Mol Gen Genet (1997) 256: 127–135
© Springer-Verlag 1997

ORIGINAL PAPER

I. Gómez · I. Chet · A. Herrera-Estrella

Genetic diversity and vegetative compatibility among *Trichoderma harzianum* isolates

Received: 27 July 1996 / Accepted: 23 April 1997

Abstract *Trichoderma harzianum* is the collective name of a set of asexual fungal strains which exhibit heterogeneity in genome structure, DNA sequence and behavior. Contour-clamped homogeneous field (CHEF) electrophoresis of the chromosomes of ten isolates of *T. harzianum* revealed six clearly distinct electrophoretic karyotypes. Of the ten isolates analyzed, four (GH12, G109, Y and YF) could be classified in a single group with identical karyotypes, while the strains T35 and 315 formed a second group. The genome size characteristic of the different isolates fell into a broad range varying from 29.6 to 56.1 Mb. Gene assignments to the resolved chromosomes showed that all genes analyzed were localized on equivalent chromosomes in the isolates belonging to the same group. Analysis of randomly amplified polymorphic DNAs from the ten isolates confirmed the classification into groups and allowed us to distinguish between isolates T35 and 315, as well as between isolates GH12, G109, Y and YF. Direct confrontation assays using isolates of the same group showed compatible interactions, whereas the same experiment carried out with isolates of different groups showed an incompatible interaction characterized by an area of cell damage. Microscopic observation of the compatible interactions showed hyphal fusions between the isolates, similar to those described for vegetative compatible groups in other fungi. The molecular karyotypes correlated well with the compatibility of the isolates. In addition, we have evaluated both electrophoretic karyotype and randomly amplified polymorphic DNAs analysis as criteria for grouping isolates within the genus according to their capacity for biocontrol of plant pathogens.

Key words *Trichoderma* · Karyotype · Vegetative compatibility · Molecular variation · Systematics

Communicated by E. Cerdá-Olmedo

I. Gómez · A. Herrera-Estrella (✉)
Centro de Investigación y Estudios Avanzados,
Plant Biotechnology and Genetic Engineering Unit,
Apartado Postal 629, 36500 Irapuato, Gto., México.
Fax: +52-462-45849-45846.
E-mail: aherrera@irapuato.ira.cinvestav.mx

I. Chet
The Hebrew University of Jerusalem,
Otto Warburg Center for Agricultural Biotechnology,
Faculty of Agriculture,
P.O. Box 12, Rehovot 76100, Israel

Introduction

The fungal genus *Trichoderma* encompasses a large set of asexual fungal strains which are heterogeneous in genome structure and behavior. Teleomorphs, where known, belong to *Hypocrea* or related genera. The species of *Trichoderma* are referred to as species group aggregates because they are made up of several genetic entities, which can not be distinguished from one another by morphological criteria. For most of the species group aggregates the corresponding teleomorphs are unknown. Thus, the definition of species is rather subjective. Within this genus the soil fungus *T. harzianum* has been shown to act as a mycoparasite against a range of economically important aerial and soil-borne plant pathogens. Different factors involved in the antagonistic properties of *Trichoderma* have been identified, including antibiotics (Dennis and Webster 1971a, b) and hydrolytic enzymes such as β-(1,3) glucanases, proteases and chitinases (Elad et al. 1984; Geremia et al. 1993). The initial interaction between *Trichoderma* and its host is characterized by the chemotropic growth of hyphae of the mycoparasite towards the host (Chet and Elad 1983). When the mycoparasite reaches the host, its hyphae often coil around it or are attached by hook-like structures (Elad et al. 1983b). Following these interactions, the mycoparasite penetrates the host mycelium, apparently by partially degrading its cell wall. Susceptible host mycelia show rapid vacuolation, collapse and disintegration (Elad et al. 1983a; Benhamou and Chet 1993).

Despite the importance of the genus, there are still many problems concerning the taxonomical classification of its constituent species. The taxonomic scheme most widely used is based on the revision of Rifai (1969), which relies on a series of morphological characters that do not allow one to distinguish the different genetic entities from one another. Several attempts have been made to use molecular techniques for the classification of *Trichoderma* spp. (Meyer 1991; Meyer et al. 1992; Herrera-Estrella et al. 1993; Muthumeenakshi et al. 1994). However, further molecular studies are needed in order to obtain a more precise view of the *Trichoderma* genome as a typological entity and increase our understanding of each independent asexual line. Furthermore, no correlations have yet emerged between the established molecular characters and the biology of the genus.

Here we report the molecular characterization of a set of *T. harzianum* isolates in relation to both their mycoparasitic properties and the physical interactions between them.

Materials and methods

Strains and plasmids

T. harzianum strains 315, ATCC32173 (ATCC), G108, G109, GH2, GH12, IMI206040 (IMI), T35, Y and YF (see Table 1), and *T. reesei* QM9414 (QM) were used in this study. *Sclerotium rolfsii*, *Pythium aphanidermatum*, *Fusarium oxysporum* f. sp. *vasinfectum* and *Rhizoctonia solani* were used to test the biocontrol abilities of the different *T. harzianum* strains in vitro and/or in greenhouse experiments. *Escherichia coli JM103* (*Sambrook et al.,* 1989) was used for DNA manipulations.

Greenhouse experiments

Experiments were carried out on cotton (*Gossypium barbardense* L.) seedlings challenged with fungal pathogens. The seedlings were grown in plastic pots, each containing 0.5 kg soil (10 plants/replicate). Experiments were performed in six replicates. The soil was a sandy loam, consisting of 82.3% sand, 2.3% silt, 15% clay and 0.4% organic matter, with a pH of 7.4 and a moisture-holding capacity of 12.2%. Temperature ranged from 27 to 30° C. Irrigation was provided daily.

In the case of plants infected with *R. solani*, the inoculum was prepared as follows: *T. harzianum* was added to soil as a wheat bran/peat preparation containing a mixture of mycelia and conidia (0.5% w/w). Chopped potato soil containing *R. solani* was prepared as previously described (Ko and Hora 1971) and used for soil infestation. *T. harzianum* and *R. solani* were added to the soil simultaneously. For challenges with *F. oxysporum*, the inoculum was prepared as follows: Erlenmeyer flasks (250 ml), each containing 50 ml of liquid YM media (0.3 g/l yeast extract, 0.3 g/l malt extract, 10 g/l dextrose and 0.5 g/l peptone in distilled water), were inoculated with mycelial disks from 72-h cultures of *F. oxysporum*. Flasks were incubated at 27° C in a rotary shaker at 120 rpm for 4 days. Conidia were collected by filtration through eight layers of surgical gauze. The conidial suspension was then centrifuged at $9150 \times g$ for 30 min at 4° C and washed. Suspensions containing microconidia were adjusted to 2×10^7 microconidia/ml. Ten milliliters of suspension was added to the sandy loam soil using an electrical soil mixer. *Trichoderma* conidia were collected from cultures grown in Erlenmeyer flasks (2 l), each containing 200 ml of potato dextrose agar. Conidia were suspended in sterile water and filtered through a 50-μm nylon mesh. Conidia were mixed with enough soil to realize an inoculum pressure of 5×10^9 conidia/kg three weeks after the infestation with *Fusarium*. Wilt symptoms on cotton seedlings were observed after 18 days. All experiments were repeated at least twice.

Disease reduction was defined as: $100(1-y/x)$, where x and y are the proportions of diseased plants in the control and treated populations, respectively.

Preparation of intact chromosomal DNA

Agarose plugs containing intact *Schizosaccharomyces pombe* chromosomal DNA were purchased from Bio-Rad (Hercules, Calif., USA). Intact *T. harzianum* chromosomal DNA was prepared as previously described (Herrera-Estrella et al. 1993). Approximately 20–30 potato dextrose agar plates were overlaid with cellophane sheets; 1×10^7 conidia were inoculated per plate and allowed to germinate (14–16 h) at 28° C. The cellophane sheets were collected and the mycelium scraped off into 75 ml of the cell wall digestion mix (1.2 M $MgSO_4$, 10 mM $NaPO_4$, 5 mg/ml Novozyme234; pH 5.6). The mycelial cells were incubated at 28° C with gentle agitation. Protoplast production was monitored microscopically; when 90% of the germinated conidia had been converted into protoplasts the suspension was filtered through a 50-μm nylon mesh. The filtrate was transferred to centrifuge tubes and carefully overlaid with an equal volume of ST buffer (0.6 M sorbitol, 10 mM TRIS-HCl, pH 7.5). The gradient was then centrifuged at $4000 \times g$ in a swinging bucket rotor for 15 min at 4° C. The protoplast band was carefully collected and mixed with 1.5 volumes of S2T buffer (1.2 M sorbitol, 10 mM TRIS-HCl pH 7.5); the protoplasts were pelleted by centrifugation at $5000 \times g$ for 10 min at 4° C and washed twice with the same buffer. The pellet was resuspended in modified GMB solution (0.125 M EDTA pH 8.0, 1.2 M sorbitol) at a concentration of $0.5–1 \times 10^9$ cells per ml. The suspension was brought to 37° C and an equal volume of 1.4% low melting agarose (FMC) in GMB was added. The mixture was then poured into a plug mold and allowed to solidify for 30 min on ice. The plugs were then immersed in NDS buffer (0.5 M EDTA pH 8.0, 10 mM TRIS-HCl pH 8.5, 1% Sarkosyl), containing 2 mg/ml proteinase K (Boehringer, Mannheim, Germany) at 50° C for at least 24 h. The chromosome plugs were washed twice in 50 mM EDTA (pH 8.0) at 50° C and stored at 4° C in the same solution.

CHEF gel conditions

Contour-clamped homogeneous field electrophoresis (CHEF) analysis was performed with the CHEF Mapper (BioRad), using the following conditions: 0.65% Megarose (Clontech Laboratories, Palo Alto, Calif., USA), 168 h running time ($0.5 \times$ TBE buffer: 0.45 M TRIS-borate, 1 mM EDTA pH 9), 1800–5400 s switching intervals with linear ramping, 120° constant angle, 1.3 V/cm, and 12° C constant temperature. In all cases the gels were stained for 30 min with a 0.5 μg/ml ethidium bromide solution and destained overnight in water.

DNA manipulations

Fungal genomic DNA was isolated according to the protocol of Raeder and Broda (1985). DNA fragments were labeled with [α-^{32}P]dCTP using a random primer DNA labeling kit (Boehringer). DNA hybridization experiments were carried out under highly stringent conditions (Sambrook et al. 1989).

Polymerase chain reaction (PCR) amplification

Randomly amplified polymorphic DNA (RAPD) analysis was carried out on *Trichoderma* genomic DNA (30 ng) using *Taq* DNA polymerase (GIBCO-BRL, Gaithersburg, Md., USA) in a total volume of 50 μl under the conditions recommended by the manu-

facturer. The amplification conditions were as follows: 94° C, 1.5 min; 30° C, 2 min; and 72° C, 3 min; for 45 cycles, with a final extension period of 7 min at 72° C. Primers used were: OPA13, 5′-CAGCACCCAC-3′; OPA03, 5′-AGTCAGCCAC-3′; OPI04, 5′-CCGCCTAGTC-3′; OPA19, 5′-CAAACGTCGG-3′; OPH13, 5′-GACGCCACAC-3′; OPG10, 5′-AGGGCCGTCT-3′; OPH16, 5′-TCTCAGCTGG-3′; and OPI12, 5′-AGAGGGCACA-3′, obtained from Operon (Alameda, Calif., USA). Aliquots (25 µl) of the reactions were subjected to agarose gel electrophoresis (1.4% agarose in TBE buffer) and DNA bands detected by ethidium bromide staining under standard conditions (Sambrook et al. 1989). Each amplification was carried out in triplicate.

Direct confrontation assays

Direct confrontation assays in vitro between the *T. harzianum* isolates and the various phytopathogenic fungi were carried out as follows. Agar plugs cut from the growing edge of a colony of each fungus were placed on opposite sides of 9-cm petri plates containing potato dextrose agar. Cultures were allowed to grow at 28° C for 4 days. The inhibitory activity of the *Trichoderma* isolates was then scored by measuring the extent to which the phytopathogenic fungus was overgrown by *Trichoderma*.

Confrontation assays in vitro between *T. harzianum* isolates were established as follows. Agar plugs cut from the edge of growing colonies of each isolate were placed on opposite sides of 9-cm petri plates containing potato-dextrose agar. Cultures were grown at 28° C for 2–3 days and then photographed.

Microscopic observation of interactions

Isolates were paired on slides placed on 2% water agar in petri dishes. The slides were covered with a thin layer of potato dextrose agar. Mycelial disks of the isolates were cultured on potato dextrose agar and placed at each end of the slide. Plates were incubated at 28° C until hyphae overlapped, usually within 48–72 h. The area of hyphal overlap was stained with 0.05% trypan blue in lactophenol, and examined under the light microscope.

Results

Biocontrol capacity of different *T. harzianum* isolates

The capacity to antagonize the growth of other fungi (biocontrol) is one of the main characteristics of the genus *Trichoderma*, as well as being of applicative value. Accordingly, we chose biocontrol ability as the phenotype to compare with the electrophoretic karyotypes and other molecular genetic criteria. Ten *Trichoderma harzianum* isolates collected in different parts of the world were used in this study. These isolates were previously classified as *T. harzianum* on the basis of the scheme proposed by Rifai (1969). In order to determine whether the ten isolates are similar or heterogeneous in their capacity to control several phytopathogenic fungi, they were tested both in vivo and in vitro. The results of this series of tests showed important degrees of variability between isolates in their capacity to control the tested plant pathogens (Table 1). Direct confrontation assays in vitro were used as an indication of the capacity of the *Trichoderma* isolates to control each of the plant pathogens. The capacity of the *Trichoderma* isolates to antagonize the growth of the host was scored by mea-

Table 1 Biocontrol capacity of *Trichoderma* isolates

Isolate	Origin	Taxonomic identification	Direct confrontation[a]					Greenhouse experiments (Disease reduction)		
			S. rolfsii	*P. aphanidermatum*	*R. solani*	*F. oxysporum* (melon)	*F. oxysporum* (cotton)	*F. oxysporum* (melon)	*F. oxysporum* (cotton)	*R. Solani*
315	Israel	*T. harzianum*	+ +	+ + +	+ + +	+	–	ND	ND	47
ATCC32173	Israel	*T. harzianum*	+	ND	+ +	ND	ND	ND	ND	ND
G108	Guatemala	*T. harzianum*	–	+ + +	+	–	–	ND	53	64
G109	Guatemala	*T. harzianum*	+	+ + +	+ +	–	–	54	ND	55
GH-2	Ghana	*T. harzianum*	–	+ + +	+	–	+	71	48	67
GH-12	Ghana	*T. harzianum*	+	+ + +	+ + +	–	–	24	ND	42
IMI206040	England	*T. harzianum*	+	ND	+ + +	–	ND	ND	ND	42
T-35	Israel	*T. harzianum*	+ + +	+ + +	+ + +	–	–	67	76	59
Y	Israel	*T. harzianum*	+	+ + +	+ + +	–	–	56	60	50
YF	Israel	*T. harzianum*	+	+ + +	+ + +	–	–	35	50	50

[a] –, No overgrowth; +, *Trichoderma* overgrew the pathogenic fungus by 5–10 mm; + +, 10–20 mm overgrowth; + + +, 20 mm overgrowth; ND, not determined

suring the degree to which *Trichoderma* overgrew the phytopathogenic fungus. The results of these tests indicated that most of the isolates were very efficient at overgrowing both *R. solani* and *P. aphanidermatum*. Isolates G108 and GH2 were exceptions, both showing a low capacity to overgrow *R. solani*. The variation in antagonistic capacity was higher when the target fungus was *S. rolfsii*. Isolates 315 and T-35 were quite active in overgrowing this species, while isolates Y, YF, ATCC32173, G109, GH12 and IMI206040 overgrew *S. rolfsii* very slowly, and isolates G108 and GH2 did not overgrow *S. rolfsii* at all. The capacity of the ten isolates to control *F. oxysporum* was in general very low. However, isolates GH2 and 315 showed an ability to overgrow *Fusarium* to some extent, although each was active against a different isolate of the pathogen.

In agreement with the in vitro tests, greenhouse experiments indicated that nine of the ten isolates were efficient at controlling *R. solani* under the conditions used. In contrast with the in vitro tests, which indicated that most of the isolates were ineffective in controlling *Fusarium*, greenhouse experiments showed significant levels of disease reduction with several of the isolates used (Table 1).

Electrophoretic karyotypes and gene assignment to separated chromosomes

To determine whether the set of ten isolates was heterogeneous at the genetic level, electrophoretic karyotypes were obtained. CHEF gel electrophoresis was performed with intact chromosomal DNA prepared from each of the ten *T. harzianum* isolates described above. Under the conditions used, it was possible to distinguish five bands (upon ethidium bromide staining; Fig. 1 and Table 2) corresponding to seven chromosomes for the strain IMI206040, as previously reported (Herrera-Estrella et al. 1993). It is noteworthy that the electrophoretic

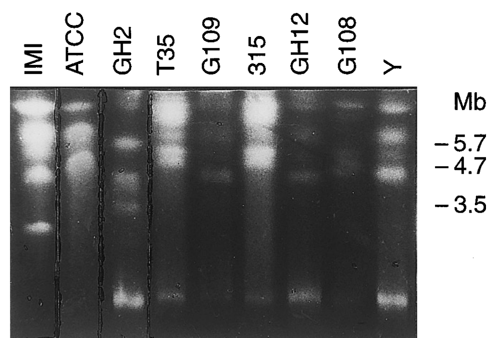

Fig. 1 *Trichoderma harzianum* electrophoretic karyotypes. Intact chromosomal DNA was prepared in agarose blocks, a block of suitable size was loaded onto the gel and subjected to CHEF gel electrophoresis. The names of the strains are indicated. Numbers and bars on the right indicate the sizes in Mb and the positions of the *S. pombe* chromosomes used as molecular weight standards, respectively

Table 2 Chromosomes and genome sizes

	Group A		Group B		ATCC32173		G108		GH-2		IMI206040	
	Chromosome[a]	Size[b]	Chromosome[a]	Size[b]	Chromosome[a]	Size[b]	Chromosome[a]	Size[b]	Chromosome[a]	Size[b]	Chromosome[a]	Size[b]
	IV	7.2	V_{ab}	7.3	IV	7.3	V_{ab}	7.3	V	7.3	V–VI	7.3
	III_{ab}	6.5	IV_{ab}	7.1	III_{ab}	6.9	IV	7.2	IV	6.0	IV_{ab}	6.5
	II	5.6	III	6.5	II	6.5	III	5.6	III_{ab}	3.5	III	5.6
	I_{ab}	3.7	II_{ab}	5.7	I_{ab}	5.1	II_{ab}	4.7	II_{ab}	3.3	II	3.7
			I_{ab}	4.7			I	4.0	I	2.7	I	2.2
Total	6	33.2	9	56.1	6	37.8	7	40.8	7	29.6	7	39.1

[a] Numbers were assigned to the different chromosomal bands in order of increasing size. Subscript letters (ab) indicate putative double chromosomal bands as estimated upon densitometric analysis of the bands observed by ethidium bromide staining of the gel. Total genome sizes were calculated adding the putative double bands twice

[b] Sizes are approximate and expressed in magabase pairs (Mb)

karyotype of this strain showed no change after six years of subculturing. Four isolates, G109, GH12, Y and YF (group A) showed indistinguishable chromosomal patterns composed of four bands (Fig. 1 and Table 2; YF data not shown). The chromosomal patterns of isolates T35 and 315 (group B) were also indistinguishable from each other, and were visualized as five bands (Fig. 1 and Table 2). Isolate ATCC32173 showed a unique pattern of four bands; isolates G108 and GH2 both gave a five-band pattern but with very different band sizes (Fig. 1 and Table 2). Based on these observations, isolates ATCC32173, IMI206040, G108 and GH2 represent four additional, independent groups. The estimated genome sizes of the different *T. harzianum* isolates analyzed were in the range of 30–40 Mb, except for isolates 315 and T35, which had a genome size of approximately 56 Mb.

In an attempt further to distinguish between those isolates that appeared to have the same karyotype, known genes were assigned to chromosomal bands by hybridization analysis. These data are also particularly valuable because it is presently impossible to construct a genetic map, due to the lack of stable genetic exchange in this genus. For this purpose we performed Southern blot analysis using as probes a series of previously cloned genes of *T. harzianum* and *T. reesei*: *ech42,* which encodes an endochitinase (Carsolio et al. 1994), *prb1*, coding for a basic proteinase (Geremia et al. 1993), *gta1*, which codes for an α-subunit of a heterotrimeric G protein (Rocha et al., in preparation) and the major cellobiohydrolase gene *cbh1* (Teeri et al. 1983). The results of this analysis (summarized in Table 3) showed that it was not possible, using this strategy, to distinguish between isolates that share the same chromosomal patterns, such as those belonging to groups A and B. Isolate GH2 showed hybridization only with *gta1* and *cbhI*. In the case of isolate G108 no hybridization signal was detected using *prb1* as a probe.

RAPD analysis

RAPD analysis is a sensitive criterion for establishing the relatedness of fungal isolates. We therefore chose RAPD analysis of *Trichoderma* genomic DNA as an alternative to molecular karyotyping. For this purpose a set of eight primers was used. The experiments were carried out with the ten isolates described above. In this case, *T. reesei* strain QM9414 and a *R. solani* isolate were included in the experiments as controls. Visual comparison of the banding patterns of RAPDs allowed us clearly to distinguish the same groups previously found, based on the electrophoretic karyotype and gene assignment to chromosomes (Fig. 2). Statistical analysis of the RAPD data carried out by the Unweighted Pair Group Method with Arithmetic Averages (UPGMAS) and the method of Skroch et al. (1992) for handling RAPD data indicated that the organisms analyzed could be separated into two major groups (Fig. 3). Group 1 contains isolates Y, YF, G109 and GH12. The members

Table 3 Chromosomal gene assignments

G109		GH-12		Y		315		T35		ATCC32173		G108		GH-2		IMI206040	
IV		IV		IV		V_{ab}	*prb1*	V_{ab}	*prb1*	IV	*prb1*	V_{ab}	*ech42*	V		V–VI	*prb1*
							ech42		*ech42*		*ech42*						*ech42*
III_{ab}	*ech42*	III_{ab}	*ech42*	III_{ab}	*ech42*	IV_{ab}		IV_{ab}		III_{ab}	*cbhI*	IV	*cbh1*	IV		IV_{ab}	
II	*cbh1*	II	*cbh1*	II	*cbhI*	III	*cbh1*	III	*cbh1*	II		III	*gta1*	III_{ab}		III	*cbh1*
I_{ab}	*gta1*	I_{ab}	*gta1*	I_{ab}	*gta1*	II_{ab}	*gta1*	II_{ab}	*gta1*	I_{ab}	*gta1*	II_{ab}		II_{ab}		II	*gta1*
	prb1		*prb1*		*prb1*												
						I_{ab}		I_{ab}				I		I	*gta1*	I	
															cbhI		

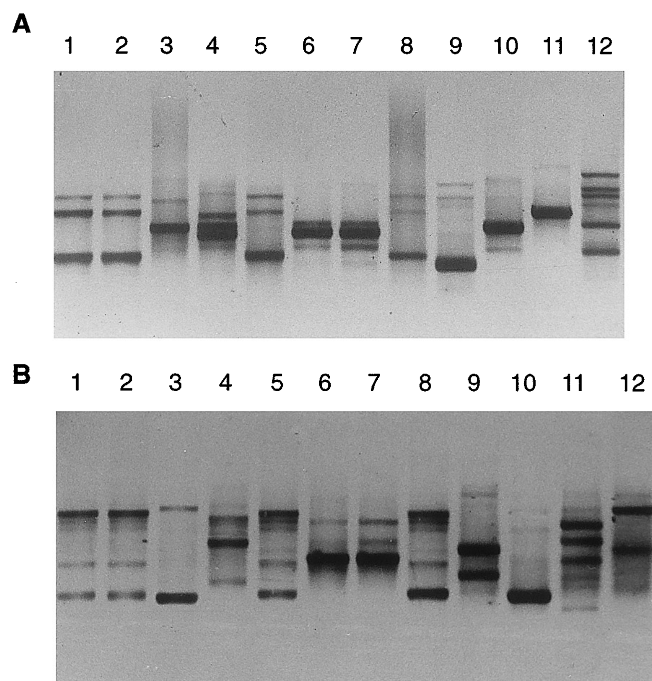

Fig. 2A, B RAPD analysis of genomic DNA. Lanes correspond to individual *Trichoderma* isolates: lane 1, strain Y; lane 2, YF; lane 3, ATCC32173; lane 4, G108; lane 5, G109; lane 6, 315; lane 7, T35; lane 8, GH12; lane 9, GH2; lane 10, IMI206040; lane 11, *T. reesei* QM9414 and lane 12, a *R. solani* isolate. The Figure shows a typical result, the primers used were: OPH13 (**A**) and OPA03 (**B**)

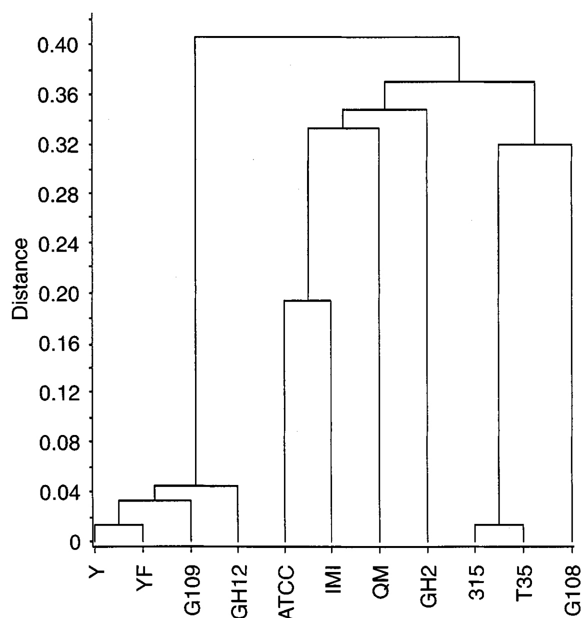

Fig. 3 Dendogram illustrating the genetic relationship between the *Trichoderma* isolates. The names of the strains are indicated at the bottom. The tree is based on the analysis of a total of 155 RAPD products

of the second major group (Group 2) are the *T. harzianum* isolates ATCC32173, IMI206040, G108, GH2, T35 and 315, and the *T. reesei* strain QM9414. Within Group 2 the situation is complex, because most of the isolates fell into this group and there is only one clear subgroup within it, formed by isolates T35 and 315. All other isolates belonging to this group were clearly distinct from their closest relative.

Trichoderma–Trichoderma interactions

An interesting question raised by the results described above is whether *Trichoderma* isolates belonging to the same group can recognize each other as the same type, and those from other groups as potential hosts. If this were the case, there should be no sign of antagonism between isolates of the same group (based on karyotype) when confronted in vitro, but antagonistic behavior should be detected in interactions between isolates from different groups. Macroscopic observation of direct confrontations of all isolates indicated that colonies from isolates belonging to the same group had very similar radial growth rates, patterns of sporulation and spore color, and that none of the isolates of the group could overgrow any other within the same group (Fig. 4B, C, E, F). In general the behavior in the direct confrontations assays within a given group (compatible interaction) was identical to that observed when two colonies of the same isolate were confronted (Fig. 4A). In the case of isolates belonging to different groups (incompatible interaction) a clear area with no aerial mycelium was observed at the border between the two colonies (Fig. 4D, G, H). This area has been described as a lysis zone (Haran et al. 1993), for mycoparasite-host interactions. However, it is very similar to the barrage described for those fungi which show vegetative incompatibility (Julián et al. 1996). One interesting observation was that, in the case of incompatible interactions, light-induced sporulation occurred with very distinct patterns. Furthermore, obvious differences in radial growth rate were also observed (data not shown). The four members of Group 1, as defined based on RAPD analysis, showed compatible interactions.

Microscopic observations of the border between the colonies allowed us to clarify the macroscopic observations. For compatible interactions, hyphal fusions were observed frequently at various points (see Fig. 5A–E). No cell death was observed in any case after cell fusion. This observation resembles what has been reported for other fungi when there is vegetative compatibility and perfect fusion (Ploetz and Correll 1988; Sneh et al. 1991). Figures 5F and G show apparently healthy hyphae of isolates G109 and 315, respectively, outside the area of direct hyphal contact. Within the area of interaction, no points of cell contact and no cell fusion were observed. Instead, cytoplasmic membrane collapse was common (Fig. 5H–I). The membrane collapse could be regarded as a symptom of damage caused by antibiosis.

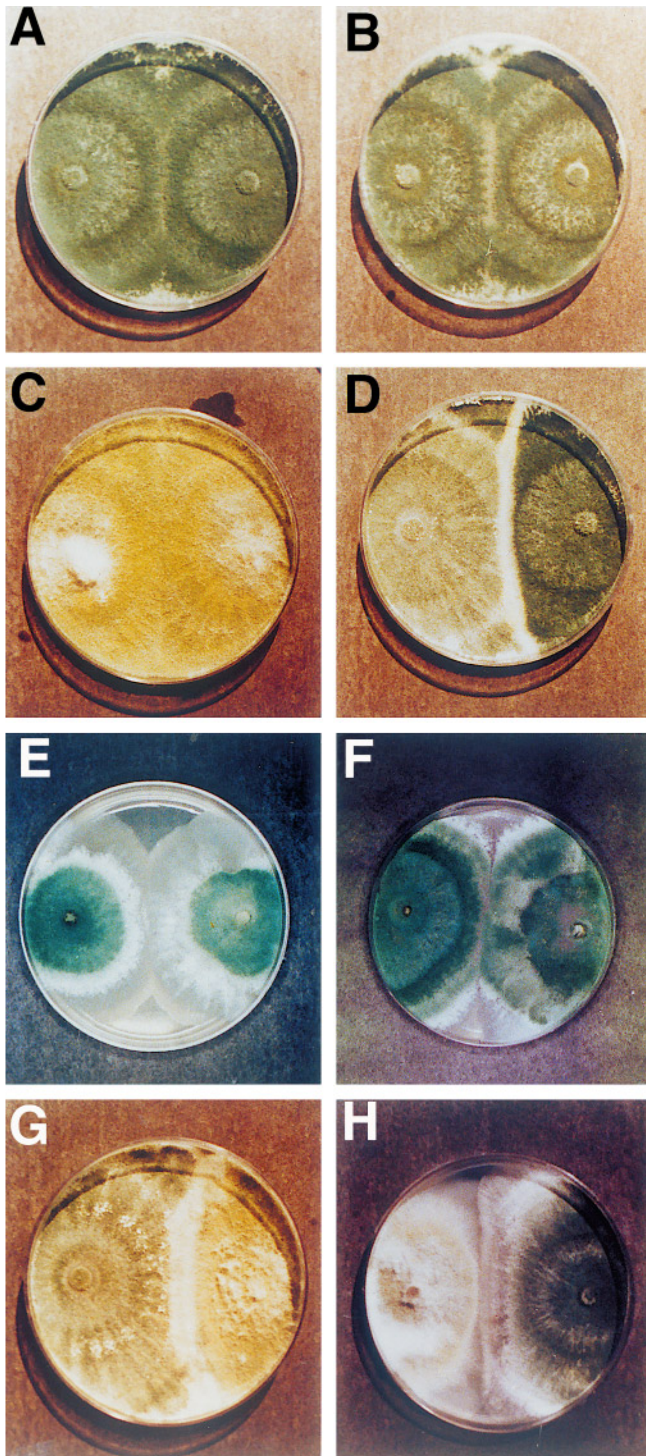

Fig. 4A–H Direct confrontation assays between *T. harzianum* isolates. **A** G109 vs G109. **B** G109 vs GH12. **C** T35 vs 315. **D** 315 vs G109. **E** GH12 vs Y. **F** G109 vs Y: **G** IMI vs T35. **H** IMI vs Y

Discussion

The taxonomic evaluation and identification of imperfect fungi is a tedious task, since in most cases only morphological and nutritional criteria can be used as markers. In this report we show that electrophoretic karyotypes and simple RAPD analysis can be used for the differentiation of isolates within the *T. harzianum* aggregate.

Highly variable intraspecific electrophoretic karyotypes have been reported among isolates of phytopathogenic fungi (for a review see Kistler and Miao 1992). These data have altered our view of fungal genomes as typological entities, as pointed out by Boehm and coworkers (1994). Usually, isolates chosen for analysis have belonged to distinct subspecific groupings based on, for example, single- and multilocus haplotypes (McDonald and Martínez 1991; Talbot et al. 1993), host specificities (Masel et al. 1990; Morales et al. 1993) and geographic origins (Kinscherf and Leong 1988).

Studies of the asexual fungus *F. oxysporum* indicate that isolates from one vegetative compatibility group are genetically distinct from those belonging to another, since they cannot form heterokaryons in culture and may share common physiological attributes (Ploetz and Correl, 1988; Moore et al. 1991), cultivar specificities (Ploetz 1990a, b), and molecular markers (Kistler et al. 1991; Ploetz 1990a; Sorensen et al. 1993). On the other hand, uniformity of electrophoretic karyotypes has been reported among geographically disparate isolates of *Gibberella fujikuroi* having different host ranges (Yan and Dickman 1992). Similarly, identical electrophoretic karyotypes were found among isolates within the same mating group of *G. fujikuroi* (Xu and Leslie 1992). However, the influence of meiosis in maintaining genomic organization has not been clearly established. Thus, it is not surprising that *T. harzianum* isolates belonging to the same group, based on the similarity of their electrophoretic karyotypes (Fig. 1), and the same subgroup, based on their RAPD patterns (Figs. 2 and 3), behave similarly when confronted with a set of phytopathogenic fungi (Table 1) and are capable of undergoing hyphal fusion with each other (Fig. 5A–E). Furthermore, isolates belonging to different groups behave differently when confronted with several plant pathogens (Table 1) and are unable to undergo hyphal fusion (Fig. 5H–I). As in the case of *G. fujikuroi,* uniformity was observed among some geographically disparate isolates of *T. harzianum,* such as those belonging to group A. This suggests either that they are in an early stage of the evolution of the asexual line, or that the many processes that could lead to variation are limited by an as yet unidentified factor.

It is especially noteworthy that, based on RAPD analysis, a *T. reesei* strain falls into the same group as some *T. harzianum* isolates (Fig. 3). This observation compounds the confusion existing in the classification of the genus *Trichoderma*. It must be noted that the *T. reesei* strain QM9414 is a mutant derived from the strain QM6a, which was subjected to mutagenesis using a linear accelerator and high voltage electrons (Mäntylä et al. 1992). However, QM9414 has been shown by RFLP analysis to be very closely related to QM6a but not to another set of *T. harzianum* isolates (Meyer et al.

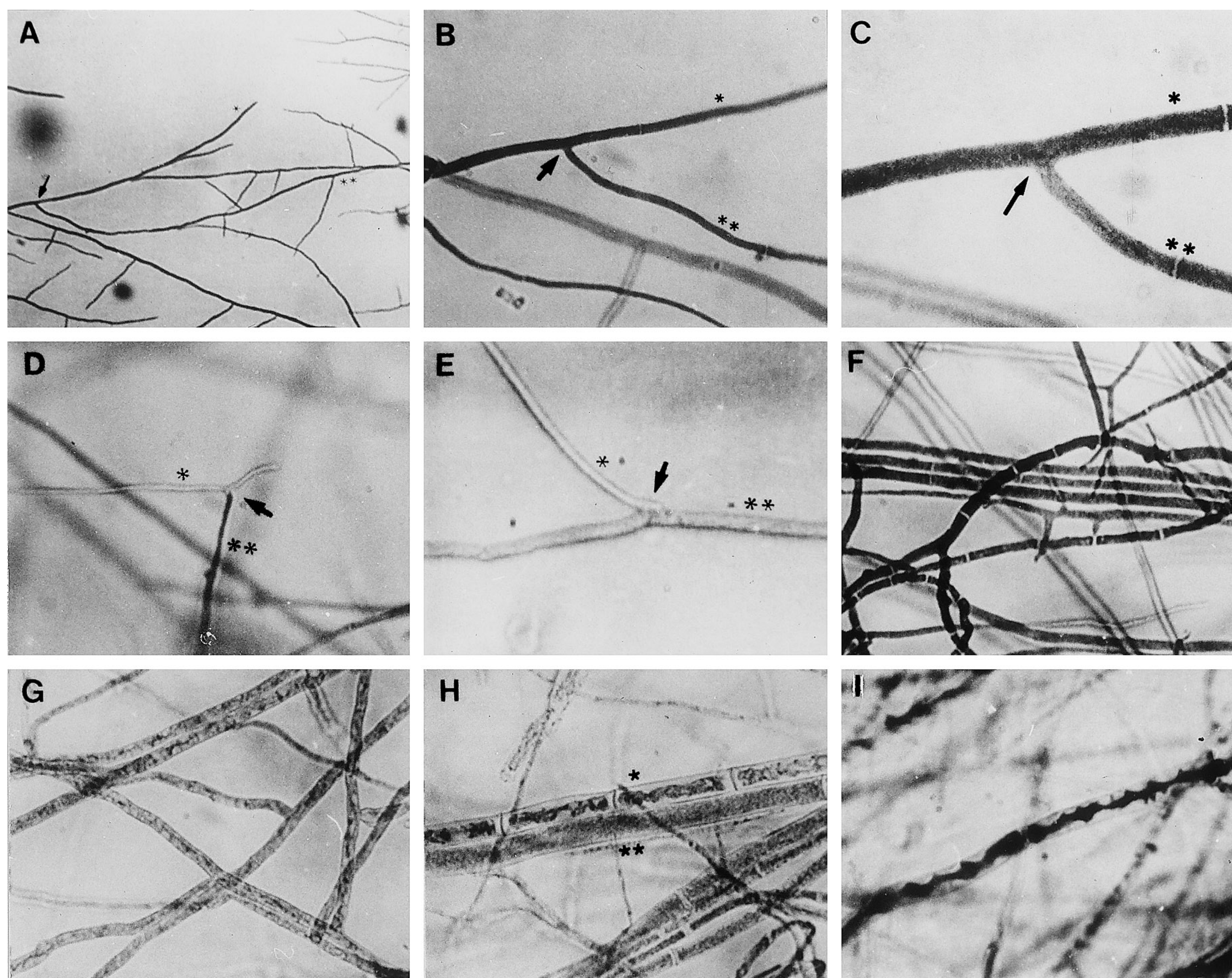

Fig. 5A–I Microscopic observations on the interaction between *T. harzianum* isolates. **A–E** Area of direct hyphal interaction between isolates G109 (marked with an *asterisk*) and GH12 (marked with a *double asterisk*). The *arrows* indicate points of fusion. **F** Isolate G109 outside the area of direct hyphal interaction. **G** Isolate 315 outside the area of direct hyphal interaction. **H, I** Area of direct hyphal interaction between isolates 315 and G109. The *star* marks the hypha of isolate G109 and the *double star* marks the hypha of isolate 315

1992). Based on our data we propose that isolates Y, YF, GH12 and G109 belong to one species or subspecies within the genus *Trichoderma*. Similarly, isolates T35 and 315 could be considered as members of a different species or subspecies. These two groups of isolates should not be classified as equal and should at least be separated into subspecies. Because of the genetic distance among all other isolates (greater than 18%), they could be considered as members of other species or subspecies, although more data are necessary to confirm this conclusion.

In summary, the data presented here offer a promising perspective for a re-appraisal of the classification of the *Trichoderma* species group aggregates (Rifai 1969), which is fundamental to the understanding of their biology. Furthermore, our data offer the possibility of selection in the formulation of products carrying several *Trichoderma* isolates for their successful use in the field. Further work should involve an extension of the present study to include many more isolates and related species.

Acknowledgments We wish to thank Drs. José Ruíz-Herrera, June Simpson, Benjamin Horwitz, George Vandemark and Gabriela Olmedo for critical reading of the manuscript, Dr. Octavio Martínez for statistical analysis of the data, Gil Virgen and Gerardo Vázquez for helpful comments and Mrs. M. Abramski for excellent assistance in the greenhouse experiments. This work was supported by EEC contract TS3-CT92-0140 and the Chais Family Charity Foundation

References

Benhamou N, Chet I (1993) Hyphal interactions between *Trichoderma harzianum* and *Rhizoctonia solani*: ultrastructure and gold cytochemistry of the mycoparasitic process. Phytopathology 83:1062–1071

Boehm EWA, Ploetz RC, Kistler HC (1994) Statistical analysis of electrophoretic karyotype variation among vegetative compat-

ibility groups of *Fusarium oxysporum* f. sp. cubense. Mol Plant Microbe Interact 7:196–207
Carsolio C, Gutiérrez A, Jiménez B, Van Montagu M, Herrera-Estrella A (1994) Characterization of *ech42*, a *Trichoderma harzianum* endochitinase gene expressed during mycoparasitism. Proc Natl Acad Sci USA 91:10903–10907
Chet I, Elad Y (1983) Mechanism of mycoparasitism. In Colloq. l′ INRA. Institute National de la Recherche Agionomique, Dijon. France, 18 35–40
Dennis C, Webster J (1971a) Antagonistic properties of species-groups of *Trichoderma*. I. Production of non-volatile antibiotics. Trans Brit Mycol Soc 57:25–39
Dennis C, Webster J (1971b) Antagonistic properties of species-groups of *Trichoderma*. II. Production of volatile antibiotics. Trans Brit Mycol Soc 57:41–48
Elad Y, Barak R, Chet I, Henis Y (1983a) Ultrastructural studies of the interaction between *Trichoderma* spp. and plant pathogenic fungi. Phytopathol Z 107:168–175
Elad Y, Chet I, Boyle P, Henis Y (1983b) Parasitism of *Trichoderma* spp. on *Rhizoctonia solani* and *Sclerotium rolfsii*. Scanning electron microscopy and fluorescent microscopy. Phytopathology 73:85–88
Elad Y, Barak R, Chet I (1984) Parasitism of sclerotia of *Sclerotium rolfsii* by *Trichoderma harzianum*. Soil Biol Biochem 16:381–386
Geremia R, Goldman GH, Jacobs D, Ardiles W, Vila SB, Van Montagu M, Herrera-Estrella A (1993) Molecular characterization of the proteinase encoding gene, *prb1*, related to mycoparasitism by *Trichoderma harzianum*. Mol Microbiol 8:603–613
Haran S, Schikler H, Pe'er S, Logemann S, Oppenheim A, Chet I (1993) Increased constitutive chitinase activity in transformed *Trichoderma harzianum*. Biol Control 3:101–108
Herrera-Estrella A, Goldman GH, Van Montagu M, Geremia RA (1993) Electrophoretic karyotype and gene assignment to resolved chromosomes of *Trichoderma* spp. Mol Microbiol 7:515–521
Julián MC, Debets F, Keijer J (1996) Independence of sexual and vegetative incompatibility mechanisms of *Thanatephorus cucumeris* (*Rhizoctonia solani*) anastomosis group 1. Phytopathology 86:566–574
Kinscherf TG, Leong SA (1988) Molecular analysis of the karyotype of *Ustilago maydis*. Chromosoma 96:427–433
Kistler HC, Miao VPW (1992) New modes of genetic change in filamentous fungi. Annu Rev Phytopathol 30:131–152
Kistler HC, Momol EA, Benny U (1991) Repetitive genomic sequences for determining relatedness among strains of *Fusarium oxysporum*. Phytopathology 81:331–336
Ko W, Hora HF (1971) A selective medium for the quantitative determination of *Rhizoctonia solani* in soil. Phytopathology 61:707–710
Mäntylä AL, Rossi KH, Vanhanen SA, Penttilä ME, Suominen PL, Nevalainen KMH (1992) Electrophoretic karyotyping of wild-type and mutant *Trichoderma longibrachiatum* (*reesei*) strains. Curr Genet 21:471–477
Masel A, Braithwaite K, Irwin J, Manners J (1990) Highly variable molecular karyotypes in the plant pathogen *Colletotrichum gloeosporioides*. Curr Genet 18:81–86
McDonald BA, Martínez JP (1991) Chromosome length polymorphism in a *Septoria tritici* population. Curr Genet 19:265–271
Meyer RJ (1991) Mitochondrial DNAs and plasmids as taxonomic characteristics in *Trichoderma viride*. Appl Environ Microbiol 57:2269–2276
Meyer W, Morawetz R, Börner T, Kubicek C (1992). The use of DNA-fingerprinting analysis in the classification of some species of the *Trichoderma* aggregate. Curr Genet 21:27–30
Moore NY, Hargreaves PA, Pegg KG, Irwin JAG (1991) Characterization of strains of *Fusarium oxysporum* f. sp. *cubense* by production of volatiles. Aust J Bot 39:161–166
Morales VM, Séguin-Swartz G, Taylor JL (1993) Chromosome size polymorphism in *Leptosphaeria maculans*. Phytopathology 83:503–509
Muthumeenakshi S, Mills PR, Brown AE, Seaby DA (1994) Intraspecific molecular variation among *Trichoderma harzianum* isolates colonizing mushroom compost in the British Isles. Microbiology 140:769–777
Ploetz RC (1990a) Population biology of *Fusarium oxysporum* f. sp. *cubense*. In: Ploetz RC (ed) Fusarium wilt of banana. American Society of Phytopathology, St. Paul, Minn., pp 63–76
Ploetz RC (1990b) Variability in *Fusarium oxysporum* f. sp. *cubense*. Can J Bot 68:1357–1363
Ploetz RC, Correll JC (1988) Vegetative compatibility among races of *Fusarium oxysporum* f. sp. *cubense*. Plant Disease 72:325–328
Raeder U, Broda P (1985) Rapid preparation of DNA from filamentous fungi. Lett Appl Microbiol 1:17–20
Rifai MA (1969) A revision of the genus *Trichoderma*. Mycol. Inst Mycol Pap 116:1–56
Sambrook J, Fritsch EF, Maniatis T (1989). Molecular cloning: a laboratory manual. (2nd edn). Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York
Skroch P, Tivang J, Nienhuis J (1992) Analysis of genetic relationships using RAPD marker data. In: Joint Plant Breeders Symposia Series, Crop Science Society of America, American Society for Horticultural Science and American Genetic Association. Minneapolis; Minnesota, USA, pp. 26–30
Sneh B, Burpee L, Ogoshi A (1991) Identification of *Rhizoctonia* species. In: American Society of Phytopathology, St. Paul, Minn., USA, pp. 17–19
Sorensen DZ, Pegg KG, Dale JL (1993) RAPD-PCR analysis of genetic variation within Australian populations of *Fusarium oxysporum* f. sp. *cubense*. In: Valmayor RV, Hwang SC, Ploetz RV, Lee SW, Rao VN (eds), International Symposium on Recent Developments in Banana Cultivation Technology. International Network for the Improvement of Banana and Plantain (Asia and Pacific Networks), Los Baños, Laguna, Philippines, pp 285–295
Talbot NJ, Salch YP, Ma M, Hamer JE (1993) Karyotype variation within clonal lineages of the rice blast fungus, *Magnaporthe grisea*. Appl Environ Microbiol 59:585–593
Teeri T, Salovuori I, Knowles J (1983) The molecular cloning of the major cellulase gene from *Trichoderma reesei*. Bio/Technology 1:696–699
Xu J-R, Leslie JF (1992) Electrophoretic karyotypes of *Gibberella fujikuroi* mating populations A-F. Phytopathology 82:1150
Yan K, Dickman MB (1992) An electrophoretic karyotype of *Fusarium moniliforme*. Phytopathology 82:1149