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Scientific Paper

GRAPEVINE TRUNK DISEASES ASSOCIATED WITH FUNGI FROM THE *DIAPORTHACEAE* FAMILY IN CROATIAN VINEYARDS*

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Grapevine trunk diseases (GTD) have a variety of symptoms and causes. The latter include fungal species from the family *Diaporthaceae*. The aim of our study was to determine *Diaporthaceae* species present in the woody parts of grapevines sampled from 12 vine-growing coastal and continental areas of Croatia. The fungi were isolated from diseased wood, and cultures analysed for phenotype (morphology and pathogenicity) and DNA sequence (ITS1, 5.8S, ITS2). Most isolates were identified as *Phomopsis viticola*, followed by *Diaporthe neotheicola* and *Diaporthe eres*. This is the first report of *Diaporthe eres* as a pathogen on grapevine in the world, while for *Diaporthe neotheicola* this is the first report in Croatia. Pathogenicity trials confirmed *Phomopsis viticola* as a strong and *Diaporthe neotheicola* as a weak pathogen. *Diaporthe eres* turned out to be a moderate pathogen, which implies that the species could have a more important role in the aetiology of GTD.

KEY WORDS: Diaporthe, Diaporthe eres, Diaporthe neotheicola, *Croatia, pathogenicity,* Phomopsis, Phomopsis viticola

In Croatia, grapevine (*Vitis vinifera* L.) is cultivated on 32,741 hectares in two vine-growing regions (coastal and continental) that include 12 subregions with numerous vineyards. Grapevine is a host to a large number of pathogenic organisms, particularly phytopathogenic fungi, among which 22 fungal species have been described in Croatia (1). Seven of these species [*Phomopsis viticola* (Sacc.) Sacc., *Macrophoma flaccida* (Viala & Ravaz) Cavara, *Botryosphaeria obtusa* (Schwein.) Shoemaker, *Eutypa lata* (Pers.) Tul. & C. Tul., *Phaeomoniella chlamydospora* (W. Gams, Crous, M.J. Wingf. & Mugnai) Crous & W. Gams, *Fomitiporia mediterranea* M. Fisch., and *Togninia minima* (Tul. & C. Tul.) Berl.] are associated with grapevine trunk diseases (GTD), which vary a lot in symptoms and aetiology. Symptoms include decline or death of plant parts and eventually of whole vines due to a variety of necroses such as cankers, dieback, browning, vascular streaking, longitudinal lesions, and cane bleaching, which affect the woody parts of the plant. Traditionally in Croatia, these symptoms have mostly been associated with the species *Eutypa lata* and *Phomopsis viticola*, but research into the aetiology of GTDs showed that symptoms can not be used to identify causes, as they often overlap (2). Various authors (3-9) have identified fungal species from the following genera as causes of GTD: *Botryosphaeria*, *Diplodia*, *Lasiodiplodia*, *Fusicoccum*, *Neofusicoccum*, *Dothiorella*, *Phomopsis*,

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Diaporthe, *Eutypa*, *Eutypella, Diatrypella*, *Diatrype*, *Cryptovalsa, Cylindrocarpon*, *Phaeomoniella*, *Fomitiporia*, *Phaeoacremonium,* and *Greeneria*. Fungal species from the genera *Phomopsis* and *Diaporthe* belong to the family *Diaporthaceae* and are known grapevine pathogens and/or endophytes (10-16). The family *Diaporthaceae* belongs to the order *Diaporthales*, phylum *Ascomycota*. According to Kirk et al. (17), the family includes five genera with 335 species. The largest number of phytopathogenic fungal species from the family *Diaporthaceae* belongs to the species from the genera *Diaporthe/Phomopsis* where the genus *Diaporthe* counts 81, while its anamorphic genus *Phomopsis* counts 234 species. According to earlier studies (18), the genus *Phomopsis* included more than 900 species, and *Diaporthe* more than 800. However, the taxonomy of the family *Diaporthaceae* and of the entire order *Diaporthales* is in constant revision (19). This particularly refers to the species of the genera *Phomopsis/Diaporthe*, where the taxonomic revision resulted in a reduction of the number of species due to a large number of synonyms (20). One of the most investigated species from genera *Phomopsis/Diaporthe* is *P. viticola,* the cause of a grapevine disease known worldwide as Phomopsis cane and leaf spot. In Croatia, the disease is known as grapevine black spot (21). This fungal species was first described in Croatia by Kišpatić (22, 23). The disease is particularly dangerous for susceptible grapevine cultivars such as Malvazija istarska, Frankovka, and Žilavka (24). Recent studies worldwide (6, 10-16, 25, 26) have determined fifteen taxa of the *Phomopsis/Diaporthe* species complex on grapevine with various degrees of pathogenicity, among which the following nine were classified to the species level: *P. viticola, Diaporthe viticola* Nitschke*, Diaporthe australafricana* Crous & Van Niekerk*, Phomopsis amygdali* (Delacr.) J.J. Tuset & M.T. Portilla*, Phomopsis vitimegaspora* K.C. Kuo & L.S. Leu (*Diaporthe kyushuensis* Kajitani & Kanem.)*, Diaporthe helianthi* Munt.-Cvetk., Mihaljč. & M. Petrov (*Phomopsis helianthi* Munt.-Cvetk., Mihaljč. & M. Petrov)*, Diaporthe ambigua* Nitschke, *Phomopsis longiparaphysata* Uecker & K.C. Kuo*,* and *Phomopsis theicola* Curzi (*Diaporthe neotheicola* A.J.L. Phillips & J.M. Santos). In Croatia, the aetiology and epidemiology of GTD have not been well investigated, particularly the association with fungal species of the family *Diaporthaceae*. Considering that only *P. viticola* and since very recently *Phomopsis cotoneastri* Punith have been reported in Croatian grapevines so far (27), it is reasonable to assume that other *Diaporthe/Phomopsis* species could be associated with GTD, especially because grapevine is cultivated in diverse geographical and climatic regions of Croatia. Therefore, the objectives of this study were to identify fungal species from the family *Diaporthaceae* and to determine their pathogenicity in Croatian grapevines.

MATERIALS AND METHODS

Collection of samples, isolation, and culturing

In field surveys of vineyards conducted at 36 localities in 12 vine-growing coastal and continental areas of Croatia between 2008 and 2010, we obtained 165 diseased wood samples from vines showing bleached canes with longitudinal lesions, dead spurs and cordons, and perennial cankers. These samples were surface-sterilised with 2 % sodium hypochlorite for 2 min, rinsed twice with sterile distilled water for 2 min, and then dried in laminar flow for 10 min. Wood chips from the margins of necrotic and healthy tissue were cut from diseased spurs, cordons, or trunks using a sterile scalpel and plated onto 90 mm Petri dishes containing potato dextrose agar (PDA, Sigma-Aldrich, USA) and streptomycin sulphate (50 μg mL^{-1}) (PDA-Strep). PDA-Strep plates were incubated in the dark at 25 °C until fungal colonies were observed. In order to obtain pure fungal cultures, hyphal tips from colony margins were transferred to fresh PDA plates and incubated under nearultraviolet (NUV) light in 12 h light-dark cycles for one to three weeks to stimulate sporulation. Diseased canes were surface-sterilised as described above, but 4 cm to 7 cm long cane pieces were incubated in moist dark chambers at 20 °C for 7 days. Upon pycnidia emergence and sporulation, oozing drops of conidia or cirrhi were spread in a sterile drop of water over the surface of 2 % water agar (WA) in 9 mm Petri dishes. Monoconidial isolates were obtained after 24 h by transferring single-growing conidia to fresh PDA plates and incubated under NUV light as described above.

Morphological identification

The *Diaporthaceae* species isolated from grapevine samples were initially separated from other fungi isolated in this study by comparing phenotypic characteristics (mycelial colour and growth) and conidial morphology (size, shape, and colour) with

those described in literature (11, 15, 28). The latter was determined by placing conidia in 100 % lactic acid and observing them with a light microscope at 1000x magnification. For every isolate, we measured the length and width of 50 conidia and calculated the averages. Based on conidial characteristics and gross colony morphology, we tentatively identified *Diaporthaceae* isolates and selected a representative subset of isolates for molecular identification.

DNA extraction, amplification, and sequencing

Total genomic DNA of isolates selected for molecular identification was extracted from pure culture mycelia cultivated on PDA. Extraction was performed using a Qiagen DNeasy Plant Mini Kit (Qiagen Inc., Valencia, USA) according to manufacturer's instructions. Extracted genomic DNA was used for polymerase chain reaction (PCR) to amplify the internal transcribed spacer (ITS) region of the nuclear ribosomal DNA (ITS1–5.8S–ITS2) using primers ITS5 and ITS4 (29). The PCR reaction mixture consisted of 1.2 Units of *i*-Taq plus DNA Polymerase (Intron Biotechnology, Korea), 1xPCR buffer, 1.5 mmol L^{-1} $MgCl_2$, 200 µmol L^{-1} of each dNTP, 12.5 pmol of each primer, approximately 50 ng of fungal genomic DNA, and was made up to a total volume of 50 µL with sterile nanopure water. PCR was performed using an Eppendorf Master Thermocycler (Eppendorf AG, Hamburg, Germany) with thermal cycler program as follows: 10 min at 95 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 55 °C, and 60 s at 72 °C, with a final extension of 10 min at 72 °C. PCR products were purified and sequenced in both directions at Macrogen sequencing facilities (Macrogen Europe, Amsterdam, Netherlands).

Molecular identification

Molecular identification was based on the comparison of sequences with reference ITS sequences from GenBank (http://www.ncbi.nlm.nih.gov) using the Basic Logical Alignment Search Tool (BLAST). All DNA sequences obtained in this study were deposited in GenBank (Table 1).

Pathogenicity trials

Three isolates of every species were used for pathogenicity trials. The first trial was done on green shoots excised from a healthy grapevine (cultivars Škrlet and Graševina) from the Faculty of Agriculture experimental vineyards at the Jazbina locality in Zagreb, Croatia. In June 2011, the collected shoots were cut in uniform pieces (~30 cm in length), with leaves, tendrils and lateral branches removed. After a short surface sterilisation in 70 % ethanol for 10 s, the shoots were dried under laminar flow for 5 min to 10 min and then wounded in the middle with a 4-mm cork borer, 2 mm deep. For each grapevine cultivar, five shoots were inoculated per tested fungal isolate by taking an agar plug from the margin of a 5-day-old fungal colony, placing it in the wound and immediately covering with parafilm. Ten shoots per each cultivar were inoculated with non-colonised, sterile agar plugs and used as negative control. Inoculated shoots were placed in Erlenmeyer's flasks covered with parafilm, with 400 mL of sterilised tap water. The flasks with inoculated shoots were then kept in a glasshouse under moist conditions at (24±1) °C for 10 days, while water was exchanged every three days. After 10 days, we measured the length of superficial necrotic discoloration up and down from the inoculation point and calculated the means. The second pathogenicity trial was conducted on dormant lignified canes collected in November 2011. Inoculation was performed as described above, but the canes were surface sterilised in 10 % sodium hypochlorite for 10 min and inoculated canes were incubated in sterile, moist dark chambers at 24 °C for 30 days under strict quarantine conditions. After 30 days, we split the canes longitudinally through the inoculation point, measured the length of necrotic discoloration, and calculated mean values. To satisfy Koch's postulates, we once again isolated the causal agents from the margins of necrotic lesions and healthy tissue and then identified them morphologically as described above. Both pathogenicity trials followed a randomized design. All plant material was autoclaved twice before disposal. Representative isolates of species identified in this study were maintained in the collection of Department of Plant Pathology, at Faculty of Agriculture, University of Zagreb, Croatia.

RESULTS

During this study, we observed a variety of GTD symptoms in all surveyed vineyards. From 165 samples of diseased grapevine wood, collected from 36 localities, we isolated 495 fungi. Phenotypic characterization helped us to separate 198 isolates belonging to the family Diaporthaceae from other

Table 1 *List of fungal species from the family* Diaporthaceae *isolated from grapevine samples*

Isolate	Identified species	Cultivar	Locality / Vine-growing subregion	GenBank
CRO_PV1005	*P. viticola*	Pinot bijeli	Jastrebarsko / Pleševica	JQ671033
CRO_PV1006	*P. viticola*	Rizvanac	Jazbina / Prigorje-Bilogora	JQ671034
CRO_PV1021	*P. viticola*	Traminac	Jazbina / Prigorje-Bilogora	JQ671035
CRO_PV1023	*P. viticola*	Pošip	Korčula, Čara / M. and S. Dalmatia	JQ671036
CRO_PV1033	*P. viticola*	Kraljevina	Sv. Ivan Zelina / Prigorje-Bilogora	JQ671037
CRO_PV1034	*P. viticola*	Trbljan	Hvar, Vrboska / M. and S. Dalmatia	JQ671038
CRO_PV1043	*P. viticola*	Teran	Pazin / Istra	JQ671039
CRO_PV1045	*P. viticola*	Vranac	Vrgorac / Dalmatinska zagora	JQ671040
CRO_PV1004*	*P. viticola*	Malvazija	Pula / Istra	JQ671041
CRO_PV1014	*P. viticola*	Vranac	Vrgorac / Dalmatinska zagora	JQ671042
CRO_PV1020	*P. viticola*	Graševina	Petrinja / Pokuplje	JQ671043
CRO_PV1022	*P. viticola*	Teran	Sv. Vincent / Istra	JQ671044
CRO_PV1035	*P. viticola*	Škrlet	Popovača / Moslavina	JQ671045
CRO_PV1037	*P. viticola*	Moslavac	Voloder / Moslavina	JQ671046
CRO_PV1042	*P. viticola*	Teran	Zminj / Istra	JQ671047
CRO_PV1007	*P. viticola*	Škrlet	Kutina / Moslavina	JQ671048
CRO_PV1026	*P. viticola*	Vugava	Vis, Volijok / M. and S. Dalmatia	JQ671049
CRO_PV1044	*P. viticola*	Plavac mali	Pelješac, Janjino / M. and S. Dalmatia	JQ671050
CRO_PV1012	*P. viticola*	Trbljan	Split / M. and S. Dalmatia	JQ671051
CRO_PV1003*	*P. viticola*	Kardinal	Ilok / Podunavlje	JQ671052
CRO_PV1008	*P. viticola*	Škrlet	Kutina / Moslavina	JQ671053
CRO_PV1010	*P. viticola*	Plavac mali	Pelješac, Dingač / M. and S. Dalmatia	JQ671054
CRO_PV1011	*P. viticola*	Škrlet	Popovača / Moslavina	JQ671055
CRO_PV1013	*P. viticola*	Vranac	Vrgorac / Dalmatinska zagora	JQ671056
CRO_PV1015	*P. viticola*	Vranac	Vrgorac / Dalmatinska zagora	JQ671057
CRO_PV1016	*P. viticola*	Vranac	Vrgorac / Dalmatinska zagora	JQ671058
CRO_PV1017	*P. viticola*	Moslavac	Štrigova / Zagorje Međimurje	JQ671059
CRO_PV1019	*P. viticola*	Pinot bijeli	Sv. Urban / Zagorje Međimurje	JQ671060
CRO_PV1027	*P. viticola*	Debit	Stankovci / Northern Dalmatia	JQ671061
CRO_PV1029	*P. viticola*	Crljenak	Stobreč / M. and S. Dalmatia	JQ671062
CRO_PV1031	*P. viticola*	Debit	Drniš / Northern Dalmatia	JQ671063
CRO_PV1032	*P. viticola*	Krkošija	Vrgorac / Dalmatinska zagora	JQ671064
CRO_PV1036	*P. viticola*	Plavac mali	Korčula, Mala kapja / M. and S. Dalmatia	JQ671065
CRO_PV1038	*P. viticola*	Frankovka	Ilok / Podunavlje	JQ671066
CRO_PV1041	*P. viticola*	Trbljan	Viš, Paršurica / M. and S. Dalmatia	JQ671067
CRO_PV1002	*P. viticola*	Vranac	Vrgorac / Dalmatinska zagora	JQ671068
CRO_PV1009*	*P. viticola*	Krkošija	Vrgorac / Dalmatinska zagora	JQ671069
CRO_PV1024	*P. viticola*	Pošip	Korčula, Mala kapja / M. and S. Dalmatia	JQ671070
CRO_PV1025	*P. viticola*	Vranac	Vrgorac / Dalmatinska zagora	JQ671071
CRO_PV1040*	*D. neotheicola*	Malvazija	Pula / Istra	JQ663433
CRO_PV1049*	*D. neotheicola*	Malvazija	Poreč / Istra	JQ663434
CRO_PV1050	*D. neotheicola*	Plavina	Stobreč / M. and S. Dalmatia	JQ663435
CRO_PV1053*	*D. neotheicola*	Gegić	Pag, Vrčići / Hrvatsko primorje	JQ663436
CRO_PV1039*	*D. eres*	Žlahtina	Krk, Vrbnik / Hrvatsko primorje	JQ663437
CRO_PV1047*	*D. eres*	Malvazija	Pazin / Istra	JQ663438
CRO_PV1048	*D. eres*	Žlahtina	Krk, Draga Baščanska / Hrvatsko primorje	JQ663439
CRO_PV1051*	*D. eres*	Frankovka	Orahovica / Slavonija	JQ663440
CRO_PV1052	*D. eres*	Gegić	Pag, Novalja / Hrvatsko primorje	JQ663441

** Isolates used in pathogenicity trials; M. and S. Dalmatia=Middle and Southern Dalmatia*

fungal families. Of those 198 isolates, 189 were identified as *P. viticola* (Sacc.) Sacc.. Their colonies were slightly raised, with prominent growth rings, predominantly buff to honey coloured with smoke-grey patches. Alpha conidia were fusoid-ellipsoidal with the apex acutely rounded, base obtuse to subtruncate, multiguttulate, sometimes biguttulate, with average dimensions (9.4 to 10.3) µm x (1.9 to 3.4) µm. Beta conidia were less frequent than alpha conidia, straight, curved or hamate, with average dimensions (20 to 24.8) µm x (0.5 to 1) µm. Judging by colony and conidial characteristics, five isolates were identified as *Diaporthe eres* Nitschke and four as *Diaporthe neotheicola* A.J.L. Phillips & J.M. Santos. *Diaporthe eres* isolates produced alpha conidia that were unicellular, fusiform, hyaline, mostly biguttulate, with average dimensions (5.6 to 7.9) µm x (2 to 2.3) µm, while beta conidia were unicellular, hyaline, filiform, hamate, with average dimensions (16.6 to 27.7) µm x (0.5 to 1.5) µm. *Diaporthe neotheicola* isolates produced alpha conidia that were unicellular, fusoid, hyaline, biguttulate with average dimensions (7.6 to 8) µm x (2.1 to 2.3) µm and beta conidia were unicellular, filiform, curved, hyaline, eguttulate with average dimensions (24.6 to 26.4) µm x (1 to 1.1) µm. Of the 189 isolates morphologically identified as *P. viticola*, 39 were selected as representative and subjected to molecular identification. All 39 were confirmed as *P. viticola*, (99 % to 100 % match with reference *P. viticola* isolate STE-U2660, GenBank: AF230751). The morphological identification of five *D. eres* isolates and four *D. neotheicola* isolates was also confirmed by molecular identification which showed 100 % match with ITS sequences from reference isolates CBS 109767 (GenBank: DQ491514) and CBS 123209 (GenBank: GQ250192), respectively. Table 1 lists all representative *Diaporthaceae* isolates identified by both methods.

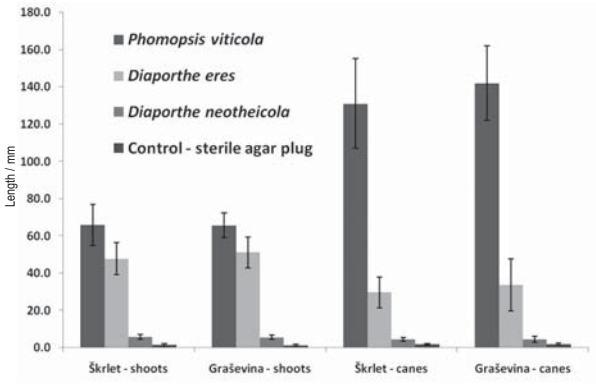

Figure 1 *Results of pathogenicity trials combining mean necrotic discoloration lengths (mean±S.D.) per tested species on either green shoots or lignified canes on two grapevine cultivars (Škrlet and Graševina)*

Table 2 shows the results of pathogenicity trials for three isolates per every species identified in either green shoots or lignified canes taken from two grapevine cultivars (Škrlet and Graševina). Combined results of pathogenicity trials per tested fungal species are shown on Figure 1. The isolates of all three species used in pathogenicity trials proved to be pathogenic on tested grapevine cultivars, but clearly differed in virulence. All three fungal species were successfully re-isolated from the inoculated grapevine plants, confirming Koch's postulates. *P. viticola* showed the highest pathogenicity in both trials and for both grapevine cultivars. *D. eres* showed moderate pathogenicity, and was considerably more pathogenic

Table 2 *Results of pathogenicity trials showing mean length (mm) of necrotic discolorations (mean±S.D.) caused by isolates of different fungal species on green shoots and lignified canes of two grapevine cultivars (Škrlet and Graševina)*

Isolate	Species	Trial with green shoots		Trial with lignified canes	
		Škrlet	Graševina	Škrlet	Graševina
CRO_PV1003	*Phomopsis viticola*	81.8±10.3	78.4±9.2	93.6±27.7	90.0±23.8
CRO_PV1004	*Phomopsis viticola*	41.4±11.7	38.4±6.1	186.4±17.7	183.0±26.2
CRO_PV1009	*Phomopsis viticola*	75.0±11.2	80.4±4.9	113.2±26.6	153.4±10.2
CRO_PV1039	*Diaporthe eres*	48.6±9.3	50.0±9.2	29.2±10.4	33.0±11.6
CRO_PV1047	*Diaporthe eres*	47.0±8.9	52.8±6.1	28.8±9.9	37.2±18.5
CRO_PV1051	*Diaporthe eres*	48.0±7.7	51.0±9.5	31.2±4.7	30.8±11.9
CRO_PV1040	*Diaporthe neotheicola*	7.2±1.3	5.4±0.9	4.4±1.1	4.4±2.1
CRO_PV1049	*Diaporthe neotheicola*	5.0±1.2	5.2±1.5	4.4±0.9	4.6±1.5
CRO_PV1053	*Diaporthe neotheicola*	5.6±1.5	6.2±0.8	4.8±0.8	4.8±1.5
	Control–sterile agar plug	1.7±0.7	1.4±0.5	1.8±0.4	2.0±0.5

than *D. neotheicola*, which turned out to be a weak pathogen or possibly an endophyte. No considerable difference in susceptibility to pathogens was found between the cultivars tested.

DISCUSSION

As only 198 of 495 isolates belonged to the family *Diaporthaceae*, while 297 isolates mostly represented species from the families *Botryosphaeriaceae* and *Diatrypaceae*, fungi from the family *Diaporthaceae* do not seem to have the primary role in the aetiology of GTD in Croatia, which is in accordance with other findings worldwide (4, 30-33). Of the altogether 16 presently known taxa of the genus *Diaporthe/ Phomopsis* on grapevine in the world, this study identified three species, of which *P. viticola* was the most prevalent species (189 of 198 isolates) followed by two much less prevalent species *Diaporthe eres* (anamorph *Phomopsis oblonga* (Desm.) Traverso) and *Diaporthe neotheicola* (anamorph *Phomopsis theicola* Curzi) (9 out of 198 isolates). *Phomopsis viticola* and *D. neotheicola* have already been identified as grapevine pathogens. To our knowledge, this study is the first to report the third species, *D. eres* as a grapevine pathogen. Until now, *D. eres* has been described as pathogenic to more than 300 woody plant species, including *Populus* spp. *Carpinus* spp., and *Magnolia* spp. Recent reports include cultivated plants like peach (*Prunus persica* (L.) Batsch) in Greece (34), blackberry (*Rubus* sp.) in Croatia (35), and butter nut (*Juglans cinerea* L.) in the United States (36). This suggests that *D. eres* is highly polyphagous and could play a considerable role in the aetiology of GTD. Our pathogenicity trials have clearly demonstrated its pathogenic potential.

Species *D. neotheicola*, (until recently known as taxon *Phomopsis* sp. 1) has been reported as a weak grapevine pathogen or endophyte in Australia, South Africa, and Portugal (11, 15). To our knowledge, this is the first report of *D. neotheicola* in Croatia. Beside grapevine, this fungal species has been reported on tea plant (*Camelia sinensis* (L.) Kuntze*)* in Italy (37) and as a weak pathogen on almond (*Prunus dulcis* (Mill.) D.A.Webb) in Portugal (38) and fennel (*Foeniculum vulgare* Mill.) in Portugal (28). Our study has demonstrated weak pathogenicity of *D. neotheicola*, which is supported by previous studies in the world (38).

Our findings have also confirmed *P. viticola* as a prevalent grapevine pathogen from the family *Diaporthaceae*. This is not surprising, since this species is known to specifically infest grapevine. Our pathogenicity trials also confirm earlier literature data (15). One *P. viticola* isolate (CRO_PV1004) was significantly less pathogenic than the other two, but such variations in pathogenicity trials are not uncommon, since isolates of the same species can vary in virulence (16) and do not suggest lower pathogenicity in general. Although previous studies worldwide report 15 different taxa on grapevine (11, 15, 16) (nine of which have been identified to the species level), the number of species found in these studies per country is similar to our own. To a certain extent, this is because some of the nine species appear to have narrow region-specific distribution, such as *Phomopsis longiparaphysata* which has only been reported in Taiwan (39), *Phomopsis vitimegaspora* (*Diaporthe kyushuensis*) in Japan and Taiwan (40), *Diaporthe australafricana* in Australia and South Africa (15), and *Diaporthe ambigua* in South Africa (11). Species *D. viticola*, *P. amygdali*, and *P. helianthi*, reported in regions closer to Croatia (Portugal, Germany, Italy, etc.) (11, 15, 20), however, have not been identified in this study. Judging from reports of fungal species *P. helianthi* and *P. amygdali* on other plant species (1, 41), these fungal species could be expected to occur in Croatian grapevines. However, considering that this is the first more comprehensive report on *Diaporthaceae*-related GTD in Croatia additional research may be needed and should include not only the aetiology, but also the epidemiology and chorology of these fungi.

CONCLUSION

Symptoms of GTD are observed in all vine-growing regions of Croatia. Three species of fungi associated with GTD, have been identified from the family *Diaporthaceae*: *P. viticola*, *D. eres*, and *D. neotheicola*. *Phomopsis viticola*, the causal agent of Phomopsis cane and leaf spot, was the most prevalent species. The two other species showed low prevalence. The most important finding of this study is that it is the first to identify *D. eres* as grapevine pathogen in the world, the 17th species from the genus *Diaporthe/ Phomopsis* and the family Diaporthaceae reported on grapevine. In Croatia, *D. eres* has only been reported as blackberry pathogen (*Rubus* spp.) until now. To our

knowledge, this is also the first study to report *D. neotheicola* in Croatia. Pathogenicity trials of *D. eres* and *D. neotheicola* showed a medium level of pathogenicity for *D. eres* and low level for *D. neotheicola*, as opposed to the high pathogenicity of *P. viticola* isolates. Our study has also shown that most necrotic grapevine wood samples contained several fungi, which points to a complex aetiology of GTD.

Further epidemiological studies should establish the prevalence of GTD in all vine-growing regions of Croatia.

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Sažetak

BOLESTI DRVA VINOVE LOZE POVEZANE S GLJIVAMA IZ PORODICE *DIAPORTHACEAE* U HRVATSKIM VINOGORJIMA

Bolesti vinove loze koje se danas u općeprihvaćenoj engleskoj fitopatološkoj terminologiji označavaju kao *grapevine trunk disease* (GTD) obuhvaćaju bolesti drva vinove loze s različitom simptomatologijom i etiologijom. Kao jedan od uzroka GTD-a navode se i fitopatogene gljive iz porodice *Diaporthaceae*. Iz ove porodice na vinovoj lozi do sada je u Hrvatskoj utvrđena samo vrsta *Phomopsis viticola* kao uzročnik bolesti crna pjegavost i *Phomopsis cotoneastri*. U svijetu se navodi još 15 vrsta koje spadaju u rod *Diaporthe*/*Phomopsis* s različitom patogenosti na vinovoj lozi pa se stoga neke vrste smatraju endofitima, a neke patogenima. Radi utvrđivanja etiologije bolesti drva vinove loze povezanih s gljivama iz porodice *Diaporthaceae* uzimani su uzorci bolesnog drva i rozgve vinove loze iz različitih vinogorja unutar svih 12 vinogradarskih podregija kontinentalne i primorske Hrvatske. Gljive su iz zaraženog drva izolirane u čistu kulturu na hranjivu podlogu. Taksonomski status izolata utvrđen je na temelju njihove fenotipske karakterizacije (karakteristike kolonija i spora) i analizom DNA-sekvencija molekularnog markera ITS (ITS1, 5.8S, ITS2). Najveći broj izoliranih gljiva identificiran je kao vrsta *Phomopsis viticola*, dok je manji dio izoliranih gljiva pripadao vrstama *Diaporthe neotheicola* i *Diaporthe eres*. Za vrstu *Diaporthe eres* ovo je prvi nalaz na vinovoj lozi u svijetu, a za vrstu *Diaporthe neotheicola* prvi nalaz u Hrvatskoj. Testovi patogenosti potvrdili su da je *Phomopsis viticola* izrazito patogena vrsta na vinovoj lozi, dok se vrsta *Diaporthe neotheicola* pokazala slabim patogenom, a vrsta *Diaporthe eres* utvrđena je kao srednje jak patogen pa bi mogla imati važniju ulogu u etiologiji GTD-a.

KLJUČNE RIJEČI: Diaporthe, Diaporthe eres, Diaporthe neotheicola, *Hrvatska, patogenost*, Phomopsis, Phomopsis viticola

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