

Thesis of University Doctoral (PhD) dissertation

**FUNGI ASSOCIATED WITH GRAPEVINE TRUNK DISEASES
AND INVESTIGATION OF POTENTIAL BIOCONTROL
OPTIONS IN TOKAJ WINE REGION**

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1. BACKGROUND AND OBJECTIVES OF THE DISSERTATION

The Grapevine Trunk Diseases (GTD) are one of the most serious diseases worldwide for the grapevine (*Vitis vinifera* L.), which are causing considerable yield and economic losses in the wine industry (Bertsch et al., 2013; Ammad et al., 2014). More and more researchers compare the disease severity of GTDs to that of phylloxera in Europe.

The grapevine growing area is 80180 hectares in Hungary (KSH, 2015). The GTD incidence increased from 1.3% in 2014 to 5.8% in 2016 in the Tokaj Wine Region according to Bihari et al. (2016).

The GTD has been described first in California in 1895. Ravaz identified the disease in France in 1898, as “folletage”. The diseases were associated with the presence of the *Stereum hirsutum* (Willd.) Pers. (1800) and the *Phellinus igniarius* (L.) Quél. (1886) (Chiarappa, 2000).

Intensive research of the GTD began from the 1950s. Hewitt et al. (1957) observed that specific symptoms cannot be detected on the diseased trunks every year. Chiarappa (1959) identified *Cephalosporium* and *Phellinus igniarius* (L.) Quél. (1886) species as potential pathogens from the symptomatic plants. Larignon and Dubos (1987) studied the question, whether the asexually reproducing (mitospore) fungi participate alone or together with basidiomycota in the evolving of the disease.

The drastic increase of the disease was observed in the 1990s in those countries, where the previously effectively used arsenite was banned (Mugnai et al., 1999). During this period the sodium-arsenite has been used in a limited extent in France, Portugal and in Spain, but the esca continues to spread in the winegrowing regions (Mugnai et al., 1999). Although the appearance and the type of GTD are different in the different wine regions, the increase of the disease became robust at the beginning of the 2000s (Graniti, 2006).

GTDs are caused by many different Ascomycetes fungi, including *Phaeoacremonium aleophilum*, *Phaeomoniella chlamydospora* and other *Phaeoacremonium* sp., *Eutypa lata*, *Diplodia seriata*, *Phomopsis viticola*, as well as Basidiomycetous fungi such as *Fomitiporia mediterranea* (Kuntzmann et al., 2010; Úrbez-Torres et al., 2014). GTD incidence may vary between closely located vineyards possibly due to microclimate, soil composition or water supply, suggesting abiotic environmental factors are likely to have a role in the evolve of the disease. The grape variety and the type of rootstock can also affect the appearance and severity of disease symptoms (Sosnowski et al., 2007; Van Niekerk et al., 2011).

Esca disease complex, Petri disease, Eutypa, Botryosphaeria, Phomopsis dieback, black dead arm (BDA) and the black foot disease are considered the major GTDs worldwide, and the

causal pathogens attack the woody perennial organs of the vine and ultimately lead to the death of the plant (Lehoczky, 1974; Fontaine et al., 2015). According to Larignon et al. (2000) the esca complex was more widespread disease comparing to Eutypa dieback in France in the 1990's. Black dead arm was new disease in some French wine regions, including Champagne in 2000 (Larignon and Dubos, 2001). The importance of esca and the BDA have been realized already in the majority of French wine regions in 2003 (Fotire Muller, 2006).

The effective control of GTD faces several problems. Although the susceptibility of the different cultivars are different (Murolo and Romanazzi, 2014) and the rootstock may also affect the frequency of the symptoms there is no grapevine cultivar known to be resistant to GTD. The protection restricts currently mainly to the prevention (healthy propagating material, prevention of pruning wounds infection).

My research work focused on the detection of the fungi associated with GTDs and the development of a potential biofungicide in the Tokaj Wine Region.

1. Monitoring GTDs in the Tokaj Wine Region to detect the disease incidence (DI%).
2. Study of the relationship between the biotic and abiotic factors and the DI%
3. Identification of the plant pathogens and the endophytes from the symptomatic and asymptomatic trunks based on morphological and genetic markers and phylogenetic studies
4. Testing some potential biocontrol strains against GTD pathogens

2. RESEARCH METHODS

2.1. Vineyard characteristics and meteorological data

The research of Grapevine Trunk Diseases was started in the Tokaj Wine Region in 2013. The collection of symptomatic trunks was carried out by the colleagues of the Research Institute for Viticulture and Oenology during 2013 and 2015 from five vineyards (Bakonyi, Dorgó, Szarvas, Szemere, Várhegy (Patrícus) vineyard) from Furmint, Hárslevelű and Zéta varieties (Figure 1., Table 1.).



Figure 1.: Sampling sites in the Tokaj Wine Region, Hungary in 2013-2015.
(1. Szemere dűlő; 2. Dorgó dűlő; 3. Várhegy dűlő; 4. Bakonyi dűlő; 5. Szarvas dűlő)

(Kovács, 2016)

Table 1.: Characteristics of sampling sites in the Tokaj Wine Region

No. of the vineyard	Studied vineyard	Year of planting	Variety	Slope	Planting distance	Soil
1	Szemere	1999	Furmint	2-3% slope (7.28°)	2 m × 0.9 m	luvisols
2	Dorgó	1994	Furmint	1-2% slope (5.87°)	2 m × 1.2 m	
3	Várhegy	2003	Zéta	1-2% slope (5.38°)	2,2 m × 0.9 m	
4	Bakonyi (F)	1992	Furmint	1-2% slope (2.79°)	2,4 m × 0.9 m	cambisols
	Bakonyi (H)		Hárslevelű	2-4% slope (4.12°)		
5	Szarvas (S)	2003	Hárslevelű	5-8% slope (8.13°)	2,4 m × 0.9 m	
	Szarvas (L)		Hárslevelű	1-2% slope (8.13°)		

The Bakonyi and Szarvas vineyards were planted on cambisols, while the Szemere, Dorgó, and Várhegy vineyards were planted on slope sediment from luvisols soil. All training systems were mid-high cordon, except for Szemere, which was Guyot. The rootstock 125AA was used only in Várhegy for the variety Zéta (*Vitis vinifera* L.), and Teleki 5C was used in all the other vineyards with varieties Furmint and Hárslevelű (*V. vinifera* L.). There was also difference on the direction of the rows on the slopes, as the Bakonyi vineyard is terraced, while the other vineyards are on steep slopes. All vineyards had only one variety, except the Bakonyi which had both Furmint (4F), and Hárslevelű (4H). Data from Szarvas vineyard was also divided into two parts: the upper part of the vineyard with 5–8% slope (5S), and a lower, flat part at the foot of the hill (5L), thus data of seven sites were drawn into the analysis (**Table 1.**).

Meteorological data were collected with MILLIMET Weather Station (Boreas Kft., Hungary) in the region. Minimum, maximum and average temperature, as well as total amount of precipitation was calculated from the data for each month.

2.2. Detection of disease incidence (DI%) and collection of samples

The evaluations of external GTD symptoms were carried out between May and August in each year from 2013-2015, with visual inspections in the vineyards. The disease incidence (DI) was calculated as the proportion of plants (%) expressing foliar symptoms with wood necrosis or dieback in each vineyard. Samples were collected at several sampling times each year between May and September from the trunks with typical GTD symptoms. Only the upper parts of the plants (cordon) were sampled to leave the plants alive for further analysis. Representative random sampling from the vineyards (with 18-19 rows) included collecting a minimum of three samples from each row. Samples were also taken from all symptomatic plants for isolation.

Woody samples were transported in a cooler (under 10°C) to the laboratory at the University of Debrecen within 48 hours to determine the colonizing fungal microflora of the wood tissues.

2.3. Isolation of fungi from woody tissues

Wood chips from debarked and surface sterilized (10% Neomagnol (chlorogen sesquihydrate) solution than washing twice with sterile distilled water) plant samples were cut from both browned and adjacent apparently healthy wood tissues, and placed on malt extract agar medium (MEA, Scharlau, Spain) in 90 mm diameter Petri dishes with a sterile scalpel, under aseptic conditions following the method of Abreo et al. (2013), with modification described in Kovács et al. (2014c). Plates were incubated at room temperature in darkness for

7 to 14 days, and mycelial fragments from emerging fungal colonies were transferred to new 2% MEA plates (Crous et al., 2006). Isolates were maintained on MEA. Mycelial or conidial suspensions were also stored in 50% glycerol at -80°C.

2.4. Morphological and molecular identification of the isolated fungi

Pure fungal cultures were used for the taxonomic identification of the fungi based on morphological and cultural features (mycelial color, cell wall structure). Pycnidia formation structures (presence, absence) on potato-dextrose agar medium with streptomycin sulfate (PDA, Scharlau, Spain) and conidia (color, shape, size) or conidiophores were examined under microscope (Kovács et al., 2014b).

Molecular identification with sequence analysis of the Internal Transcribed Spacer (ITS) region (ITS1, ITS2 (Schoch et al., 2012) of the rDNA was performed for isolates whose sporulation was not observed. Randomly chosen isolates from each morphological group were also sequenced. We used different molecular markers for the identification of endophytes and GTD pathogens (**Table 2.**).

Table 2.: Molecular markers and annealing temperature used for the PCR amplification of endophytes and GTD pathogens

Molecular marker	Species	Primer	Sequence	Reference	T* (°C)
ITS1/ITS2	Endophytes and GTD pathogens	ITS4	TCCTCCGCTTATTGATATGC	White et al., 1990	53
		ITS5	GGAAGTAAAAGTCGTAACAA GG		
	<i>Trichoderma</i> sp.	SR6R	AAGTAGAAGTCGTAACAAGG		50
		LR1	GGTTGGTTTCTTTTCCT		
<i>tef1</i>	<i>Trichoderma</i> sp.	EF1 728F	CATCGAGAAGTTCGAGAAGG	Carbone and Kohn, 1999	59
		EF1 986R	TACTTGAAGGAACCCTTACC		
chi18-5 (ech42)	<i>Trichoderma harzianum</i>	Chit42-1a	GCTYTCCATCGGTGGCTGGAC	Kullnig-Gradinger et al., 2002	55
		Chit42-2a	GGAGTTGGGGTAGCTCAGC		

* Annealing temperature

Producer: IBA, Germany

PCR products were purified with NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel). DNA concentrations were measured with NanoDrop 2000 (Thermo Scientific) from 2 µl DNA. Purified amplification products were sequenced by Microsynth Austria GmbH and Eurofins MWG GmbH (Germany). The sequences were manually aligned and compared with those

deposited in the NCBI GenBank database using the BLAST program (Altschul et al., 1990; Kovács et al., 2014a). The ITS sequences were also submitted to the NCBI GenBank for the species identification. Sequences were aligned with Clustal-X program (Higgins and Sharp, 1988; Thompson et al., 1997; Larkin et al., 2007) then we used the GeneDoc program for the refinement of the sequences (Nicholas et al., 1997). The phylogenetic tree were prepared with MEGA 6.0 program (Tamura et al., 2013). The ITS sequences were deponated into the NCBI database and KU377167-KU377290 accession numbers were obtained.

2.5. Statistical analysis

The variables were calculated following the data collection and the laboratory examinations. The number of the removed trunks was few (<1%), so we did not take into consideration the removed and the replanted plants for the statistical analysis. The determination of the Infection Rate (IR%) was based on the microbiological analysis of 2015 year for the proportion of the GTD pathogens (*Diaporthe* sp., *D. seriata*) in each examined vineyards. Representative random sampling was performed from the vineyards included collecting a minimum of three samples from each row except from the Bakonyi (4) vineyard, where all plants were examined in each year. Binominal tests were used to analyze deviation of the infection rate in the sites from the summarized proportion in the whole sample. The Disease Incidene (DI%) was calculated based on the ratio of the plants with GTD symptoms detectable on the leaf and in woody tissues (Murolo and Romanazzi, 2014). The relationship between IR and DI was analyzed with correlation analysis. Beyond that the effect of locations (seven studied sites), age of plantations (below or above 15 years old in 2013), vintage (three studied years), topology (slope and plain), soil types (luvisols and cambisoils) and grapevine variety (Furmint and Hárslevelű) on the DI and IR were analyzed. Three categories were used to characterize the temporal stability of symptom appearance between 2013 and 2015: occasional – symptoms appeared only one year, stable – symptoms appeared twice and continuous – symptoms appeared in all three studied years. The proportion of the three categories was calculated for each site and the effect of above mentioned biotic and abiotic parameters on it was analyzed.

The Kolmogorov-Smirnov test for normality and the Levene test were used to test normality and the equality of variance assumptions of parametric tests. Considering that our data did not meet these assumptions in case of multiple comparison of groups Kruskal-Wallis nonparametric test were used. Pairs showing significant differences were compared by Mann-

Whitney U-test. The paired comparisons were also made by Mann-Whitney U-test (Reiczigel et al. 2007). Statistical analysis were performed by SPSS 21.0 (Ketskeméty et al., 2011).

Besides the mentioned comparative analysis we determined the Isolation Incidence (IsI %) of the endophytes and GTD pathogens based on the total number of symptomatic samples with isolated fungi (received for microbial analysis from the vineyards).

3. RESULTS

3.1. GTDs incidence in the examined vineyards between 2013 and 2015

The percentage of DI of GTD varied from 0.17 to 42.11% in the three-year study according to observations made on 22794 grapevines (**Table 3., Figure 2.**). Mean DI showed significant differences (Kruskal-Wallis test: $H=19.375$ $df=6$, $n=21$, $p=0.004$) in the different vineyards. The highest mean DI was detected in the Bakonyi vineyard (**Figure 2.**).

Table 3.: Number of plants with GTD symptoms and the Disease Incidence (DI) in the different vineyards between 2013 and 2015 at the Tokaj Wine Region, Hungary

Sampling sites*	Location	No. of observed plants**			No. of symptomatic plants			DI (%)		
		2013	2014	2015	2013	2014	2015	2013	2014	2015
1	Szemere	1630	1635	1635	9	19	16	0.55	1.16	0.98
2	Dorgó	1763	1759	1759	72	48	33	4.08	2.73	1.88
3	Várhegy	1741	1744	1744	3	3	3	0.17	0.17	0.17
4F	Bakonyi	209	209	209	72	86	88	34.45	41.15	42.11
4H	Bakonyi	283	270	266	39	44	44	13.78	16.30	16.54
5S	Szarvas	1266	1266	1266	27	63	43	2.13	4.93	3.40
5L	Szarvas	714	713	713	36	42	44	5.04	5.89	6.17

* Number of the sampling sites: according to Fig. 2. 4 F: vineyard 4 with Furmint variety, 4H: vineyard 4 with Hárslevelű variety, 5 S: vineyard 5 on slope, 5 L: vineyard 5 on low, horizontal field.

**number of planted trunks (without remove, with replantation)

The highest mean DI (13.78-42.11%) was detected in the Bakonyi (4F; 4H) vineyard. Here both Furmint (4F) and Hárslevelű (4H) varieties showed significantly higher DI than other vineyards and there was also significant difference between them. An intermediate mean DI value was observed in the Szarvas vineyard with significant difference between higher (5S) and lower (5L) part of this site (2.13-6.17%). The Dorgó vineyard also showed intermediate DI, while the Szemere and Várhegy vineyards had the lowest mean DI (**Figure 2.**). The soil type (cambisols) plantation distance, training system and rootstock type (Teleki 5C) were the same in both examined areas. The DI% (5.04-6.15%) was significantly higher in the terraced, plain Szarvas vineyard (5L) than in the top part (5S) on 5-8% slope. The DI value was between 1.88 and 4.08% in the Dorgó vineyard. This value was much lower than in the Bakonyi (4F, 4H) vineyard with the same age, cultivation system and rootstock.

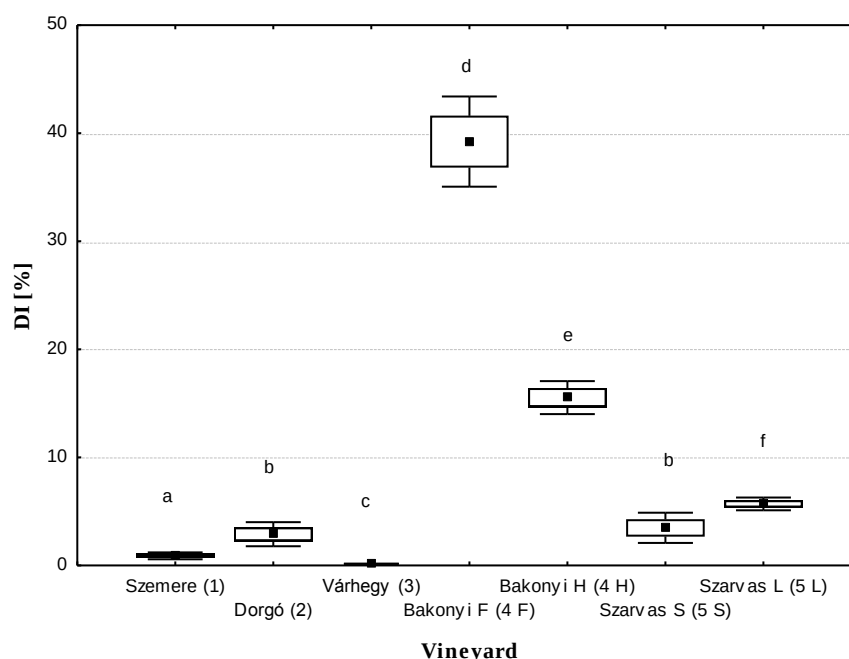


Figure 2.: Mean disease incidence (DI% $\pm$ SE/SD) at the different vineyards in the Tokaj Wine Region, Hungary between 2013 and 2015.

Letters indicate significant differences ($p < 0.05$) according to Mann-Whitney U-test. Number of sampling sites according to Figure 1.

The disease incidence was the highest in Bakonyi vineyard in each year (**Table 3., Figure 2.**). The Szemere and the Szarvas (5S, 5L) vineyards had similar plantation characteristics (rootstock, variety). The DI rate was much lower (0.55-6.17%) in these vineyards than in the Bakonyi vineyard (4F, 4H), in each year. The lowest DI (0.17%), and the fewest symptomatic plants were detected in the Várhegy vineyard. This area had unique features, regarding both the variety (Zéta) and the rootstock (125AA). The plantation was one of the youngest (12 years in 2013), but the disease incidence was significantly lower, than in the Szarvas vineyard with the same age and training system (mid-high cordon) (**Table 1., Figure 2.**). The oldest Furmint plantations (19 and 21 years in 2013) were in the Dorgó and the Bakonyi (4F) vineyards. However their average DI showed significant difference ($P < 0.05$; $P = 0.0495$) (**Table 3.**).

In conclusion, the disease incidence showed significant difference in the different examined years in each area. The changes of the DI did not show obvious trend in the Dorgó, Bakonyi and Szarvas vineyards during the three years (**Figure 2.**).

3.2. The DI value between 2013 and 2015 in each vineyard

There was no significant difference ($P > 0.05$, $P = 0.9528$) between the DI value and the vintages (2013, 2014, 2015) regarding all examined vineyards. Furthermore, there was no significant difference between the two Bakonyi sites and the examined years ($P > 0.05$,

P=0.8759). However there was significant difference between the varieties (Furmint, Hárslevelű) and the three-years ($P<0.05$, $P=0.0015$) in the Bakonyi vineyard.

3.3. The different varieties and the evolution of the DI in each, examined vineyard

The Furmint variety was examined in different soils. The soil type did not affect the IR. We found that the DI value of the Furmint variety was significantly different ($P<0.05$, $P=0.000934$) in three, examined years from the Hárslevelű variety in the Bakonyi vineyard, but significant difference was not experienced in the analysis of all vineyards.

3.4. Comparison of the different soil and DI in the vineyards

The DI value was significantly higher ($P<0.05$, $P=0.000048$) in the cambisols soil (which is in a volcanic bedrock), than in the luvisols soil in the different vineyards. Cambisols is considered as a soil with high water capacity (Rhoton and Markewich, 2006), the rate of the disease was also higher in these areas (Bakonyi (4F, 4H) and Szarvas (5S, 5L)).

3.5. The effect of the plantation age on the DI

The DI value ($P<0.05$, $P=0.004$) was higher in the older plantation (2, 4F, 4H) comparing to the younger ones ((1, 3, 5S, 5L) in the examined period.

3.6. The effect of the topology on the DI

The location of the vineyards was different. The Bakonyi (4F, 4H) areas are perpendicular to the slope and microterraced in the Szarvas (5L) vineyard the rows run down on the slope. There was a significant difference between the upper (5S) and the lower (5L) part of the Szarvas vineyard in 2013 ($P<0.05$, $P=0.0326$) and in 2015 ($P<0.05$, $P=0.0389$). The DI value did not show significant difference between the different parts ($P>0.05$, $P=0.2203$) in 2014, due to the rainy period, the DI was also higher in the slope area. The direction of the rows were similar in Szemere, Dorgó, Várhegy and the Szarvas (5L). The DI value was lower ($P<0.05$, $P=0.000007$) in the plantations on slopes areas (4F, 4H, 5S), than in the plain areas (Kovács et al., 2016).

3.7. The incidence of GTD pathogens and endophytes in plantations with different age during 2013 and 2015

The incidence of the GTD pathogens and the endophytic fungi were different in the examined vineyards in each year. GTD pathogens were isolated from half of the symptomatic trunks. Mainly *Diplodia seriata* and *Diaporthe* species were identified from the Tokaj Wine Region. *Diplodia mutila* was isolated from seven trunks with partial necrosis in 2013. Regarding GTD pathogens, the *D. seriata* was isolated in higher proportion than *Diaporthe* species. Significant difference was not detected in the rate of the isolated GTD pathogens ($P>0.05$, $P=0.2797$), GTD+Endophytes ($P>0.05$, $P=0.6227$) and the Endophytes species ($P>0.05$, $P=0.1582$) in each year. The detection rate of *Diplodia* in the different vintage was significantly different ($P<0.05$, $P=0.4673$). GTD pathogens were isolated from the symptomatic trunks in the Bakonyi (4F) vineyard in all years. GTD pathogens was not detected in the other areas in each year.

The age of the plantation did not affect significantly the rate of the GTD pathogens ($P>0.05$, $P=0.8309$). There was no significant difference either in the combined incidence rate of the GTD pathogens and the endophytes ($P>0.05$, $P=0.8029$), or the incidence rate of the endophytes ($P>0.05$, $P=0.5220$) and *D. seriata* ($P>0.05$, $P=0.1766$) in the examined vineyards during the three year examination. The **Table 4.** shows that the Bakonyi (4F, 4H) vineyard had the highest isolation rate, because the most samples were received from these areas between 2013 and 2015.

Table 4.: Isolation rate of the fungi (GTD pathogen and Endophytes) (2013-2015)

Vineyard	Isolation rate (%)		
Vintage	2013	2014	2015
Szemere (1)	0.25	0.73	0.98
Dorgó (2)	3.86	1.14	0.63
Várhegy (3)	0.11	0.11	0.00
Bakonyi (4F)	39.71	72.73	97.61
Bakonyi (4H)	10.95	7.78	12.78
Szarvas (5S)	2.61	2.29	2.21
Szarvas (5L)	4.90	4.49	1.54

One or more GTD pathogens were isolated in all vineyards in 2013, but we cannot isolate in 2014 and 2015. We did not found GTD pathogens in the Zéta variety in 2014. GTD pathogens were detected in the highest proportion from Furmint variety in 2015, but the soil type did not affect the ration.

The overall conclusion is, that *D. seriata* was identified as GTD pathogen with the highest percentage from all vineyards in all studied years.

3.7.1. Isolation rate of *Diplodia seriata* and *Diaporthe* sp. and simultaneous occurrence of symptomatic plants in the examined plantations (2013-2015)

D. seriata could be isolated from most of the symptomatic plants. Its isolation rate varied between 94 and 100% (**Table 5.**). *Diaporthe* species were isolated with much lower frequencies, while other *Botryosphaeria* sp. (*D. mutila*) were detected only in one year (2013) from one vineyard (4F).

Table 5.: Isolation rate (%) of two GTD pathogens *D. seriata* and *Diaporthe* sp. and ratio of their parallel occurrence (D*D) between 2013 and 2015 from symptomatic grapevine wood tissue in the Tokaj Wine Region, Hungary

Sampling site*	Isolation rate (%)		
	<i>Diplodia seriata</i>	<i>Diaporthe</i> sp.	D*D
1	100.00	0.00	0.00
2	98.78	4.88	3.66
4F	96.10	2.44	1.95
4H	94.92	5.08	0.00
5S	94.23	5.77	0.00
5L	97.56	4.88	2.44
mean ($\pm$ SD)	96.93 (2.25)	3.84 (2.20)	1.34 (1.57)

*1: Szemere, 2: Dorgó, 3: Várhegy, 4F: Bakonyi Furmint, 4H: Bakonyi Hárslevelű, 5S: Szarvas slope, 5L: Szarvas plain

3.7.2. Isolation rate of the endophytic fungi in the examined vineyards (2013-2015)

Besides the GTD pathogens, different endophytic fungi were isolated from the trunks (*Alternaria* sp., *Aspergillus* sp., *Aureobasidium* sp., *Bionectria* sp., *Epicoccum* sp., *Fusarium* sp., *Mucor* sp., *Phoma* sp., *Peniophora* sp., *Penicillium* sp., *Trichoderma* sp.). Rábai et al. (2008) also found the same endophytes besides the GTD pathogens in the trunk. Bruez et al. (2014) also proved their presence with metagenomics analysis. The symptom severity may increase in the presence of *Alternaria* and some other fungi (Larignon and Dubos, 1997; Moreno-Sanz et al., 2013). *Alternaria* species were present with remarkable frequencies in the symptomatic plants. According to Moreno-Sanz et al. (2013) these species occur in the dead or decaying parts of the trunk. These fungi are not pathogens, but saprophytic fungi in the woody tissues. We isolated non-GTD pathogens in 16-65% between 2013 and 2015 from each

vineyard. We had few sample from Várhegy (3) plantation in 2013 and 2015, so their frequency was 0%, while the incidence rate was 100% in 2014. Different endophytic fungi were isolated from the vineyards in 2013. The isolation incidence of *Alternaria* species was the highest in the Bakonyi and the Szarvas areas (2-40%). We could identify *Fusarium* species from all vineyards except Szemere. Endophytes were present in high proportion in Várhegy area, where the number of samples was the fewest. The detection rate of the most frequently isolated endophytic fungi (*Alternaria* sp.) decreased in Bakonyi (4F, 4H) and Szarvas (5S, 5L) vineyards in 2014 (4-20%). We did not isolate *Alternaria* sp. in the Szemere and Várhegy vineyards. Besides *Alternaria* species *Fusarium* sp. were identified with the highest proportion from the Bakonyi (4F, 4H), Dorgó and Szarvas (5S) vineyards (1-50%) between 2013 and 2015. We could not isolate these species from the Szemere vineyards in the examined years. *Fusarium* species were detected from Várhegy plantation just in 2013, while it also occurred in the Szarvas (5L) vineyard in 2013 and 2014, but we did not isolate it in 2015. The incidence rate of the isolated *Mucor* sp. was more common (9-30%) in 2014 compared to 2013 (5-20%). These fungi were not isolated from the Szemere vineyard, but they were present in Várhegy area in 2013 and 2014. The detection rate of *Trichoderma* sp. was similar between 2013 and 2015. The rate of the most common isolated endophytic fungi (*Alternaria* sp., *Fusarium* sp.) decreased in 2015 compared to the previous years. *Aureobasidium* species were isolated from the Bakonyi vineyard (2%) only in 2015.

3.8. Phylogenetic analysis of *Trichoderma* strains based on ITS és *tef1* sequences

The isolated *Trichoderma* sp. was identified first based on the ITS sequence (**Figure 3/A.**). It is necessary to determine the *tef1* (translation elongation factor alpha 1) gene sequence for the exact taxonomical identification of *Trichoderma* strains. Maximum Likelihood (ML) analysis was used for the analysis of the *tef1* sequence (**Figure 3/B.**), where sequences of deponated GenBank extype strains were also used for the analysis. The strains were distinct well from each other with the *tef1* sequence analysis. Most of our *Trichoderma* isolates belonged to the *T. harzianum* clade (TR01-05, TR07, TR09-10). One isolate was (TR06) *T. orientale*, and another (TR08) *T. viride*.

In case of *T. harzianum*, *tef1* (**Figure 4/A.**) and chi 18-5 (GH18: glycoside-hydrolase 18) (**Figure 4/B.**) were also used for the exact taxonomical identification.

Our isolates were to the *T. harzianum* subclade: *T. harzianum* subclade (TR07, TR10), „*H. lixii*” subclade (TR01-04), Subclade II (TR05). In case of investigation of the *tef1* gene

sequence was TR09 isolate in the Subclade III, while it was in case of the examination of the chi18-5 sequence in the Subclade IV.

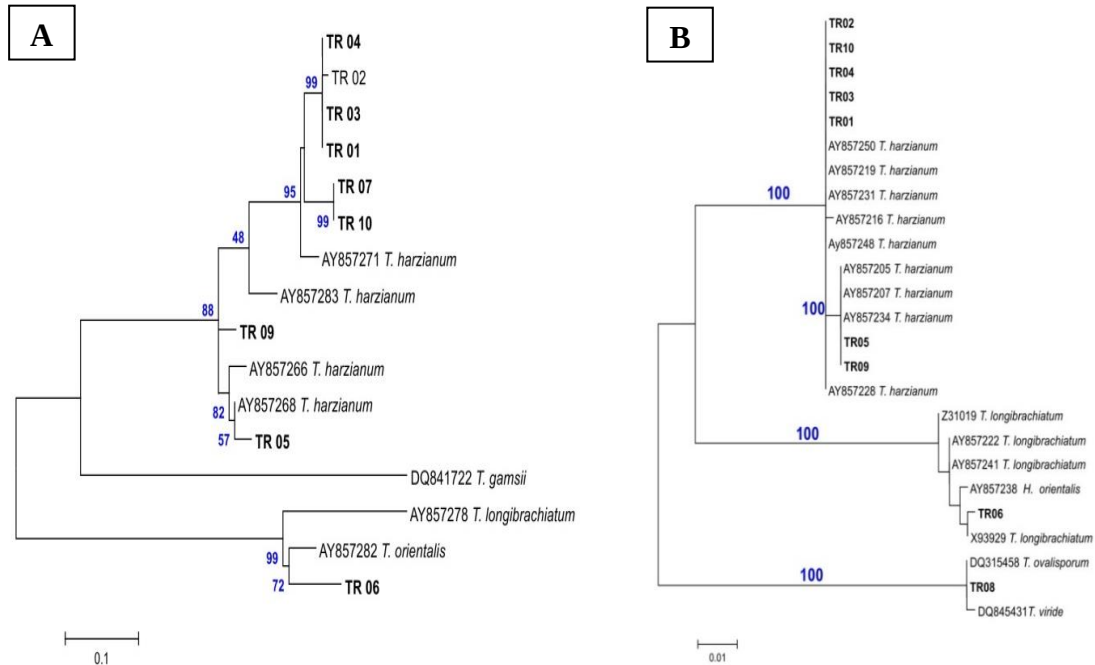


Figure 3.: ITS (A) and *tef 1* (B) phylogenetic trees of *Trichoderma* isolates

The length of branches is proportional to the number of nucleotide differences in the sequences, the scale is under the dendrogram. The blue number shows the results of the bootstrap analysis (it means we had 1000 replicates) next to the branches. The Accession Number is before species name. TR01-TR10 show our isolates.

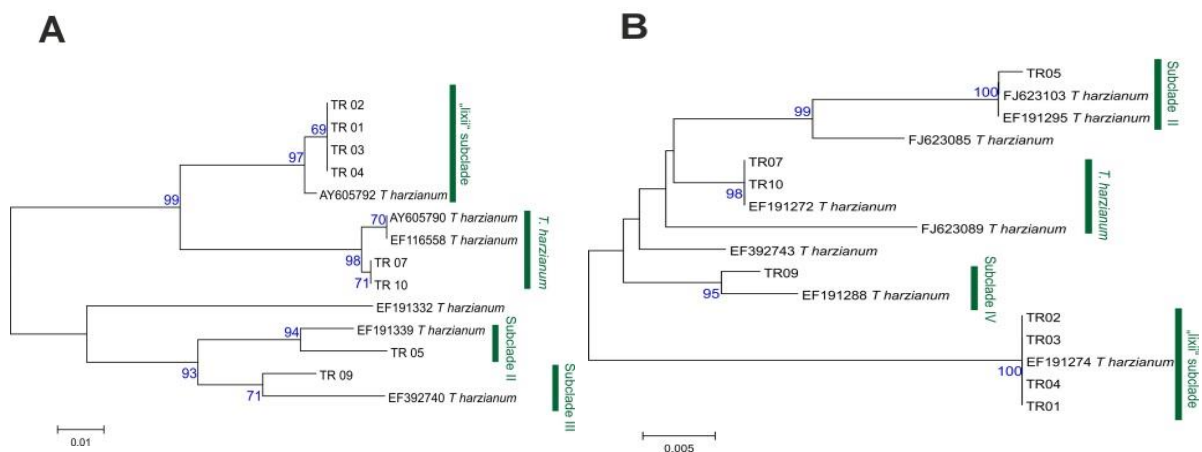


Figure 4.: Phylogenetic trees of *T. harzianum* strains based on *tef1* (A) and chi18-5 (B) genes

The length of branches is proportional to the number of nucleotide differences in the sequences, the scale is under the dendrogram. The blue number shows the results of the bootstrap analysis (it means we had 1000 replicates) next to the branches. The Accession Number is before species name. TR01-TR10 show our isolates.

3.9. Development of potential biofungicide against the GTD pathogens

3.9.1. Mycelial growth of endophytic *Trichoderma* isolates

The *T. harzianum* isolates could be classified in different phylogenetic groups (Subclade, **Figure 4.**) (TR01 – *H. lixii* subclade, TR05 – Subclade II., TR07 – *T. harzianum* subclade, TR09 – Subclade III. (investigation of *tef1* gene, IV. examination of *chi18-5* gene)) and the *T. viride* (TR08). These isolates had slower growth at the highest examined temperature (37°C), than between 18.5 and 30°C. (**Figure 5., Figure 6.**).

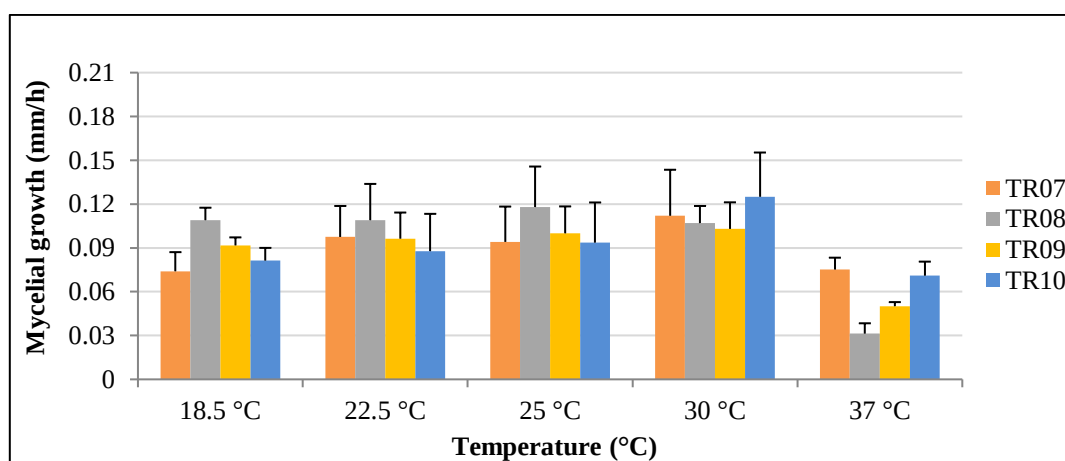


Figure 5.: Mycelial growth activity of TR07-TR10 *Trichoderma* isolates between 18.5 and 37°C

The *T. orientale* (TR06) isolate had faster mycelial growth, than the *T. harzianum* and *T. viride* isolates (**Figure 6.**). The *T. orientale* had the highest growth activity. The TR01 and TR05 *T. harzianum* isolates had low growth rate at 37°C (**Figure 5.**). TR08 *T. viride* and TR09 *T. harzianum* isolates also had lower mycelial growth at the highest temperature (**Figure 5., 6.**).

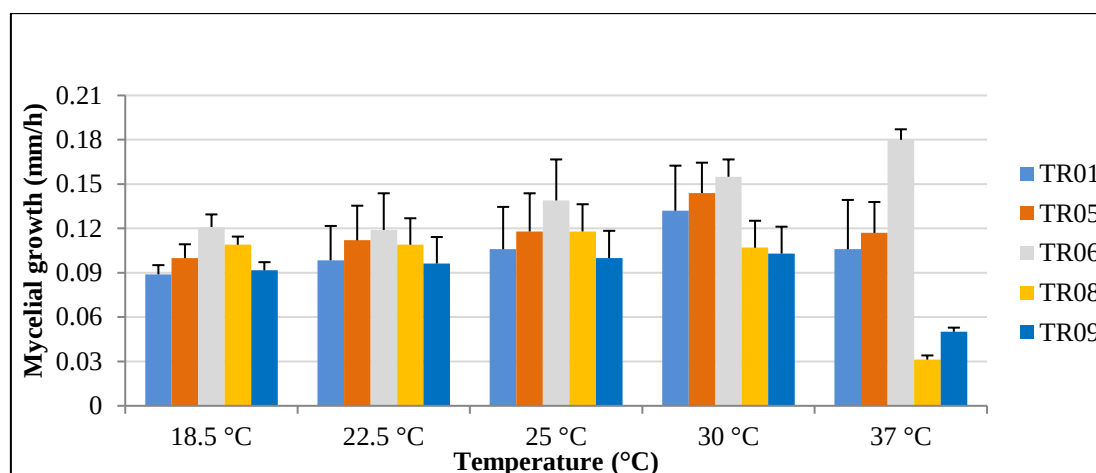


Figure 6.: Mycelial growth rate of *T. harzianum* (TR01, TR05, TR09), *T. viride* (TR08) and *T. orientalis* (TR06)

The growth of the isolates started after one week adaptation at 5°C. The TR01 (*T. harzianum*, H. lixii subclade) isolate showed one of the most intensive growth. The TR07 and the TR10 isolates (*T. harzianum* subclade) showed significantly lower growth (**Figure 7**).

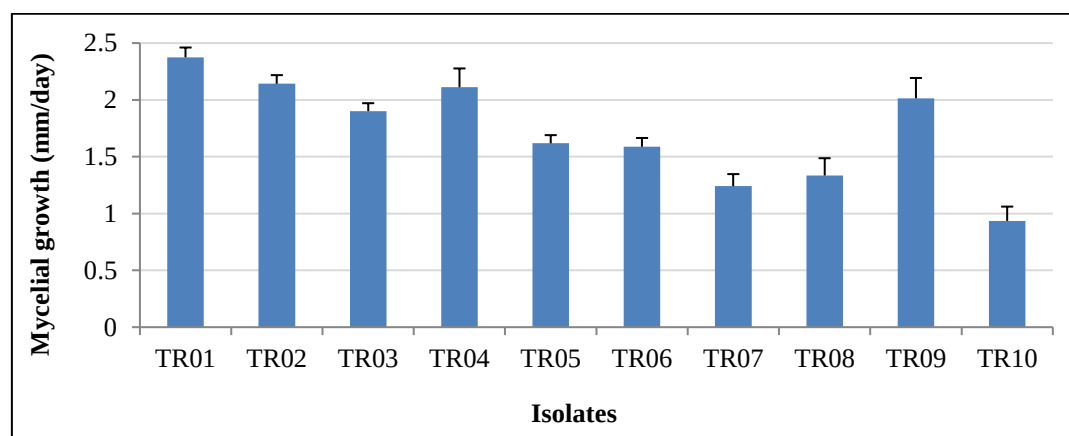


Figure 7.: Mycelial growth of *Trichoderma* isolates at 5°C

3.9.2. Mycoparasitic ability of the different *Trichoderma* isolates

The competition ability of *Trichoderma* isolates were tested against GTD pathogens (*D. seriata*, *Neofusicoccum parvum*), and *Fusarium oxysporum*. All isolates had 100% Biocontrol Index, so the *Trichoderma* isolates overgrew the pathogens. The devastating effect of the mycoparasite was observed. The *T. harzianum* coiled around the hyphae of the plant pathogen *D. seriata* and formed appressorium (Kotze et al., 2011) (**Figure 8**).

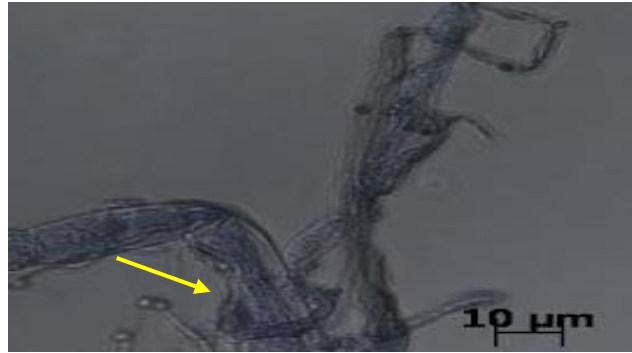


Figure 8.: The *T. harzianum* coiled around the hyphae of *D. seriata*
(Kovács, 2014)

3.9.3. Field test of *Trichoderma* spore solution

The field tests were set up in the experimental plantations of the Research Institute for Viticulture and Oenology, Tarcál. The spore concentration (10^7 cell/ml) was determined with Thoma chamber. The three *Trichoderma* isolates (*T. harzianum* - TR04, TR05) were different at their cardinal temperatures. We sprayed the spore suspension once or twice on the surface of the fresh pruning wounds 06.05.2014. The treated/untreated trunks were checked monthly, and symptom appearance was examined. The samples were collected again three months following the treatment. **Figure 9/A.** shows the Tarcál 26 variety with/without *Trichoderma* spore suspension treatment in 2014. We could reisolate the *Trichoderma* three months later and even one year following the treatment. Trunks treated with *Trichoderma* spore suspension showed more intensive sprout growth in 2015 compared to the control trunks, and the difference was also observed in the size of the clusters (**Figure 9/B.**). TR04 and TR05 (*T. harzianum*) isolates were reisolated from the spore suspension.

Four of the treated trunks with GTD foliar symptoms after the *Trichoderma* treatment did not show any visible symptoms later on, and the *Trichoderma* strains could be isolated again from their woody tissues. The disease-specific symptoms decreased on all the other treated samples, except one (III. row 1. trunk). We concluded that this trunk had been destroyed even before the *Trichoderma* application.

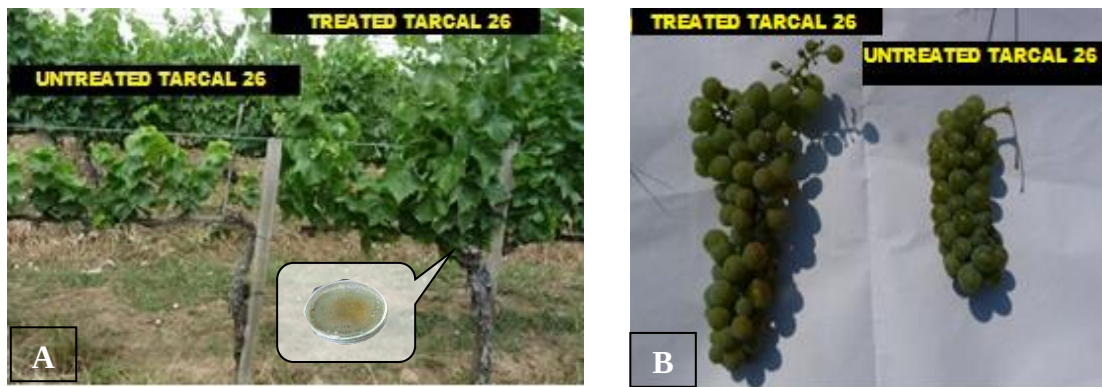


Figure 9.: Tarcal 26 variety with/without *Trichoderma* spore solution one year after the treatment (A), Clusters of the Tarcal 26 variety (B)
(Kovács, 2014)

4. NEW SCIENTIFIC RESULTS OF THE DISSERTATION

1. The soil type of the plantations affected the DI%. We proved, that the DI was significantly higher in vineyards on cambisols, than on luvisols. The age of the plantations affected DI. It was significantly higher in the older plantations, than in the younger plantations. The topology of the vineyards (slope, plain) also affected the DI; it was lower in the slope area, than in the plain or microterraced areas.

2. Most frequently isolated GTD pathogen was *D. seriata* based on large number of the samples in the Tokaj Wine Region. We could isolate *Diaporthe* sp. with lower rate. *D. mutila* pathogen was identified from seven trunks showing partial necrosis of the Furmint variety.

3. Different Endophytes were also identified first (*Alternaria* sp., *Aspergillus* sp., *Epicoccum* sp., *Fusarium* sp., *Mucor* sp., *Penicillium* sp., *Peniophora* sp., *Pleosporales* sp., *Trichoderma* sp., *Xanthomendoza* sp., *Xylaria* sp.) from the monitored vineyards. Their presence was variable during the three studied years.

4. *Trichoderma* species were isolated from asymptomatic trunks. The taxonomical identification of *Trichoderma* strains was carried out by marker sequences (ITS, *tef1*, and *chi18-5*). The isolated *Trichoderma* sp. belonged to three taxons: *T. orientale*, *T. viride*, *T. harzianum*.

5. The mycelial growth of the identified *Trichoderma* species was tested *in vitro*. Our isolates had different growth rate at different temperatures (5; 12; 18,5; 22,5; 25; 30; 37°C). Several isolates showed intensive growth rate at low temperature, so we concluded, that these isolates can be dispersed during the end of the winter - early spring period.

6. The mycoparasitic activity of *Trichoderma* isolates was determined by Biocontrol Index (BCI). All isolates had 100% BCI. Therefore these isolates are potential biopesticides against GTD pathogens.

5. PRACTICAL UTILIZATION OF THE SCIENTIFIC RESULTS

1. We concluded that *D. seriata* was the most frequently isolated GTD pathogen in the upper part (cordon and the top of the trunk) based on the survey of the Grapevine Trunk Diseases in all examined vineyards in Tokaj Wine Region during 2013 and 2015.
2. The use of the isolated *Trichoderma* species as biopesticide was successful in preliminary field test, but this was only a small scale field experiment. GTD symptoms were suppressed by *Trichoderma* spore suspension spraying on the pruning wounds early May.
3. We tested the *Trichoderma* species adaptability by the examination of their mycelial growth and their optimal temperature range for Hungarian climatic conditions. We found that several isolates (TR01, TR02, TR04, TR05, TR06) are potential biopesticides, because they can grow in wide temperature range. The TR04 isolate showed intensive growth especially at low temperatures, which is very important for the application at early pruning time.
4. The isolates are suitable for the effective defense against GTD, because they had 100% Biocontrol Index, so they are able to destroy the pathogens.
5. The sprayed *Trichoderma* species decreased the disease-specific symptoms on the vegetative organs in *in vivo* experiments and were reisolated from four of the treated trunks three months and one year following the treatment. The symptomatic, control trunks did not show the trunk disease symptoms, probably due to the treatment of the trunks in close proximity.
6. The *Trichoderma* strains, isolated from the woody tissues of the grapevine could efficiently colonise the plant tissues. In case of successful colonisation we can expect long-term positive effects (stimulation of grape growth, promoting of the different nutrient uptake, bioantagonistic and mycoparasitic effects against plant pathogens).

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7. LIST OF PUBLICATIONS RELATED TO THE DISSERTATION



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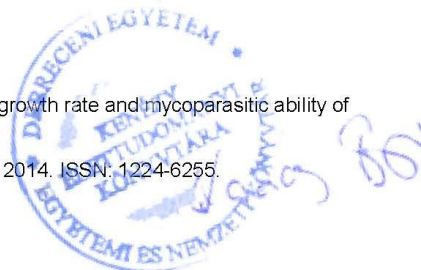
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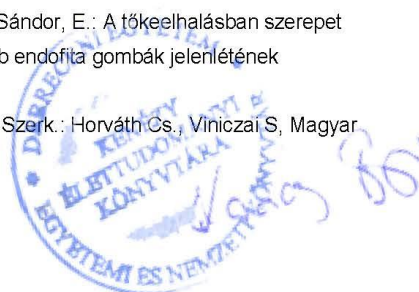
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