ANALYSIS OF MYCELIAL GROWTH RATE AND MYCOPARASITIC ABILITY OF DIFFERENT *TRICHODERMA* ISOLATES FROM GRAPEVINE TRUNKS

Kovács Csilla, Sándor Erzsébet, Peles Ferenc

University of Debrecen, Faculty of Agricultural and Food Sciences and Environmental Management, Food Science Institute, Böszörményi Street 138., H-4032 Debrecen, Hungary
kovacs.csilla@agr.unideb.hu

Abstract

Grapevine trunk diseases (GTD) are one of the most important diseases in vineyards worldwide, which can be found in Hungarian vineyards as well. Trichoderma used as biopesticides against pathogen fungi. Trichoderma fungi are belongs to the Ascomycota phylum, Sordariomycetes class and the Hypocreaceae family. The aim of the study was to examine the growth rate of the isolates from grape trunks at different temperatures. Ten Trichoderma isolates from the Tokaj wine region (TR01-TR10) were tested at different temperatures (18.5; 22.5; 25; 30 and 37 °C). The mycelial growth was determined from the two average colony diameter in Petri-dishes with potato-dextrose agar, measured for each Trichoderma isolates for 4 days in three replicates.

The TR05 and TR06 are capable for active growth under broad temperature range, therefore these isolates are potentially good biopesticides at temperate region against grape pathogens. Trichoderma species are considered effective biocontrol agents providing the reduction of chemical pesticide application in horticulture.

Key words: *Trichoderma* sp., biopesticide, mycelial growth, mycoparasitic ability, grapevine trunks

INTRODUCTION

Grapevine trunk diseases (GTD) are one of the most important diseases in vineyards worldwide, which can be found in Hungarian vineyards as well. GTD are caused by a variety of pathogen fungi, like: *Phaeoacremonium aleophilum, Phaeomoniella chlamydospora, Eutypa lata, Fomitiporia mediterranae, Diplodia seriata* (Bertsch et al., 2012).

The most spores of the plant pathogens come by pruning of trunks to the plants. The infection can be reduced by the possible of protection (application of hygienic disinfection, phytotechnical procedures (wound treatment, appropriate cultivation and pruning methods).

Currently there is no effective fungicide, which could be used against the disease.

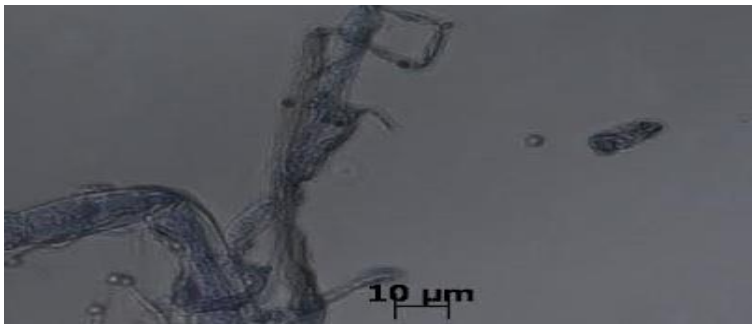
Previously used the sodium arsenite for the treatment of disease in Western Europe (Mugnai et al., 1999) but it was banned because of it is carcinogenic effect and the toxic effect on the environment.

Today, the research and development focused on the protection of pruning of wounds with different fungicides or biological control options (Hallen et al., 2010).

The *Trichoderma (Hypocrea)* species are mycoparasites with antagonistic and mycotoxic metabolite production properties (Kubicek et al., 2007). They are used as biopesticides against pathogen fungi. *Trichoderma* fungi are belongs to the *Ascomycota* phylum, *Sordariomycetes* class and the *Hypocreaceae* family. Their conidia are single-celled with white or green colour. The thin hyphae are indicated in the genus name from Greek „tricho" (hair) word. The optimum growth temperature is between 25-30 °C. The species have rapid growth, so they displace the plants pathogens, and mycoparasitic activity also restrict the growth of the pathogens. The *Trichoderma* species are well known biocontrol agents since 1930. *Trichoderma* has also reported to induce systemic and local defense in the plant (Harman et al., 2004).

Studies proved that incidence of the disease caused by *Eutypa lata* reduced significantly with *Trichoderma* treatments significantly compared to the control samples (Tran, 2010).

Weindling (1932) has been described the way of the destruction of pathogenic fungi by these hyperparasites. The *T. harzianum* is coiling around the hyphae of the plant pathogens and forming haustorium. The produced chitinase enzyme finally enables the penetration of *Trychoderma*'s hyphae through the cell wall of the pathogen fungi (*Diplodia seriata*) (*Picture 1*) (Kotze et al., 2011).



Picture 1: The *Trichoderma harzianum* coiled around the hyphae of *Diplodia seriata*
Photo: Csilla Kovács

The aim of the study was to examine the growth rate of the isolates from grape trunks at different temperatures, and to identify them.

MATERIAL AND METHOD

Microbiological tests were carried out in the Microbiological Laboratory of Food Science Institute.

The ten *Trichoderma*s were isolated from the Tokaj wine region (TR01-TR10).

Mycelial growth

Trichoderma isolates were tested at different temperatures (18.5; 22.5; 25; 30 and 37 °C). The mycelial growth was determined from the two average colony diameters in Petri-dishes with potato-dextrose agar (PDA), measured for each *Trichoderma* isolates for 4 days in three replicates.

Mycoparasitic ability of different *Trichoderma* species

The examinations of mycoparasitic ability of different Trichoderma isolates were performed according to the method of Szekeres et al. (2006). Two days old *Trichoderma* and three days old *Diplodia seriata* colonies grown on potato-dextrose agar were used for inoculation. The antagonist species were put next to the pathogen, and pictures were taken following the 10 days incubation at room temperature at malt-extract agar (MEA) (Schubert et al., 2008). First plant pathogen hyphal plug was inoculated 1.5 cm from the middle of Petri-dish. Following 24 hour incubation, the tested *Trichoderma* mycelia plug was inoculated 3 cm from the pathogen on the same plate. The experiments were carried in three replicates.

DNA Isolation

Fungal DNA was disrupted with MagNaLyser (Roche), than DNA isolation was carried with NucleoSpin Plant II (Macherey-Nagel). The DNA concentrations were measured by NanoDrop (Thermo Scientific). The isolated DNA was checked with run on 0.8% agarose gel in TAE buffer.

PCR amplification and sequence analysis

Ribosomal DNA region, containing ITS1 and ITS2 was amplified with SR6R and LR1 universal primers (White et al., 1990; Thiericke, 2003).

PCR products were purified with NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel). Purified amplification products were sequenced by Microsynth Austria GmbH.

Sequences were blasted into the *Trichoderma* databases (TrichoBLAST http://isth.info/tools/blast/index.php).

Sequences were aligned with Clustal-X program (Higgins-Sharp, 1988; Thompson et al., 1997; Larkin et al., 2007) and further corrected with GeneDoc (Nicholas et al., 1997). Phylogenetic tree was created with MEGA5.5 (Tamura et al., 2011).

Statistical analysis

Analysis of variance (ANOVA) and Tukey-test or nonparametric Kruskal-Wallis test and comparative Dunn's test were used for the statistical analysis of the resulted data. Differences were considered statistically significant at the 95% confidence level ($P<0.05$). All statistical analysis was performed using Microsoft Excel and GraphPad Prism 3.02 (Motulsky, 1999) statistical programs.

RESULTS AND DISSCUSIONS

The mycelial growth of ten *Trichoderma* isolates was measured.

Figure 1 shows the mycelial growth of TR01, TR02, TR03, TR04 and TR05 isolates. The optimum growth temperature of these isolates was 30 °C and reduced mycelial growth was detected at 37 °C temperature. Difference was not significant compared to the average growth rate at 30 °C.

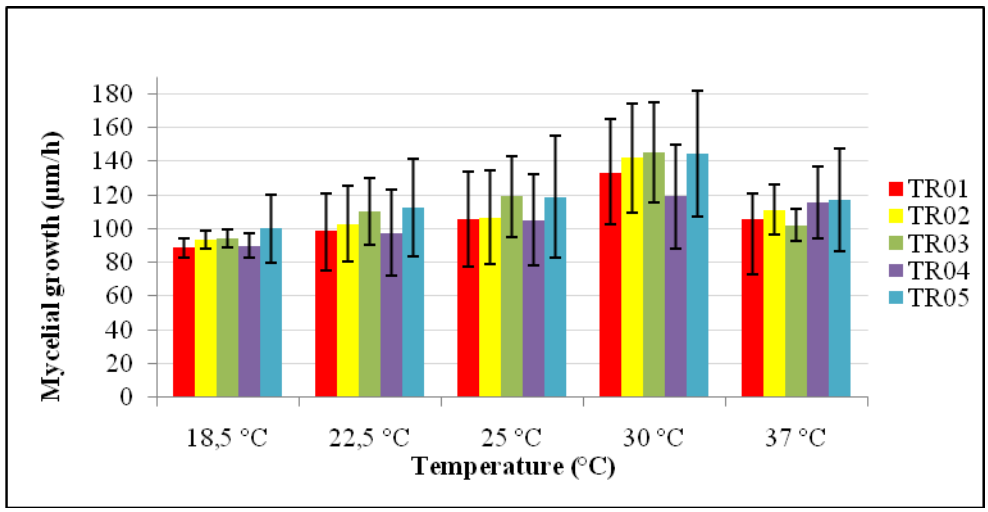


Fig. 1: Mycelial growth of TR01-TR05 *Trichoderma* isolates at different temperatures

The mycelial growth of TR07-TR10 *Trichoderma* isolates showed significantly lower growth ($P<0.05$) than TR01-TR05 isolates (*Figure 2*). They had relatively constant growth between 18.5 and 30 °C.

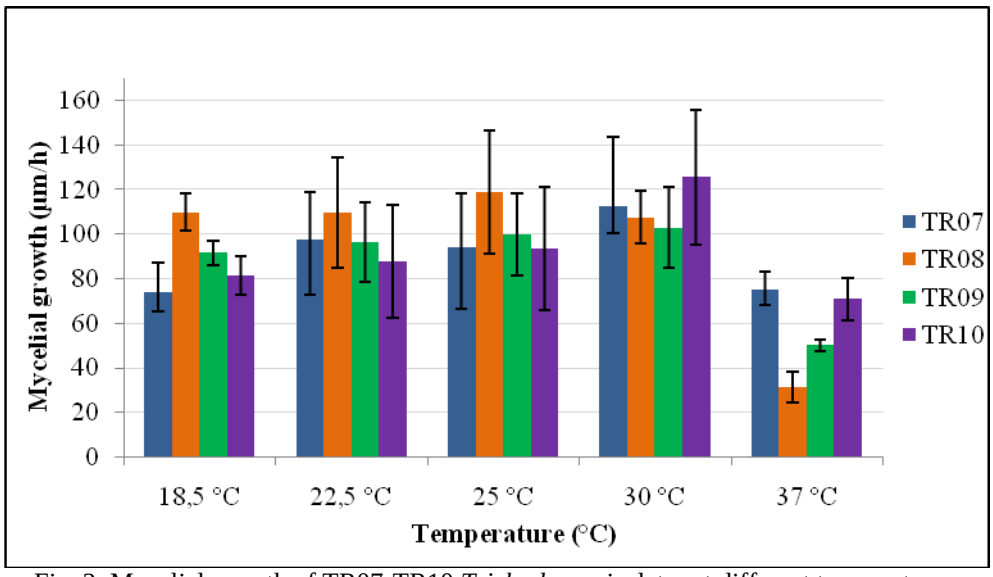


Fig. 2: Mycelial growth of TR07-TR10 *Trichoderma* isolates at different temperatures

The TR07-TR10 *Trichoderma* isolates showed a significantly higher growth rate ($P<0.05$) than TR06 isolate at each tested temperatures. The TR01 and TR04 showed slower growth at 37 °C than at lower temperatures, however differences were not significant. The TR08 and TR09 isolates had significantly the lowest growth activity among the tested isolates (*Figure 3*).

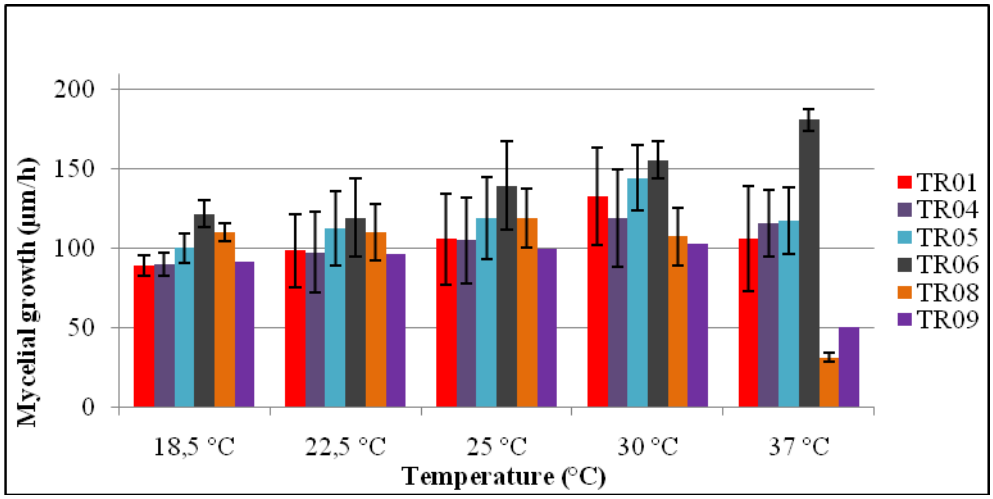


Fig. 3: Mycelial growth of TR01, TR04-TR06, TR08 and TR09 *Trichoderma* isolates at different temperatures

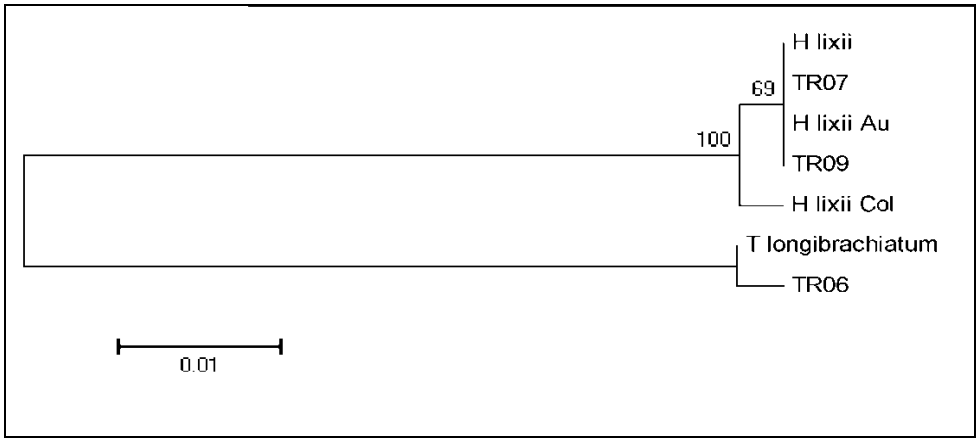
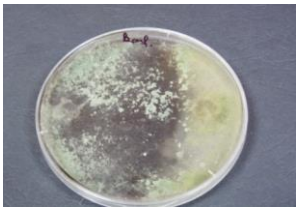


Fig. 4: Molecular Phylogenetic analysis by Maximum Likelihood method of TR06-07 and 09 isolates from grapevine trunks, Hungary. H. lixii, H. lixii AU and H. lixii indicates *Hypocrea lixii* deponated sequences AY605730, AY605727 and EU280075, while T. longibrachiatum refers EU280095 sequence. Phylogenetic tree was created with MEGA5.5. Numbers above the lines indicate the result of bootstrap analysis with 1000 replicates. Branch length is correlated with differences among the sequences.

Maximum Likelihood method based on the Jukes-Cantor model (Jukes-Cantor, 1969) was used for the phylogenetic analysis (*Figure 4.*). The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analysed (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analyses were conducted in MEGA5.5 (Tamura et al., 2011). Two of the three analysed ITS sequences of TR 07 and 09 isolates showed similarity to the *Hypocrea lixii*, belonging to the *T. harzianum* phylogenetic group (Nagy et al., 2007). The ITS sequence of the third *Trichoderma* isolates (TR06) however belongs to the *T. longibrachiatum* group.

Mycoparasitic activity was detected 10 days later, when the *Trichoderma* species destroyed the pathogen (*Picture 2*).



Picture 2: The *Trichoderma harzianum* (TR07) interaction with *Diplodia seriata*
Photo: Csilla Kovács

The Biocontrol Index was 100 % for all of the examined *Trichoderma* isolates against the pathogen *Diplodia seriata*.

CONCLUSIONS

Our results indicated that the growth of TR01, TR02, TR03 and TR04 isolates was slower at lower temperature value (18.5 °C), while the TR07, TR09 (*T. harzianum* phylogenetic group), TR08, and TR10 isolates was slower at the highest examined temperature (37 °C). TR05 and TR06 (*T. longibrachiatum* group) isolates had the highest growth rate at the examined temperature range. Their growth was very intensive even at 30 and 37 °C.

Consequently, the TR05 and TR06 are capable for active growth under broad temperature range, therefore these isolates are potentially good biopesticides at temperate region against grape pathogens. However their mycoparasitic activity also should be tested. *Trichoderma* species are considered effective biocontrol agents providing the reduction of chemical pesticide application in horticulture.

For further identification of *Trichoderma* species TEF-1 marker sequences will also use. The isolates within the *T. harzianum* group also will be analysed with the chitinase gene (Nagy et al., 2007).

Acknowledgments

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