FIRST REPORT ON MOLECULAR DETECTION OF FUNGAL TRUNK PATHOGENS IN GRAPEVINE WOOD FOCUSING ON MORAVIAN CERTIFIED ROOTSTOCK MOTHER PLANTS

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Grapevine trunk diseases (GTD) are one of the most destructive complex diseases affecting grapevine and causing significant economical losses to the wine industry worldwide. The symptomatic manifestation can differ, from the most common leaf discolorations and deformations to wilting of shoots, cordon and trunk dieback due to canker formation in the vascular tissue. As the disease progresses, the yield of the afflicted plant decreases due to the loss of productive wood which often shows dead arms, diebacks or black foot symptoms. The main aim of this study was to determine the spectrum of fungal pathogens responsible for GTD in Czech Republic, more specifically in the wine region Moravia. The wood from the rootstock mother plants was chosen as the point of focus due to their initial role in the possible infection spreading. Two certified mother rootstocks of Selection Oppenheim 4 (SO4) were used for detection of fungi. Samples were obtained by two different methods, fungal isolation from mixture of wooden material and isolation from symptomatic parts of wood. From these samples, a spectrum of fungal isolates was obtained, resulting in the acquirement of sequences of pathogens generally recognized as a part of the GTD complex causal agents and also some of the unknown ones, not recognized by the NCBI/GenBank. This study presents the pilot project on uncovering of the spectrum of fungal pathogens causing GTD in the Czech Republic.
Keywords: Esca, grapevine trunk disease, molecular detection, PCR, phylogeny

Erster Bericht über den molekularen Nachweis von holzzerstörenden Pilzen im Rebholz zertifizierter Mutterunterlagsreben in Mähren. Der Krankheitskomplex der Holzkrankheiten gehört zu den verheerendsten Krankheiten der Weinrebe und verursacht erhebliche wirtschaftliche Verluste in der Weinindustrie weltweit. Die auftretenden Symptome können sich unterscheiden, sie reichen von den am häufigsten vorkommenden Blattverfärbungen und -verformungen über das Welken der Triebe bis zum Absterben der Kordone und des Stammes aufgrund der Zerstörung des Gefäßgewebes. Wenn die Krankheit fortschreitet, sinkt der Ertrag der betroffenen Rebe wegen des Verlustes an produktivem Holz, was sich am Absterben von Stockteilen oder ganzen Rebstöcken sowie Schwarzfußkrankheit zeigt. Das Hauptziel dieser Studie war es, das Spektrum der Pilzerreger, die in Tschechien, genauer gesagt in der Weinbauregion Mähren, Holzkrankheiten an der Rebe verursachen, zu bestimmen. Das Holz aus den Schnittanlagen für Unterlagsreben stand besonders im Fokus aufgrund deren Rolle bei der möglichen Verbreitung der Infektion. Zwei zertifizierte Mutterunterlagsreben der Sorte Selektion Oppenheim 4 (SO4) wurden für den Nachweis von Pilzen verwendet. Die Holzproben wurden mittels zweier verschiedener Methoden analysiert, einmal durch Pilzisolierung aus einer Mischung von holzigem Material und einmal durch Isolierung aus symptomtragenden Holzteilen. Aus diesen Proben ergab sich ein Spektrum von Pilzisolaten. Die Analyse dieser Isolate ergab Gensequenzen von Pathogenen, die bereits als Verursacher von Holzkrankheiten beschrieben wurden. Darüber hinaus wurden aber auch Gensequenzen unbekannter Pathogene, die nicht von der NCBI/GenBank erfasst sind, festgestellt. Diese Studie stellt ein Pilotprojekt zur Identifizierung des Spektrums von Schaderregern dar, die am Krankheitskomplex der Holzkrankheiten der Rebe in der Tschechischen Republik beteiligt sind.
Schlagwörter: Esca, Holzkrankheiten der Rebe, molekulare Erkennung, PCR, Phylogenie

In the early nineties of the 20th century the incidence of fungal diseases affecting grapevine wood (*Vitis vinifera L.*) has increased worldwide. It is a complex disease which can be caused by many species of various fungi, collectively known as GTD – grapevine trunk diseases (Gramaje and Armengol, 2011). These diseases manifest a variety of symptoms.

The Esca disease, Petri disease, Black foot disease, Dead arm, Eutypa or Botryosphaeria dieback are considered to be the GTD complex (Augustí-Brisach et al., 2014; Bruez et al., 2014; Fleurat-Lessard et al., 2014).

Spectrum of pathogen species varies both within individual infested grapevines, and also within geographic location.

ESCA AND PETRI DISEASE

Esca and Petri disease are caused mainly by genus *Phaeoacremonium, Phaeomoniella* and *Cadophora* (Gramaje et al., 2010). Symptoms include "apoplexy", or sudden wilting of the vine, with the shedding of some or all leaves and fruit, superficial brown to purple spots on the berry surface (measles), vascular discolouration of wood, typical tiger-striped patterns on leaves and tip dieback of shoots (Eskalen et al., 2007). In some studies, F*omitiporia mediterranea M.* Fisch, *Phomopsis viticola* (Sacc.) *Sacc. or Stereum hirsutum* (Willd.) Pers. are also ranked to Esca complex (Fischer and Kassemayer, 2003; Sánchez-Torres et al., 2008). In several cases *F. mediterranea* was isolated out of darkened, very hard wood, colonized by the other fungi (Fischer and Kassemayer, 2003) and recently this fungus is ranged more as the secondary fungus in GTD complex.

EUTYPA DIEBACK

Eutypa dieback is caused mainly by *Eutypa* spp., *Eutypella* spp., *Dyatripe* spp. and *Cryptovalsa* spp. The main symptoms are slowing of shoot growth and reduction of fruit size, eventually causing the whole bush to die down (Rolshausen et al., 2014; Trouillas et al., 2010).

BLACK FOOT DISEASE

Black foot disease can be caused mainly by genus *Campylocarpon, Cylindrocarpon* and *Ilyonectria*. Growth of affected grapevines slows down and differences in vigor are typified by reduced caliper size of the trunk, shortened internodes, reduced foliage, and reduced leaf size. Grapevines that appear sound in cross section at planting begin their first season with adequate vegetative vigor and development. However, during the subsequent 3 to 5 years after planting, the onset of foliar symptoms may appear as interveinal chlorosis, followed by necrosis, and early defoliation. When trunks of declining grapevines are viewed in cross section, dark-brown to black streaking in the vascular elements is evident. This discoloration may occur in a few to most of the vascular elements. Uneven wood maturity, which is usually associated with a rapid desiccation event, is another symptom. Below ground, symptoms include a reduction in total root biomass, low numbers of feeder roots, and sunken, necrotic root lesions (Agustí-Brisach et al., 2014; Halleen et al., 2006).

DEAD ARM

Dead arm is caused by the two main fungi, *Phomopsis viticola* (Sacc.) Sacc. and *Diplodia mutila* (Fr.) Mont. Fungus *P. viticola* (syn. *Fusicoccum viticolum* Reddick) was originally associated with a syndrome named "necrosis of the grapevine" in the state of New York and was renamed dead arm disease of grapes a few years later. *P. viticola* was recently reported from grapevine perennial cankers in grape-growing regions in the northeastern United States (Baumgartner et al., 2012).

BOTRYOSPHAERIA DIEBACK

Botryosphaeria dieback is caused by a complex consisting of genera *Botryosphaeria, Diplodia, Dothiorella, Lasiodiplodia* or *Neofusicoccum*. In young vineyards, *Botryosphaeria* spp. can cause 'grapevine decline', seen as stunted, chlorotic growth, with delayed budburst and necrosis of leaf and flower buds. In older vineyards, the growing canes can die back often due to the infection of summer trimming wounds. In autumn, infected canes are bleached and have superficial, raised, black fruiting bodies (pycnidia). In spring, infected shoots and canes may produce stunted and chlorotic leaves and then die. These pathogens can also cause a soft brown rot on berries, which become shrivelled with surface pycnidia.

The infection of bunches and buds can spread into supporting shoots, causing dieback (Úrbez-Torres et al., 2008; Yan et al., 2013).

SPREADING OF GTD

One way of spreading is probably the utilization of planting material from infected mother plants and another is dissemination by aerial spores. Contamination of tools (during cutting) or the presence of pathogens in the soil are other ways which these fungi can use to enter its host body directly on site (Gramaje and Armengol, 2011). The protection consists of preventive measures, such as the use of non-infectious propagating material and keeping appropriate agronomical practices; generally appropriate measures are the ones leading to the reduction of the stress factor impact (Gramaje et al., 2009; Mundy et al., 2012). Antagonistic fungi of the genus *Trichoderma* might reduce the spread of Esca pathogens and thus provide protection of pruning wounds and protection in nurseries (Mutawila et al., 2011; Fourie and Halleen, 2006). Chemical compounds aiming to protect pruning wounds such as Benomyl, Pyraclostrobin, Tebuconazol or Thiophanate-methyl based pesticides (Díaz and Latorre, 2013) are also used. The banned sodium arsenite solution was used historically as a unique curative agent (Bertsch, 2013). So-called 'Hot Water Treatment' seems to be an effective measure in control of Black foot and Petri disease in grapevine propagating material (Gramaje and Armengol, 2011). GTD is a globally significant threat to the viticultural practice considering that there is no full protection available against the whole complex of these pathogens. The spectrum of the GTD fungal pathogens present varies greatly depending on the location.

The aim of this study is to gain information on the fungal species present in trunk diseased vines in the Czech Republic. It is one of the first reports on the spectrum of fungal diseases targeting grapevine wood in the Czech Republic (wine region Moravia), focusing on SO4 certified rootstock mother plants as a possible starting point of infection.
The combination of utilizing the cultivation of pure fungal colonies together with the methods based on molecular genetic analysis appears to be the most suitable for the identification of fungi.

MATERIALS AND METHODS

SAMPLING

The SO4 grapevines were obtained from a commercial nursery in Geisenheim (Germany) and grown in the wine region Moravia (Czech Republic) as rootstock mother plants. We selected two symptomatic rootstocks for the study. The grapevines were ten years old plants which were stunted in growth, had short internodes and showed interveinal chlorosis transitioning from yellow to red and brown. This symptom is usually called a „tiger-stripe leaf". Described symptoms indicating GTD were observed on both monitored plants.

ISOLATION OF FUNGI

For the isolation of fungi from the two symptomatic certified SO4 rootstock mother plants, two methods of extraction were used.

PLANT SO4 NO. 1

The first plant was horizontally cut in half through the trunk head, surface-sterilized with ethanol (96 %) and washed with distilled water. Infected wood was scooped out, ground in distilled water and the obtained suspension was plated on potato dextrose agar (PDA; Sigma-Aldrich, St. Louis, MO, USA).

PLANT SO4 NO. 2

The second sample plant was cut into 2 cm segments, washed with ethanol (96 %) and sterilized by flame. For the testing, 7 fragments of each symptomatic wood area shown in Table 1 were carefully removed by a sterile blade and placed on PDA supplemented with 0.5 g/l streptomycin sulfate. Exudate flowing out from the cut trunk was placed on PDA, too.

All plates were incubated at 25 °C in the dark. The mycelial growth was observed daily. Samples of mycelia from woody fragments were transferred to a Petri dish with PDA and then transferred to a new dish with PDA again to obtain pure cultures of the acquired fungi.

Table 1: Description of purification. Only the samples examined at all levels are included. Sample designated as * had identical sequence to the NCBI/GenBank

Sample	Symptoms in the wood	Plant	Isolate	Fungus detected by sequencing	GenBank Acc. Nos.
1	wood mixture as described in 'Isolation of fungi'	SO4 n. 1	S1A	*Truncatella angustata* (Pers.) S. Hughes	KP255347
2	as sample 1	SO4 n. 1	S2A	*Botrytis cinerea* Pers. : Fr.	KP255348
3	as sample 1	SO4 n. 1	S1B	*Clonostachys rosea* f. *rosea* (Link : Fr.) Schroers et al.	KP255349
4	vascular secretion - black dots	SO4 n. 2	11	*Fomitiporia mediterranea* M. Fisch	KP255350
5	canker, necrotic wedges	SO4 n. 2	24	*Fomitiporia mediterranea* M. Fisch	KP255350*
6	circular necrosis	SO4 n. 2	35	*Bjerkandera* spp.	KP255353
7	exudate from cut wood	SO4 n. 2	48A, 48B	Unknown fungus	KP255354, KP255355
8	soft wood	SO4 n. 2	610	*Talaromyces trachyspermus* (Shear) Stolk & Samson	KP255351
9	soft wood	SO4 n. 2	63	*Phomopsis* spp.	KP255352
10	soft wood	SO4 n. 2	79	*Basidiomycetes* spp.	KP255356

DNA EXTRACTION

Total DNA of cultured samples was extracted by NucleoSpin Tissue kit (Macherey-Nagel, Düren, Germany) according to manufacturer's instructions. We used 20 mg of fungal culture collected from the agar plates for DNA isolation. In order to evaluate the concentration of obtained DNA, the fluorometric measurement was used (Rengarajan et al., 2002). Resulting DNA concentration was between 35 to 60 ng/ml.

PCR FOR ITS AMPLIFICATION AND ITS SEQUENCING

The extracted DNA was amplified in the total volume of 20.8 µl. The reaction mix for PCR consisted of 10.5 µl of water (HPLC purity), 4 µl of 5× Colorless GoTaq Flexi Buffer for polymerase (Promega, Madison, USA), 1.2 µl of 25mM MgC12 (Promega, Madison, USA), 0.2 µl of 10 µ M dNTP mixture (Invitek, Berlin, Germany), 0.2 µl of GoTaq G2 Flexi DNA polymerase (5 U/µl) (Promega, Madison, USA), 1 µl of both primers (10 µ M) ITS3 and ITS4 (White et al., 1990), 0.7 µl of Flexi 5× Green GoTaq Flexi Buffer (Promega, Madison, USA) and 2 µl of DNA template.

The PCR was carried out as follows. After an initial denaturation for 3 min at 95 °C, the amplification was performed within 30 cycles of 2 min denaturation at 95 °C, 25 sec of primer annealing at 50 °C and 2 min at 72 °C for extension, followed by the final step at 72 °C for 5 min.

PCR fragments were separated on 1.5 % agarose gels, stained by GelRed (Biotium, Hayward, USA) and visualized under UV light. Blank and negative controls were included in each test.

The PCR products corresponding to the size of approx. 330 bp (depending on fungal species) were sequenced as described by Eichmeier et al. (2010). Sequencing was done in both directions using primers ITS3 and ITS4 (White et al., 1990). The obtained nucleotide sequences were analysed using CLC Main Workbench 5.0 (CLC bio, Aarhus, Denmark) and ClustalW2. Multiple alignments and phylogenetic relationship were determined.

PCR FOR SPECIFIC DETECTION OF GENUS *PHAEOACREMONIUM, PHAEOMONIELLA, EUTYPA, FOMITIPORIA* AND *FAMILY BOTRYOSPHAERIACEAE*

The PCR reaction mixtures for detection of 5 main GTD groups were prepared in a total volume of 20 µl and con-

tained 1 × colorless GoTaq Flexi buffer (Promega, Madison, USA), 1.5 mM MgCl2, 0.2 mM dNTPs (Promega, Madison, USA), 1U GoTaq G2 Hot Start Polymerase (Promega, Madison, USA) and 0.2 ng of template DNA for each reaction. The presence of the fungal pathogens was assessed by application of primers specific for a particular pathogen (genus, family). For *Phaeoacremonium* spp. testing, primers Pm1 and Pm2 were used (Aroca and Raposo, 2007) in concentration of 0.2 µM (each). *P. chlamydospora* was tested using 1.5 µM of each primer Pch1 (F) and Pch2 (R) (Tegli et al., 2000). Multispecies specific primers BOT100F and BOT472R (Ridgway et al., 2011) in concentration of 5 µM were used for testing of the fungi from the family Botryosphaeriaceae. *E. lata* was tested by 0.2 µM of each primer Lata 1 and Lata 2-2 (Lecomte et al., 2000). Primers FM and FMP designed by Pilotti et al. (2010) were used in 0.3 µM concentration to detect *F. mediterranea.*

PCR fragments were separated on 1.5 % agarose gels, stained by GelRed (Biotium, Hayward, USA) and visualized under UV light. Blank and negative controls were included in each test. Positive control samples (provided by Universidad Politécnica de Valencia – Spain) and blank controls were included in each test.

RESULTS AND DISCUSSION

The results obtained by combined analysis of all data from both grapevine plants were indicative of spectra of fungal pathogens causing GTD. The results, being the first of its kind on grapevine, present the first draft of the GTD pathogens spectra in the Czech Republic at molecular level.

TRUNK SYMPTOMS OF INFECTION

Analysed rootstock mother plants had a short trunk (about 40 cm), the head was relatively low to the ground. White rot was most evident in the uppermost part of the trunk next to the pruning wounds. It was also found to extend into the more basal parts of the plants. Here, it was often limited to the area around the pith or it spread along a sector, eventually reaching the surface of the trunk. The infection of both plants was very distinct, clearly visible on the cut about 10 cm below the head of the grapevine. The cuts of shoots on the grapevine heads could be a possible point of infection for some detected fungal species.

MORPHOLOGICAL CHARACTERISTICS

The cultures from 6 different parts of infected wood were cultivated on 20 Petri dishes (Table 1). After transferring the culture to a new set of dishes, the samples were evaluated at morphological level by comparison with already determined isolates (provided by Universidad Politécnica de Valencia – Spain). Samples 1 to 3 from plant SO4 no. 1 were not evaluated at morphological level because of the high inconsistence of samples. Moreover, these samples (1 to 3) did not look like GTD pathogens. Sample 1 was white semolina without clear edges, sample 2 was white changing into dark brown and sample 3 was white regular mycelium. Sample 4 (vascular secretion-black dots) formed creamy yellow to light brown colonies with airy mycelium and on morphological level it showed resemblance to the Basidiomycete *F. mediterranea.* Sample 5 (canker, necrotic wedges) showed similar characteristics. Sample 6 (circular necrosis) was established as another Basidiomycete but undetermined on species level. These colonies had a fast growth rate and they were white to pale yellow. Sample 7 (exudate from cut wood) differed from other isolates. It was not possible to determine these colonies on the basis of morphological parameters, they showed different characteristics on individual dishes; the colour, the rate of growth and the density of hyphae were variable. Determination based only on its morphological properties was also not possible in case of sample 8 (soft wood). Sample 9 (soft wood) seemed to belong to *Diatrypaceae* and sample 10 (soft wood) was determined as belonging to *Basidiomycetes* or a *Diatrypaceous* fungi. Fungal isolations are depicted in Figure 1.

PCR RESULTS

The specific PCR detection allowed the identification of *Fomitiporia mediterranea* in samples 4 and 5, in all other samples no GTD pathogens were detected by this method.

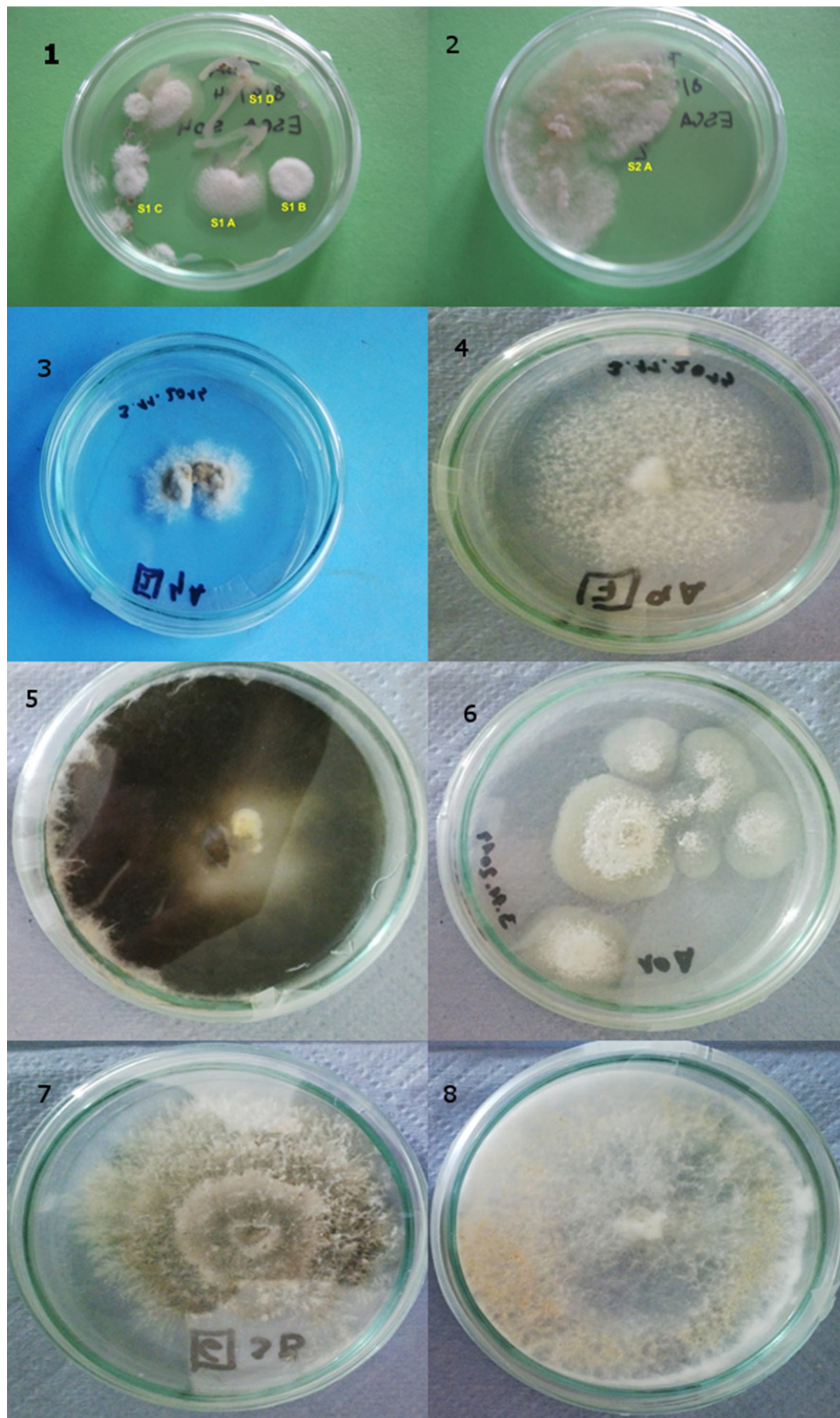

Fig. 1: Isolated fungi from different parts of wood. 1: S1A (*Truncatella angustata*), S1B (*Clonostachys rosea*), S1C and S1D (undetermined); 2: S2A (*Botrytis cinerea*); 3: *Fomitiporia mediterranea*; 4: *Bjerkandera* spp.; 5: undetermined fungus isolated from exudate; 6: *Talaromyces trachyspermus*; 7: *Phomopsis* spp.; 8: undetermined Basidiomycete sp. The types of infected wood from which these fungi were isolated are highlighted in Table 1.

PHYLOGENETIC ANALYSIS

The ITS2 genomic portion is a part of non-functional RNA situated between structural ribosomal RNAs (rRNA). This fungal genomic portion was successfully amplified in case of all samples and the obtained sequences were submitted to NCBI/GenBank under Acc. Nos. KP255347 to KP255356. The region is probably the most suitable for a phylogenetic analysis for this purpose (Ihrmark et al., 2012).

The phylogenetic analysis was performed on all obtained ITS sequences. Each sequence was subjected to BLASTN analysis. According to the multiple sequence alignment, the phylogenetic tree was made using ClustalW2 software (Fig. 2). The parameters for the tree construction are stated in the Figure 2 caption. The tree is divided into three main clusters. The two sequences of an unidentified fungus obtained from the plant SO4 no. 2 are grouped in the first cluster, suggesting that these sequences belong to previously undescribed species. These sequences were obtained from DNA that was isolated by a commercial kit from fungal culture isolated from drops of exudate after cutting of grapevine wood. The fungus from exudate was not conclusively established (sequences KP255354, KP255355). The occurrence of an exudate was already determined as the symptom of GTD (Graniti et al., 2001; Stamp, 2001) but it has never been described which fungus is fully responsible for exudate oozing.

Phomopsis spp., obtained from plant SO4 no. 2, is situated in the second cluster. Due to the ambiguity of the sequence and also the still unclear taxonomic situation regarding *Phomopsis* spp., it was only possible to classify the pathogen at the genus level. The rest of the obtained sequences is grouped in the third cluster, with closer relationship between *B. cinerea* and *C. rosea f. rosea* from plant SO4 no. 1 and another unidentified sample belonging to Basidiomycetes, possibly a wood decaying fungus *Peniophora* sp. from plant SO4 no. 2. The sequences of *F. mediterranea* and *Bjerkandera* spp. are situated in the same subcluster, both of which are Basidiomycetes and also wood decaying fungi. This cluster also includes two remaining sequences, identified as *Truncatella angustata* (plant SO4 no. 1) and *Talaromyces trachyspermus* (plant SO4 no. 2). The sequence of *T. angustata* is situated in the middle of the phylogenetic tree and it is the only fungus with parasitic character from the sequences found; the other fungi are saprophytes.

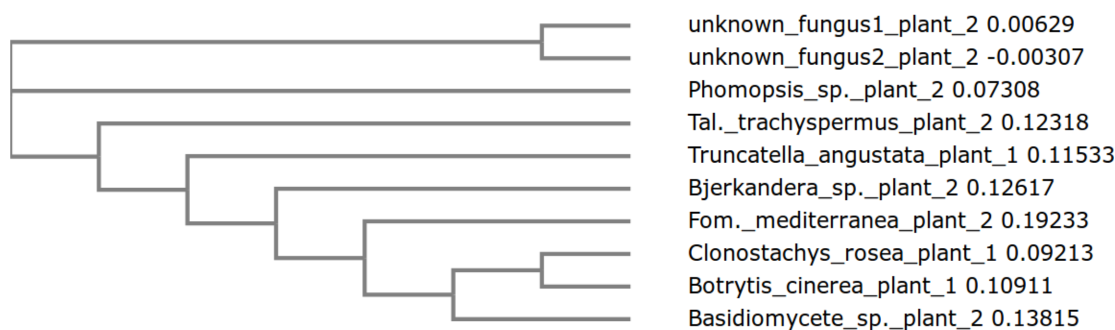

Fig. 2: Phylogenetic tree created by ClustalW2. Neighbour joining clustering and DNA Weight Matrix was carried out by ClustalW. All ten obtained fungal sequences of ITS2 were aligned and phylogenetic tree was constructed.

EVALUATION OF FOMITIPORIA MEDITERRANEA PHYLOGENY

The *F. mediterranea* sequence was examined in detail because of its importance as the GTD pathogen in all countries where this disease is located. All available sequences for ITS2 of this pathogen were downloaded from NCBI/GenBank. On the basis of their multiple alignment, a phylogenetic tree (Fig. 3) was made using CLC Main Workbench 5.0 (CLC bio, Aarhus, Denmark). The phylogenetic tree has two main clusters and five subclusters. In one of the subclusters the sequence of *F. mediterranea* obtained in this study is located. The phylogenetically closest sequences are EU477470, EU477473, EU477476, EU477462, EU851119 obtained from the fungi isolated in Italy from the fruit body on common hazel (*Corylus avellana* L.). The EU477466 sequence is of the same origin, but was isolated directly from hazel wood.

Another sequence present in this cluster is an AY854080 sequence. This sequence of *F. mediterranea* was acquired from a grapevine plant grown in the Kaiserstuhl area near Freiburg im Breisgau, Germany. The important fact is that the AY854080 *F. mediterranea* sequence was obtained by isolation from the grapevine in Germany and the plants examined in this study grown in spatial isolation in Moravia had also been obtained in Geisenheim, Germany. It is therefore possible that *F. mediterranea* from Geisenheim was introduced into the Czech Republic through infected SO4 mother plants. This would confirm that one of the main ways of the pathogen's spreading is through propagating material (Cloete et al., 2014; Cortesi et al., 2002; Gramaje and Armengol, 2011).

The phylogenetic tree (Fig. 3) determines the phylogenetic classification of *F. mediterranea* isolated from plant SO4 no. 2. Nucleotide sequences of *F. mediterranea* obtained from sample 4 and sample 5 were identical (Table 1). Sample 4 was isolated from vascular secretion-black dots, which is a typical symptom for the pathogens causing Petri disease *P. chlamydospora*, *Phaeoacremonium* spp. (Mugnai et al., 1999) or *Cadophora luteo-olivacea* (Gramaje et al., 2011), therefore the detection of *F. mediterranea* only from this sample comes as a surprise. Regarding the temporal dynamics of fungal communities (Bruez et al., 2014) it is possible that *F. mediterranea* could overgrow the other slow-growing fungi such as *Phaeoacremonium s*pp. on the plates and that therefore these slower growing species might have been not detected during analysis. Sample 5 was obtained from canker and necrotic wedges, which are the characteristic symptoms of *Eutypa* spp. (Luque et al., 2009).

The Basidiomycete *F. mediterranea* is often the predominant fungus in locations of Central Europe as described (Fischer and Kassemayer, 2003) in German vineyards (Baden-Württemberg, Rhineland-Palatinate, Hesse, Bavaria, and Saxony).

CONCLUSION

In this study, nine different fungi were obtained from the wood, suggesting *F. mediterranea* as an important GTD member, followed by *Phomopsis* spp. and probably also *Bjerkandera* spp., all of them belonging to Basidiomycetes. The rest of the detected fungi are not considered as serious GTD members. Furthermore, three closer unclassified fungi were found, one of them probably being *Peniophora* spp. (Basidiomycetes). *Peniophora* spp. was already found in grapevine wood (Fischer and Kassemayer, 2003).

The study results confirmed variability of GTD pathogen spectrum which is different worldwide and this study is the start of exploring the fungal spectrum in the Czech Republic. Entirely new information concerning grapevine region in the Czech Republic was obtained. Continuing to bring new insights to fungal spectrum in grapevine certified mother plants is crucial because of its probability as the starting point for subsequent spreading of the significant fungal pathogens. The study of GTD spectra in the Czech Republic needs to be extended in near future because of growing losses in Moravian vineyards.

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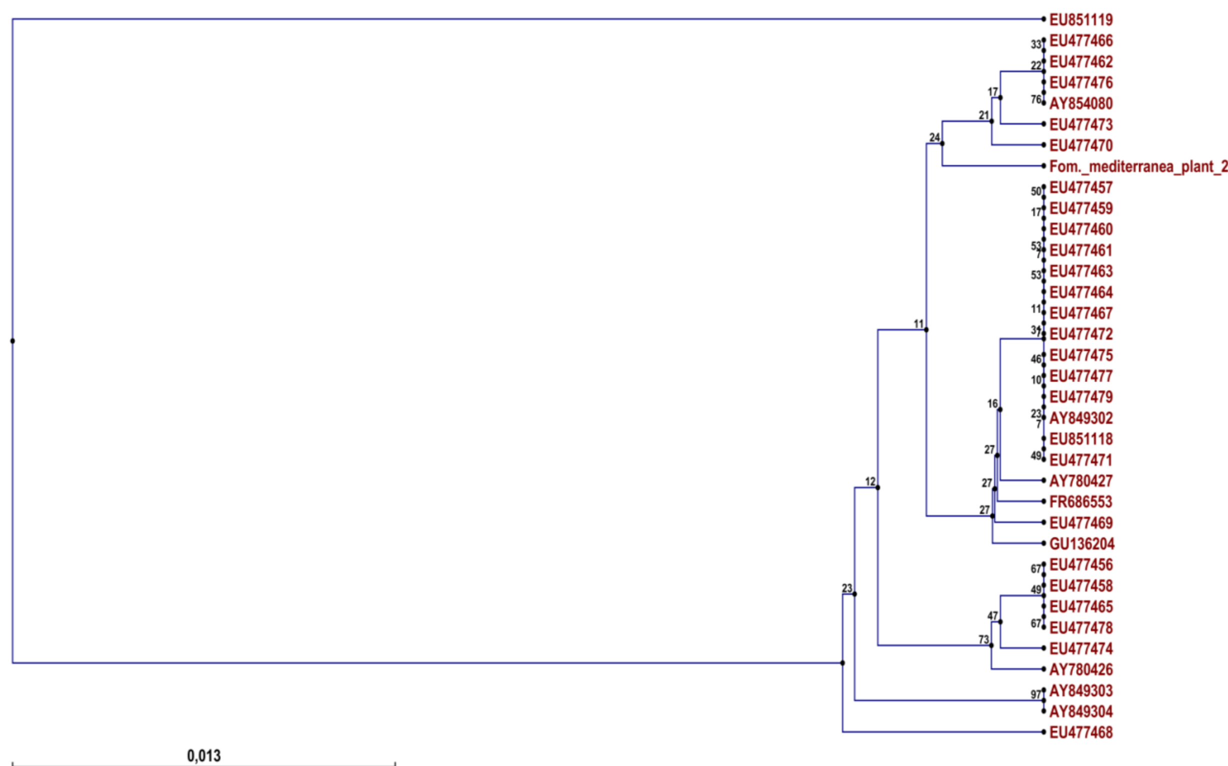

Fig. 3: Species specific phylogenetic tree created by CLC Main Workbench 5.0 (CLC bio, Aarhus, Denmark). The algorithm UPGMA and nucleotide distance measure Jukes-Cantor with bootstrapping 100 replicates were used. There are 2 main clusters, one is represented only by sequence EU851119 which belongs to fungal isolate from Spain (Sánchez-Torres et al., 2008). In the second cluster, there are 5 subclusters where the sequence of *F. mediterranea* obtained from plant SO4 no. 2 is located. Scale bar represents a genetic distance of 0.013.

Surprisingly, in the present study not many of the main fungal species described as GTD agents were detected in surveyed plants. This study is the first pilot draft where we tried to detect the fungal spectrum in case of two SO4 certified mother plants in the Czech Republic. The spectrum of pathogens can vary depending on the site of cultivated plants (Essakhi et al., 2008). Probably, the Czech Republic may have its own spectrum of GTD pathogens as well as the other countries (Fischer and Kassemayer, 2003; Gramaje and Armengol, 2011; Mugnai et al., 1999; Urbez-Torres et al., 2014).

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