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ORIGINAL PAPER

Molecular polymorphism in Romanian isolates of *Trichoderma spp.*with antifungal properties

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Abstract

Plant fungal disease control using environmental friendly (agents) compounds is the aim of various studies. Different commercial products are currently available but the selection and characterization of new, more efficient biological control agents still remains an open field of researches.

Several new strains of Trichoderma spp. selected in our laboratory proved an increased activity against various pathogenic fungi (Botrytis, Fusarium, Rhizoctonia, Pythium etc). The use of some of these strains in bio-products formulation is conditioned by a good characterization of biological control agents and, by the possibility of monitoring them after the release. 9 new strains of Trichoderma spp. isolated from various sources (soil, vegetal material, seeds) were subjected to molecular characterization by various methods (ITS-PCR, IGS PCR, RFLP). The molecular methods allow to emphasize a possible intra-specific polymorphism in fungi tested strains as well as to study the possibility of identification of some strain-specific molecular markers. The electrophoretic pattern of the restriction fragments shown that tested Trichoderma strains could belong to some different species.

Keywords: *Trichoderma* species, antagonism, *Botrytis sp., Phytophtora spp.,* molecular tests

Introduction

Plant diseases caused by soil-borne pathogens like *Phytium, Botrytis, Rhizoctonia, Fusarium* and *Phytophtora* play an important role in the destruction of natural resources in agriculture. Chemical pesticides have been extensively used for control fungal plant disease but their employment favored the selection of fungicides resistant strains as well as negative effect on non-target organisms and environment [1]. In this respect, the development of alternative methods for plant pathogens control is of great interest not only for scientists but also for agriculture. Biological control agents are risk free both for environment and non-target organisms, and could reduce the use of chemical products. Several commercial biological products based on antagonistic microorganism are available now on the market [2] but the interest for selection of new antagonists is not diminished [3, 4]. The filamentous fungi *Trichoderma* (Ascomycetes, Hyprocreales) have attracted the attention because the inhibitory action against various plants pathogens and the diversity of mechanisms of action [5].

The aim of our work was to examine, through applying different molecular techniques (ITS-RFLP, IGS-RFLP), the genetic polymorphism of the selected strains, in order to identify them at species level and to examine the possibility of identify possible molecular markers for monitoring the target strains.

Material and Methods

1. **Cultures of *Trichoderma spp.***
The fungal antagonists were isolated using dilution plate techniques on TSM medium [6] from soil samples provided by ICDPAPM Bucharest, Romania. The fungal isolates were purified and obtained single spore cultures and their identification was based on morphological characters. All cultures were maintained on potato dextrose agar slants at 4^{o}C. In the experiments were also used strains of some plant pathogens (table 1) in order to establish the antifungal action of the *Trichoderma* new isolates.

Table 1. Plant pathogens used in the experiments

No.	Plant pathogens	Origin
1	*Botrytis cinerea P1*	Isolated from strawberry fruit
2	*Botrytis cinerea P2*	Isolated from plants of *Eustoma grandiflorum* obtained by *in vitro* cultures
3	*Phytophtora sp. P1*	Dr.Maria Oprea, IPP Bucharest

2. ***In vitro* antagonism** test was performed by dual cultures technique on Petri dishes containing PDA supplemented or not, with 100 μg/mL $FeCl_3$. Petri dishes were inoculated with mycelial disc of 7-day-old culture of the pathogen and antagonistic strains at equal distance from the periphery. Inoculated plates were incubated for 6 days at 25^{o}C and the radial growth of the pathogen was measured. From the zone of interaction between the antagonist and plant pathogen, the mycelial mats were gently removed with a needle and examined under microscope for hyphal interaction [4]. Inhibition was observed as presence of inhibition zones prior to any mycelial contact. The percent RI was calculated as follows: RI= 100x (R2-R1)/R2. R1 was the distance between the inoculums of pathogen and the edge of the colony (after 6 days at 25^{o}C) measured in the direction of the inoculum of inhibitory strain. R2 is the colony growth of pathogen measured in the direction of maximum radius [7].
3. **DNA extraction**.
Total DNA was extracted by the method described by Siddiquee et al. [3] and the DNA samples were prepared in TE (10mM Tris-Hydrochloric acid and 1mM EDTA, pH – 8.0) and stored at -20^{o}C in small aliquots.
5. **PCR amplification**
Different types of primers, obtained from Biosearch Technologies, were used in our experiments (table 2).

Table 2. Primers used in the experiments

Primer	Nucleotide sequence of the primer	Target	References
ITS1	TCC GTA GGT GAA CCT GCG G	Internal transcribed sequences from rDNA	White et al., 1990)
ITS4	TCC TCC GCT TAT TGA TAT GC		
IGS1(CNL12)	CTG AAC GCC TCT AAG GTC AG	Intergenic region of rDNA	Kim et al., 2001
IGS2 (CNS1)	GAG ACA AGC ATA TGC CTA CTG		
TharzF1	TTG CCT CGG CGG GAT	*Trichoderma harzianum*	Haugland et al., 2002
TharzR1	ATT TTC GAA ACG CCT ACG AGA		
TviriF1	CCC AAA CCC AAT GTG AAC CA	*Trichoderma viride/atroviride/koningii*	Haugland et al., 2002
TviriR1	TCC GCG AGG GGA CTA CAG		

Polymerase chain reactions (PCRs) were performed in 25ml with 1,25 unit *Taq* DNA polymerase (Promega), 1x *Taq* buffer (Mg2+ plus), 0.2mM of dNTPs, 0.02mM of each primer, and 60ng of the genomic DNA. Reactions were performed by denaturation for 4 min at 92°C followed by 35 cycles (92°C – 1 min.; 52°C – 1 min.; 72°C – 2 min) and a final extension at 72°C for 10 min. Aliquots (5 µl) were analyzed by electrophoresis in 1.5% (w/v) agarose gel in 1x TBE buffer (0,04M Tris-borate and 0,001M EDTA) buffer at 70V for 2 h.

Results and Discussions

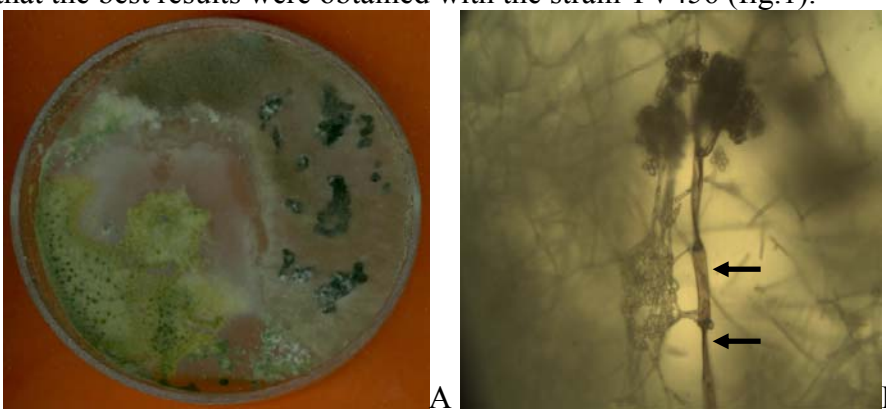
Soil samples collected from different agricultural fields and forests were inoculated on Petri plates with potato dextrose agar (PDA) medium following dilution plate technique. After 7 days incubation period at 25°C, colonies determined to belong to *Trichoderma* genus, according to Samuels et al. [8] were purified. Seven distinct strains with inhibitory action against other fungi present in samples were selected in order to test them against plant pathogens: strains *Trichoderma spp.*P8 and *Trichoderma spp.* P456 were isolated from two forest soils (Ilfov district), *Trichoderma spp.*SB6 from soil under maize cultivated in biological agriculture system (Arges district), *Trichoderma spp.*S37 from an agricultural soil fertilized with composted sewage sludge (Caracal) and *Trichoderma spp.* TV1 and TV2 from garden soil (Bucharest). The strain *Trichoderma spp.* UV was selected after UV treatment of the strain TV1 (data not shown). Preliminary identification was performed using morphological and microscopic criteria, that allowed the proposal of *T.harzianum* for the strains SB6 and P8, *T.atroviride* for the strain S37 and *T.viride* for TV1, TV2 and TV456 strains.

The antifungal activity was detected by plate confrontation assays on PDA and the results obtained allow the conclusion that the isolates TV456 and P8 are very efficient in the inhibition of *Botrytis* strains as well as of *Phytophtora* isolate (table 3)

Table 3. Antifungal activity of the *Trichoderma spp* new Romanian isolates

Antagonist	Plant pathogens		
	B.cinerea P1	*B.cinerea P2*	*Phytophtora* sp. *P1*
T. viride SP 456	*90%*	*80%*	*97%*
T.harzianum P8	*56%*	*46%*	*94%*

The examination of the plates containing fungal strains and the microscopically observations of the interactions between *Trichoderma* strains and the pathogen determine de conclusion that the best results were obtained with the strain TV456 (fig.1).



A B

Figure 1. Interactions between *Botrytis cinerea* P1 vs. *Trichoderma harzianum* P8: A – inhibition of plant pathogen alter 14 days of cultivation; B – attachment of the *Trichoderma* hyphae on the hyphae of *Botrytis cinerea* (300x)

Molecular characterization of fungal isolates

The genus *Trichoderma* is one of the most important fungal groups from practical point of view due to the ability of various strains, belonging to different species, to inhibit fungal plant pathogens. In order to use certain strain in a bioproduct useful for plant protection it is necessary to characterize it and to identify the species. The influences of environmental conditions on morphological and physiological characteristics have made accurate identification very difficult [3]. For a better characterization of the strains, molecular techniques are very useful, until now being tested various arbitrary primers, ITS type primers as well primers considered as group or species specific [9, 10]. Internal transcribed spacer (ITS) primers and intergenic spacer (IGS) primers for ribosomal DNA region (rDNA) were used to amplify specific sequences of this region in order to distinguish Romanian *Trichoderma* isolates.

625 bp single amplicon was obtained for each strain of *Trichoderma* and one of 575bp for *Penicillium sp.A2* when the primer pair ITS/ITS4, for ITS1–5.8S–ITS2 rDNA fungal region, was used (fig.2).

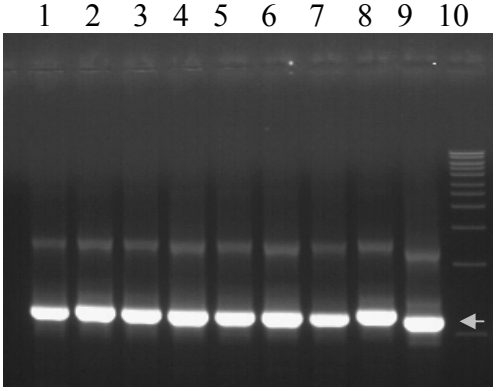


Figure 2. Amplification products obtained with ITS1/ITS4 primer pair: 1= *T.atroviride* S37, 2= *Trichoderma sp.* SP9; 3= *T.viride* TV456, 4 = *Trichoderma sp.*TV2; 5= *Trichoderma sp.TV1*; 6= *Trichoderma sp.*TV.UV.; 7= *T.harzianum* SB6; 8= *T.harzianum* P8; 9= *Penicillium sp.A2;* 10 = 1kb ladder DNA (Gibco BRL)

When the ITS amplicons were subjected to restriction with MspI and HaeIII, different restriction profiles were obtained (fig.3).

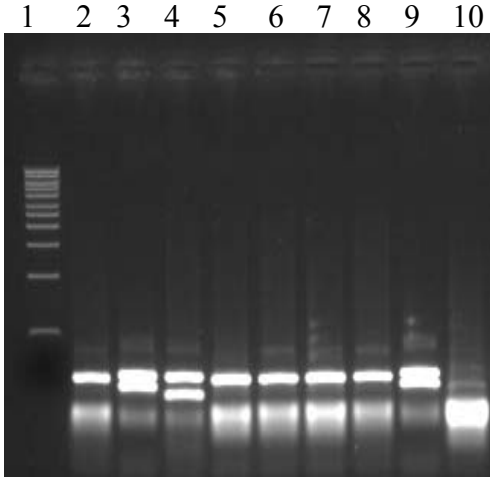


Figure 3. Electrophoretic pattern of restriction products generated by MspI action: 1 = 1 kb ladder DNA (Gibco BRL); 2 = *T.atroviride* S37, 3 = *Trichoderma sp.* SP9; 4 = *T.viride* TV456, 5 = *Trichoderma sp.*TV2; 6 = *Trichoderma sp.*TV1; 7 = *Trichoderma sp.*TV.UV.; 8 = *T.harzianum* SB6; 9 = *T.harzianum* P8; 10 = *Penicillium sp.A2;*

Similar restriction patterns were obtained for the strains S37, TV1 and SB6, and also for TV2 and TV.UV. Moreover, strains SP9 and P8 presented identical restriction pattern, instead the strains TV456 and A2 that shown significant differences.

The use of IGS1/IGS2 primer pair allowed the amplification of various fragments, originated from rDNA genomic region, their length being larger than 2kb (fig.4).

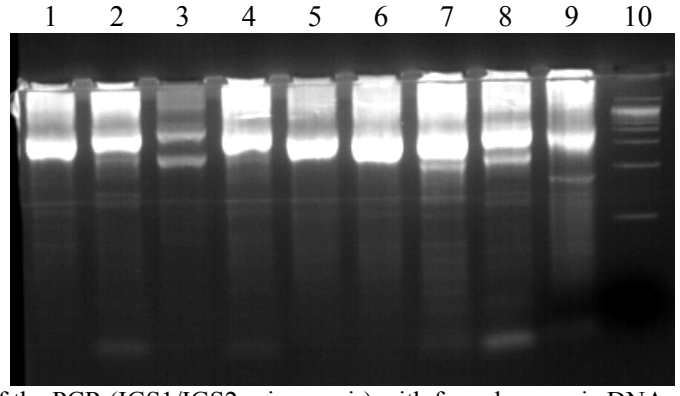


Figure 4. Sensitivity of the PCR (IGS1/IGS2 primer pair) with fungal genomic DNA: 1= *T.atroviride* S37, 2= *Trichoderma sp.* SP9; 3= *T.viride* TV456, 4 = *Trichoderma sp.*TV2; 5= *Trichoderma sp.TV1*; 6= *Trichoderma sp.*TV.UV.; 7= *T.harzianum* SB6; 8= *T.harzianum* P8; 9= *Penicillium sp.A2;* 10 = 1kb ladder DNA (Gibco BRL)

It is obvious that with this primer pair, differences between the amplicons can be seen: large, single significant amplification product was obtained in S37, TV2, TV1, TV.UV, instead at least two amplicons were resolved by electrophoresis in TV4546, SB6, P8 and A2. Digestion of these IGS amplification products with MspI restriction enzyme (IGS-RFLP analysis) resulted in polymorphic patterns of restriction fragments (fig.5).

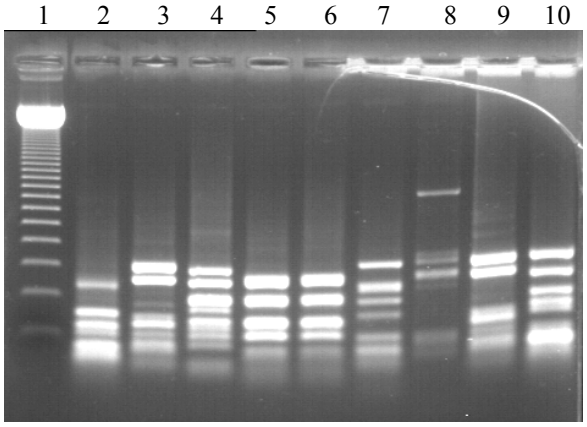


Figure 5. Electrophoretic pattern of restriction products generated by MspI action: 1 = 123 bp ladder DNA (Invitrogen); 2 = *T.atroviride* S37, 3 = *Trichoderma sp.* SP9; 4 = *T.viride* TV456, 5 = *Trichoderma sp.*TV2; 6 = *Trichoderma sp.*TV1; 7 = *Trichoderma sp.*TV.UV.; 8 = *T.harzianum* SB6; 9 = *T.harzianum* P8; 10 = *Penicillium sp.A2*

The results suggest that this approach allows for the observation of a molecular polymorphism among *Trichoderma* isolates. Some of the strains could be grouped by their restriction profile: strains TV2 and TV1 seem to be similar, as well as SP9 and P8. These strains could belong to the same species: *T.viride* and *T.harzianum,* respectively. The other strains presented different restriction profiles, but this do not means that they are belonging to different species. Similar results were obtained in other laboratories where was shown that

species of *Trichoderma*, including *T.viride* and *T.harzianum*, are very heterogenous as molecular characteristics [9, 10, 11].

Another approach for examining the diversity of the fungal isolated was the use of species-specific primers. In this order, two primer pairs were used: TharzF1/TharzR1 primer pair for *T.harzianum* and TviriF1/TviriR1 recommended for the group *Trichoderma viride/ atroviride/koningii* [12]. With the primer pair TharzF1/TharzR1, various products of amplification were obtained. Similar fragments were observed in P8 and SP9, confirming the previous results (fig.6).

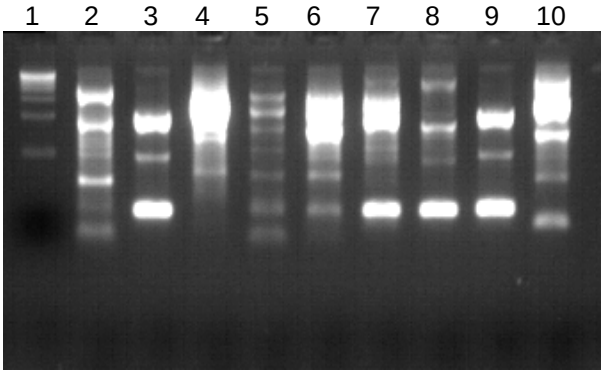


Figure 6. Sensitivity of PCR using TharzF1/TharzR1 primer pair: 1= 1 kb ladder DNA; 2 = *Penicillium spp.A2;* 3 = *T.harzianum* P8; 4 = *T.harzianum* SB6; 5 = *Trichoderma spp.*TV1.1; 6 = *Trichoderma spp.*TV1; 7 = *Trichoderma spp.*TV2; 8 = *T.viride* TV456; 9 = *Trichoderma spp.* SP9; 10 = *T.atroviride* S37

Different profiles of amplicons were obtained with these primers in the other strains. The results could be due, probably, to a reduced sensitivity of these primers for *T.harzianum* or, less probably, to the fact that none of the strains belong to this species.

The polymorphism of the amplification products obtained by the use of TviriF1/ TviriR1 primer pair, recommended for the group *Trichoderma viride/atroviride/koningii,* is more reduced, comparing with that of previous primers: an unique fragment of about 400 bp was observed in S37, SP9, TV2 and TV1 strains, and one fragment of 500 bp was detected in P8 and TV.UV strains. For the strains TV456 and SB6, larger fragments were observed, in addition to the main fragment of 400bp, and 500bp respectively (fig.7).

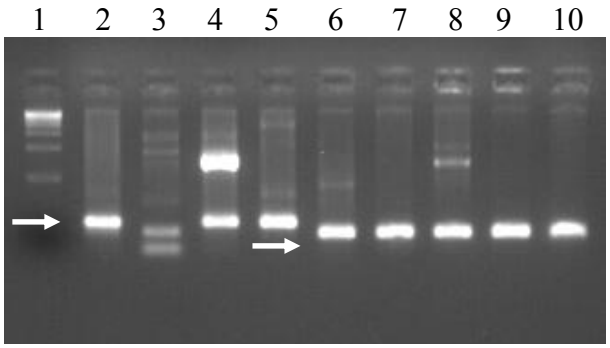


Figure 7. Sensitivity of PCR using TviriF1/TviriR1 primer pair: 1= 1 kb DNA ladder; 2 = *T.harzianum* P8, 3 = *Penicillium sp.A2*, 4 = *T.harzianum* SB6, 5 = *Trichoderma sp.*TV1.1; 6 = *Trichoderma sp.*TV1; 7 = *Trichoderma sp.*TV2; 8 = *T.viride* TV456; 9 = *Trichoderma sp.* SP9; 10 = *T.atroviride* S37

The results obtained with these primers are more uniform, comparing with the previously ones, confirm the expectations for some strains (TV1, TV2, TV456 and S37) but are contradictory for others (SP9 and P8, for example). It is interesting that the strain TV456

presented an additional band, of about 1kb, that could be characteristic. This assumption will be proved correct in the following experiments, the electrophoretic pattern obtained with TviriF1/ TviriR1 primer pair could be used for the identification of this strain, among other strains of *Trichoderma*.

As a general conclusion, the aim of this work was succeed: the *Trichoderma* strains presented an increased molecular polymorphism, both inter- and intraspecific, based to the molecular approach. The molecular techniques are very useful in the characterization of the new fungal isolates, allowing grouping the strains. In the absence of reference strains we are not able, in this moment, to have clear conclusions regarding to which species the isolates belong. Moreover, complex studies (microbiological, biochemical and molecular) are necessary when the identification of new fungal isolated is the purpose of the experiments.

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