Incidence of grapevine trunk diseases is influenced by soil, topology and vineyard age, but not by *Diplodia seriata* infection rate in the Tokaj Wine Region, Hungary

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Abstract

Grapevine Trunk Diseases (GTD) are of great importance worldwide, including Hungary, a Center European country with long wine producing history. Several GTD pathogens have been described till now in Europe, but only a few from Hungary. The presence of a GTD pathogen in the vine does not necessarily result in the immediate appearance of disease symptoms, and information on the importance of environmental factors related to disease incidence are still limited. The aim of this research was to assess the occurrence of GTD in the Tokaj Wine Region, and to determine the biotic and abiotic factors influencing disease incidence. Five vineyards within 15 km radius – each with different topology, soil types, varieties and age – were studied for three consecutive years (2013 – 2015). The incidence of GTD-infection was determined every year for each vineyard. *Diplodia seriata* was isolated with incidence ranging from 50 to 100 %, while *Diaporthe* spp. were the only other – minor – GTD pathogen found. Topology and soil type appeared to be major abiotic factors affecting incidence of GTD symptom. Disease incidence was also positively correlating with the age of the vineyards, and it was in fact found to be the definitive biotic factor regarding incidence. In contrast, *D. seriata* infection rate appeared unrelated to disease incidence or to any of the biotic or abiotic factors investigated.

Key words: disease incidence, Furmint, Hárslevelű, *Diplodia seriata*

Introduction

Grapevine trunk diseases (GTDs) restrict the productivity and longevity of vineyards, causing significant economic losses (Scheck et al. 1998; Siebert 2001). Eutypa, Botryosphaeria, Phomopsis dieback, Esca disease complex, and Petri 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; Larignon and Dubos 1997; Rolshausen et al. 2004; Úrbez-Torres et al. 2006; Kotze et al. 2011; Úrbez-Torres 2011; Bertsch et al. 2012; Úrbez-Torres et al. 2013; Úrbez-Torres et al. 2014; Fontaine et al. 2015).

Grapes are the largest fruit crop in Hungary, with high economic value (Hungarian Central Statistical Office, 2014). Moreover there is also great cultural importance in the historic Tokaj Wine Region, which is listed on the UNESCO World Heritage since 2002 as a producer of the world's oldest botrytized "aszú" wines. The actual size of cultivated areas in the Tokaj Wine Region is 5050 hectares, growing almost inclusively indigenous varieties (Furmint, Hárslevelű, Sárgamuskotály (yellow muscat), Zéta, Kövérszőlő, Kabar) (Hungarian Central Statistical Office, 2014). GTDs have significant effects on the profitability of grape production in the Tokaj Wine Region of Hungary, causing almost 110000 USD (32 million HUF) in losses annually (Bihari et al. 2015).

Hewitt et al. (1957) observed that specific symptoms cannot be detected on the diseased trunks every year. Latest results have also proved that abiotic factors affect the appearance and the severity of the disease (Lecomte et al. 2011). GTDs presents in different forms: (i) slow but chronic disease development over many years (grapevine leaf stripe disease, young esca or 'Phaeotracheomycosis of grapevine'), or (ii) rapid, acute disease development (apoplexy, esca proper) within one season (Surico et al. 2006). GTDs are caused by many different Ascomycete fungi, including Diatrypaceaous and Botryosphaeriaceous species, *Phomopsis* species, *Phaeomoniella* and *Phaeoacremonium* species, as well as Basidiomycetous fungi such as *Fomitiporia mediterranea* (Kuntzmann et al. 2010; Kotze et al. 2011; Bertsch et al. 2012; Úrbez-Torres et al. 2014). GTD incidence has increased during the last few decades (Úrbez-Torres et al. 2014). Esca incidence has reached 60% to 80% in some old vineyards in

southern Italy (Pollastro et al. 2000; Sidoti et al. 2000; Surico et al. 2000; Romanazzi et al. 2006; Calzarano and Di Marco 2007). 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 development of the disease (Lecomte et al.; 2011; Bertsch et al. 2012).

GTDs have been reported previously in Hungary. Lehoczky (1974) described the black dead arm disease of grapevine, which is caused by *Botryosphaeria stevensii* (R. A. Schoemaker), in the Tokaj Wine Region. Later *Phaeoacremonium hungarium* (S. Essakhi, L. Mugnai, G. Surico & P. W. Crous (Pers.)) was identified as a new *Phaeoacremonium* species in the Tokaj Wine Region (Essakhi et al. 2008).The disease incidence of the esca was reported to increase from 2% to almost 13%, between 2003 and 2007 in Hungary (Dula 2011). However, there is limited information about the current extent and distribution of GTD in Hungary, especially in the Tokaj Wine Region.

The effective control of GTD faces several problems. Although the susceptibility of the different cultivars are different (Marchi 2011; Romanazzi et al. 2009; Andreini et al. 2014; Murolo and Romanazzi 2014) and the rootstock may also affect the frequency of the symptoms (Marchi 2011; Murolo and Romanazzi 2014) there is no grapevine cultivar known to be resistant to GTD. Once GTD pathogens have entered the woody tissues, they proliferating inside the vine, where fungicides may have difficulty reaching them. Sodium arsenite, the previously used most effective chemical (Mugnai et al. 1999), was banned because of its human carcinogenic, and environmental toxic properties (Kuntzmann et al. 2010). Prevention of planting material (Gramaje et al. 2015) and appropriate protection of pruning wounds (Eskalen et al. 2007; Sosnowski et al. 2008; Rolshausen et al. 2010; Kotze et al. 2011; Sosnowski et al. 2013) are the most important techniques to prevent disease. The appropriate time for pruning by avoiding spore dispersal periods (Petzoldt et al. 1983; Rooney-Latham et al. 2005; Eskalen et al. 2007; Úrbez-Torres and Gubler 2008; Rolshausen et al. 2010; Fontaine et al. 2015) and pathogen free planting materials (Billones-Baaijens et al. 2015) may also prevent infection and decrease disease incidence.

The aims of this research were (i) to investigate the incidence of GTD in different vineyards of the Tokaj Wine Region, Hungary; and (ii) to identify possible biotic (age, variety, endophytic fungi) and abiotic (plantation characteristics, soil, weather conditions, vintage) factors affecting disease incidence.

Material and Methods

Vineyard characteristics and meteorological data

The survey was carried out in five vineyards (Bakonyi, Dorgó, Szemere, Szarvas and Várhegy) in the Tokaj Wine Region during 2013 and 2015, located close to each other, within 15 km (Fig. 1).

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 (Table 1.). The rootstock 125AA was used only in Várhegy for the variety Zéta (*Vitis vinifera*), and Teleki 5C was used in all the other vineyards with varieties Furmint and Hárslevelű (*V. vinifera*). There was also difference on the direction of the rows on the slopes, as the Bakonyi vineyard is teracced, while the other vineyards are on steep slopes. All vineyards had only one variety, except 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 draw 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.

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 ratio of the number of plants expressing foliar symptoms with wood necrosis or dieback in each vineyard over the number of all vines and multiplying by 100. Samples were collected from each vineyard 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. Samples were taken from all symptomatic plants for isolation. Moreover representative random sampling was performed to determine the infection rate of *D. seriata* in each vineyard. A minimum of three samples were collecting from each row for this representative sampling. We sampled at least 3% of vines in each vineyard except Bakonyi, where all plant were analyzed.

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.

Isolation of fungi from woody tissues

We established the infection rate (IR) (percentage of infected vines in the sample) of *D. seriata* and *Diaporthe* sp. to characterize and compare the infection level in vineyards on the basis of microbiological analysis made in 2015. Wood chips from debarked and surface sterilized (10% Noemagnol 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. (2014). 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.

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 streptomycine sulfate (PDA, Scharlau, Spain) and conidia (color, shape, size) or conidiophores were examined under microscope. The morphological identification of *Diplodia seriata* were based on Crous (2006), and *Phomopsis* species on Mostert et al. (2001) and Van Niekerk et al. (2005).

Molecular identification with sequence analysis of the Internal Transcribed Spacer (ITS) region of the rDNA was performed for isolates whose sporulation was not observed. Randomly chosen isolates from each morphological group were also sequenced.

Fungal DNA was isolated from fresh mycelium scraped at the surface of PDA described previously (Assadollahi et al. 2013). DNA concentrations were measured by NanoDrop 2000 (Thermo Scientific).

Amplifications of 25 µl PCR reaction containing 12.5 µl 2 X PCR Master Mix (GoTaq Green Master (Promega), 40-40 pmol of each primer, 20-40 ng of genomic DNA and nuclease free water were run. ITS4 and ITS5 primers (White et al. 1990) (Integrated DNA Technologies, Inc.) were used to amplify the full length of ITS region, with the following amplification protocol: 3 min initial denaturing at 95°C, followed by 5 cycles of 1 min at 95°C, 1 min annealing at 50°C, 1 min at 72°C and 25 cycles of 1 min at 90°C, 1 min annealing at 50°C, 1 min at 72°C and 15 min final extension at 72°C. Purified amplification products were sequenced by Microsynth Austria GmbH.
The sequences were manually aligned and compared with those deposited in the NCBI GenBank database using the BLAST program (Altschul et al. 1990). The ITS sequences were also submitted to the NCBI GenBank (www.ncbi.nlm.nih.gov) for the species identification and KU377167-KU377290 accession numbers were obtained.

Statistical analyses

Binominal tests were used to analyze deviation of the infection rate in the sites from the summarized proportion in the whole sample. The relationship between IR and DI was analyzed with correlation analysis. Beyond that the effect of location (seven studied site), age of plantations (below or above 15 years old in 2013), vintage (three studied years), topology (slope and horizontal), soil types (luvisols and cambisoils) and grapevine variety (Furmint and Hárslevelű) on the DI were analyzed. The analyses were made on the whole samples in each case except the Várhegy vineyard, where only Zéta variety is grown, so it was excluded from the comparison of different grapevine variety.
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 Kruskall-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 analyses were performed by SPSS 21.0 (Ketskeméty et al. 2011).

Results

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 2). Large areas of chlorosis and deterioration between the veins were observed on the leaves of the diseased plants together with small black spots on the cross section of the cordon. 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. 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)

193 and lower (5L) part of this site. The Dorgó vineyard also showed intermediate DI, while the Szemere and Várhegy
194 vineyards had the lowest mean DI (Fig. 2.).
195 The most frequently identified GTD pathogens from the upper part of the symptomatic grapevine woody tissues
196 was *D. seriata*, while *Diaporthe* sp. was identified with much lower rates from the 558 samples taken in 2015. The
197 mean infection rate (IR) of *D. seriata* was 76% with large variance among studied sites. Infection rate of each site
198 significantly differed from the mean value (Binomial test $p<0.05$). The mean IR of *Diaporthe* sp. was 4% without
199 significant deviation from this mean (Binomial test $p>0.05$) (Table 2). There was low correlation between IR and
200 DI ($r^2=0.4818$). The success of isolation was established in the ratio of infected plant to symptomatic plants (*D.*
201 *seriata*: 75.51%, 406/558 plants; *Diaporthe* sp.: 3.71% 19/558 plants; see details in Table 3).
202 *D. seriata* could be isolated from most of the symptomatic plants. Its isolation rate varied between 94 and 100%.
203 *Diaporthe* species were isolated with much lower frequencies, while other *Botryosphaeria* sp. were detected only
204 in one year (2013) from one vineyard (Bakonyi).
205 Non-GTD pathogen, endophytic fungi (*Trichoderma* sp., *Alternaria* sp., *Mucor* sp., *Penicillium* sp, *Epicoccum*
206 sp., *Fusarium* sp. and *Aspergillus* sp.) were also isolated with up to 100% isolation rates from symptomatic plants.
207 *Alternaria*, and *Fusarium* species were the most frequently isolated non-GTD pathogens isolated with 43-100%,
208 and 0-55% of the symptomatic plants.
209 Among the studied abiotic and biotic factors topology, soil type and age of the vineyard showed significant effect
210 on the disease incidence (DI) (Table 4). However variety and vintage (year) did not have a significant effect on
211 the DI. There was no significant difference on the average DI of the vineyards at the different vintages (Kruskall-
212 Wallis test: H=0.0967, df=2, n=21, p=0.953). DI was the highest in 2014 in Szemere and Szarvas (sloped), but
213 decreased during the three years in Dorgó, but did not change in Várhegy.
214 In case of topology, significantly lower DI was detected in vineyards planted on slopes (Dorgó,Szemere, Várhegy),
215 than in the terraced Bakonyi. This difference could be seen even within the same vineyard (Szemere) with different
216 topology (5S and 5L, see above in the text and Fig. 2. and Table 1.). Plantations on luvisols soil also had
217 significantly lower DI than on the cambisols with similar differences in mean values to that seen with topology
218 (Table 4).
219 Although in the Bakonyi vineyard there was a large difference in mean DI between two varieties Furmint (4F) and
220 Hárslevelű (4H) (Fig 2). However DI of Furmint and Hárslevelű varieties was not statistically different across all
221 vineyards with different age. There were no differences in the DI between years either. Because of the relatively
222 short study period, the trends in temporal changes of DI could not been analysed but different tendencies could be
223 observed in the different vineyards (Table 1. The Várhegy vineyard which was planted with the variety Zéta on
224 rootstock 125AA, had the lowest disease incidence (0.17%) of all those examined (Table 1., Fig. 2.).
225 The age of vineyard had an effect, with DI in the older vineyards (over 15 years) were significantly higher,
226 comparing to the younger ones (under 15 years) (Table 4.).
227 Neither studied abiotic or biotic factors, nor DI showed significant effect on *D. seriata* infection rate of the
228 vineyards. Similarly no correlation was found between isolation rate of *D. seriata* from symptomatic plants and
229 any of the studied factors.
230 Only eight symptomatic plants could be detected in the Várhegy vineyard during the three years of the study, with
231 one vine expressing symptoms over two years, but none with continuous disease incidence was observed (Table
232 5). Therefore it was excluded from the statistical analysis of the symptom stability. The ratio of occasional disease

incidence was significantly (Mann-Whitney U test, p=0.0244) higher in younger vineyards (younger than 15 years at 2013), than stable or continuous disease incidence (Figure 3.).

Discussion

Grapevine trunk diseases are the most threatening problem to wine industries with increasing disease incidence worldwide (Bertsch et al. 2012; Úrbez-Torres et al. 2013). It is a complex disease with unique characteristics, infection can be latent without visible disease symptoms for years (Lehoczky 1974; Cristinzio 1978; Phillips 2002; Auger et al. 2004; Marchi et al. 2006; Van Niekerk et al. 2006; Savocchia et al. 2007; Rego et al. 2008), but the factors affecting the disease appearance are still only suspected. The influence of the weather conditions has been reported may affect symptom appearance. Increased disease incidence was reported in case of irrigation (Bertsch et al. 2012) or high amount of precipitation (Lehoczky 1974; Hewitt 1988). Bruno et al. (2007); Marchi et al (2006) and Andolfi et al. (2009) explained that increase with facilitated transport of the fungal toxins responsible for foliar symptoms. Moreover Surico et al. (2006) and Bertsch et al. (2012) reported increased disease appearance when drought weather was followed by rainy period. Sosnowski et al. (2007) reported that both temperature and rainfall were related to eutypa dieback symptom development. Different biotic factors, like variety (Marchi 2011; Maher et al. 2012; Murolo and Romanazzi 2014), rootstock type (Marchi 2011; Murolo and Romanazzi 2014) age of the plant (Mugnai et al. 1999) was also indicated may affect the disease incidence.

The detection rate of the reported GTD pathogen *D. seriata* was high, but variable (50 – 100 %) in the vineyards when both symptomatic and asymptomatic plants were included in the test. This infection rate was similar to previously reported isolation rate of GTD pathogens, particularly *D. seriata* in other studies from symptomatic vines (Bruez et al. 2014). It was concluded that infection rate did not affect symptom appearance, expressed as disease incidence rate in this study. It must be mentioned, however that only the upper part was sampled for fungal isolation, therefore GTD pathogens that may exist in the lower parts of the vine were not included.

There was significant difference in the disease incidence of GTD at the different vineyards. DI was much higher in the Bakonyi vineyard, than in Dorgó vineyard with similar characteristics except soil type and topology. The Bakonyi vineyard is terraced, whereas the other examined vineyards with significantly lower disease incidence were planted with rows on the slopes. The difference of the DI could be detected even within the same vineyard (Szemere) with different topology. The DI was significantly higher on horizontal (5L) part, than on 5-8% slope (5S). Surico et al. (2000), and Robotic and Bosancik (2007) similarly found higher disease incidence in vineyards on gentler slope, than on sleep ones.

Soil type was the other tested abiotic factor for GTD. Szemere, Dorgó, Várhegy vineyards were on luvisols, while vineyards Bakonyi and Szarvas on cambisols. DI of the vineyards on cambisols was significantly higher, comparing with the other soil type. Cambisol is considered as a soil with high water capacity (Rhoton and Markewich 2006). Soil with high water reserve was reported to enhance esca symptoms (Guérin-Dubrana et al. 2005), and DI of GTD (Kovács et al. 2016).

Regarding the varieties, higher disease incidence was observed on Furmint, than on Hárslevelű at Bakonyi vineyard in each year. However Furmint had lower disease incidence in the other two younger (age > 15 years) vineyards (Szemere and Dorgó), than in Szarvas vineyard planted later (age < 15 years) with Hárslevelű variety (Table 2, Fig. 2). Interestingly, Várhegy vineyard 3 had the lowest disease incidence (0.17%), among the examined ones. It had unique characteristics regarding variety (Zéta) and rootstock (125AA), comparing to the others. It was

among the youngest examined plantation (12 years old in 2013), and its disease incidence was lower, than Szemere vineyard with the same age.

Although the weather conditions were different in the three years of the study, neither temperature nor amount of precipitation resulted differences of the DI The trends of the DI changes were different in vineyards on slope (1-3 and 5S), however vineyards 4F, 4H, and 5L, with horizontal topology showed an increasing DI between 2013 and 2015. There was significant difference in DI of 5L and 5S (different topology) at 2013, and 2015, while the difference in the GTD symptom expression has disappeared at 2014, when higher amount of precipitation was observed during the summer (green berries growth) period on the slopes as well (Table 2).

The majority of vines expressed disease symptoms in only one of the three years. The average disease incidence rate, regarding the individual vineyards was also the highest (62.42%) for the plants expressing symptoms only once. Occasional disease incidence ranged above 50% (55.17-80%) in all vineyards, except the 4H (Bakonyi with Hárslevelű variety), where stable disease expression occurred at 68%. Interestingly, not this vineyard had the highest DI value.

It was concluded, that topology and soil type, and were the most important abiotic factors for disease incidence (DI) of the GTD symptom appearance expressed by foliar symptoms or dieback. The higher DI may have resulted by increased pathogen activity (growth or toxin production), or facilitated toxin transport (Bruno et al 2007; Marchi et al. 2006). Among the biotic factors, age of the vineyards had the highest detectable impact for the DI, resulting a significantly higher rate of diseased plants in the vineyards with older plants. It also may have caused by several factors, e.g. cumulated infection of the different GTD pathogens, moreover the change of the plant physiology and plant resistance, or the endophytic microbiota. Further studies are necessary to explain the role of the soil type, topology in the increase of the diseased plants in the vineyards.

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Legend for figures

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

Fig. 2 Mean disease incidence (DI% ±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 Fig 1.

Fig. 3 Ratio of different disease stability categories (mean ±SE/SD) in the studied sampling sites with different age in the Tokaj Wine Region, Hungary, between 2013 and2015.