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Physiological and Biochemical Characterization of *Trichoderma harzianum*, a Biological Control Agent against Soilborne Fungal Plant Pathogens

I. GRONDONA,[1] R. HERMOSA,[1] M. TEJADA,[1] M. D. GOMIS,[1,2] P. F. MATEOS,[1] P. D. BRIDGE,[3] E. MONTE,[1]* AND I. GARCIA-ACHA[1]

Departamento de Microbiología y Genética, CSIC/Universidad de Salamanca, 37007 Salamanca,[1] and AMC Chemical, 41920 San Juan de Aznalfarache, Seville,[2] Spain, and International Mycological Institute, Egham TW20 9TY, United Kingdom[3]

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Monoconidial cultures of 15 isolates of *Trichoderma harzianum* were characterized on the basis of 82 morphological, physiological, and biochemical features and 99 isoenzyme bands from seven enzyme systems. The results were subjected to numerical analysis which revealed four distinct groups. Representative sequences of the internal transcribed spacer 1 (ITS 1)-ITS 2 region in the ribosomal DNA gene cluster were compared between groups confirming this distribution. The utility of the groupings generated from the morphological, physiological, and biochemical data was assessed by including an additional environmental isolate in the electrophoretic analysis. The in vitro antibiotic activity of the *T. harzianum* isolates was assayed against 10 isolates of five different soilborne fungal plant pathogens: *Aphanomyces cochlioides*, *Rhizoctonia solani*, *Phoma betae*, *Acremonium cucurbitacearum*, and *Fusarium oxysporum* f. sp. *radicis lycopersici*. Similarities between levels and specificities of biological activity and the numerical characterization groupings are both discussed in relation to antagonist-specific populations in known and potential biocontrol species.

The genus *Trichoderma* was introduced by Persoon almost 200 years ago and consists of anamorphic fungi isolated primarily from soil and decomposing organic matter. Isolates of *Trichoderma* are ubiquitous and are relatively easy to isolate and culture. In addition, isolates grow quickly on many substrates, produce metabolites with demonstrable antibiotic activity, and may be mycoparasitic against a wide range of pathogens. Mycoparasitic activity and antibiotic production were first demonstrated in *Trichoderma* by Weindling in 1932 and 1934 (52, 53), and many modern biotechnological applications of these fungi as biocontrol agents (9, 18, 44) are derived from these early works.

Most species of the genus grow rapidly in artificial culture and produce large numbers of small green or white conidia from conidiogenous cells situated at the ends of widely branched conidiophores. This characteristic allows a relatively easy identification of *Trichoderma* as a genus, but the species concepts are difficult to interpret and there is considerable confusion over the application of specific names. Rifai (40) divided *Trichoderma* into nine species aggregates on the basis of morphological features. Bisset (2) revised the genus and also included some *Hypocrea* anamorphs in the genus, resulting in the establishment of five new sections. Species concepts within *Trichoderma* are very wide, and this has resulted in the establishment of many specific and subspecific taxa (42).

Trichoderma harzianum Rifai is a species aggregate which includes a plethora of strains that can be used as biological control agents of plant pathogenic fungi and viral vectors (10, 51). The taxonomic status of this species is imprecise, and the criteria used to classify and identify strains so far do not provide sufficient discrimination, especially with those isolates of interest in biocontrol programs. There have been many studies aimed at the characterization of these microorganisms on the basis of their antifungal activity, either antibiotic (13, 20, 38), lytic (18), or a combination of both (44).

T. harzianum can be divided in three (33, 45), four (16, 50), or five subspecific groups (24, 56), depending on the strains and on the attributes studied. This disparity of criteria makes it difficult to search for, and above all characterize, new biological control agents within the species and to reidentify them in a natural environment once they have been freed in a pathosystem.

The current work is an integrated study which combines some novel physiological and biochemical attributes, isoenzyme analysis, and sequencing of the internal transcribed spacer 1 (ITS 1)-ITS 2 region in the ribosomal DNA (rDNA) gene cluster to determine if there are distinct functional groups within *T. harzianum* which correlate with biological activity and which may provide characteristics that could be used to select isolates as biological control agents (30, 35). The utility of this approach in identifying characteristics that may aid in the clarification of species and in the sub-specific delimitation of the genus is also investigated.

MATERIALS AND METHODS

Strains. Forty-five single-spore cultures were isolated from 15 cultures of *T. harzianum* Rifai (Table 1). Cultures were maintained at −20°C on potato dextrose agar (PDA) slants and deposited in the Spanish Type Culture Collection (CECT, Burjassot, Valencia, Spain). A 16th culture, ThVA, was included in the isoenzyme analysis only. This was a recent environmental isolate and was included to test whether further cultures would be more likely to show similarities with existing groups.

Morphology. Colony size on malt extract agar (MEA) was recorded after 14 days at 25°C. Chlamydospore production and size of conidiogenous cells and conidia were studied under the same conditions. Morphological results are the mean values determined from at least 12 plates.

Physiological techniques. Growth and sporulation in liquid medium with glucose, ethanol, lactic acid, citric acid, ammonium oxalate, or ammonium tartrate as the sole carbon source; aesculin and gelatin hydrolysis; and analysis of growth and colony texture in the presence of 0.005 or 0.001% crystal violet, 0.032%

* Corresponding author. Mailing address: Departamento de Microbiología y Genética, Universidad de Salamanca, Edificio Departamental, Lab. 208, Avenida Campo Charro s/n, 37007 Salamanca, Spain. Phone: 34 23 294532. Fax: 34 23 294515. E-mail: emv@gugu.usal.es.

TABLE 1. Strains of *T. harzianum* used

Strain	Source	Origin	Year isolated	IMI no.[a]
2923	Soil	Harare, Zimbabwe	1983	281112
2924	Soil	Gujurat, India	1985	293162
2925	Soil	Carimagh, Colombia	1985	296235
2926	Soil	Worthing, United Kingdom	1986	298371
2927	Soil	Worthing, United Kingdom	1986	298372
2928	Soil	Worthing, United Kingdom	1986	298373
2929	Soil	Worthing, United Kingdom	1986	298374
2930	*Beta vulgaris*, soil	Pantnagar, India	1986	304056
2931	*Lycopersicon esculentum*, soil	Pantnagar, India	1986	304057
2932	*B. vulgaris*, soil	Pantnagar, India	1986	304058
2933	Soil	Gunnaruwa, Sri Lanka	1986	300082
11[b]	*B. vulgaris*, soil	Montpellier, France	1990	
3[b]	*B. vulgaris*, soil	Montpellier, France	1990	
24	*B. vulgaris*, soil	Salamanca, Spain	1984	352940
260	University of Strathclyde	Strathclyde, United Kingdom	1964	110150

[a] IMI, International Mycological Institute (Egham, United Kingdom).
[b] P. Davet, INRA (Montpellier, France).

sodium selenite, or 0.05 or 0.001% copper sulfate were performed according to Bridge (6). Colony growth and radial growth inhibition (RI) by 50, 100, and 200 mg of Cu (as copper sulfate)/liter were calculated by the methods of Baath (1). Growth and sporulation on creatine, ammonium oxalate, sodium nitrite, glycine, or urea as the sole nitrogen source and pigmentation on 25% glycerol agar, nitrite agar, Czapek-ammonium agar and MEA were analyzed as described by Bridge et al. (7). Growth at 4°C was tested in MEA after 30 days, and thermal resistance of conidial suspensions was recorded after a 5-min incubation at 75°C.

Biochemical techniques. API ZYM test strips (API-bioMerieux) were inoculated with spent culture fluid from cultures grown for 9 days in GYM medium (32).

Isoenzyme sample preparation. All single-spore isolates were grown on PDA for 3 days at 25°C in the dark. Blocks of agar medium were removed from the margins of the colonies and used as inocula of GYM medium (32) in 250-ml Erlenmeyer flasks at 25°C, without shaking. Mycelia were harvested after 9 days, washed with sterile water, and centrifuged at 2,000 × *g* for 10 min at 4°C. The resultant mycelial mat (approximately 1 to 2 g [wet weight]) was squeezed and crushed with a pestle and shaken for 5 min with 0.45-mm-diameter Ballotini glass beads in a chilled potter with 1.5 ml of sterile water. Cell disruption was checked by light microscopy. The cell homogenates were transferred with a Pasteur pipette to microcentrifuge tubes and centrifuged at 13,000 × *g* for 2 min at 4°C. The resulting supernatant was filtered through a 0.45-μm-pore-size Sterivex H-A filter (Millipore, Bedford, Mass.) and stored in small aliquots at −20°C until isozyme analysis.

Electrophoresis. Samples for electrophoresis were prepared at a final concentration of 100 mg of protein (5) by suspending 30 ml of mycelial extract in 10 ml of 0.5 M Tris-HCl, pH 6.8, containing 1.2 ml of 0.5% bromophenol blue in glycerol (1:4 dilution) as tracking dye. Enzymes were separated by vertical 7 to 10% polyacrylamide gel (0.7-mm thickness) electrophoresis, according to the method described by Laemmli (22), with a discontinuous buffer system (electrode buffer, 1 M Tris-glycine [pH 8.2]; stacking gel buffer, 0.126 M Tris-HCl [pH 6.8]; gel buffer, 0.37 M Tris-HCl [pH 8.8]). In replicate runs of the same strain, mycelium from the same liquid culture was used.

Enzyme staining. Enzyme staining solutions were prepared according to Shaw and Prasad (46) and Paterson and Bridge (34). Isoenzyme patterns were determined for alkaline phosphatase (ALP) (EC 3.1.3.1), acid phosphatase (ACP) (EC 3.1.3.2), catalase (CAT) (EC 1.11.1.6), malate dehydrogenase (MDH) (EC 1.1.1.37), alcohol dehydrogenase (ADH) (EC 1.1.1.1), and esterase (EST) (EC 3.1.1.1) activities. Cellulase [1,4-(1,3;1,4)-β-D-glucan-4-glucanohydrolase] (CEL) (EC 3.2.1.4) was detected by preparing an overlay gel of 1% agarose in 0.05 M acetic acid-acetate buffer, pH 5.2, on a gel bond film (Sigma, St. Louis, Mo.), which was then stained with Congo red according to the method of Mateos et al. (25). The isoenzymes used gave well-resolved bands in all single-spore isolates examined in this study.

DNA extraction. Mycelium from five *T. harzianum* strains (2925, 2931, 11, 24, and 260) was grown in liquid shake cultures (120 rpm) at 25°C in potato dextrose broth (Difco) for 48 h. DNA was extracted from 300 mg of freeze-dried mycelial powder following the method of Raeder and Broda (37).

PCR amplification. The ITS 1 region of rDNA was amplified with the ITS 1 and ITS 2 primers described by White et al. (55) and synthesized in an Applied Biosystems model 391 PCR-Mate DNA synthesizer. PCR mixtures contained diluted genomic DNA (40 to 60 ng) reaction buffer, 200 μM (each) deoxynucleoside triphosphate, 0.2 μM (each) primers 1 and 2, and 2.5 U of *Tfl* DNA polymerase; they were subjected to 30 cycles of 1.5 min at 94°C, 2 min at 45°C, and 3 min at 72°C in a Perkin-Elmer Cetus thermal cycler. PCR products were visualized in 1.5% (wt/vol) agarose gels (electrophoresis buffer, 40 mM Tris-acetate–2 mM EDTA, pH 8) stained with ethidium bromide (0.5 μg/ml). The molecular size marker was ϕX179-*Hae*III (Promega, Madison, Wis.).

Nucleotide sequence determination. The amplified fragments were cloned in pBluescript SK(+) and KS(+) vector (47) digested with *Sma*I. Single-stranded DNA was sequenced by the dideoxynucleotide chain termination procedure (43) by using a T7 DNA polymerase kit (Pharmacia, Uppsala, Sweden). The sequence data were aligned by using the 1989–1992 DNASTAR package (DNASTAR, London, United Kingdom). The percent divergence between nucleotide sequences was calculated as follows: divergence = $[(Ts+Tv+I/D)/L] \times 100$, where *Ts* is the number of transitions, *Tv* is the number of transversions, *I* is the number of insertions, *D* is the number of deletions, and *L* is the sequence length.

Numerical analysis. Physiological and biochemical tests were coded as follows: 0, negative; 1, weak reaction; and 2, strong positive reaction. Morphological tests were coded on an "all present" system where 0 is negative and 2 is predominantly positive.

The results obtained from all of the isoenzyme analyses were combined and coded according to Monte et al. (26). Banding patterns were recorded according to their relative mobilities (Rf) and were coded as follows: 0, band absent; 1, band faint or variable; and 2, band present. Coded results for all strains were compared by using Gower's general similarity coefficient, with matching negative results excluded, and the cosine ϕ (cos ϕ) coefficient (14). Dendrograms were produced by unweighted average linkage (UPGMA) clustering (48). Principal coordinates analysis was undertaken by the method of Gower (15).

Assays of fungal inhibition in vitro. Biocontrol assays were determined on PDA plates by inoculating the *T. harzianum* isolates with 10 isolates of five different soilborne fungal plant pathogens (Table 2) according to Royse and Ries (41). Inhibition was observed as presence of inhibition zones prior to any mycelial contact. The percent RI was calculated as follows: $RI = 100 \times (R_2 - R_1)/R_2$. R_1 was the distance between the inoculum (5-mm-diameter agar plug) of pathogen and the edge of the colony (after 7 days at 25°C) measured in the direction of the inoculum of the *T. harzianum* isolate. Each *T. harzianum* isolate used was inoculated 5 cm from the inoculum of pathogen either at 4 days later for *Acremonium cucurbitacearum* or at 48 h later for the other pathogens. R_2 is the colony growth of pathogen measured in the direction of maximum radius. RI values correspond to the mean values of three monosporic lines and six repetitions per strain.

Nucleotide sequence accession numbers. Sequences for the ITS 1-ITS 2 region of rDNA from *T. harzianum* strains used in this study are available in the EMBL database under accession numbers Y13570 (strain 2925), Y13571 (strain 2931), Y13572 (strain 11), Y13573 (strain 260), and Y13574 (strain 24).

RESULTS

Reproducibility. No individually significant variations were observed between single-spore isolates derived from single strains. Results are therefore presented from the 15 named isolates.

Morphological, physiological, and biochemical features. All the *T. harzianum* cultures studied gave no change in the pH of the medium with glucose or ethanol as the sole carbon source. They did not show growth in medium with either ammonium

TABLE 2. Strains of pathogens used

Strain[a]	Source	Origin	Year isolated
A. cochlioides			
IMI300493	Sugar beet	Queensland, Australia	1986
CECT20200	Sugar beet	Salamanca, Spain	1990
P. betae			
IMI327291	Sugar beet	Italy	1984
CECT20198	Sugar beet	Salamanca, Spain	1989
CECT20199	Sugar beet	Salamanca, Spain	1989
R. solani			
Sa1	Sugar beet	Salamanca, Spain	1990
A. cucurbitacearum			
CECT20202	Muskmelon	Valencia, Spain	1994
CECT20203	Muskmelon	Murcia, Spain	1994
CBS52593	Muskmelon	Ciudad Real, Spain	1991
F. oxysporum f. sp. *radicis lycopersici*			
CECT20201	Tomato	Canary Islands, Spain	1994

[a] IMI, International Mycological Institute (Egham, United Kingdom); CECT, Colección Española de Cultivos Tipo (Valencia, Spain); CBS, Centraalbureau voor Schimmelcultures (Baarn, The Netherlands).

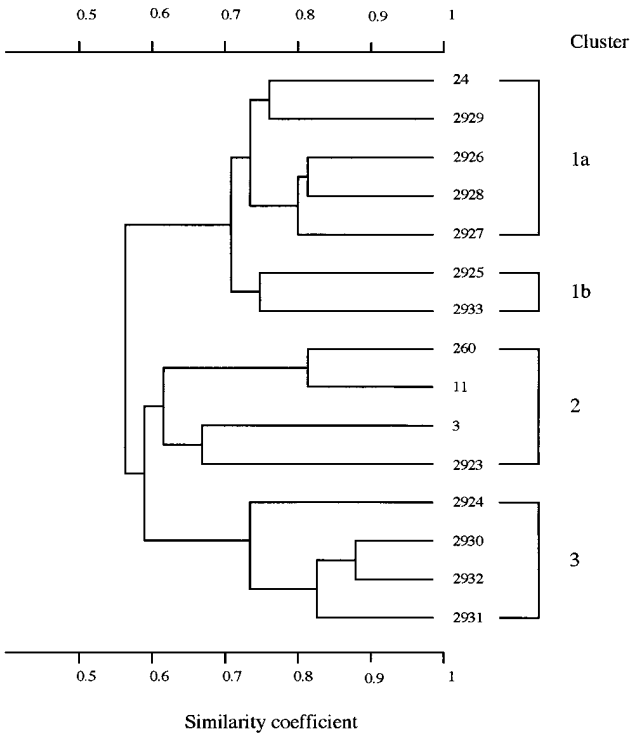

FIG. 1. UPGMA dendrogram, based on Gower's coefficient, derived from physiological, biochemical, and morphological attributes of 45 single-spore isolates from 15 strains of *T. harzianum*.

oxalate or ammonium tartrate as the sole carbon source. None of the cultures displayed either a shift of the bromocresol purple indicator to yellow or surface growth in medium with creatine as the sole nitrogen source, and under the assay conditions, no growth was observed in the medium with sodium nitrite as the sole nitrogen source. In addition, culture fluid assayed with the API ZYM strips did not exhibit α-fucosidase activity and did not produce significant amounts of extracellular lipases, valine arylamidase, cystine arylamidase, α-chymotrypsin, or β-glucuronidase. These characteristics were not considered in the numerical analysis.

The results for the 15 cultures from the morphological characteristics (6), physiological tests (45), and biochemical tests (18) were combined in a single cluster analysis producing an UPGMA dendrogram derived from Gower's coefficient (Fig. 1).

The dendrogram gave three clusters in which overall group similarity ranged from 57 (cluster 1) to 61% (clusters 2 and 3). Further analysis based on the cos ϕ coefficient gave one discrepancy in that two cultures of *T. harzianum* (2925 and 2933) which grouped at 70% to cluster 1 in the Gower's coefficient dendrogram (Fig. 1) were recovered as a fourth separate cluster, designated 1b, in the cos ϕ-derived dendrogram (not shown). The characteristics of each of the clusters are shown in Table 3.

Cluster 1a grouped strains from the United Kingdom together with a good biological control agent isolated in Salamanca, Spain (17). All the strains had intercalary chlamydospores, hydrolyzed gelatin and produced a yellow pigment on MEA.

Cluster 1b consisted of one isolate from Sri Lanka and one isolate from Colombia which produced spores in medium with ethanol as the sole carbon source and which differed from the strains in cluster 1a in that they lacked intercalary chlamydospores and did not produce a yellow pigment on MEA.

Cluster 2 contained cultures from a mixed geographic distribution and grouped two French strains (3 and 11) and one British strain (260), of proven efficiency in the biological control of phytopathogenic fungi, together with another strain from Zimbabwe (2923) which showed low levels of extracellular enzyme activity (19) and low antagonist activity. All members of this cluster produced colonies of less than 8 cm in diameter on MEA and conidia with a diameter greater than 2.4 μm; they developed aerial mycelia on Czapek-ammonium agar, did not form mats in the 0.032% sodium selenite medium, and produced an orange pigment on 25% glycerol agar.

Cluster 3 included three cultures from India that produced conidia with a diameter smaller than 2.4 μm; they produced aerial mycelia in Czapek-ammonium agar and in nitrite agar, hydrolyzed gelatin, produced an orange pigment, and developed a yellow color on MEA.

Isoenzyme analysis. In order to test whether these methods could be used to group new, unassigned isolates, an additional environmental isolate of *T. harzianum* (ThVA), recovered from soil in a sugar beet field in Matapozuelos (Valladolid, Spain), was included in the isoenzyme analysis.

The seven enzymes selected were polymorphic among strains with the exception of ADH, which was only detected in strain 2923. All isolates exhibited activity in the other six enzyme systems, except strains 2926 and 2929 for ALP, CAT, and MDH, strain 3 for ALP, strain 2928 for MDH, and strain 2932 for ACP.

Gels stained for ALP gave 6 bands (Fig. 2a), for ACP gave 5 bands (Fig. 2b), for CAT gave 4 bands (Fig. 2c), for MDH gave 15 bands (Fig. 3a), and for CEL gave 17 bands (Fig. 3b). All cultures gave complex band patterns for EST activity, and 51 different bands were identified (Fig. 3c). All strains studied had EST activity.

An UPGMA dendrogram was derived from Gower's coefficient for the combined electrophoresis data (Fig. 4). The application of different similarity coefficients did not alter the stability of the results (data not shown). Five clusters were obtained at similarities ranging from 69.7 (cluster 2b) to 74.9% (clusters 1a and 1b). These clusters were equivalent to those

TABLE 3. Number of strains positive for selected characteristics

Characteristic	No. of strains with characteristic by cluster			
	1a (5)[a]	1b (2)	2 (4)	3 (4)
Production of intercalary chlamydospores	5	0	3	1
Production of terminal chlamydospores	4	1	4	3
Chlamydospore diameter of >10 μm	0	0	3	0
Mean conidiogenous cell length of >10 μm	1	0	3	0
Mean conidiogenous cell breadth of >3 μm	2	0	1	0
Conidial diameter of >2.4 μm	1	0	4	0
Superficial growth on glucose	0	1	2	1
Superficial growth on citric acid	4	2	4	4
Sporulation on citric acid	0	0	2	0
Purple coloration on citric acid	4	2	2	4
Superficial growth on ethanol	3	1	3	3
Sporulation on ethanol	0	1	0	0
Superficial growth on lactic acid	1	0	2	3
Sporulation on lactic acid	0	0	1	3
Purple coloration on lactic acid	0	0	1	0
Superficial growth on glycine	0	1	4	4
Purple coloration on glycine	5	2	2	0
Superficial growth on ammonium oxalate	1	2	4	4
Sporulation on ammonium oxalate	0	0	2	2
Purple coloration on ammonium oxalate	5	2	0	3
Superficial growth on urea	0	2	4	4
Aerial cottony mycelium on urea	0	0	1	0
Sporulation on urea	4	2	0	1
Purple coloration on urea	5	2	4	3
Sporulation on creatine	3	0	3	1
Growth on crystal violet (0.01 g/liter)	0	1	0	0
Mycelial mats on selenite	1	2	0	4
Colonies with rosetta shape on selenite	0	0	2	1
Colony diameter of >3.1 cm on MEA with Cu (200 mg/liter)	1	0	0	2
Colony diameter of 2.5–3.1 cm on MEA with Cu (200 mg/liter)	4	2	1	2
Colony diameter of <2.5 cm on MEA with Cu (200 mg/liter)	0	0	3	0
Colony growth reduction of >21% on MEA with Cu (200 mg/liter)	0	1	1	1
Colony growth reduction of 9–21% on MEA with Cu (200 mg/liter)	3	1	2	3
Colony growth reduction of <9% on MEA with Cu (200 mg/liter)	2	0	1	0
Colony diameter of >8 cm on MEA	1	2	0	4
Colony diameter of 6.5–8 cm on MEA	3	0	2	0
Colony diameter of <6.5 cm on MEA	1	0	2	3
Yellow pigment on MEA	5	0	0	4
Aerial mycelium on Czapek-ammonium agar	0	0	1	2
Sporulation on Czapek-ammonium agar	0	0	3	4
Hydrolysis of gelatin	4	1	1	0
Sporulation on gelatin	0	0	3	4
Orange pigment on gelatin	1	0	0	4
Growth on nitrite agar	0	0	2	4
Sporulation on nitrite agar	0	0	4	4
Aerial mycelium on nitrite agar	0	0	1	4
Growth on glycerol agar	3	2	3	1
Orange pigment on glycerol agar	2	0	0	4
Sporulation on esculin	0	0	3	4
Spore resistance to heating (75°C for 5 min)	2	1	1	3
Growth at 4°C on MEA (30 days)	0	0	2	0
API ZYM ALP	5	2	4	4
API ZYM EST (C_4)	0	0	1	0
API ZYM ESTlipase (C_8)	4	0	0	1
API ZYM leucine arylamidase	5	1	1	4
API ZYM trypsin	0	0	0	2
API ZYM ACP	3	2	3	4
API ZYM naphthol-AS-BI-phosphate	4	2	4	4
API ZYM α-galactosidase	3	2	2	4
API ZYM β-galactosidase	0	2	0	0
API ZYM α-glucosidase	0	1	2	2
API ZYM β-glucosidase	4	2	3	4
API ZYM *N*-acetyl-β-glucosidase	4	2	2	1
API ZYM α-mannosidase	1	0	0	2

[a] Numbers in parentheses are numbers of *T. harzianum* strains in each cluster.

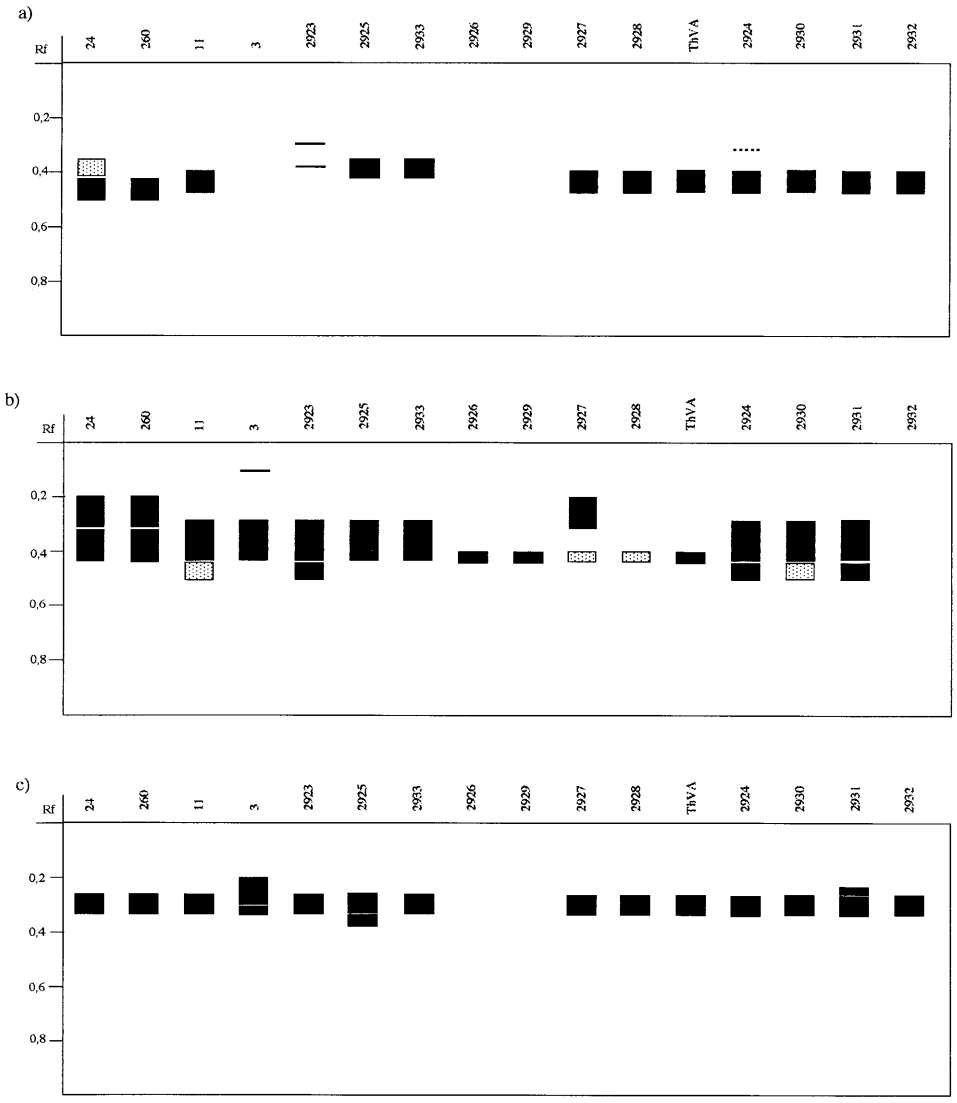

FIG. 2. Schematic representation of isoenzyme bands of ALP (a), ACP (b), and CAT (c) activities of 16 isolates of *T. harzianum*. ■, clear band; ▨, faint band.

obtained from the morphological, physiological, and biochemical analyses (Fig. 1), with the exception of cluster 2 which split into two subclusters, the first of which formed with isolate 24, which had grouped in cluster 1a in the earlier dendrograms from morphological, physiological, and biochemical characteristics.

A principal components analysis was also undertaken with the electrophoretic data and showed the same five groups in the first two dimensions (data not shown). Isolate 24 was again recovered with isolates 260 and 11 from cluster 2a but differed from these isolates in the third (*z*) dimension.

The environmental isolate ThVA grouped with other European isolates in cluster 1a. Isolates in cluster 1a can be distinguished by an ACP band at Rf 0.40 to 0.44 and an EST band at Rf 0.52. Cultures in cluster 1b were the only ones to show EST bands at Rf 0.39, 0.41, 0.43, and 0.45.

The split in cluster 2 resulted in two subclusters of which cluster 2a consisted of the two cultures (2923 and 3) isolated from soil in Zimbabwe and France. Culture 2923 was the only one in this group to show an ADH band, but it was an ineffective biocontrol agent (see above). Culture 3 exhibited a good antagonist activity, mainly at field level (35). The representative bands detected in this cluster were CEL bands at Rf 0.40 and 0.69 to 0.82 and an EST band at 0.50.

Cluster 2b grouped the most powerful biocontrol agents included in this study, with good CEL and 1,3-β-glucanase activity (19) and the capability of growing at low temperatures under natural conditions (16, 29). The strains of this cluster were positive for EST bands at Rf 0.25 and 0.78.

Cluster 3 was distinguished from the others by MDH patterns, and all members showed MDH bands at Rf 0.22 to 0.23, 0.27, 0.30, and 0.32.

DNA sequence analysis. Sequences of the ITS 1-ITS 2 region of the rDNA of five representative *T. harzianum* isolates representing each cluster, derived from physiological and biochemical data, showed that all clusters had ITS regions of different sizes (202 bp for cluster 1a, 198 bp for cluster 1b, 184 bp for cluster 2, and 200 bp for cluster 3) and composition (Fig. 5) and that the cluster 3 isolates had identical sequences.

The representative sequence from cluster 1a showed 1.5,

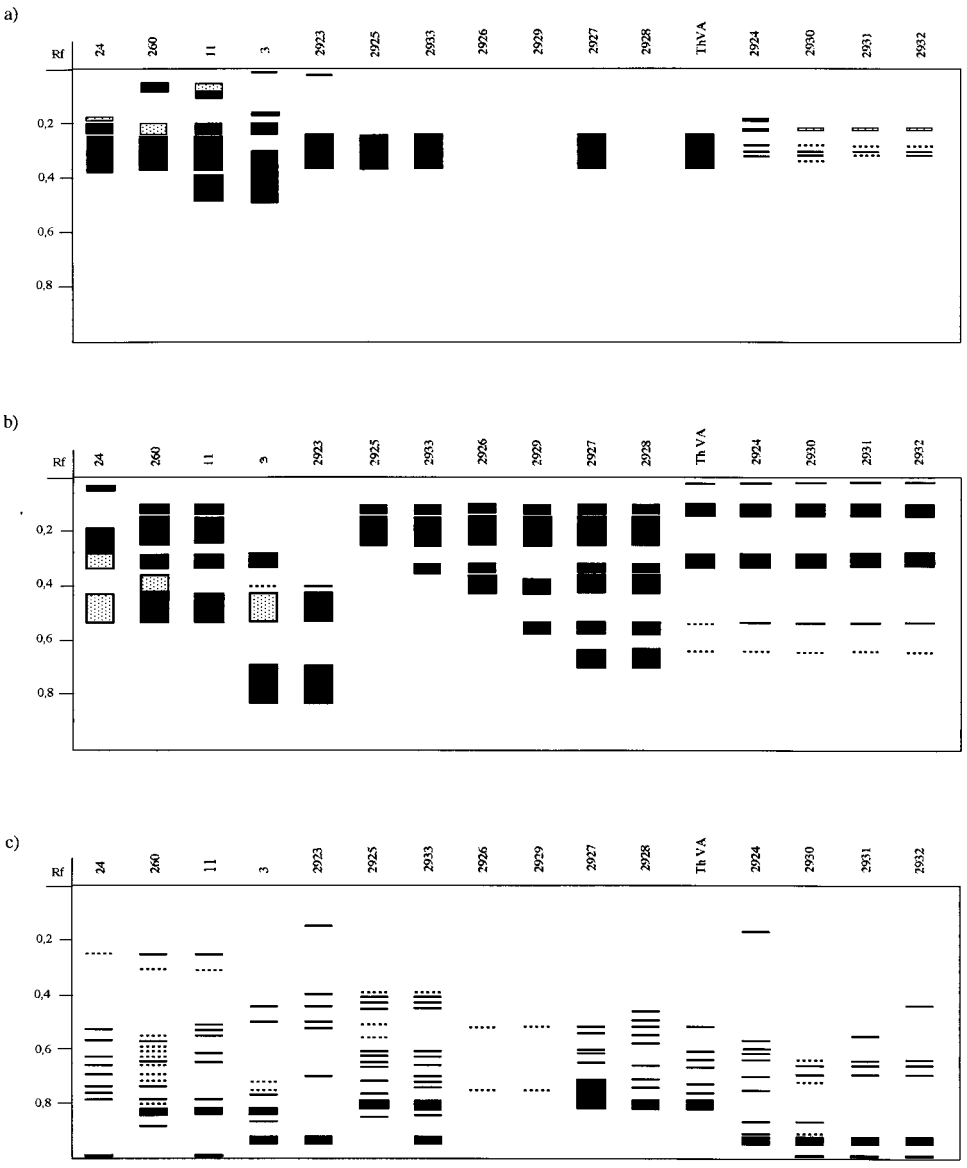

FIG. 3. Schematic representation of isoenzyme bands of MDH (a), CEL (b), and EST (c) activities of 16 isolates of *T. harzianum*. ■, clear band; ▨, faint band.

18.3, and 0.5% divergence from those representative of clusters 1b, 2, and 3, respectively. Cluster 1b showed 16.8% divergence from cluster 2 and 1% divergence from cluster 3. The divergence between clusters 2 and 3 was 17.8%.

The percent divergences between the nucleotide sequences analyzed in this study and the same ITS 1-ITS 2 region published for other *T. harzianum* isolates in former studies (33) are shown in Table 4. Sequences obtained with representative isolates of clusters 1a, 1b, and 3 showed divergences lower than 2% from rDNA group 1, divergences between 4.4 and 5.4% from those of rDNA group 2, and divergences greater than 16.8% from rDNA group 3 (33). Isolates 11 and 260, from cluster 2, had divergences of 17.8 and 20.3% from rDNA groups 1 and 2, respectively, and the same sequence as the six isolates of rDNA group 3 sequenced by Muthumeenakshi et al. (33).

Antifungal activity. In vitro inhibition of the growth of five phytopathogenic fungi was carried out by confronting the antagonist microorganism and the pathogen simultaneously under the same conditions. Under these conditions marked inhibition of the growth of the five fungi occurred in the presence of most of the cultures of *T. harzianum* studied. Therefore, the pathogens were preincubated for 48 h without the antagonists in order to allow the pathogens to become established. *A. cucurbitacearum* was preincubated for 4 days due to the small radial growth of the colonies after 2 days of incubation, which made the measurement of RI very difficult. The presence of inhibition zones prior to any mycelial contact indicates that inhibition may be due to the production of diffusible components by *T. harzianum*. Subsequent inhibition after contact may be due to enzymatic activity (18). This method showed differences in the percentage of inhibition of radial growth of the phytopathogen colonies by the different cultures of *T. harzianum*. The highest mean inhibition values, above 50% RI, were obtained against *Aphanomyces cochlioides* and the lowest were obtained against *Fusarium oxysporum* f. sp. *radicis lycopersici*:

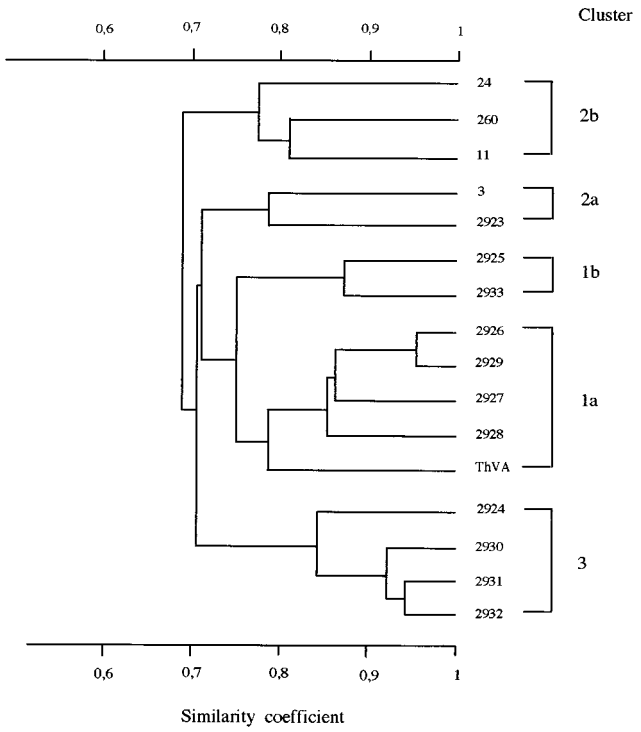

FIG. 4. UPGMA dendrogram, based on Gower's coefficient, derived from isoenzyme band characteristics for 16 isolates of *T. harzianum.*

more than 20% RI, despite the 48-h delay allowing for the establishment of the pathogen.

Table 5 shows the mean percentages of RI of the phytopathogens. Maximum RI percentages were obtained with strains 2932 (69.1% against *A. cochlioides* and 32.3% against *F. oxysporum* f. sp. *radicis lycopersici*), 2925 (40.9% against *Rhizoctonia solani*), and 11 (45.3% against *Phoma betae*). Strain 2925 also gave the maximum mean inhibition value against *A. cucurbitacearum* with simultaneous (31.5%) and 48-h delay (17.6%) confrontation. The mean RI values for the colonies of *P. betae* and of both *R. solani* and *A. cucurbitacearum* were higher for members of clusters 2 and 1b, respectively, whereas strains in cluster 3 had a maximum RI value against *A. cochlioides* and *F. oxysporum* f. sp. *radicis lycopersici*. The differences in the mean RI percentages for the four individual clusters were evident for the different phytopathogens, and the in vitro findings have been confirmed in independent field experiments against *P. betae*, *R. solani*, and *A. cochlioides* involved in sugar beet damping-off (16, 35).

TABLE 4. Percent divergences among nucleotide sequences of the ITS 1-ITS 2 regions of *T. harzianum* groups

Cluster	% Divergence from rDNA group[a]		
	1	2	3
1a	0.5	4.4	18.3
1b	2.0	5.4	16.8
2	17.8	20.3	0.0
3	1.0	4.9	17.8

[a] Classification of rDNA groups (ITS 1-ITS 2) according to Muthumeenakshi et al. (33).

DISCUSSION

The integration of physiological and biochemical attributes, together with classic morphological criteria, has served to establish new infraspecific groups within *T. harzianum* which can be related to different levels of biological activity and be directed against different phytopathogenic fungi.

Cluster	rDNA group[a]		
			10 20 30 40 50 60
1a	1	24	CCGAGTTTACAACTCCCAAACCCAATGTGAACGTTACCAAACTGTTGCCTCGGCGGGATCTCTGCCCCG
1b	1	2925	----------
3	1	2931	----------
-	1	T28JF[a]	----------
-	2	ThI[a]	----------C----------
2	3	11	----------CA----------G--A-*------
2	3	260	----------CA----------G--A-*------
-	3	Th3(4)[a]	----------CA----------G--A-*------
			70 80 90 100 110 120 130
1a	1	24	GGTGCGTCGCAGCCCCGGA*CCAAGGCGCCCGCCGGAG*GACCAACCTAAAACTCTTATTGTATACCCC
1b	1	2925	----------*----------*--------*---------T----------
3	1	2931	----------*----------*--------*----------
-	1	T28JF[a]	----------*----------*----------
-	2	ThI[a]	--C----------*----------C----------
2	3	11	----------A---*----------G--------*---CTCT--TC----*GT---
2	3	260	----------A---*----------G--------*---CTCT--TC----*GT---
-	3	Th3(4)[a]	----------A---*----------G--------*---CTCT--TC----*GT---
			140 150 160 170 180 190 200
1a	1	24	CTCGCGGGTTTTTTTT*ATAATCTGAGCCTT*CTCGGCGCCTCTCGTAGGCGTTTCGAAAATGAATCA
1b	1	2925	----------***----------*----------*----------
3	1	2931	----------**----------*----------
-	1	T28JF[a]	----------**----------T----------
-	2	ThI[a]	----------A-----*-CT----------*---------C-----G----------
2	3	11	-------ACG--A--TCT--**-**---***---GA--*********-AAAA--C**----------
2	3	260	-------ACG--A--TCT--**-**---***---GA--*********-AAAA--C**----------
-	3	Th3(4)[a]	-------ACG--A--TCT--**-**---***---GA--*********-AAAA--C**----------

[a] rDNA groups and ITS 1-ITS 2 sequences published by Muthumeenakshi *et al.* (1994).

FIG. 5. Nucleotide sequences of the ITS 1-ITS 2 region in the rDNA gene cluster of *T. harzianum* isolates with depiction of their pertinence to the clusters described in this study and to rDNA groups previously established. Isolates T28JF, ThI, and Th3(4) were considered representative of rDNA groups 1, 2, and 3, respectively. *, insertion or deletion of one nucleotide.

TABLE 5. *T. harzianum* inhibition of phytopathogens

Phytopathogen (no. of isolates)	% RI by *T. harzianum* cluster[a]											
	1a			1b			2			3		
	Min	Mean	Max	Min	Mean	Max	Min	Mean	Max	Min	Mean	Max
A. cochlioides (2)	52.20	56.46 ± 4.35	62.40	59.90	56.90 ± 4.24	53.90	31.80	37.57 ± 12.84	61.50	51.90	60.47 ± 7.44	69.10
R. solani (1)	33.20	36.48 ± 2.06	38.30	39.80	40.35 ± 0.78	40.90	28.70	32.50 ± 2.73	35.00	32.70	35.95 ± 3.47	40.50
P. betae (3)	32.80	36.18 ± 3.03	40.80	34.70	34.75 ± 0.07	34.80	26.80	39.10 ± 8.38	45.30	33.70	37.30 ± 2.83	40.50
A. cucurbitacearum (3)	15.20	15.98 ± 0.48	16.50	17.60	17.60 ± 0.00	17.60	15.00	16.37 ± 0.98	17.30	11.40	13.87 ± 2.63	16.30
F. oxysporum f. sp. *radicis lycopersici* (1)	18.50	24.40 ± 4.37	27.70	23.10	23.40 ± 0.42	23.70	18.70	22.10 ± 2.80	25.50	22.50	27.90 ± 4.49	32.30

[a] Mean values are shown ± standard deviations. Min, minimum percent RI; Max, maximum percent RI.

The phenotypic stability of the different replicates of the monospore cultures seen here contrasts with the morphological variability as regards the texture and pigmentation observed in different monospore lines of other filamentous fungi such as *Penicillium* spp., *Rhizoctonia*, and *Phoma* used in similar studies (7, 27, 31).

It was not possible to perform a numerical analysis of the morphological characteristics studied independently due to the small number of variable morphological features observed between the isolates of *T. harzianum* assayed. However, both the size of the spores (40) and the presence and arrangement of the chlamydospores (23) are characteristics that have been used by other authors. The differentiating limits considered in this work for the chlamydospores (10-μm-diameter), phialides (10 by 3 μm), and conidia (2.4-μm-diameter) of *T. harzianum* lie within the range of values accepted by Bisset (3) for this species: 4- to 12-μm diameter for the chlamydospores, 3.5 to 10 by 2.5 to 3.8 μm for the phialides, and 2 to 3 by 1.5 to 2.5 μm for the conidia. The conidia of the strains grouped in cluster 2, with a diameter of 2.4 to 3 μm, are larger than those of the rest of the isolates studied, although they are smaller than the conidia of *Trichoderma parceramosum* (which also possess a smooth wall but which are mostly 2.5 to 4.2 by 2.2 to 3.6 μm) (4). The presence of subglobose to ellipsoidal chlamydospores which are intercalary or terminal on short branches (54) may be of primary importance for the survival of the biological antagonists in the soil and proved to be of use for establishing groupings in this study (Table 3).

All the strains used in the present study were able to assimilate ethanol and grow and sporulate in lactic acid as the sole carbon source. These results are not consistent with those published by Manczinger and Polner (24) for several species of *Trichoderma*. However, these differences may be accounted for by differences in assay conditions (agar plugs with hyphal tips in liquid medium, pH 4.5, and 25°C temperature in this study versus conidial suspensions on agar plates, pH 5 to 6, and 30°C temperature in the study reported by Manczinger and Polner). The growth and alkalinization of the culture media in the assimilation assays with nitrogen sources did not prove as useful here as they are for terverticillate penicillia (8). Despite this, it is interesting that, with the exception of isolate 3, good growth in the nitrogen sources assayed was always accompanied by a shift in the indicator from yellow to purple.

In the agar-based assays of copper sulfate, this salt accumulated on the edge of the petri dish since the apices of the hyphae were able to drag the inhibitory agent to external zones of the colonies. This phenomenon has previously been observed in *P. betae* as a result of an extracellular polysaccharide matrix that wraps around the hyphae like a sheath (28). Because of this difficulty in quantifying the concentration of copper in the successive zones of active growth of the colony, the inhibitory effect of copper sulfate on the growth of *T. harzianum* was measured in the form of the percentage of reduction of the diameter of the colonies with respect to a control without inhibitory compounds. This type of assay may be useful in the identification and classification of filamentous fungi, as long as the inhibitor is used at a suitable concentration.

The production of yellow pigment in MEA proved to be taxonomically significant as well as serving to demonstrate that this medium can be used for systematic ends in addition to the simply morphological purposes reported by Rifai (40). Gelatin hydrolysis, sporulation, and the production of a diffusible orange pigment in the medium with gelatin were the assays that yielded the maximum discrimination among the strains studied.

With the exception of isolate 2925, all isolates showed similar activity profiles in the API ZYM system, and this technique did not permit the establishment of infraspecific groups.

The stability of the dendrogram groupings was independent of the different similarity coefficients employed, pointing to a correct application of the coding system used for the individual characters. The isolates were recovered in groups with very low similarity levels (Fig. 1 and 4), which confirms the heterogeneity of the set of species known as *T. harzianum*. This has previously been demonstrated by Manczinger and Polner (24), who placed 24 strains of *T. harzianum* into five groups belonging to two subclusters, according to conidial germination and growth in different carbon sources; by Seaby (45), who delimited three groups in *T. harzianum* on the basis of growth rate and sporulation in culture; and by Stasz et al. (50), who placed 16 strains in four clusters in a numerical phylogenetic study using parsimony of allozyme phenotypes at 16 enzyme loci. Muthumeenakshi et al. (33) described three groups within *T. harzianum* as "DNA-based species" on the basis of ITS 1-ITS 2 sequences, restriction fragment length polymorphisms of mitochondrial DNA and rDNA, and randomly amplified polymorphic DNA analysis. This study was restricted to only strains isolated from a single substrate in the British Isles but can provide some useful comparison with our wider range of isolates. The ITS 1-ITS 2 sequences of strains 24, 2925, 2931, 260, and 11 were determined, and of these, isolates 24 (cluster 1a), 2925 (cluster 1b), and 2931 (cluster 3) are very similar to the sequences given by Muthumeenakshi et al. as group 1, with divergences of 0.5, 2.0, and 1.0%, respectively. Isolates 260 and 11 (cluster 2) have an ITS 1-ITS 2 sequence identical to that described as group 3 (33). Although these are all European isolates, this comparison provides some support to the identification of these groups as DNA species with a worldwide distribution and provides further non-DNA characteristics for their delineation.

The molecular similarity among clusters 1a, 1b, and 3 suggests that there are three physiological groups of *T. harzianum*

within rDNA group 1 with different but very similar ITS 1-ITS 2 sequences and a worldwide distribution: Europe, South America, and Asia. However, the existence of a constant sequence for isolates pertaining to rDNA group 3, as well as their relation to the same physiological cluster, suggests a common origin for this subspecific group. Nevertheless, isolate 2923 was from a different geographical origin and further work is needed to explain the possible origin of these molecular groups, including the wider distribution of rDNA group 2, and to interpret their relationship with the isoenzymatic data.

The characteristics used in this study can be considered to be a mixture of phenotypic and genotypic characteristics. As such, groupings derived from them could be expected to represent both functional and genetic relationships. An example of this is seen with the correlation obtained between in vitro antifungal activity and the clusters from the dendrograms. The electrophoretic data agreed well with this with the exception of isolate 24. This isolate grouped with cluster 1 on the basis of both its antifungal activity and its morphological, physiological, and biochemical properties. However, it was placed with cluster 2 isolates on the basis of its isoenzyme band patterns. This discrepancy may represent an example of an isolate that has arisen from one genetic line (cluster 2) but has developed properties closer to another line (cluster 1), perhaps through selection imposed by particular environmental or ecological conditions. This result could also support the theory of evolution of subspecific groups of *T. harzianum* from cluster 2 (group 3 of Muthumeenakshi et al. [33]), as all strains from this rDNA group which have been sequenced (33 and this study) have identical sequences with percent divergences of 17.3 to 18.3% from sequences of isolates from rDNA group 1 and 22.9% from rDNA group 2.

Powell (36) stated that the established view of biological control is that it is less efficient and less reliable but safer than chemical control. It has a narrower range of activity and can be limited by the type of crop or the environment. To be realistic we should not expect a very broad range of pest or disease control from biological agents in terms of controlling major pests or pest complexes in major crops in a wide range of environments. Since biological control agents are, by their nature, more limited than the equivalent chemicals, their use needs to be targeted carefully, based upon an appropriate characterization of the different strains to enable their discriminant selection.

In this study we have demonstrated that the behavior of different isolates of *T. harzianum* varies according to the target fungus, as shown by Elad et al. (12). The greater control of *A. cochlioides*, a fungus whose cell wall is composed of cellulose, in comparison to the inhibition of the growth of *P. betae*, *R. solani*, *A. cucurbitacearum*, and *F. oxysporum* f. sp. *radicis lycopersici*, which have chitin and glucan cell walls, was perhaps due to the production of cellulases by almost all the strains of *T. harzianum* studied. Isolate 2923 was the only strain that did not reduce the growth of *P. betae* and *A. cochlioides* to the expected levels. In turn, this isolate was deficient in protein secretion and showed the lowest levels of lytic enzymatic activities against other fungi. This strain gave the lowest values of RI and cannot be considered a real biocontrol agent. This isolate was the only one that showed low extracellular cellulase activity, and this could be the reason for the weak inhibition against *A. cochlioides*. A direct relationship between antagonist capacity and enzymatic activities of *Trichoderma* has been reported previously (11).

Given the variability within *Trichoderma*, it is difficult to use the names assigned by Rifai (40) or Bisset (2) for biological control agents, since these names involve species concepts that are too broad for practical situations. In a practical biological control situation differentiation is required to define populations within a single species name. A particular strain of *T. harzianum* may be a good or bad biological control agent depending upon the intended target and the functions required. It will therefore be necessary to select the most efficient strain for each individual pathosystem.

The results presented here support the existence of "cryptic species with similar morphologies" (49) and "DNA-based species" (33), and the proposed attributes may be used for the selective choice, as biological control agents, of new strains of *T. harzianum* within the four clusters described. Similar results have been obtained for other fungal biocontrol agents, such as the insect pathogens of the genera *Beauveria* and *Tolypocladium* (32, 39) and entomopathogenic, fungicolous, and plant-pathogenic strains of the genus *Verticillium* (21).

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