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Incidence of symptoms and fungal pathogens associated with grapevine trunk diseases in Czech vineyards: first example from a north-eastern European grape-growing region

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Summary. Grapevine trunk diseases (GTD) are one of the most important constraints for grapevine and wine production worldwide. Most of the published studies on these diseases in Europe relate to the major vine growing regions mainly located in southern Europe. This is the first study of GTD incidence and associated fungal pathogens in Czech vineyards, as an example of the north-eastern European grape-growing region. Annual incidence of GTD symptoms in vineyards of cvs. ‘Chardonnay’ and ‘Cabernet Sauvignon’ in three Czech locations was surveyed. On average, 3.7% of the ‘Chardonnay’ plants showed GTD symptoms and 0.7% suddenly collapsed each year. Within the ‘Cabernet Sauvignon’ vineyards, 10.1% of the plants showed GTD symptoms and 2.5% suddenly collapsed. Several fungal pathogens were associated with the suddenly collapsed grapevines, and the most frequent was *Phaeoconiella chlamydospora* (77% of analyzed grapevines), followed by *Eutypa lata* (62%), *Diaporthe ampelina* (31%), *Botryosphaeria dothidea* (15%) and *Fomitiporia mediterranea* (15%).

Key words: *esca*, *Eutypa lata*, *Phaeoconiella chlamydospora*, north-east Europe.

Introduction

Grapevine trunk diseases (GTDs) induce decline in grapevine vigour that usually leads to death of the plants. Current data show that GTD incidence increases steadily in the main grape growing regions worldwide. For example, 64% of ‘Sauvignon Blanc’ vines in California are reported to be infected with GTD caused by *Botryosphaeriaceae* (Gubler *et al.*, 2005), close to 60% of vineyards surveyed in Tuscany report GTD symptoms with incidence ranging from 20 to 40% (Cortesi *et al.*, 2000), and the average annual increase of incidence is estimated to be 45% (Mugnai

et al., 1999). Similar observations have been made in Spain, France, Greece and Portugal (Rego *et al.*, 2000; Armengol *et al.*, 2001; Redondo *et al.*, 2001; Rumbos and Rumbou, 2001; Bruetz *et al.*, 2013). For these reasons GTDs have been considered the most destructive diseases of grapevine for the past three decades, and some sources compare their negative impacts with the outbreaks of *Phylloxera* at the end of 19th century (Smart 2013).

Dactylonectria torresensis, *Eutypa lata*, *Phaeoconiella chlamydospora*, *Phaeoacremonium minimum*, *Diaporthe ampelina* (syn. *Phomopsis viticola*), *Fomitiporia mediterranea* and several *Botryosphaeriaceae* species are considered to be the major pathogens causing GTDs (Graniti *et al.*, 2000; Urbez-Torres 2011; Bertsch *et al.*, 2013; Gramaje *et al.*, 2018). Symptoms are vari-

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able and inconsistent, but generally involve wood and leaf necrosis as well as poor growth and/or establishment of vines, and in acute cases, vine death (Graniti *et al.*, 2000; Mostert *et al.*, 2006a; Lecomte *et al.*, 2012). Individual roles of GTD pathogens and the interactions between them and with other microorganisms, together with the role of weather factors in disease development, have not been fully determined.

The common situation in GTD-affected vineyards is that the proportions of symptomatic plants can vary from one year to the next (Marchi *et al.*, 2006; Di Marco and Osti, 2009; Calzarano *et al.*, 2018). The greatest economic damage results from decreased grape yields from symptomatic vines, and from death of severely affected plants and the associated need for replanting (Calzarano *et al.*, 2014). External factors are likely to induce increased manifestation of symptoms and plant death (Surico *et al.*, 2004; Calzarano *et al.*, 2018). Due to the characteristics of GTD infections, where fungal pathogens gradually colonise vascular tissues, it has been suggested that meteorological factors play important roles by influencing water balance in plants (Surico *et al.*, 2000).

Most of the published studies on GTDs in Europe have originated from southern countries such as Spain, France, Greece, Portugal and Italy. In contrast, information about the situation in northerly located wine regions is less extensive. For example, few studies have described the situation in Germany (e.g., Fischer and Kassemeyer, 2003), while there is no available information from the Czech Republic and Slovakia on GTD incidence. Information from northern European areas will add to the complex picture of GTD spread. Moreover, relevant local information can indicate the economic losses in this region, which will highlight the importance of the GTD complex. Comprehensive characterization of fungal pathogens associated with GTDs in the Czech wine-producing region is also necessary for winegrowers to adjust their cultural practices and control measures to decrease the economic impacts of these diseases.

The objectives of the present study were firstly, to determine the prevalence of GTDs in selected Czech vineyards as indicated in three consecutive annual field surveys, and secondly, to identify fungal pathogens present in suddenly collapsed grapevine plants.

Materials and methods

Vineyard surveys

Six 'Cabernet Sauvignon' and 'Chardonnay' vineyards in the Moravia wine region of the Czech Republic were selected for the surveys. This region is the most important grape-producing region of the country, comprising 95 % of the production from the Czech Republic. Vineyard details are presented in Table 1. The vineyards were of similar age, from 17 to 19 years. The two cultivars were selected because they are grown worldwide and are useful reference for other studies. Vineyards were located in three different sub-regions of Moravia (two vineyards per sub-region), and are about 40 km apart.

Evaluation of symptoms occurrence

The selected vineyards were monitored for GTD symptom expression in the 2014, 2015 and 2016 growing seasons. Symptoms were evaluated each year at the end of flowering (end of June) and at fruit ripening (mid-September). Observations focused on characteristic symptoms of GTD infection, including weak and stunted growth of annual shoots and shortened internodes (typical for *Eutypa lata*), unusual shape of leaves (wrinkled, ripped, small), which are also typical signs of *Eutypa lata* infections, tiger-stripe discolouration of leaves (typical for esca), and shrivelled shoots. At fruit ripening, plants in each vineyard showing symptoms of sudden collapse were also recorded. On the basis of these observations the numbers of plants with GTD symptoms and their mortality in given years were assessed. The differences between the numbers of recorded symptomatic or collapsed plants in individual years were also tested for their statistical significance by using STATISTICA 12 software (StatSoft, Inc.).

Isolation and identification of fungi from severely symptomatic plants

Thirteen severely symptomatic plants showing typical GTD symptoms during the 2016 season were selected from different surveyed vineyards for pathogen isolation and identification. Seven of these plants already showed strong decline (stunted growth of the shoots, shortened internodes) in the spring of 2016. The other six plants collapsed suddenly during late summer of 2016.

Table 1. Details of vineyards surveyed for grapevine trunk diseases in the Czech Republic.

Vineyard	Sub-region	Town	Cultivar	Date planted	Site features
CH1	V. Pavlovice	Kobylí	Chardonnay	2001	260 MASL* southeast slope gravely sandy soil
CS1	V. Pavlovice	Kobylí	Cabernet Sauvignon	2000	225 MASL southeast slope gravel on sandstone
CH2	Mikulov	Perná	Chardonnay	1997	250 MASL southeast slope limestone cliff / clay-loam soil
CS2	Mikulov	Perná	Cabernet Sauvignon	1998	290 MASL western slope limestone hill, clay-loam soil
CH3	Znojmo	Vrbovec	Chardonnay	1998	230 MASL slight south slope clay loam soil on loess
CS3	Znojmo	Vrbovec	Cabernet Sauvignon	1998	235 MASL slight south slope clay loam soil on loess

*MASL = meters above sea level.

Six trunk segments, each 2 cm thick, were cut from each symptomatic plant. Two segments were from the trunk base, two from the middle and two from the upper part. The segments were debarked, washed with distilled water, disinfected for 1 min. in sodium hypochlorite (2% solution) and washed twice with sterile distilled water. From each segment eight pieces of necrotic or discoloured woody tissue were plated in two Petri dishes containing malt extract agar (MEA, Sigma-Aldrich) supplemented with 0.5 g L⁻¹ of streptomycin sulphate (Biosynth). The plates were incubated in darkness for 10–20 d at 25°C. Hyphal tips of fungi growing from the tissues were subcultured onto potato dextrose agar (PDA, Sigma-Aldrich), and were maintained under the same conditions.

Total DNA was extracted from 20 mg of mycelium from PDA subcultures with the NucleoSpin Tissue kit (Macherey-Nagel) according to the manufacturer's instructions. The ITS2 region was amplified with primers ITS3 and ITS4 (White *et al.*, 1990), and part of the actin gene was amplified with primers ACT-512F and ACT-783R (Carbone and Kohn, 1999). PCR conditions were those described by Eichmeier *et al.* (2016). PCR products corresponding to approx. 330 bp for ITS2 and 270 bp for actin (depending on fungal

species) were sequenced as described by Eichmeier *et al.* (2010). Sequences were analysed using CLC Main Workbench 5.0 (CLC bio) and deposited in GenBank/NCBI. Identification of isolates proposed by CLC Main Workbench were subsequently confirmed by BLASTN searches within GenBank/NCBI, using holotype or ex-holotype isolates of respective GTD pathogens as reference.

Results

Evaluation of GTD incidence in vineyards

Appearance of GTD symptoms in vineyards in individual years is summarized in Table 2. The mean annual incidence of symptoms in 'Chardonnay' was 3.7%, and 0.7% of plants suddenly collapsed each year. Greater incidence was recorded in 'Cabernet Sauvignon', where 10.1% of the plants showed symptoms while the mean annual incidence of suddenly collapsed plants was 2.5%.

All symptoms registered in spring corresponded to those commonly associated with *Eutypa dieback* (weak and stunted growth of annual shoots and shortened internodes). Symptoms typical for *Eutypa*

Table 2. Proportions of vines with grapevine trunk disease (GTD) symptoms in six Moravian vineyards during three growing seasons (2014, 2015 and 2016).

Vineyard	No. of plants per vineyard (2014)	Mean percentages of vines with symptoms of GTD ^a / <i>Eutypa</i> dieback ^b / collapsed plant ^c		
		2014	2015	2016
CH1	770	0.00/0.00/0.00	0.00/0.00/0.00	0.52/0.00/0.13
CS1	610	6.23/0.49/0.82	4.59/0.82/3.11	3.61/0.49/2.30
CH2	544	3.13/0.55/0.55	3.49/1.84/1.65	4.60/0.18/1.29
CS2	435	5.52/4.14/1.84	14.94/5.75/5.52	13.56/2.76/1.84
CH3	542	4.98/0.55/0.00	4.43/1.85/0.55	16.79/5.90/2.77
CS3	535	12.34/0.56/0.93	11.03/2.43/4.49	19.81/4.86/3.36

^a GTD is the sum of symptomatic plants registered in spring (corresponds to *Eutypa* dieback cases) and late summer of respective years.

^b Values correspond to spring GTD incidence because all registered symptoms corresponded to *Eutypa* dieback.

^c Values represent plants showing collapse at the late summer assessment.

dieback were registered in 24% of all vines recorded with GTDs. The remaining 76% of GTD cases, representing 5% of the plants in the surveyed vineyards, were accompanied by symptoms other than *Eutypa* dieback. The symptoms predominantly appeared in late summer, mainly as tiger-stripe symptoms on leaves.

The year-on-year differences between numbers of symptomatic or collapsed plants in individual vineyards are indicated in the Table 2. After calculating averages of these data for individual years, a statistical analysis was also made to determine differences between the total numbers of symptomatic and collapsed plants in individual years. No statistically significant difference was found between numbers of symptomatic plants in individual survey years (data are not shown). However, there were significant increases ($P \leq 0.05$) in the mean values of collapsed plants between 2014 (mean = 0.69%) and 2015 (2.53%) or 2016 (2.00%). Differences between numbers of collapsed plants in 2015 and 2016 were not statistically significant ($P > 0.05$). The assumption that the number of collapsed plants in individual years was proportional to the number of symptomatic plants was also tested by comparing their ratios achieved in individual years of survey. Significant differences ($P \leq 0.05$) in the ratios of collapsed to symptomatic plants was found for 2014 results (0.14) compared with 2015 (0.41) and 2016 (0.27). Differences between the 2015

and 2016 ratios were not statistically significant ($P > 0.05$). This indicates that factors causing plant collapses were weakly expressed in 2014 compared with 2015 and 2016.

Fungi associated with severely symptomatic plants in the evaluated vineyards

Seventy-three fungal colonies were obtained from trunk tissues of 13 collapsed plants. Twenty-four isolates (the 33% of total) were identified as *P. chlamydospora*. The second most frequently isolated fungus was *E. lata* (23 isolates, 32%), followed by *B. dothidea* (7%), *F. mediterranea* (4%) and *D. ampelina* (18% of samples) (see Tables 3 and 4). The most prevalent GTD fungus in the analysed plants was *P. chlamydospora*, which was isolated from 77% of the grapevines, followed by *E. lata* (62%), *D. ampelina* (31%), *B. dothidea* (15%) and *F. mediterranea* (15%). One isolate of *Cytospora* sp. (1% of total isolates), newly considered as part of grapevine trunk-disease complex, was also found. Sequences of ITS2 regions originating from isolates assessed in this study are available in GenBank/NCBI as follows: KY270963, KY270964, KY270965 and KY270966 for *D. ampelina*; KY270983 and KY270984 for *F. mediterranea*; KY270985 and KY270986 for *B. dothidea*; KY270967, KY270968, KY270969, KY270970, KY270971 and KY270972 for *E. lata*, and KY270973, KY270974,

Table 3. Fungi isolated from trunk woody tissues of grapevines severely affected by grapevine trunk diseases in spring 2016.

Vine No.	Cultivar/vineyard	Fungal species ^a
1	Cabernet Sauvignon – CS2	<i>Diaporthe ampelina</i> (2) <i>Eutypa lata</i> (6) <i>Fomitiporia mediterranea</i> (2) <i>Phaeomoniella chlamydospora</i> (3)
2	Cabernet Sauvignon – CS1	<i>Botryosphaeria dothidea</i> (2) <i>Eutypa lata</i> (3) <i>Phaeomoniella chlamydospora</i> (2)
3	Chardonnay – CH3	<i>Botryosphaeria dothidea</i> (3) <i>Diaporthe</i> sp. (1) <i>Eutypa lata</i> (1) <i>Phaeomoniella chlamydospora</i> (1) <i>Tumularia</i> sp. (1)
4	Cabernet Sauvignon – CS2	<i>Coniothyrium</i> sp. (1) <i>Eutypa lata</i> (4) <i>Neofabraea kienholzii</i> (1) <i>Phaeomoniella chlamydospora</i> (2)
5	Cabernet Sauvignon – CS3	<i>Eutypa lata</i> (1) <i>Phaeomoniella chlamydospora</i> (3)
6	Cabernet Sauvignon – CS3	<i>Diaporthe ampelina</i> (9) <i>Eutypa lata</i> (1)
7	Chardonnay – CS1	<i>Eutypa lata</i> (4)

^a Number of isolates in parentheses.

KY270975, KY270976, KY270977, KY270978, KY270979, KY270980, KY270981, and KY270982 for *P. chlamydospora*. Other fungi, generally not considered as grapevine trunk pathogens, were also isolated, including *Tumularia* sp. (three isolates from two plants), *Coniothyrium* sp. (one isolate), *Neofabraea* sp. (one isolate) and *Cryptococcus* sp. (one isolate). Details are shown in Tables 3 and 4, and Figure 1.

The spectrum of fungal species detected within plants showing strong decline in the spring of 2016 and those which suddenly collapsed by the autumn of 2016 are shown in Figure 1. *Eutypa lata* prevailed in the plants that showed strong symptoms in spring. This fungus was isolated from all plants already

showing strong symptoms in the spring. In the plants that collapsed in late summer, *P. chlamydospora* predominated, but other fungi from the GTD complex, including *F. mediterranea* and *D. ampelina*, were also found.

Discussion

This study is the first survey of grapevines affected by GTDs in the Czech Republic, to determine prevalence of these diseases in vineyards and the fungal pathogens present in affected plants. In the three years of the surveys, averages of 4% of 'Chardonnay' plants and 10% of 'Cabernet Sauvignon' plants with

Table 4. Fungi isolated from grapevine trunk woody tissues of collapsed vines in late summer 2016.

Vine No.	Cultivar/vineyard	Fungal species ^a
8	Cabernet Sauvignon – CS1	<i>Eutypa lata</i> (3)
9	Chardonnay – CH3	<i>Phaeomoniella chlamydospora</i> (3) <i>Fomitiporia mediterranea</i> (1) <i>Cytospora</i> sp. (1)
10	Cabernet Sauvignon – CS3	<i>Phaeomoniella chlamydospora</i> (2) <i>Diaporthe ampelina</i> (1)
11	Cabernet Sauvignon – CS2	<i>Phaeomoniella chlamydospora</i> (2)
12	Chardonnay – CH1	<i>Phaeomoniella chlamydospora</i> (1) <i>Cryptococcus</i> sp. (1)
13	Cabernet Sauvignon – CS2	<i>Phaeomoniella chlamydospora</i> (5)

^a Number of isolates in parentheses.

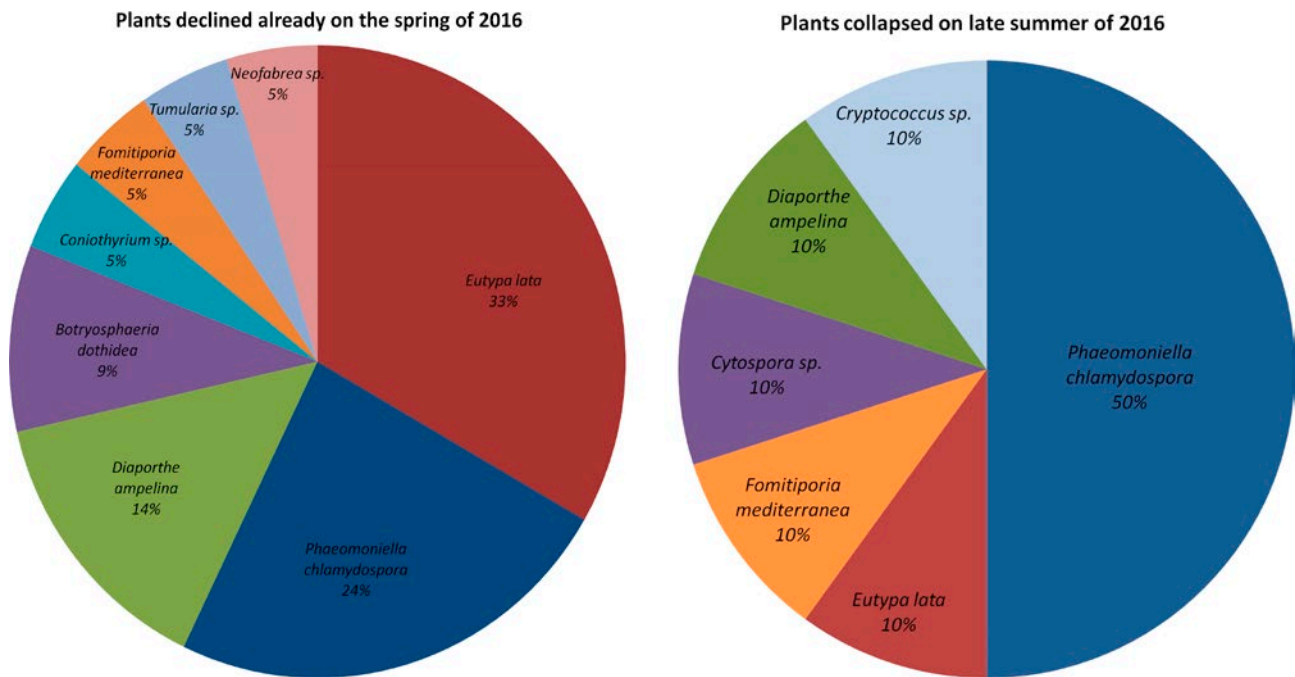


Figure 1. The proportions of fungal taxa isolated from grapevine plants showing symptoms of decline in the spring of 2016 (from seven plants, a total of 21 fungal taxa were identified; repeated occurrences of individual taxa in more plants were also counted), and grapevines that collapsed in the late summer of 2016 (in six plants, a total of ten fungal taxa were identified; repeated occurrences of individual taxa in more plants were also counted).

symptoms of the disease were recorded. Quaglia *et al.* (2009) in a 2-year evaluation of vineyards in central Italy reported incidence of GTD symptoms on 'Cabernet Sauvignon' greater than 50% and approx. 15% for 'Chardonnay'. In another study in Italy, Marchi *et al.* (2006) reported 10–25% incidence of GTDs in 'Cabernet Sauvignon' vineyards, depending on region and vintage year. Greater incidence than in the present was also described by Andreini *et al.* (2014), where incidence of symptoms in vineyards in Tuscany (Italy) over 6 years averaged 45% for 'Cabernet Sauvignon' and 8% for 'Chardonnay'. These studies were made on for emerging GTD situations in the surveyed regions, so these incidence levels probably do not reflect the situation throughout Italy. Results from an extensive survey in France (Bruez *et al.*, 2013) showed similar incidence of GTD symptoms to that in Czech vineyards. For 'Chardonnay' vines in the Yonne department of the Bourgogne region, 4% had GTD symptoms and 1% of 'Chardonnay' vines in the Côte d'Or in the same region were similarly affected.

There are likely to be several factors that influence the different rates of GTD incidence in these different regions, with lower incidence recorded in northern areas of Europe than those in the south. Confirmation of this difference requires much more comprehensive comparison of data from different regions.

Another relevant aspect arising from our field surveys was that numbers of symptomatic plants do not necessarily increase year on year; in some cases there was a noticeable decrease in year-to-year comparisons. Examples are comparison of the situation in vineyards CS1 in 2014, 2015 and 2016, CH3 in 2014 and 2015 and CS3 in 2014 and 2015. For disease incidences obtained after the subtraction of the *Eutypa dieback* cases from the total number of GTD, which correspond to incidence of esca, we confirm the phenomenon of "hidden esca" described by Marchi *et al.* (2006). This is where diseased vines do not show symptoms in a particular year, leading to the erratic frequency of esca symptom expression in different years.

Within individual years of the survey the ratios between symptomatic and collapsed plants were different. This indicates that the number of suddenly collapsed plants was not influenced by the numbers of symptomatic plants during the season. Annual fluctuations have been previously indicated in most of the few studies reporting annual comparisons of incidence of GTD symptoms (Marchi *et al.*, 2006; Qua-

glia *et al.*, 2009; Bruez *et al.*, 2013; Di Gennaro *et al.*, 2016; Calzarano *et al.*, 2016; 2017a; 2017b). Different hypotheses have been proposed to explain this phenomenon, but none have yet been fully confirmed by pivotal study. One possible explanation is through meteorological effects and specifics for individual years, but very few studies have focused on this aspect (Surico *et al.*, 2000; Marchi *et al.*, 2006; Andreini *et al.*, 2014). For visual symptoms, Marchi *et al.* (2006) and Calzarano *et al.* (2018) considered that cool growing seasons favoured their expression, while dry seasons resulted in low proportions of vines showing symptoms. Furthermore, the only study also considering with incidence of dead plants (Surico *et al.*, 2000) assumed that the acute form of esca appears in summer accompanied by water stress and hot weather, whereas cool rainy summers favour the development of chronic esca.

The most prevalent fungus isolated from grapevines in this field survey was *P. chlamydospora*, which has been frequently reported to be associated with esca in mature grapevines (Larignon and Dubos, 1997; Scheck *et al.*, 1998; Khan *et al.*, 2000; Mostert *et al.*, 2006b; Eskalen *et al.*, 2007), and with Petri disease in young (<7-year-old) grapevines (Díaz and Latorre, 2014).

Eutypa dieback caused by *E. lata* is also one of the most common grapevine trunk pathogens. This fungus was detected in 62% of all analyzed grapevines, but in 100% of plants showing severe decline in spring (stunted growth of the shoots and leaves, shortened internodes). These observations are in accordance with the known characteristics of *Eutypa dieback* symptoms, and their timing, in grapevines infected by *E. lata* (Trouillas *et al.*, 2010; Rolshausen *et al.*, 2014). Strong representation of *E. lata* in the regional complex of GTDs has also been reported in other studies. For example, Bruez *et al.* (2013) showed that in Charantes (one of the most important wine-producing region in France), incidence of *Eutypa dieback* outweighed that for esca symptomatic plants.

Diaporthe ampelina was detected in 31% of the analysed grapevines. Typical symptoms caused by this fungus are dead arms (Mostert *et al.*, 2001; Baumgartner *et al.*, 2012; Morales-Cruz *et al.*, 2015), and this symptom has been very frequently recorded, and was often noticeable in Czech vineyards.

From the species most commonly associated with GTDs, we also found a basidiomycete *F. mediterranea*, a causal agent of the trunk soft rot of the trunk. This

fugus was detected in 15% of analysed plants. Eichmeier *et al.* (2016) also described *F. mediterranea* from rootstock mother plants in Czech Republic.

Botryosphaeria dothidea is the causal agent of cankers in many trees and shrubs (Phillips 1998; Castillo-Pando *et al.*, 2001; Taylor *et al.*, 2005). While this fungus is best known as a pathogen, it has also been identified as an endophyte, existing in association with symptomless plant tissues (Pérez *et al.*, 2010). This pathogen belongs to the group causing *Botryosphaeria dieback* (Úrbez-Torres 2011), and was detected in only 15% of samples. In contrast, the survey of two viticulture regions in the USA showed significant occurrence of *B. dothidea* in local plants, at amounts greater than *P. chlamydospora* (Úrbez-Torres *et al.*, 2012). Some other important and very common *Botryosphaeria dieback*-associated pathogens, such as *Neofusicoccum parvum* or *Diplodia seriata* (Luque *et al.*, 2009; Bénard-Gellon *et al.*, 2015), were not identified in the present study. Confirmation of this state will be assessed in future Czech GTD surveys.

One isolate of *Cytospora* sp. was also detected. *Cytospora* species have been recently described as part of the GTD complex in eastern North America (Lawrence *et al.*, 2017).

In conclusion, this study extends knowledge of GTD occurrence in Czech vineyards, as an example of north-eastern wine-producing region in Europe. From the field survey of GTD incidence it is evident that economic losses from these diseases are significant in Czech vineyards, especially for grapevine varieties with high susceptibility such as ‘Cabernet Sauvignon’. Preliminary information on the spectrum of fungi associated with GTDs in the surveyed vineyards in the Czech Republic has been obtained. This knowledge is important for local grape producers, to ensure that they do not underestimate the importance of GTDs and adjust their management strategies to reduce the negative impacts of these diseases. The results also indicate important aims for future research, particularly variety cultivar susceptibility, impacts on harvest quality, and the establishment of efficient control measures for nurseries and vineyards.

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