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ORIGINAL PAPER

Characterization of novel *Trichoderma* spp. isolates as a search for effective biocontrollers of fungal diseases of economically important crops in Argentina

Verónica Fabiana Consolo · Cecilia Inés Mónaco · Cristina Alicia Cordo · Graciela Lidia Salerno

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Abstract Monoconidial cultures of 33 isolates of *Trichoderma* from Buenos Aires Province, Argentina were characterized on the basis of twenty eight morphological, physiological and biochemical features. All of them were screened for proteinase, endochitinase and β-1,3 glucanase activity. Universally primed PCR (UP-PCR) and inter-simple sequence repeat (ISSR) techniques were used to examine the genetic variability among isolates, which resulted in 127 bands for the total number of isolates. These results were subjected to numerical analysis revealing 20 haplotypes grouped in five clusters. The ability of *Trichoderma* isolates to antogonize soil-borne fungal plant pathogens using a dual culture assay was done against five fungal species: *Alternaria* sp., *Bipolaris sorokiniana, Fusarium graminearum*, *F. solani*, and *Pyricularia oryzae*. The highest inhibition values (85% RI) were obtained against *B. sorokiniana* and *P. oryzae*. Three isolates of *T. harzianum* named as FCCT2, FCCT3 and FCCT9 were capable of causing a high growth inhibition on four of the fungal species assayed, which was in agreement with their higher extracellular hydrolytic activity. Our results suggest that these isolates have the potential to be effective agents for biocontrol of cereal and tomato fungal pathogens.

V. F. Consolo (✉) · G. L. Salerno
Centro de Estudios de Biodiversidad y Biotecnología (INBA-CONICET), Centro de Investigaciones Biológicas, Fundación para Investigaciones Biológicas Aplicadas (CEBB-CIB-FIBA), C.C. 1348, 7600 Mar del Plata, Argentina
e-mail: faconsolo@fiba.org.ar

C. I. Mónaco · C. A. Cordo
CIDEFI, Facultad de Ciencias Agrarias y Forestales, Universidad Nacional de La Plata, Calle 60 y 119, 1900 La Plata, Argentina

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Introduction

Species of the genus *Trichoderma* (Ascomycota, Hypocreales) comprise a great number of fungal strains with economic importance mainly as biological control agents (BCAs). These soil-borne free-living microorganisms are non pathogenic fungi, which can also colonize roots of numerous plants. They are recognized for their important benefits to agriculture such as their ability to protect crops against diseases and to increase crop yield under field conditions (Harman et al. 2004). So far, *Trichoderma* spp. are among the biocontrollers commercially marketed as biopesticides, biofertilizers and soil amendments (Harman et al. 2004; Lorito et al. 2004). Several mechanisms have been suggested as being responsible for the control of plant disease by *Trichoderma* isolates. It was reported that they are capable of producing extracellular lytic enzymes such as chitinases, glucanases and proteases (Chet and Chernin 2002; Viterbo et al. 2002). This observation, together with the fact that chitin and β-1,3 glucan are the main structural components of fungal cell wall, suggests that chitinases and β-1,3 glucanases produced by some *Trichoderma* isolates are key enzymes in the lysis of cell walls during mycoparasitic action, being most frequently considered playing an important role in biocontrol (Kullnig et al. 2000; Kubicek et al. 2001). On the other hand, *Trichoderma* proteases have been reported to play a role in the host lysis by attacking lipids and proteins, which are also part of the cell–wall skeleton. Also *Trichoderma* spp. strains produce antifungal antibiotics (Ghisalberti and Rowland 1993), may be competitors to fungal pathogens (Grondona et al. 1997),

promote plant growth (Vinale et al. 2004), and induce plant defence responses in plants (Harman et al. 2004).

Most *Trichoderma* species grow rapidly in artificial culture media and produce large number of small green or white conidia from conidiophores. The most common strains of *Trichoderma* genus used as BCAs are *T. virens*, *T. viride* and above all, *T. harzianum* (Grondona et al. 1997). There is an increasing interest in this genus because of its biological properties against soil-borne plant pathogenic fungi and commercialization as biopesticides, biofertilizers and soil amendments (Harman et al. 2004; Lorito et al. 2004).

Knowledge concerning the behaviour of *Trichoderma* as antagonists is not only essential for their effective use but also because there is a need for a more precise characterization of the isolates. Several attempts to assess genetic diversity have been used for the characterization and grouping of isolates including isozyme analysis (Stasz et al. 1989; Leuchtmann et al. 1996), polymerase chain reaction (PCR) fingerprinting and sequence analysis (Zimand et al. 1994; Kuhls et al. 1995; Turner et al. 1997; Bulat et al. 1998), random DNA (RAPD)-like PCR fingerprinting method and universally primed PCR (UP-PCR) (Cumagun et al. 2000). Another method that could be used to assess genetic diversity within and among fungi is non-anchored inter simple sequence repeat (ISSR). This technique consists on the amplification of DNA sequences between simple sequence repeats by means of homologous primers without previous knowledge of the sequence, generating specific and reproducible patterns (Bornet and Branchard 2001). The use of these techniques for genetic diversity assessments helps to eliminate duplicated strains in programs for microbial selection (Samson 1995), and during mass production and maintenance of long-term cultures, which could lead to contamination (Markovic and Markovic 1998) or mutation (Becker and Schwinn 1993).

Although around the world several fungicides based on formulation of *Trichoderma* have been commercialized in the last few years, there is still appreciable interest on finding and formulating more efficient products based on this fungus. Particularly, in Argentina commercial products based on *Trichoderma* have been recently registered and used as BCA and plant growth promoters. To select more effective and antagonistic strains for biocontrol ability, a completely characterization under a range of conditions, and strictly selection and evaluation should be done. Understanding both the genetic diversity of strains within *Trichoderma* species and their mechanisms of biocontrol will lead to improve the application of the different strains as BCAs. Because the value of a selected BCA will also depend on the applicability of isolates to different ecozones (Grondona et al. 1997) there is still interest in finding novel native strains with potential biological control activity.

The aim of this study was to isolate and select novel native strains of *Trichoderma* spp. from different agricultural systems of Buenos Aires Province, Argentina, with high quality as potential plant fungal controllers. We examined morphological, biochemical, physiological and molecular features of the isolates in addition to their ability as antifungals against five plant pathogens, important fungal pests of agricultural products.

Materials and methods

Isolation and identification of *Trichoderma* spp.

Nineteen soil samples were collected using a cylindrical tube, from different agroecosystems in Buenos Aires Province, Argentina, during 2007–2008 (Table 1). Each sample was taken randomly at 20 cm soil depth and stored at 4°C within a period of 1 month. The serial dilution technique was used for *Trichoderma* isolation (Elad et al. 1981). One ml of 10^{-3} soil dilution was plated on selective medium using rose bengal and pentachloronitrobenzene, and incubated at 28°C for 5–7 days. From colonies determined as *Trichoderma* according to Samuels et al. (2009), monoconidial isolates were obtained by transferring a germinated conidium on potato dextrose agar medium (PDA).

DNA extraction

Each *Trichoderma* isolate was grown for 5 days at room temperature in 50 ml of liquid malt extract medium on a orbital shaker. Mycelia were harvested under vacuum and lyophilized. About 100 mg of powered mycelia were used for DNA extraction following a modified version of the cetyltrimethylammonium bromide (CTAB) method (Murray and Thompson 1980), as follows. In total, 750 µl of extraction buffer (100 mM Tris–HCl, pH 8, 100 mM EDTA, 250 mM NaCl, and 2% CTAB) and 15 µl of 2-mercaptoethanol were added to the lyophilized mycelia and incubated at 65°C for 30 min. Cellular proteins were precipitated with 300 µl of 3 M potassium acetate (pH 4.8). After centrifugation at maximum speed in a microcentrifuge (Hermle Labortechnik GmbH, Wehingen, Germany), the supernatant was transferred to a new tube and extracted with 500 µl of phenol:chloroform:isoamyl alcohol (25:24:1) and chloroform:isoamyl alcohol (24:1) until the interface became transparent. Nucleic acids were precipitated by adding 750 µl of cold isopropanol followed by incubation at 4°C for 30 min. After centrifugation at 17,500 *g* for 5 min, the pellet was rinsed twice with 500 µl of 70% ethanol, air-dried and dissolved in 100 µl of Tris–EDTA buffer (10 mM Tris–HCl pH 8 and 1 mM EDTA). DNA quality was determined by electrophoresis on 0.9% agarose gel.

Table 1 Identification of *Trichoderma* isolates from soils of Buenos Aires Province, Argentina

Strain	Soil source	Field location	Year of collection	Closest species match	Accession code	Similarity (%)
FCCT1	Soybean	Azul	2007	*T. harzianum*	HE601545	99
FCCT2	Soybean	Azul	2007	*T. harzianum*	HE601546	98
FCCT3	Soybean	Azul	2007	*T. harzianum*	HE608884	98
FCCT4	Soybean	Azul	2007	*T. harzianum*	HE608893	98
FCCT5	Soybean	Azul	2007	*T. harzianum*	HE608885	98
FCCT6	Soybean	Azul	2007	*T. koningii*	HE608894	98
FCCT7	Soybean	Azul	2007	*T. koningii*	HE608895	98
FCCT8	Soybean	Azul	2007	*T. harzianum*	HE608896	99
FCCT9	Soybean	Azul	2007	*T. harzianum*	HE608897	99
FCCT10	Soybean	Azul	2007	*T. harzianum*	HE608886	86
FCCT11	Rice	La Plata	2007	*T. virens*	HE608898	98
FCCT12	Rice	La Plata	2007	*T. virens*	HE608899	98
FCCT13	Rice	La Plata	2007	*T. virens*	HE608900	98
FCCT14	Corn	Sierra de Los Padres	2008	*T. harzianum*	HE608901	99
FCCT15	Corn	Sierra de Los Padres	2008	*T. harzianum*	HE608902	99
FCCT16	Corn	Sierra de Los Padres	2008	*T. harzianum*	HE608911	99
FCCT17	Corn	Balcarce	2008	*T. harzianum*	HE608892	80
FCCT18	Corn	Balcarce	2008	*T. harzianum*	HE608912	99
FCCT19	Corn	Balcarce	2008	*T. harzianum*	HE608887	99
FCCT20	Corn	Balcarce	2008	*T. harzianum*	HE608890	99
FCCT21	Corn	Sierra de Los Padres	2008	*T. harzianum*	HE608888	99
FCCT22	Corn	Sierra de Los Padres	2008	*T. harzianum*	HE608913	99
FCCT23	Corn	Sierra de Los Padres	2008	*T. harzianum*	HE608914	99
FCCT24	Corn	Sierra de Los Padres	2008	*T. harzianum*	HE608891	98
FCCT25	Corn	Sierra de Los Padres	2008	*T. harzianum*	HE608889	99
FCCT26	Corn	Sierra de Los Padres	2008	*T. virens*	HE608903	89
FCCT27	Corn	Sierra de Los Padres	2008	*T. virens*	HE608904	89
FCCT28	Corn	Sierra de Los Padres	2008	*T. virens*	HE608905	99
FCCT29	Corn	Sierra de Los Padres	2008	*T. virens*	HE608906	97
FCCT30	Corn	Sierra de Los Padres	2008	*T. virens*	HE608907	97
FCCT31	Corn	Sierra de Los Padres	2008	*T. virens*	HE608908	97
FCCT32	Corn	Sierra de Los Padres	2008	*T. virens*	HE608909	97
FCCT33	Corn	Sierra de Los Padres	2008	*T. virens*	HE608910	97

PCR amplification of fungal-specific genes

Polymerase chain reaction (PCR) amplification of internal transcribed spacers (ITS) of the ribosomal DNA (rDNA) region from each *Trichoderma* isolate was performed with primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White et al. 1990). Each amplified fragment includes the ITS1, the 5.8S rDNA gene and the ITS2 regions. PCR was conducted 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 (Invitrogen, Carlsbad, CA, USA). Thermal cycling conditions involved an initial denaturation step at 94°C for 2.5 min, followed by 40 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 (Invitrogen Carlsbad, CA, USA) in 1× TAE buffer (40 mM Tris–HCl, pH 8, and 2 mM EDTA). Gels were run for 1 h at 120 V, stained with ethidium bromide and photographed with a Fotodyne system (Hartland, WI, USA).

DNA sequencing and data analysis

PCR amplification products were purified and sequenced by Macrogen (Korea) using primer ITS4. Each sequence was entered in Genbank and compared with those of known origin by using the BLAST search program (Altschul et al. 1997). All the sequences obtained in this

study were deposited in the European Molecular Biology Laboratory (EMBL) and assigned accession numbers listed in Table 1.

Morphology

Colony diameter on PDA, mycelium and chlamydospore production, and conidium sizes were examined and registered.

Physiological characterization

Growth at different temperatures and pH values

The ability of *Trichoderma* isolates to grow at 4, 37 and 40°C over 14 days was tested on PDA, and the thermal resistance of the spore suspensions was determined after 5 min incubation at 75°C (Grondona et al. 1997). Growth at pH 2, 10 and 12 was tested in liquid medium containing 0.05 g bromocresol purple l^{-1} (Bridge 1985).

Assimilation of nitrogen sources

Growth, sporulation and pigment production were determined on solid media containing ammonium oxalate, sodium nitrite, creatine or urea. Plates were incubated at 24 ± 2°C and evaluated after 14 days (Bridge 1985; Grondona et al. 1997).

Assimilation of carbon sources

Growth and sporulation in liquid medium with citric acid, glucose, ethanol or lactose as sole carbon sources were performed in the presence of 0.005 or 0.001% crystal violet, according to Bridge (1985).

Assay of inhibition of plant pathogenic fungi on agar

The fungi *Alternaria* sp., *Bipolaris sorokiniana, Fusarium graminearum* were selected because they cause major foliar disease and grain discoloration on cereals. They mainly affect wheat yield in Buenos Aires Province (Galich 1997). Conversely, *F. solani* causes wilt and basal root rot on tomato (Dal Bó et al. 2010). All these fungi were kindly provided by Centro de Estudios en Fitopatologia (CIDEFI), Facultad Agronomia, Universidad Nacional de La Plata. The fungus *Pyricularia oryzae* is responsible for the most important rice disease (blast) in Argentina (Consolo et al. 2008). The *P. oryzae* isolate used in the bioassays was from the Fundación para Investigaciones Biológicas Aplicadas Culture Collection (FCC). Fungal isolates were incubated on PDA at 25°C for 6 days, before being used in inhibition assays.

Biocontrol assays were performed on PDA plates using a dual culture technique (Grondona et al. 1997) by inoculating each *Trichoderma* isolate with one of the five fungal plant pathogens mentioned above. Each host fungus and the *Trichoderma* isolates were grown on PDA plates for a week at 26 ± 2°C. Blocks of the target fungus were cut from the periphery and transferred to a petri dish. After 48 h of growth of the target fungus, a block of each *Trichoderma* isolate was transferred in the same plate separated by about 3 cm. Plates were incubated at 26 ± 2°C for 7 days and observed periodically. Inhibition was registered as presence of an inhibition zone prior to any mycelial contact. The percentage of ratio inhibition (RI) was calculated as follows: $RI = 100 \times (R_2 - R_1)/R_2$ (Watts et al. 1988), where R_1 was the distance between the inoculum of the pathogen and the edge of colony measured in the direction of the inoculum of the *Trichoderma* isolate, and R_2 was the colony growth of the pathogen measured in the direction of maximum radius. RI value was the mean of three replicates per isolate. Microscopic examinations were done on the area of intermingling contact (pathogen–antagonist).

Determination of enzyme activities from *Trichoderma* spp.

Aesculin, starch, Tween 80, gelatin hydrolysis and reduction of tetrazolium were tested on solid media as previously described (Lynch et al. 1981; Bridge 1985; Grondona et al. 1997).

To determine specific activity of secreted proteinase, endochitinase and β-1,3 glucanase, isolates were grown on Vogel′s Medium for 48 h at room temperature at 150 rev min^{-1} and centrifuged. Culture filtrates were used for enzymatic activity determination. The activity of β-1,3 glucanase was assayed in the presence of 0.5% (w/v) laminarin (Sigma) in 50 mM sodium acetate buffer (pH 5.5) and reducing-sugar content was quantified by Somogyi and Nelson′s method after 30 min incubation at 37°C (de la Cruz et al. 1995). Endochitinase activity was detected using the highly sensitive substrate 4-methylumbelliferyl β-D-*N, N′, N″*-triacetylchitotriose (Sigma) after 30 min incubation at 37°C (Baek et al. 1999). Proteinase activity was determined in the presence of the synthetic substrate *N*-Succinyl-Ala–Ala-Pro-Phe *p*-nitroanilide (Sigma) after 30 min incubation at 37°C (St. Leger et al. 1987; Bindschedler et al. 2002). One unit of β-1,3 glucanase or endochitinase activity was defined as the amount of enzyme required to release 1 μM of glucose equivalents or of 4-methyl umbelliferone per min, respectively, and one unit of protease activity was defined as an increase of 1 OD unit at 410 nm produced by 10 μg of protein in 30 min.

Protein concentration was determined by the Bradford method using bovine serum albumin as standard.

Genetic variability of *Trichoderma* isolates

Twelve ISSR primers (Stenglein and Balatti 2006; Moreno et al. 2008) and four UP-PCR primers (Cumagun et al. 2000) were tested to analyse genetic diversity among *Trichoderma* isolates (Table 2). PCRs for ISSR primers were performed in an Eppendorf Gradient Mastercycler in a 20 µl final volume, 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, 2.5 mM of each primer, and 1 unit of Taq polymerase (Invitrogen, Carlsbad, CA). 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. The same procedure was followed for amplification reactions for UP-PCR primers except for primer annealing that was 56°C. Fifteen µl of PCR products were separated by electrophoresis in a gel containing 1.5% agarose (Invitrogen Carlsbad, CA, USA) in 1× TAE buffer (40 mM Tris–HCl, pH 8, and 1 mM EDTA). Gels were run for 3–4 h at 100 V, stained with ethidium bromide, and photographed with a Fotodyne system (Hartland, WI, USA).

Data analysis

Morphological tests were coded as on an "all present" system, where 0 is negative and 1 is positive. Physiological and biochemical tests on solid media were coded as 0 or negative and 1 as positive reaction. Twenty eight characters were evaluated from morphological, physiological and biochemical test.

To determine the genetic relationship among isolates, the presence or absence of amplified fragments was scored manually and was converted into binary data. Two similarity matrices were calculated using the DICE coefficient (SD) (Sneath and Sokal 1973). One of the matrices was based on morphological, physiological and biochemical features, and the other one was constructed with fingerprint data. Cluster analysis based on the unweighted pair group method of averages (UPGMA) (Sneath and Sokal 1973) was performed using NTSYS software, version 2.0 (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 combined set of morphological, physiological, biochemical and fingerprint data matrices were used to analyse the relationship between characters. A matrix comparison with 1,000 permutations was conducted using the Mantel test (Mantel 1967).

Results

Morphological features

Thirty three isolates of *Trichoderma* were obtained from nineteen samples collected on soybean, corn and rice soils from different fields located in Buenos Aires Province, Argentina. The identification based on ITS sequences and BLAST searches revealed three *Trichoderma* species: *T. harzianum* (20 isolates), *T. virens* (11 isolates) and *T. koningii* (2 isolates) (Table 1). Morphological characters were generally found to be highly variable within and between *Trichoderma* species. All isolates produced colonies larger than 8.1 cm on PDA medium, and developed aerial mycelia and conidia >2 µm diameter. Only the isolate named FCCT1 was able to produce abundant chlamydospores of about 11.5 µm.

Physiological features

It was observed that *Trichoderma* strains showed different growth at different temperatures. Except for FCCT2, FCCT19 and FCCT31 that only grew at 26°C, the other strains developed not only at 26°C but also at 37°C. In addition, none of the isolates was capable of growing at 4 or 40°C, but spores of them resulted resistant to temperatures as high as 75°C. The isolates did not show growth on media at pH 2, 10 or 12.

In solid media containing different nitrogen sources, only three isolates were capable of growing with ammonium oxalate or creatine but none of them grew with sodium nitrite or urea. Whereas all isolates developed on the media containing the different carbon sources, only nine isolates produced orange pigments on medium containing citric acid, glucose or ethanol.

In vitro inhibition assays

When we observed in vitro growth of five plant fungus pathogens, marked inhibition occurred in the presence of most of the 33 *Trichoderma* isolates. This test showed variations in the percentage of inhibition of radial growth of the soil-borne fungal plant pathogens (Table 2). The highest inhibition values (85% RI) were obtained against *B. sorokiniana* and *P. oryzae* with the isolates FCCT3 and FCCT9, respectively. Maximum RI percentage (62.5%) against *Alternaria* sp. was observed with isolates FCCT5 and FCCT8. For *F. graminearum* and *F. solani* the highest RI values were obtained with isolates FCCT2 and FCCT9 (61%), and FCCT2 and FCCT3 (57%). On the area of intermingling contact (pathogen–antagonist) differences in hyphal morphology of the pathogen as vacuolization of hyphae, plasmolysis of mycelium or coiling were observed.

Table 2 Inhibition effects of *Trichoderma* isolates on some phytopathogenic fungi

Trichoderma isolates	*Alternaria* sp.	*Bipolaris sorokiniana*	*Fusarium graminearum*	*F. solani*	*Pyricularia oryzae*
FCCT1	37.50 ± 0.00	51.00 ± 0.70	45.00 ± 0.50	51.00 ± 0.01	38.00 ± 0.00
FCCT2	37.50 ± 0.01	62.50 ± 0.45	61.00 ± 0.00	57.00 ± 0.00	72.00 ± 0.70
FCCT3	50.00 ± 0.00	85.00 ± 0.00	25.50 ± 0.10	57.00 ± 0.01	69.00 ± 0.51
FCCT4	50.00 ± 0.11	42.00 ± 0.01	32.50 ± 0.45	42.00 ± 0.40	69.00 ± 0.00
FCCT5	62.50 ± 0.00	71.00 ± 0.01	32.50 ± 0.50	37.00 ± 0.30	69.00 ± 0.10
FCCT6	32.50 ± 0.00	71.00 ± 0.20	42.00 ± 0.01	50.00 ± 0.40	52.00 ± 0.25
FCCT7	52.00 ± 0.22	42.00 ± 0.02	50.00 ± 0.00	50.00 ± 0.02	64.60 ± 0.50
FCCT8	62.50 ± 0.15	42.00 ± 0.25	32.50 ± 0.25	50.00 ± 0.01	69.00 ± 0.20
FCCT9	37.50 ± 0.10	28.00 ± 0.00	61.00 ± 0.40	57.00 ± 0.0	85.00 ± 0.01
FCCT10	45.00 ± 0.00	45.00 ± 0.00	30.00 ± 0.10	42.00 ± 0.01	72.00 ± 0.35
FCCT11	27.50 ± 0.20	62.50 ± 0.00	35.00 ± 0.40	51.00 ± 0.20	42.00 ± 0.20
FCCT12	37.50 ± 0.11	45.00 ± 0.15	35.00 ± 0.10	28.00 ± 0.25	42.00 ± 0.01
FCCT13	37.50 ± 0.50	85.00 ± 0.15	32.50 ± 0.25	50.00 ± 0.10	71.00 ± 0.02
FCCT14	32.50 ± 0.50	74.00 ± 0.20	51.00 ± 0.20	45.70 ± 0.10	35.00 ± 0.22
FCCT15	28.50 ± 0.30	62.50 ± 0.15	35.00 ± 0.20	42.00 ± 0.00	51.50 ± 0.20
FCCT16	28.50 ± 0.20	71.00 ± 0.10	45.00 ± 0.25	45.70 ± 0.00	45.50 ± 0.42
FCCT17	28.50 ± 0.25	42.00 ± 0.70	41.50 ± 0.15	28.00 ± 0.40	80.00 ± 0.00
FCCT18	28.50 ± 0.40	71.00 ± 0.65	38.00 ± 0.25	28.00 ± 0.25	74.60 ± 0.01
FCCT19	28.50 ± 0.40	70.00 ± 0.50	35.00 ± 0.30	40.00 ± 0.20	72.00 ± 0.02
FCCT20	28.50 ± 0.11	74.00 ± 0.00	48.00 ± 0.01	37.00 ± 0.10	78.40 ± 0.35
FCCT21	31.40 ± 0.15	62.50 ± 0.01	27.50 ± 0.30	45.70 ± 0.15	41.50 ± 0.12
FCCT22	57.10 ± 0.35	62.50 ± 0.01	32.00 ± 0.40	37.00 ± 0.30	39.50 ± 0.25
FCCT23	17.40 ± 0.45	62.50 ± 0.02	48.50 ± 0.30	42.00 ± 0.10	74.60 ± 0.35
FCCT24	42.80 ± 0.11	62.50 ± 0.01	45.00 ± 0.00	42.00 ± 0.01	74.60 ± 0.22
FCCT25	42.80 ± 0.15	28.00 ± 0.00	31.50 ± 0.00	42.00 ± 0.00	74.60 ± 0.14
FCCT26	28.50 ± 0.70	54.20 ± 0.20	29.50 ± 0.01	42.00 ± 0.25	55.50 ± 0.10
FCCT27	28.50 ± 0.25	62.50 ± 0.60	42.00 ± 0.20	50.10 ± 0.0	50.10 ± 0.02
FCCT28	40.00 ± 0.45	60.00 ± 0.50	37.00 ± 0.01	42.80 ± 0.20	45.00 ± 0.00
FCCT29	25.00 ± 0.10	35.00 ± 0.20	35.00 ± 0.20	50.10 ± 0.20	35.10 ± 0.00
FCCT30	28.50 ± 0.10	52.00 ± 0.15	34.50 ± 0.40	28.50 ± 0.40	39.30 ± 0.45
FCCT31	35.00 ± 0.25	55.00 ± 0.25	21.00 ± 0.01	40.00 ± 0.00	55.00 ± 0.30
FCCT32	37.50 ± 0.25	57.00 ± 0.00	32.50 ± 0.05	42.80 ± 0.15	52.00 ± 0.02
FCCT33	37.50 ± 0.25	62.50 ± 0.00	32.50 ± 0.40	42.80 ± 0.10	55.00 ± 0.00

Values are an average for two repetitions and are expressed as percentage of inhibition

Enzyme activities

By using aesculin hydrolysis for β-glucosidase activity determination, it was found that all isolates produced mycelium. Most of the isolates (27) produced spores; however, there was no relationship between β-glucosidase activity, mycelium production and sporulation. In experiments to determine esterase activity, all isolates were capable of hydrolysing Tween 80. The determination of protease and cellulase activity showed that 79% of the isolates hydrolysed gelatin. Additionally, hydrolytic activities were measured in isolate culture filtrates. The proteinase, endochitinase and β-1,3 glucanase activities of isolates that caused higher values of inhibition against fungal plant pathogens are summarized in Table 3. The highest level of proteinase and chitinase activity was measured with isolate FCCT9 and that of β-1,3 glucanase activity was observed with isolate FCCT2.

Genetic diversity of *Trichoderma* isolates

Whereas the four UP-PCR primers tested yield between 4 and 8 polymorphic bands, among the twelve ISSR primers used, eight amplified more than 12 polymorphic DNA fragments (Table 4), showing consistent and reproducible banding patterns. Thus, ISSR primers were selected to analyse diversity of *Trichoderma* isolates. A total of 127 fragments was generated using the primers $CT(AC)_8$,

Table 3 Total enzyme activity from 48 h VMS filtrates of representative *Trichoderma* strains

Trichoderma isolates	Proteinase activity (U/mg)	β-1,3 Glucanase activity (U/mg)	Endochitinase activity (U/mg)
FCCT2	15.63	356.27	37.19
FCCT3	14.31	61.00	20.55
FCCT5	24.25	1.04	4.39
FCCT8	1.55	15.02	5.97
FCCT9	66.25	4.86	40.88

Values are an average for two repetitions and are expressed as units of activity per milligram of protein

Table 4 Total number of polymorphic bands generated by PCR using UP-PCR or ISSR primers from thirty three isolates of *Trichoderma* spp.

Primer sequence (5′–3′) used	Total number of polymorphic bands
UP-PCR	
3-2-TAAGGGCGGTGCCAGT	8
AA2M2-CTGCGACCCAGAGCGG	8
L45-GTAAAACGACGGCCAGT	5
L21-GGATCCGAGGGTGGCGGTTCT	4
ISSR	
$CT(AC)_8$	21
$(AG)_8TA$	21
$ACA(CAA)_5$	17
$(GA)_8ACC$	15
$(AG)_8$	14
$(AATG)_4$	14
$(CAA)_5ACG$	13
$TCA(GT)_8$	12
$(GCC)_5$	2
$(AG)_8CG$	0
$GAG(CAA)_5$	0

$(GA)_8ACC$, $(AG)_8TA$, $TCA(GT)_8$, $(CAA)_5ACG$, $(AATG)_4$, $(AG)_8$ and $ACA(CAA)_5$ ranging in size from 400 to 2800 bp, with an average of 14 bands per primer. A representative banding pattern using the primer $(AG)_8TA$ is shown in Fig. 1. To analyse the genetic distances between isolates, a dendrogram was constructed using the DICE coefficient. Cluster analysis of ISSR data defined 20 haplotypes among the 33 isolates analysed, grouped in five clusters with an average similarity between groups of 51% (Fig. 2). The cophenetic correlation coefficient for the dendrogram ($r = 0.95$) points to an excellent fit between clustering.

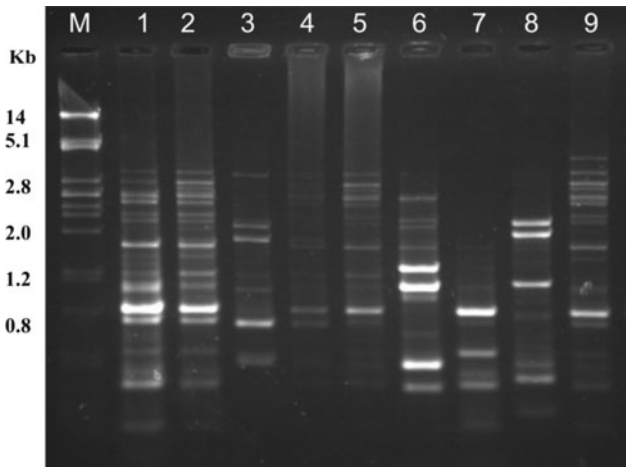

Fig. 1 PCR amplification patterns of nine *Trichoderma* isolates using the ISSR primer $(AG)_8TA$. *Lanes 1–9* : FCCT1, FCCT2, FCCT6, FCCT8, FCCT9, FCCT10, FCCT14, FCCT17, and FCCT18 isolate, respectively. *M* molecular size marker (*Pst*I-digested λ DNA), in kilobases (kb)

While all *T. virens* isolates (11) group in cluster 3, *T. harzianum* isolates group mainly in clusters 1, 2 and 4 (7, 11 and 2 isolates, respectively). Particularly, four from the 33 isolates (FCCT10 and FCCT17, FCCT6 and FCCT7, identified as *T. harzianum* and *T. koningii*, respectively) exhibited a completely different genetic pattern, as shown in Fig. 2.

Finally, no correlation was found between morphological, biochemical or physiological features and genetic diversity of the *Trichoderma* isolates ($r = -0.089$).

Discussion

The integration of physiological, biochemical and molecular attributes, together with classic morphological criteria, have been used in this study to characterize a group of *Trichoderma* isolates from different regions of Buenos Aires Province in Argentina. From 33 isolates, three species could be certainly identified as *T. harzianum, T. koningii* and *T. virens.*

From the evaluation of morphological and physiological aspects there are some interesting points to be emphasized. Only *T. harzianum* FCCT1 was capable of producing subglobose and abundant chlamydospores of diameter more than 10 μm, an important feature of sporulation. As reported, chlamydospore formation and their primary importance for the survival of the fungus in the soil have a potential role in biocontrol (Chet 1987). None of the isolates grew on media at extreme pH values (pH 2, 10 or 12), which is in accordance with previous results for *T. harzianum*. The maximal linear growth of the isolates occurred at pH values higher than 6.5 (Papavizas 1985). In general, a

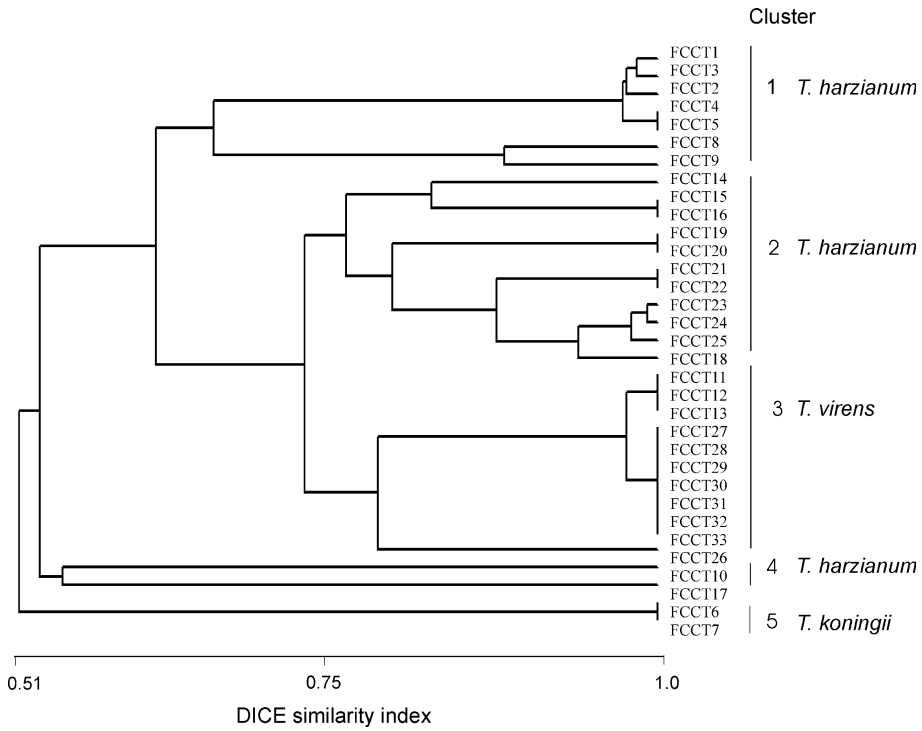

Fig. 2 UPGMA dendrogram based on Dice (SD) similarity index illustrating the genetic relationship among thirty three *Trichoderma* isolates from Buenos Aires Province, Argentina

poor growth or sporulation was observed for all isolates with the different nitrogen sources tested. It has been demonstrated that with ammonium salts, glycine or urea, the medium pH increases, which may be ascribed to the accumulation of ammonia. Under these conditions, the fungi may be particularly prone to autolysis and release ammonium compounds (Bridge 1985). Assimilation of carbon sources showed that all isolates of *Trichoderma* were capable of growing on media containing different compounds. Grondona et al. (1997) evaluated several *Trichoderma* strains, showing that all of them were able to assimilate glucose or ethanol, and grow and sporulate in lactose as the sole carbon source. The only assay that yielded discrimination between our isolates was sporulation on lactose, which allowed differentiate *T. koningii* isolates, being the only capable of sporulating on this medium.

Development of *Trichoderma* isolates on different solid media for determination as β-glucosidase, esterase, protease and cellulase, have shown variations depending on enzyme activity. Whereas all isolates hydrolysed aesculin and Tween 80, 28 isolates hydrolysed gelatin. It was shown that *Trichoderma* spp. produce cellulase, β-1-3 glucanase and chitinase enzymes, and extracellular hydrolases that are involved in the degradation of glucans of the walls of plant pathogens (Howell 2003; Woo et al. 2006). Screening of the 33 isolates of *Trichoderma* spp. resulted into the identification of isolates FCCT2 and FCCT9 as the best producers of β-1-3 glucanase and chitinase, respectively. Furthermore, isolate FCCT9 produced high levels of proteinase (Table 3). These properties were partially in agreement with the aggressiveness of the different *T. harzianum* isolates against the fungal plant pathogens assayed. Particularly, FCCT2 that showed high levels of β-1-3 glucanase and chitinase, caused high inhibition against *F. graminearum* (61%) and *F. solani* (57%), two related fungal species. FCCT9 that showed both high proteinase and chitinase activity values was capable of causing high inhibition against *P. oryzae* (85%), *F. graminearum* (61%), and *F. solani* (57%). FCCT3 was the second best producer of β-1-3 glucanase (Table 3), being capable of causing high inhibition in the growth of *B. sorokiniana* (85%) and *F. solani* (57%). However, as was reported by Mischke (1996), the specific activity of proteases produced by the strains of *Trichoderma* did not correlate with their biocontrol ability.

On the other hand, isolates FCCT5 and FCCT8 even though they have shown high inhibition against *Alternaria* sp. (over 60%), this behaviour did not correlate with the hydrolytic enzyme activity exhibited (Table 3). Although a direct relationship has been reported between antagonist capacity and enzyme activities of *Trichoderma* (Davet 1987), other studies have shown that these fungi (especially *T. harzianum* and *T. viride*) exhibit considerable variability among strains with respect to their production of lytic enzymes, biocontrol activity and host range (Sivan and Chet 1992). For example, an isolate of *T. harzianum* strain TH 250 produced high levels of chitinase and β-1-3 glucanase when grown on mycelia of *Rhizoctonia solani*, whereas it produced low levels of these enzymes on those

of *Sclerotium rolfsii* (Elad et al. 1982; Grondona et al. 1997). It could be suggested that a similar mechanism could be operating between *Trichoderma* isolates FCCT5 and FCCT8, and *Alternaria* development, or that other compounds with biological activity could be being produced by these strains, causing the inhibition in the fungus growth (Sivasithamparam and Ghisalberti 1998).

Although some isolates have shown differences in growth inhibition of the five fungal isolates evaluated, *T. harzianum* was the most aggressive species against all phytopathogens assayed (Tables 1, 2), suggesting its high potential as effective agent for the biocontrol of fungal pathogens as previously reported (Ghisalberti and Sivasithamparam 1991). Additionally, *P. oryzae* and *B. sorokiniana* were found to be the most susceptible pathogens to *Trichoderma* isolates used in our experiments (85% of inhibition).

The molecular characterization of *Trichoderma* isolates using UP-PCR primers revealed poor information about genetic diversity among the isolates; thus, the generated data were not taken into account for further analysis. In contrast, ISSR markers allowed discrimination of molecular genetic variation among the 33 isolates. It was possible to define 20 haplotypes grouped in five clusters. *T. harzianum* was one of the most representative species found in this study. Eighteen isolates grouped in two main clusters (Fig. 2, clusters 1 and 2) and another two isolates that exhibited a completely different genetic pattern, grouped separately (Fig. 2, cluster 4). Our results indicate an intraspecific genetic variation among both *T. harzianum* and *T. virens* isolates, respectively. However, the origin of this molecular variation 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. Furthermore, other mechanisms such as mutations, recombination and migration could explain the genetic diversity observed in this study (Chaverri et al. 2003; Sharma et al. 2009). Remarkably, cluster 1 grouped not only isolates FCCT2, FCCT3 and FCCT9, which showed high levels of hydrolytic enzyme activities and strong antagonism action but also isolates FCCT5 and FCCT8, which were capable of causing strong antagonism against *Alternaria* sp. but had low levels of hydrolytic enzymes (Fig. 2, Table 3).

In conclusion, a partial correspondence was observed between polymorphism patterns and aggressiveness of isolates. This discrepancy may represent an example of isolates that have arisen from one genetic line but have developed properties closer to another line perhaps through selection imposed by particular environmental or ecological conditions (Sharma et al. 2009). From the screening of the 33 *Trichoderma* isolates, three *T. harzianum* isolates were the most aggressive ones: FCCT2 against *F. graminearum* and *F. solani*, FCCT3 against *F. solani* and *B. sorokiniana* and FCCT9 against *P. oryzae*, *F. graminearum* and *F. solani*. This behaviour was in agreement with their extracellular hydrolytic activity levels. 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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