

Investigation of *Trichoderma* strains isolated from winter wheat rhizosphere

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Abstract: Species in the filamentous fungal genus *Trichoderma* are of great economic importance as sources of enzymes and antibiotics, plant growth promoters, degraders of xenobiotics, and most importantly, as commercial biofungicides. One hundred and forty *Trichoderma* strains were isolated from roots of winter wheat grown in agricultural fields of southern Hungary, and the identity of species was examined based on both morphological and molecular characters. The morphological data were collected by measuring structure and shape of conidiophores, phialides and conidia. For the investigation of molecular diversity, cellulose-acetate electrophoresis mediated isoenzyme analysis was applied. After initial testing of 12 enzymes for activity and resolution of bands, SIX of them (glucose-6-phosphate dehydrogenase, glucose-6-phosphate isomerase, 6-phosphogluconate dehydrogenase, peptidase A, peptidase B and phosphoglucomutase) proved to be appropriate for the analysis of the full sample set. Comparing the different electrophoretic types of the defined isolates, four of these enzymes could be used as molecular markers for the identification of FOUR *Trichoderma* spp.

Key words: taxonomy, isoenzyme analysis, cellulose-acetate electrophoresis, biocontrol, *Trichoderma*

Introduction

There is a worldwide need to adopt the practice of sustainable agriculture, using strategies that are environment-friendly, less dependent on agricultural chemicals and less damaging to soil and water resources. One of the key elements of such sustainable agriculture is the application of biocontrol agents for plant protection. *Trichoderma* species are filamentous fungi with teleomorphs belonging to the *Hypocreales* order of the *Ascomycota* division. Members of this genus are potent biocontrol agents, the proposed mechanisms resulting in biocontrol are including the stimulation of the defensive mechanisms of the plants (Benítez et al., 1998), competition for the substrate (Naár & Kecskés, 1998) as well as antibiosis by the production of antifungal metabolites and mycoparasitism by the action of cell-wall degrading enzymes (Benítez et al., 1998; Manczinger et al., 2000).

Identification of *Trichoderma* species based on morphological and cultural characteristics is sometimes difficult, because their traits exhibit variations on a continuous scale that may overlap between the species. The use of molecular markers for species-specific detection assays has become widespread. One of the methods, the cellulose-acetate electrophoresis mediated isoenzyme analysis has already been reported as useful and rapid method for the identification of *Ganoderma* (Smith & Sivasithamparam, 2000) and *Phytophthora* species (Oudemans & Coffey, 1991).

The aims of the present study were the isolation of *Trichoderma* strains from Hungarian soil samples, their morphological characterisation, and the detection of isoenzymes, which could be used routinely for their identification at the species level.

Materials and methods

Isolation of Trichoderma strains from Hungarian soil samples

The chopped roots of winter wheat were placed to Petri dishes containing selective medium (5 g/l peptone, 1 g/l KH_2PO_4 , 10 g/l glucose, 0.5 g/l $\text{MgSO}_4 \times 7\text{H}_2\text{O}$, 0.5 ml/l 0.2% dichloran-ethanol solution, 0.25 ml/l 5% Rose Bengal, 20 g/l agar). Growing *Trichoderma* strains were transferred from the plates onto yeast extract medium (2 g l^{-1} yeast extract, 5 g l^{-1} KH_2PO_4 , 20 g l^{-1} agar). All isolates were grown from single conidia for identification. The taxonomic identity was examined according to an interactive key available for the genus *Trichoderma* at the web site of the United States Department of Agriculture, Agricultural Research Service (<http://nt.ars-grin.gov/taxadescriptions/keys/TrichodermaIndex.cfm>).

Cellulose acetate electrophoresis

Inoculation of 250 ml liquid yeast extract broth media was performed with conidial suspensions to a final concentration of 10^5 conidia/ml. Cultures were incubated for four days on an orbital shaker at 200 rpm and 25 °C. Mycelia were filtered through filter paper, washed three times with distilled water and lyophilized. Dried mycelia were grinded to dust with a pestle in a mortar. Protein extraction was performed according to the method described by Ládáy & Szécsi (2001). Cellulose acetate electrophoresis (CAE) was conducted as described by Hebert & Beaton (1993), the CAE system was purchased from Helena Laboratories (Beaumont, TX). Titan III cellulose acetate gels were soaked for at least 30 min in electrophoresis buffer (0.25 mmol Tris-glycine, pH 8.5) in a bufferizer and blotted dry between sheets of filter paper. The protein extracts were applied from the sample plate to the gel with a Super Z-12 Applicator. Electrophoresis was carried out at 180 V for 20 min. Gels were stained for 12 enzyme systems (Table 1), enzyme activities were detected using agar overlays (Hebert & Beaton, 1993). The relative mobility (R_f value) of each isoenzyme band was calculated using the anodally moving band of *T. harzianum* T66, which was arbitrarily chosen as the standard. For each enzyme assay, bands were designated by the abbreviations of enzymes and the percentage mobility of the band relative to the standard band.

From the isoenzyme data, matrices were created based on the presence or absence of a given activity band. Simple matching coefficients were calculated with the PHYLTOOLS software package (version 1.32). Phylogenetic trees were prepared by the neighbor-joining method (Saitou & Nei, 1987) using the program NEIGHBOR of the PHYLIP software package, version 3.57c, (Felsenstein, 1995). Data were analyzed using the outgroup rooting method with a *Neosartorya glabra* strain designated as the outgroup taxon.

Results and discussion

One hundred and forty *Trichoderma* strains were isolated on selective media from roots of winter wheat from thirty-one test holes of six agricultural fields in southern Hungary.

From the isolates, 79 were identified based on morphological characters of conidiophores, phialides and conidia, and colony radius on both potato dextrose agar and corn meal agar. These strains were included in species *T. viride* (34 strains), *T. virens* (32 strains), *T. longibrachiatum* (5 strains) and *T. atroviride* (8 strains). *T. virens* was assigned to section *Pachybasium* (Sacc.), *T. viride* to section *Trichoderma*, while *T. longibrachiatum* and *T.*

atroviride to section *Longibrachiatum*. The undefined isolates are also belonging to the *Trichoderma* genus, but their exact morphological analysis is still in progress.

Table 1. Enzyme systems tested and their Enzyme Commission (E.C.) numbers

	Abbreviation	E.C. no.	Activity	No. of bands with different R _f values
6-Phosphogluconate dehydrogenase	6PGDH	1.1.1.44	+	7
Aconitase	ACN	4.2.1.3	-	-
Glucose-6-phosphate dehydrogenase	G6PDH	1.1.1.49	+	7
Glucose-6-phosphate isomerase	GPI	5.3.1.9	+	6
Glycerol-3-phosphate dehydrogenase	GPDH	1.1.1.8	-	-
Malate dehydrogenase	MDH	1.1.1.37	-	-
Peroxidase	PRX	1.11.1.7	-	-
Peptidase A (Gly-Leu)	PEP A	3.4.11/13	+	5
Peptidase B (Leu-Gly-Gly)	PEP B	3.4.11/13	+	6
Phosphoglucomutase	PGM	5.4.2.2	+	11
Shikimate dehydrogenase	SKDH	1.1.1.25	-	-
Succinate dehydrogenase	SUD	1.3.99.1	-	-

Twelve enzyme systems were tested initially for activity, resolution, and consistent appearance of the bands. Six enzymes which showed clear, reproducible banding patterns were selected for the full sample set, from which 42 distinct bands were scored and used in the analysis (Table 1). A single band common to all isolates was detected for GPI, PEP A, PEP B and PGM enzyme systems. Electromorphs of G6PDH were double and triple banded in the isolates observed.

All six enzymes were polymorphic for the full sample set, PGM, G6PDH, PEP B and 6PGDH showed interspecific, while GPI and PEP A showed intraspecific polymorphisms. The patterns of the enzymes were used to group the defined strains into different electrophoretic types (ETs). With this grouping we obtained 25 different ETs. In the case of the 79 defined isolates the patterns of enzymes fell into seven common ETs: ET I, ET II and ET III for *T. viride*, ET IV and ET V for *T. virens*, ET VI for *T. atroviride* and ET VII for *T. longibrachiatum*. In the cases of *T. virens* and *T. viride*, GPI and PEP A patterns split the species to the mentioned ETs, while PGM, G6PDH, PEP B and 6PGDH patterns proved to be appropriate to identify the four species.

When a dendrogram was created from the diagnostic isoenzyme data, the whole sample set clustered in eleven major groups and the morphologically characterised strains clustered in four distinct groups according to the corresponding species (Figure 1). The phylogenetic distance between species is in correlation with taxonomic categories, however, the final phylogenetic analysis requires more isoenzyme data.

There is a great number of different enzyme systems potentially available for use in isoenzyme analysis, therefore it is highly probable that an excellent molecular marker can be found for the differentiation and practical diagnostics of different *Trichoderma* species. Based on the results of the present study, cellulose acetate electrophoresis mediated isoenzyme analysis seems to be promising method for the rapid and accurate identification and classification of *Trichoderma* isolates.

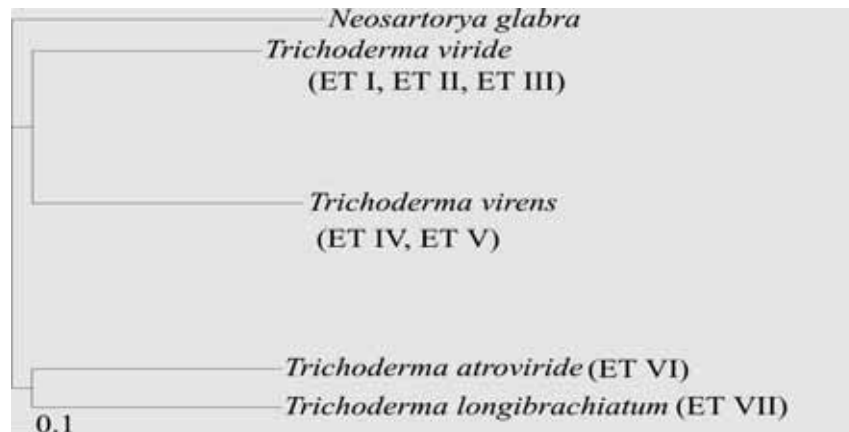


Figure 1. Neighbour joining dendrogram resulting from the analysis of the diagnostic isoenzymes of the defined isolates with the different electrophoretic types

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