# *TRICHODERMA* COMMUNITIES OF THE WINTER WHEAT RHIZOSPHERE

**KREDICS[1] LÁSZLÓ LÁDAY MIKLÓS [2], KÖRMÖCZI[1] PÉTER, MANCZINGER[1] LÁSZLÓ, RÁKHELY[3] GÁBOR, VÁGVÖLGYI[1] CSABA AND SZEKERES[4] ANDRÁS**

[1]Department of Microbiology, Faculty of Science and Informatics, University of Szeged, Szeged, Közép fasor 52., H-6726 Szeged, Hungary
kredics@bio.u-szeged.hu
[2]Plant Protection Institute, Hungarian Academy of Sciences, Budapest, P.O. Box 102., H-1525 Budapest, Hungary
[3]Department of Biotechnology, Faculty of Science and Informatics, University of Szeged, Szeged, Közép fasor 52., H-6726 Szeged, Hungary
[4]FumoPrep LtD., Vállalkozók útja 1/B., 6782 Mórahalom, Hungary

**ABSTRACT – *Trichoderma* communities of the winter wheat rhizosphere in the Pannonian Plain**
A total of 116 *Trichoderma* strains were isolated from roots of winter wheat from test holes of five agricultural fields in the Pannonian Plain. The identity of the strains was examined based on the sequence analysis of the internal transcribed spacer (ITS) region by *TrichO*KEY 2.0. The examined wheat field rhizosphere samples could be characterized with a remarkable biodiversity. The 11 taxa detected in the samples were *T. harzianum, T. pleuroticola, T. tomentosum/T. cerinum, T. virens, T. rossicum, T. spirale, T. brevicompactum, T. atroviride, T. gamsii, T. koningiopsis/T. ovalisporum* and *T. longibrachiatum/H. orientalis*. The most frequently isolated species was *T. harzianum* with 41 isolates representing a series of known ITS genotypes as well as 2 genotypes that were firstly obtained during this study. Both *T. virens* (31 isolates) and *T. atroviride* (9 isolates) could be classified into 2 ITS-genotypes, one of them being identical with that of the ex-type strains in the cases of both species. Ten isolates proved to belong to 2 genotypes of *T. rossicum*, one of them has not been found so far. The remaining 7 species were isolated with a lower frequency. Several species could be characterized with well-defined isoenzyme patterns during cellulose-acetate electrophoresis.

**Keywords:** *Trichoderma*, biodiversity, winter wheat rhizosphere

## 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. The efficient control of fungal plant pathogens causing substantial losses in agricultural production is an important issue for all plant cultivation systems. Species of the genus *Trichoderma* (Ascomycota, Hypocreales, Hypocreaceae) are predominant components of the soil mycota in various soils (KLEIN and EVELEIGH, 1998). The genus involves promising biocontrol candidates with excellent antagonistic abilities against a number of plant pathogenic fungi. Several modes of action have been proposed to play roles in biocontrol capabilities, including antibiosis by the production of antifungal metabolites, competition for space and nutrients, plant growth promotion, induction of the defense responses in plants and mycoparasitism (HARMAN, 2004). These processes are supposed to act synergistically (SCHIRMBÖCK et al., 1994).
A number of studies are available in the literature about the distribution of *Trichoderma* species in different soil and rhizosphere ecosystems. Data presented in the early studies

about the biodiversity of the genus (DANIELSON and DAVEY 1973, WIDDEN and ABITBOL, 1980, NELSON, 1982) are hard to interprete as the identification of the species was based on morphological characters and a series of *Trichoderma* species were not yet described those times. Recent studies examined the *Trichoderma* communities of different habitats by molecular methods, including ITS (internal transcribed spacer) sequence-based identification with the aid of *TrichO*key (DRUZHININA et al., 2005) and BLAST simlarity searches performed with *Tricho*BLAST (KOPCHINSKIY et al., 2005), both programmes available online at the homepage of the International Subcommission on *Trichoderma* and *Hypocrea* Taxonomy (www.isth.info). Natural ecosystems investigated in details by molecular methods for *Trichoderma* biodiversity include a mid-European, primeval floodplain-forest (WUCZKOWSKI et al., 2003), soils from Russia, Nepal, northern India (KULLNIG et al., 2000), south-east Asia (KUBICEK et al., 2003), Sardinia (MIGHELI et al., 2009) and South America (HOYOS-CARVAJAL et al., 2009). A series of new genotypes as well as new phylogenetic species of *Trichoderma* have been recognized during these studies.

Besides the natural ecosystems, the investigation of agricultural soils may also reveal interesting data about *Trichoderma* biodiversity (GHERBAWY et al., 2004, MULAW et al., 2010), especially from the point of view of biocontrol applications, as the rhizosphere of agricultural soils is an ideal source of potential biocontrol agents. The aim of this study was to assess the biodiversity of the genus *Trichoderma* in the rhizosphere of winter wheat fields in the Pannonian Plain.

## MATERIAL AND METHOD

Soil samples with winter wheat seedlings were collected from five agricultural fields (Algyő, Deszk, Rúzsa, Kunszentmiklós and Tiszasziget) in the Pannonian Plain by a 5 cm x 5 cm square sampler in random sampling order. The chopped roots of wheat were placed to plates with Rose Bengal medium (5 g $l^{-1}$ peptone, 1 g $l^{-1}$ $KH_2PO_4$, 10 g $l^{-1}$ glucose, 0.5 g $l^{-1}$ $MgSO_4X7H_2O$, 0.5 ml $l^{-1}$ 0.2% dichloran-ethanol solution, 0.25 ml $l^{-1}$ 5% Rose Bengal, 20 g $l^{-1}$ agar supplemented with 0.1 g $l^{-1}$oxytetracyclin, 0.1 g $l^{-1}$ streptomycin and 0.1 g $l^{-1}$ chloramphenicol to inhibit bacteria). Growing *Trichoderma* strains were transferred to solid yeast extract medium (2 g $l^{-1}$ yeast extract, 5 g $l^{-1}$ $KH_2PO_4$ and 20 g $l^{-1}$ agar in distilled water – YEGS) supplemented with the above mentioned antibiotics. Monospore cultures of the isolated strains were deposited in the Microbiological Collection of the University of Szeged (SZMC; *Table 1*).

For the isolation of genomic DNA, *Trichoderma* isolates were cultured in 200 ml liquid YEG medium in 250 ml Erlenmeyer flasks which were inoculated to an end concentration of $10^5$ conidia $ml^{-1}$. Cultures were shaken with 200 rpm for 4 days at 25°C. Mycelia of the isolates were subjected to DNA isolation, PCR amplification of the internal transcribed spacer (ITS1-5.8S rDNA-ITS2) region, and automatic DNA sequencing as described previously (ANDERSSON et al., 2009). Sequences were analysed by the program *TrichO*Key 2.0 (DRUZHININA et al., 2005) available online at the homepage of the International Subcommission on *Trichoderma* and *Hypocrea* Taxonomy (www.isth.info).

Protein extraction was performed as described by LÁDAY and SZÉCSI (2001). CAE was as described by Hebert & Beaton (1993), with a CAE system from Helena Laboratories (Beaumont, TX, USA). Titan III cellulose-acetate gels (Helena Laboratories) were soaked for 30 min in electrophoresis buffer (0.25 mM Tris-glycine, pH 8.5) and were then 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. When the staining activity was low, the extracts were blotted two or three times. Electrophoresis was carried out at 180 V for 20 min. The gels were stained for 5 enzyme systems and the enzyme activities were detected using agar overlays. Staining protocols were as described previously (HEBERT AND BEATON 1993). All samples were extracted and analysed on three occasions in separate runs.

## RESULTS

A total of 116 *Trichoderma* strains were isolated from 18 sampling sites of 5 agricultural fields (Algyő, Deszk, Rúzsa, Kunszentmiklós and Tiszasziget) in the Pannonian Plain (Table 1). Isolations were performed directly from the roots of winter wheat. The number of isolated strains was the highest in the case of sample K1 (location: Kunszentmiklós, number of isolates: 15) and the lowest for sample R2 (location: Rúzsa, number of isolates: 1). The average number of isolates per sampling site was 6.4.

The 11 taxa detected in the samples were *T. harzianum*, *T. pleuroticola*, *T. tomentosum/T. cerinum*, *T. virens*, *T. rossicum*, *T. spirale*, *T. brevicompactum*, *T. atroviride*, *T. gamsii*, *T. koningiopsis/T. ovalisporum* and *T. longibrachiatum/H. orientalis*. The most frequently isolated species was *T. harzianum* with 41 isolates representing a series of known ITS genotypes as well as 2 genotypes that were firstly obtained during this study. Both *T. virens* (31 isolates) and *T. atroviride* (9 isolates) could be classified into 2 ITS-genotypes, one of them being identical with that of the ex-type strains in the cases of both species. Ten isolates proved to belong to 2 genotypes of *T. rossicum*, one of them has not been found so far. The remaining 7 species were isolated with a lower frequency.

*Figure 1* shows the species diversity at the particular sampling sites. *H. lixii/T. harzianum* was the most abundant species (35.3%) and it was found in 12 of the 18 samples examined. It was the most frequent *Trichoderma* species found at three locations (Algyő, Deszk, Rúzsa) and in seven samples (A1, D2, D3, R1, R6, T1, T3). The next two most abundant species were *T. virens* and *T. rossicum* (26,7% and 8,6% of all isolates, present in 12 and 5 samples, respectively). *T. virens* was most abundant in the agricultural fields examined at Tiszasziget, where it accounted for 45.5% of the isolates. *T. rossicum* dominated sample K2 and could also be found in samples R1, R4, K1 and T4. *T. atroviride* occured with a frequency of 7,8% of all isolates, this species could be found in 6 samples. *T. gamsii* could be found in 4 samples at 3 locations (5.17% of all isolates). The other taxa occurred with a frequency of less than 5%: *T. longibrachiatum/H. orientalis*, *T. brevicompactum*, *T. pleuroticola* and *T. tomentosum/cerinum* with 4.3%, 3.4% 2.6% and 2.6%, respectively, each of them found in 2 geographic locations and 3 samples; while *T. koningiopsis/T. ovalisporum* (2.6%) and *T. spirale* (0.9%) occured in single samples only (T3 and R1, respectively).

The highest biodiversity of *Trichoderma* species was detected in a sample from Kunszentmikós (sample K1: *T. harzianum* and 5 further species among 15 isolates) and in a sample from Rúzsa (sample R4: 5 species among 8 isolates, no *T. harzianum*).

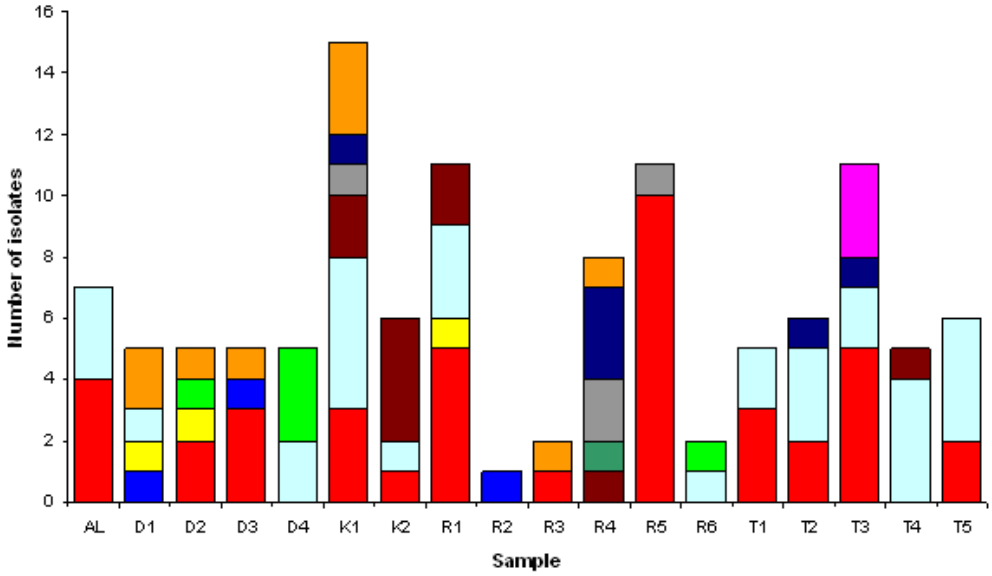


**Fig. 1. Biodiversity of the genus *Trichoderma* in the examined winter wheat rhizosphere samples. Different colors indicate different *Trichoderma* species: *T. harzianum* ■, *T. pleuroticola* ■, *T. tomentosum/T. cerinum* ■, *T. virens* ■, *T. rossicum* ■, *T. spirale* ■, *T. longibrachiatum/H. orientalis* ■, *T. brevicompactum* ■, *T. atroviride* ■, *T. gamsii* ■, *T. koningiopsis/T. ovalisporum* ■**

Source: own calculation

Sample R5 was characterized with a relatively poor biodiversity due to a large number of isolates from an individual species (*T. harzianum*). Only two species were detected in 8 out of 18 samples (A1, D4, R3, R5, R6, T1, T4, T5), in these samples either one of the two most abundant species (*T. harzianum* or *T. virens*), or both of them (A1, T1, T5) were present.

During the CAE-based isoenzyme analysis performed for the full set of isolates, banding patterns of five enzymes, 6-phosphogluconate-dehydrogenase (6PGDH), glucose-6-phosphate dehydrogenase (G6PDH), glucose-6-phosphate isomerase (G6PI), peptidase B (Leu-Gly-Gly) (PEPB) and phosphoglucomutase (PGM) were selected for analysis based on the results of a previous study (SZEKERES et al., 2006). A total of 38 electromorphs were registered in the population (*Table 1*). Several species could be characterized with well-defined isoenzyme patterns during cellulose-acetate electrophoresis.

**Table 1: Number of electrophoretic patterns for the examined enzymes**

| **Enzyme** | **Abbreviation** | **Activity** | **Number of electrophoretic patterns** |
|---|---|---|---|
| 6-phosphogluconate-dehydrogenase | 6PGDH | + | 7 |
| glucose-6-phosphate dehydrogenase | G6PDH | + | 6 |
| glucose-6-phosphate isomerase | GPI | + | 8 |
| peptidase B (Leu-Gly-Gly) | PEPB | + | 6 |
| phosphoglucomutase | PGM | + | 11 |

Source: own calculation

## DISCUSSION

The community of *Trichoderma* in the rhizosphere of winter wheat in the Pannonian Plain proved to be highly diverse. Beneficial taxa widely used as biocontrol agents against plant pathogenic fungi (e.g. *T. harzianum, T. virens, T. atroviride*) could be isolated from the samples examined during this study, indicating that the winter wheat rhizosphere may be a rich source of potential biocontrol isolates. The most frequent species isolated was *T. harzianum* followed by *T. virens*, which is in congruence with previous data (KULLNIG et al. 2000, MIGHELI et al. 2009, WUCZKOWSKI et al. 2003). *T. gamsii* and *T. rossicum* were also found in the winter wheat rhizosphere samples. Both of these species were previously shown to have a widespread distribution, occurring also in Central Europe (HOYOS-CARVAJAL et al. 2009, MIGHELI et al. 2009, WUCZKOWSKI et al. 2003). Only a single isolate of *T. spirale* was found in this study. This species was shown to be the dominant *Trichoderma* in the carbon-rich forest soil of Badde Salighesones in Sardinia (MIGHELI et al. 2009). *T. hamatum*, a species which was found to be subdominant in several Sardinian soils (MIGHELI et al. 2009) and *T. asperellum*, a predominant species of neotropic regions (HOYOS-CARVAJAL et al. 2009) could not be isolated during this study. On the other hand, *Trichoderma* species known as potential opportunistic pathogens in humans (*T. longibrachiatum/H. orientalis*) (DRUZHININA et al. 2008) and as causal agents of the green mould disease in mushroom cultivation (*T. pleuroticola*) (KOMOŃ-ZELAZOWSKA et al. 2007) could be detected in the examined samples. The development of biocontrol products from isolates of these potentially harmful species should be avoided.

## ACKNOWLEDGEMENTS

The Project named „TÁMOP-4.2.1/B-09/1/KONV-2010-0005 – Creating the Center of Excellence at the University of Szeged" is supported by the European Union and co-financed by the European Regional Fund. The SOILMAP project is co-financed by the European Union through the Hungary-Romania Cross-Border Co-operation Programme 2007-2013 (HURO/0901/058/2.2.2).

## REFERENCES

ANDERSSON M.A., MIKKOLA R., RAULIO M., KREDICS L., MAIJALA P., SALKINOJA-SALONEN M.S. (2009): Acrebol, a novel toxic peptaibol produced by an *Acremonium exuviarum* indoor isolate. Journal of Applied Microbiology 106: 909-923.

DANIELSON R.M., DAVEY C.B. (1973): The abundance of *Trichoderma* propagules and the distribution of species in forest soils. Soil Biology and Biochemistry 5: 486-494.

DRUZHININA I.S., KOPTCHINSKI A., KOMON M., BISSETT J., SZAKACS G., KUBICEK C.P. (2005): A DNA-barcode for strain identification in *Trichoderma*. Fungal Genetics and Biology 42: 813-828.

DRUZHININA I.S., KOMOŃ-ZELAZOWSKA M., KREDICS L., HATVANI L., ANTAL Z., BELAYNEH T., KUBICEK C.P. (2008): Alternative reproductive strategies of *Hypocrea orientalis* and genetically close but clonal *Trichoderma longibrachiatum*, both capable of causing invasive mycoses of humans. Microbiology (UK) 154: 3447-3459.

GHERBAWY Y., DRUZHININA I., SHABAN G.M., WUCZKOWSKY M., YASER M., EL-NAGHY M.A., PRILLINGER H.J., KUBICEK C.P. (2004): *Trichoderma* populations from alkaline

agricultural soil in the Nile valley, Egypt, consist of only two species. Mycological Progress 3: 211-218.

HARMAN G.E., HOWEL C.R., VITERBO A., CHET I., LORITO M. (2004): *Trichoderma* species – opportonunistic, avilurent plant symbionts. Nature Reviews Microbiology 2: 43-55.

HOYOS-CARVAJAL L., ORDUZ S., BISSETT J. (2009): Genetic and metabolic biodiversity of *Trichoderma* from Colombia and adjacent neotropic regions. Fungal Genetics and Biology 46: 615-631.

KLEIN D., EVELEIGH D.E. (1998): Ecology of *Trichoderma*. Trichoderma and Gliocladium, *Vol. 1. Basic Biology, Taxonomy and Genetics* (Kubicek CP & Harman GE , eds), pp 57-74. Taylor and Francis Ltd., London

KOMOŇ-ZELAZOWSKA M., BISSETT J., ZAFARI D., HATVANI L., MANCZINGER L., WOO S., LORITO M., KREDICS L., KUBICEK C.P., DRUZHININA I.S. (2007): Genetically closely related but phenotypically divergent *Trichoderma* species cause world-wide green mould disease in oyster mushroom farms. Applied and Environmental Microbiology 73: 7415-7426.

KOPCHINSKIY A., KOMOŇ M., KUBICEK C.P., DRUZHININA I.S. (2005): *Tricho*BLAST: A multilocus database for *Trichoderma* and *Hypocrea* identifications. *Mycol Res* 109: 657-660.

KUBICEK C.P., BISSETT J., DRUZHININA I., KULLNIG-GRADINGER C.M., SZAKACS G. (2003) Genetic and metabolic diversity of *Trichoderma*: a case study on South East Asian isolates. Fungal Genetics and Biology 38: 310-319.

KULLNIG C.M., SZAKACS G., KUBICEK C.P. (2000): Molecular identification of *Trichoderma* species from Russia, Siberia and the Himalaya. *Mycol. Res.* 104, 1117-1125.

LÁDAY M., SZÉCSI Á. (2001): Distinct electrophoretic isoenzyme profiles of *Fusarium graminearum* and closely related species. Systematic and Applied Microbiology 24: 67-75.

MIGHELI Q., BALMAS V., KOMOŇ-ZELAZOWSKA M., SCHERM B., FIORI S., KOPCHINSKIY A.G., KUBICEK C.P., DRUZHININA I.S. (2009): Soils of a Mediterranean hot spot of biodiversity and endemism (Sardinia, Tyrrhenian Islands) are inhabited by pan-European, invasive species of *Hypocrea/Trichoderma*. Environmental Microbiology 11: 35-46.

MULAW T.G., KUBICEK C.P., DRUZHININA I.S. (2010): The rhizosphere of *Coffea Arabica* in its native highland forests of Ethiopia provides a niche for a distinguished diversity of *Trichoderma*. Diversity 2: 527-549.

NELSON E.E. (1982): Occurrence of *Trichoderma* in a Douglas-fir soil. Mycologia 74: 280-284.

SZEKERES A., LÁDAY M., KREDICS L., VARGA J., ANTAL Z., HATVANI L., MANCZINGER L., VÁGVÖLGYI C., NAGY E. (2006): Rapid identification of clinical *Trichoderma longibrachiatum* isolates by cellulose-acetate electrophoresis-mediated isoenzyme analysis. Clinical Microbiology and Infection 12: 369-375.

WIDDEN P., ABITBOL J.J. (1980): Seasonality of *Trichoderma* species in a spruce-forest soil. Mycologia 72: 775-784.

WUCZKOWSKI M., DRUZHININA I., GHERBAWY Y., KLUG B., PRILLINGER H., KUBICEK C.P. (2003): Species pattern and genetic diversity of *Trichoderma* in a mid-European, primeval floodplain-forest. Microbiological Research 158: 125-133.