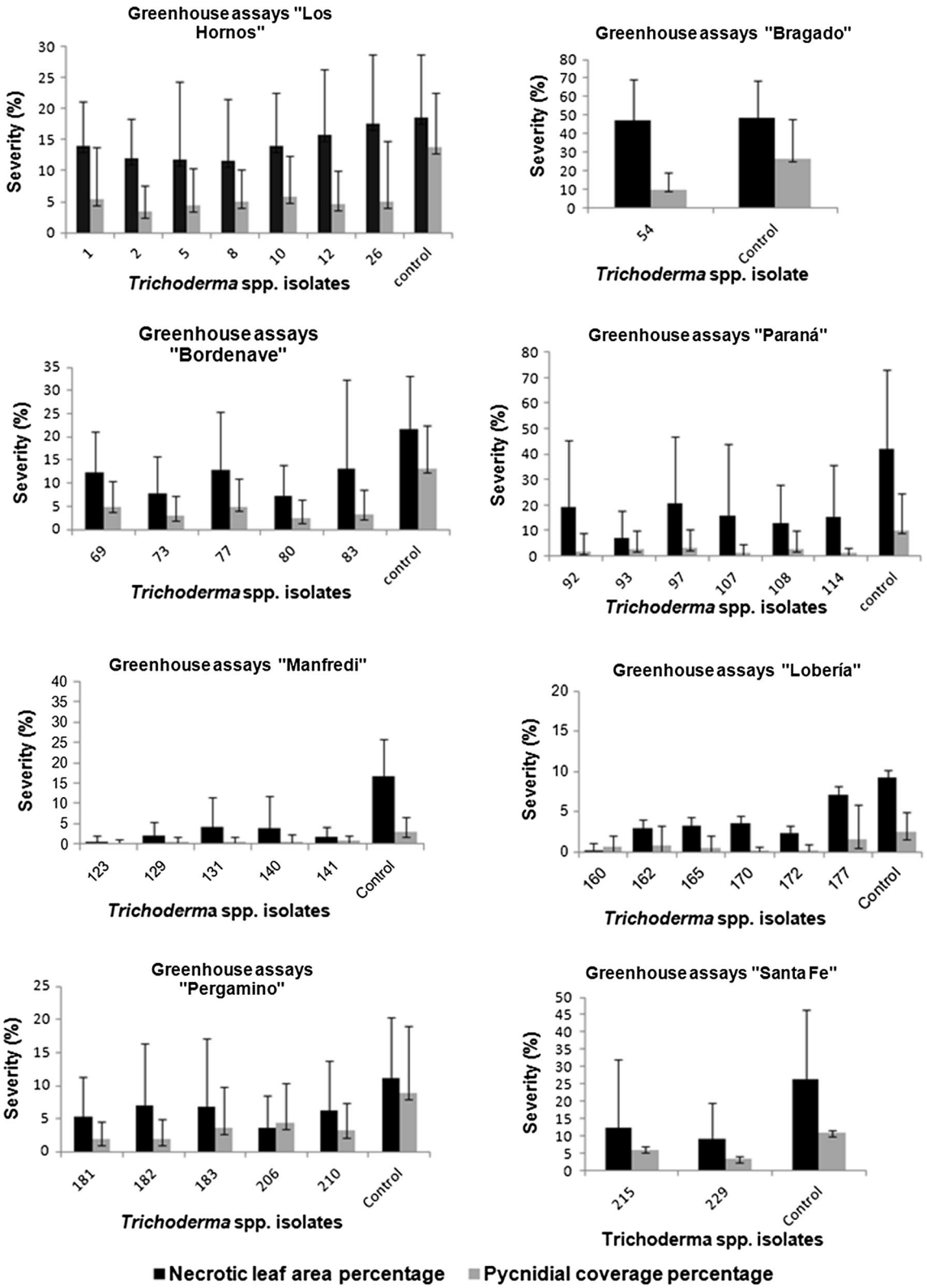

Fig. 2 Reduction of the disease severity (necrotic and pycnidial coverages) caused by Z. *tritici* in plants pretreated with *T. harzianum* isolates obtained from the soil of eight localities of the Argentine wheat area. Control treatment was represented by plants coming from seeds without the antagonist. *Vertical lines* on bars represent the standard deviation of each treatment. Significant differences between treatments were obtained by LSD test ($p \leq 0.05$)

Table 2 ISSR primer sequences and polymorphic bands generated by PCR from 37 isolates of *T. harzianum* isolates

ISSR sequence	Number of total loci	Number of polymorphic loci	Polymorphism information content (PIC)
$CT(AC)_8$	30	25	0.318
$(AG)_8TA$	25	20	0.289
$ACA(CAA)_5$	19	14	0.243
$(GA)_8ACC$	22	20	0.342
$TCA(GT)_8$	24	20	0.248
$(CAA)_5ACG$	12	7	0.153

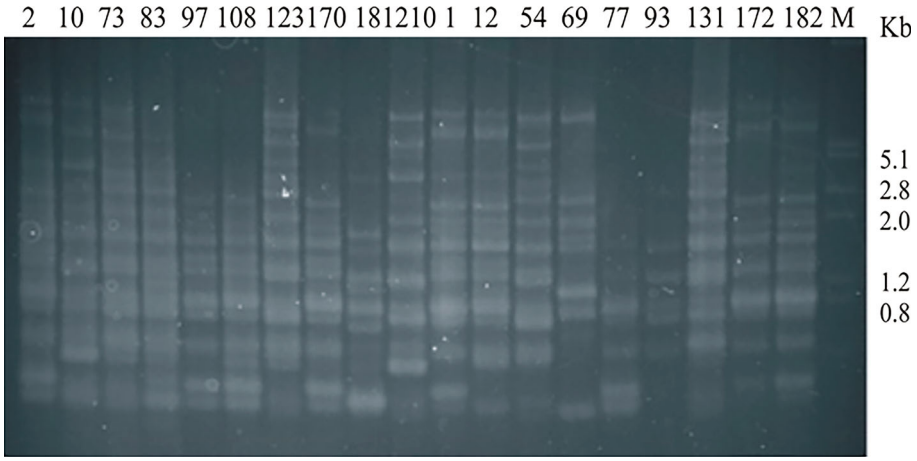

Fig. 3 PCR amplification patterns of 19 *Trichoderma harzianum* isolates using the ISSR primer TCA(GT)8. M molecular size marker (*Pst*I-digested λ DNA), in kilobases (kb)

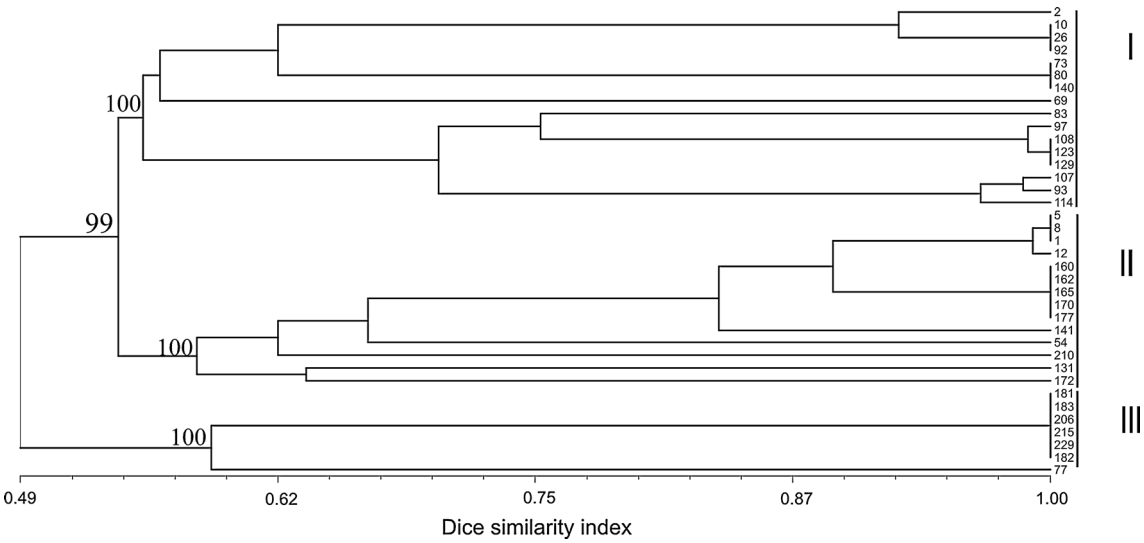

Fig. 4 UPGMA dendrogram based on Dice (SD) similarity index, illustrating the genetic relationship among 37 *Trichoderma harzianum* isolates from Argentina

The most important biocontrol *Trichoderma* species are *T. harzianum*, *T. virens*, and *T. viride* (Hermosa et al. 2004), especially useful in the biocontrol action of pathogens of different crops such as maize, cotton, wheat, tobacco, tomato, cucumber and lima bean (De Meyer et al.1998; Engelberth et al. 2001; Harman 2000; Howell et al. 2000; Perelló et al. 1997; Rotblat et al. 2002; Viterbo et al. 2007).

In Argentina, studies on the biological control of foliar wheat diseases have shown that *T. harzianum*, *T.*

aureoviride and some *T. koningii* strains are effective antagonists against phylloplane pathogens, reducing the leaf blotch caused by *Z. tritici* (Cordo et al. 2007; Perelló et al. 1997, 2001, 2003, 2006). Although results in controlling this disease is promising, in Argentina, the development of commercial biological products based on *T. harzianum* is scarce. So far, only two products have been registered: Plant shield HC, originated in the USA and commercialized by Wassington Argentina, and Trat Bac, from Argentina and commercialized by Bea Tecno Bio S.A. (Rivera and Wright 2014).

Several reports have indicated that the biocontrol efficiency of *Trichoderma* spp. against different pathogens may vary in different regions of the world, i.e., that a species that is highly antagonistic against a particular pathogen in a given region may react poorly to the same pathogen in another place (Ashrafizadeh et al. 2002; Hajieghrari et al. 2008; Howell 2003; Otadoh et al. 2011). For this reason, in biocontrol programs, it is very important to use native strains to develop effective products containing species that are compatible with the environment or with the characteristics of the region where they will be applied (Rabeendran et al. 2006). Moreover, to control *Z. tritici* on wheat leaves, the antagonists selected in this research were obtained from soils of the wheat cultivated areas of Argentina.

In the present study, the importance of reducing the pycnidial coverage on leaf blotch disease with native strains of *Trichoderma* is based on the inoculum reduction of the pathogen available for a new infection. Epidemics are generated when large amounts of inoculum are reproduced for fast growth and are in contact with very susceptible plants (Stubbs et al. 1986). Regarding SLB, the conidia contained in the pycnidia of Z. *tritici* from the lower leaves of the crop reach the top of the canopy by share rainsplash drops (Lovell et al. 1997). As a consequence, the reduction of the pycnidial coverage by *T. harzianum* biocontrol release fewer conidia for dispersion and made slower the progression of the disease in plants. Recent results with strains of *Trichoderma* spp. have confirmed this through the reduction of the area under the disease progress curve (AUDPC). Treatments with *Trichoderma* spp. on wheat plants as biofungicides, with at least two applications (seed pelleting and an aerial application at tillering), not only decrease the severity of the disease until doughy grain but also increase yield (kg ha^{-1}) and reduce the rate of increase of SLB (Cordo et al. 2012). Our results indicate that strains 123 and 170 are native isolates adapted to the ecological conditions of the wheat crop in Argentina. These strains have been identified and characterized genetically and are considered important for their great potential as biocontrol agents. For this reason, they could be good candidates to be considered in the formulation of a biological product. Further studies should be conducted to evaluate their behavior in the field.

Several reports indicate that *Trichoderma* species have evolved as opportunistic avirulent plant symbionts and that they are able to establish interactions that induce metabolic changes in plants. These changes increase resistance to a wide range of phytopathogenic fungi (Harman et al. 2004). *Trichoderma* isolates added to the rhizosphere protect plants because they induce resistance mechanisms (Benitez et al. 2004; Haggag 2008; Harman et al. 2004). Cordo et al. (2007) established that the seed coating technique is more effective against leaf blotch than the leaf spraying technique. Perelló et al. (1997, 2003, 2009) established that the *Trichoderma* spp. population decreased rapidly when the aerial inoculum was applied on the most advances growth stages of wheat. They concluded that this may be explained by differences in the tolerance of *Trichoderma* conidia to climate conditions such as high temperature, ultraviolet light and less wetness (Perelló et al. 2009). On the other hand, the seed coating technique used in this work to inoculate *Trichoderma* is simple, advantageously and reproducible to be applied under field conditions.

In this study, the 37 *T. harzianum* strains identified through ITS sequencing and ISSR markers allowed the determination of genetic variation among the isolates. It was able to define 19 haplotypes, grouped in three clusters, showing an intermediate variability among the isolates (PIC value 0.265). It is important to note that the genetic variability was analyzed in a small group over a total of 240 strains, which were previously selected for their good biocontrol potential against *Z. tritici*. This pre-selection of the strains could explain the intraspecific genetic variation. The origin of this molecular variability is unclear. Since *Trichoderma* is a diploid and heterothallic fungus (Seidl et al. 2009), different strains are likely to recombine and evolve rapidly depending on the frequencies of mating type. Other studies have indicated that *T. harzianum* has a complex speciation history, which reveals overlapping of the isolated biological species with sexual reproduction, recent agamospecies and numerous relict lineages with unresolved phylogenetic positions (Druzhinina et al. 2010).

The three UPGMA clusters observed in this study shared strains of different geographical origins, isolated in different years. However, the origin of the isolates and the genetic group were partially related. All isolates from Paraná were in cluster I, all isolates from Lobería were in cluster II, and all isolates from Pergamino and Santa Fe were in cluster III. Other studies have found no relationship between polymorphism and the origin or habitat of the strains (Góes et al. 2002; Gherbawy et al. 2014; Sharma et al. 2009). Thus, to confirm this relationship, we will need to increase the number of isolates studied in some locations.

Biocontrol properties and molecular techniques are important tools to identify and characterize *T. harzianum* isolates. Furthermore, the importance of using native strains in biocontrol programs could be advantageous for the preparation of biopesticides adapted to agroecological conditions. Molecular techniques could help to eliminate duplicates strains and to detected contamination or mutation of them during mass production and long-term maintenance. Further experimental work is needed to determine the effectiveness of these isolates under different field conditions and consider them as candidates for mass production as an alternative to chemical pesticides.

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