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GRAPEVINE TRUNK DISEASES PATHOGENES IDENTIFICATION ON GRAPEVINE ROOTSTOCKS IN UKRAINE

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The objective *of this study was isolation of fungi from the grapevine trunks, DNA extraction and identification of potential esca pathogens in Ukraine.* ***Methods.*** *Isolation and identification of fungal cultures based on morphology and DNA sequencing. Total DNA of fungal colonies was extracted by NucleoSpin Tissue kit (Macherey-Nagel, Düren, Germany) according to manufacturer's instructions. To amplify ITS region, ITS1 and ITS4 primers were used. The PCR products corresponding to the size of approx. 550 bp. were sequenced and the obtained nucleotide sequences were analysed using CLC Main Workbench 5.0 (CLC bio, Aarhus, Denmark).* ***Results.*** *Determination of potential pathogens complex showed the presence of species associated with "trunk diseases". Among specific esca pathogens 3 isolates of Cadophora luteo-olivacea were found out. Esca-affected samples also showed the presence of Eutypa lata (causal agent of Eutypa dieback) and Botryosphaeria dothidea, (causal agent of Botrioshaeria dieback).* ***Conclusions.*** *DNA identification of fungal pathogens showed a discrepancy in species composition between asymptomatic plants and esca-affected plants. Esca-affected cultivars showed the presence of Eutypa lata and Botryosphaeria dothidea, which were absent in asymptomatic plants.*

Keywords: grapevine trunk diseases, esca, Cadophora luteo-olivacea, PCR, DNA sequencing.

Grapevine trunk diseases (GTD) are currently considered one of the most relevant challenges for the viticulture (Fontaine et al. 2016) from which viticulture of European countries, including Ukraine, has been suffering during the last 20 – 30 years [1, 2]. These diseases are mostly chronic and leads to a progressive decrease in yield and grapevine longevity [3]. In Ukraine, esca has been found in almost all wine-growing regions (Odessa, Mykolaiv, Kherson, and rarely Transkarpation region). It heavily affects cv. Cabernet Sauvignon and its descendant – Odessa black (Alibernet) cultivar.

Esca is caused mostly by a complex of fungal pathogens including *Phaeomoniella chlamydospora*, *Phaeoacremonium* species, *Cadophora* ssp. and *Fomitiporia mediterranea* [4].

Foliar symptoms mainly appear in *Vitis vinifera* L. cvs, whereas in rootstock varieties endophytic lesions manifest more clearly.

Esca symptoms vary each year and depend on a range of factors, primarily genotype resistance, meteorological conditions in a certain year, etc. [5, 6].

In many wine-growing countries of the world, the control of this disease includes sanitary selection in the systems of certified plant material production, first of all, laboratory diagnostics of esca pathogens [7].

In Ukraine, as the number of infected vineyards has increased, esca screening has become mandatory for sanitary control in the certification system of plant material [8]. However, detection of esca pathogens has not been previously conducted using DNA analysis such as sequencing.

The **objective** of this study was i) isolation of fungi from the grapevine trunks ii) DNA extraction and identification of potential esca pathogens in Ukraine.

Materials and methods. Grape clones and varieties bred at National Scientific Center "Tairov Research Institute of Viticulture and Wine-Making" were studied. The visual sanitary selection of each plant was conducted on clone trial plots and repository of clones in glass-house. In order to determine the sanitary state of rootstock varieties, a degree of endophytic wood damage was evaluated. Isolation and identification of fungal cultures based on morphology and DNA sequencing was carried out.

Isolation and identification of fungi from severely symptomatic plants. For isolation of fungal cultures a two-year old shoots from upper parts of vine plants were used. Shoots were debarked and cut to 2 cm long segments. These segments were washed in distilled water, disinfected one minute in 2% sodium hypochlorite and washed two times with sterile distilled water. Segments were longitudinally cut and small discoloured or necrotic pieces of the wood were aseptically placed on malt extract agar, MEA (Sigma-Aldrich; St. Louis, MO, USA) supplemented with 0.5 g/l of streptomycin sulphate (Biosynth, Staad, Switzerland) and cultivated in dark up to 20 days at 25°C. From each segment three petri dishes with nine pieces of wood were prepared. To obtain fungal isolates growing mycelia were replaced to PDA (HiMedia, Mumbai, India) and cultivated at the same conditions. For DNA isolation fungal isolates were morphologically grouped and single-spored.

Total DNA of fungal colonies was extracted by NucleoSpin Tissue kit (Macherey-Nagel, Düren, Germany) according to manufacturer's instructions; 20 mg of fungal culture were collected from the PDA plates for DNA isolation. Identification of cultivated fungi was performed via amplification and sequencing of genes for internal transcribed spacer (ITS). To amplify ITS region, ITS1 and ITS4 primers were used (White *et al.,* 1990) [9]. Conditions for PCR amplification were used as described in Eichmeier et al. (2016) [10]. The PCR products corresponding to the size of approx. 550 bp. were sequenced as described by Eichmeier *et al.* (2010) [11]. The obtained nucleotide sequences were analysed using CLC Main Workbench 5.0 (CLC bio, Aarhus, Denmark) Assignments of individual cultures proposed by CLC main workbench was subsequently confirmed by blast within NCBI using "reference genome sequence" database and by using sequences uploaded by Westerdijk Fungal Biodiversity Institute (former CBS-KNAW) as a reference. Samples were also controlled at morphological level by comparison with already determined isolates provided by Universidad Politécnica de Valencia in Spain.

Results. *Esca foliar and endophytic symptoms manifestation.*

To visually determine a level of esca infestation of each plant, distribution of affected plants into 4 groups by a degree of foliar symptoms manifestation (1– pre-esca, 2 – 30% of foliage damage, 3 – 4 up to 50 and up to 100% of foliage damage, respectively) was proposed. The visual state of Dobrinja rootstock plants, which belong to the first and fourth groups, is presented in Fig. 1–2.

Dobrinja rootstock variety derived from susceptible to esca Cabernet Sauvignon and resistant to this disease Rupestris du Lot cultivars. It responds to the infection by both the foliar symptoms and endophytic lesions.

Fig.1 Symptoms of pre-esca on Dobrinja rootstock variety

Fig. 2. Symptoms of esca (group 4) on Dobrinja rootstock variety

To evaluate the state of rootstock varieties, assessment of endophytic symptoms, which most often appeared in the form of concentric xylem lesions, was used (Fig. 3).

Identification of grapevine trunk disease pathogens based on sequencing. Determination of potential pathogens complex (Table 1, 2) showed the presence of species associated with “trunk diseases”. Among specific esca pathogens 3 isolates of Cadophora luteo-olivacea were found out.

Fig. 3. Concentric xylem lesions on Dobrinja variety

Table 1

DNA identification fungal isolates on asymptomatic samples of rootstock clones bred at National Scientific Center "Tairov Research Institute of Viticulture and Wine-Making", 2018

Clone or code	Cultivar	Identification
5BB 9191	Berlandieri x Riparia Kober 5 BB	*Acaromyces sp.*
5BB 211161	Berlandieri x Riparia Kober 5 BB	*Scopulariopsis sp., Sarocladium sp., Alternaria alternata, Cadophora luteo-olivacea*
101-14 4923	Riparia x Rupestris 101-14	*Alternaria sp., Acremonium spp.*
41B 3721	Chasellas white x Berlandieri 41 B	*Alternaria sp.*
CO4 1791	Berlandieri x Riparia CO4	*Alternaria sp., Cladosporium sp.*
CO4 97101	Berlandieri x Riparia CO4	*Diplodia seriata**
Riparia Gloire 5941	Riparia Gloire	*Alternaria sp.*

Table 2

DNA identification fungal isolates on Dobrinja rootstock variety and Cabernet Sauvignon reference variety with esca symptoms (NSC "Tairov Research Institute of Viticulture and Wine-Making", 2018)

Clone or code	Cultivar	Identification
Dobrinja 1-1-1	Dobrinja	*Diaporthe viticola*, Eutypa lata**
Dobrinja 1-1-2	- / -	*Quambalaria sp.*
Dobrinja 1-1-3	- / -	*Alternaria sp.*
Dobrinja 2-1-4	- / -	*Alternaria sp., Fusarium sp.*
Dobrinja 3-1-1	- / -	*Botryosphaeria dothidea**
Dobrinja 9-2-1	- / -	*Pyrenochaeta sp*
CS-1	Cabernet Sauvignon	*Aureobasidium pullulans*
CS-2	Cabernet Sauvignon	*Epicocum nigrum*

*fungal pathogens associated with GTD diseases other than ESCA (Phomopsis dieback, Eutypa dieback and Botryosphaeria dieback, respectively)

Discussion. Recent studies have demonstrated that *Phaeoacremonium spp*., *Phaeomoniella chlamydospora* [12] and *Fomitiporia mediterranea* [13] are associated with the esca disease. However, often *E. lata* and *Botryopshaeriaceae* are also present in the wood of esca affected grapevine [14]. The main fungal agents associated with Petri disease ("young vine decline") are *P. chlamydospora and Phaeoacremonium spp*. [15], but it also related with *Cadophora luteo-olivacea. C. luteo-olivacea* has been isolated from the wood of grapevine with internal symptoms both esca and Petri disease [16].

Hofstetter et al. (2012) published that *Cadophora luteo-olivacea* were present in approximately the same ratio (nearly 70 % of samples) both in esca-affected and in asymptomatic plants. *Eutypa lata* and *Alternaria infectoria* were more often isolated from plants with esca symptoms [17].

Ukrainian samples without esca symptoms showed a presence of *Cadophora luteo-olivacea,* (3 isolates) one of the typical species associated with esca and Petri diseases. Esca-affected samples also showed the presence of *Eutypa lata* (causal agent of Eutypa dieback) and *Botryosphaeria dothidea* (causal agent of Botrioshaeria dieback) which were absent in asymptomatic plants. On symptomless samples *Diaporthe viticola,* the causal agent of Phomopsis dieback was found out, too.

However, other typical species associated with esca in European wine-growing countries (*Phaeomoniella chlamydospora*, *Phaeoacremonium* sp., *Fomitiporia mediterranea*) were not detected on the samples.

It could be caused by the fact that we used only two years old wood of grapevines in this study and many of known trunk pathogens could be detected in the older wood that is called trunk.

Therefore, in order to obtain the whole picture regarding the esca pathogens in Ukraine, it is advisable to conduct additional research using European varieties from different vineyards of Ukraine.

Conclusions. Visual assessment of clones of rootstock varieties bred at NSC "Tairov Research Institute of Viticulture and Wine-Making" showed the absence of external and endophytic symptoms of esca disease. In Dobrinja rootstock variety both external (from pre-esca to overall canopy damage) and endophytic symptoms were manifested.

DNA identification of fungal pathogens showed a discrepancy in species composition between asymptomatic plants and esca-affected plants. Esca-affected varieties showed the presence of *Eutypa lata* and *Botryosphaeria dothidea*, which were absent in asymptomatic plants. To some extent, it allows to assume their connection with esca disease development. One of the typical species associated with esca, *Cadophora luteo-olivacea* (3 isolates) were found out.

ВИЯВЛЕННЯ ПАТОГЕНІВ ХВОРОБ БАГАТОРІЧНОЇ ДЕРЕВИНИ ВИНОГРАДУ НА ПІДЩЕПНИХ СОРТАХ ВИНОГРАДУ В УКРАЇНІ

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Резюме

Метою роботи було виділення грибів з виноградних штамбів, виділення ДНК та виявлення потенційних патогенів ески в Україні. **Методи.** Виділення та ідентифікацію грибних культур проведено на основі морфології та секвенування ДНК. Загальна ДНК грибних колоній була екстрагована набором NucleoSpin Tissue (Macherey-Nagel, Düren, Німеччина) згідно з інструкціями виробника. Для ампліфікації ITS-області використовували праймери ITS1 і ITS4. Продукти ПЛР, що відповідали розміру близько 550 bp, секвенували. Отримані нуклеотидні послідовності аналізували за допомогою CLC Main Workbench 5.0 (CLC bio, Aarhus, Denmark). **Результати.** Визначення видового складу потенційних збудників комплексу ески показало присутність видів, які пов'язані із хворобами багаторічної деревини винограду. При цьому було виявлено 3 ізоляти *Cadophora luteo-olivacea,* яка відноситься до специфічних патогенів комплексу ески. Зразки, уражені ескою, показали також присутність виду *Eutypa lata* (збудник відмирання винограду – еутипозу – eutipa dieback) і *Botryosphaeria dothidea* (збудник відмирання – botrioshaeria dieback). **Висновки.** ДНК-ідентифікація грибних патогенів показала різницю у видовому складі між безсимптомними рослинами винограду та рослинами, ураженими ескою. Зразки із симптомами ески продемонстрували наявність видів *Eutypa lata* і *Botryosphaeria dothidea,* які були відсутні в безсимптомних рослинах.

Ключові слова: хвороби багаторічної деревини винограду, еска, *Cadophora luteo-olivacea,* ПЛР, секвенування ДНК.

ВЫЯВЛЕНИЕ ПАТОГЕНОВ БОЛЕЗНЕЙ МНОГОЛЕТНЕЙ ДРЕВЕСИНЫ ВИНОГРАДА НА ПОДВОЙНЫХ СОРТАХ ВИНОГРАДА В УКРАИНЕ

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Резюме

Целью работы было выделение грибов из виноградных штамбов, выделение ДНК и выявление потенциальных патогенов эски в Украине. **Методы.** Выделение и идентификация грибных культур проведены на основе морфологии и секвенирования ДНК. Общая ДНК грибных колоний была экстрагирована набором NucleoSpin Tissue (Macherey-Nagel, Düren, Германия) согласно инструкциям производителя. Для амплификации ITS-области использовали праймеры ITS1 и ITS4. Продукты ПЦР, соответствующие размеру около 550 bp, секвенировали. Полученные нуклеотидные

последовательности анализировали с помощью CLC Main Workbench 5.0 (CLC bio, Aarhus, Denmark)**. Результаты.** Определение видового состава потенциальных возбудителей комплекса эски показало присутствие видов, связанных с болезнями многолетней древесины винограда. При этом было выявлено 3 изолята *Cadophora luteo-olivacea,* которая относится к специфическим патогенам комплекса эски. Образцы, пораженные эской, показали также присутствие вида *Eutypa lata* (возбудитель отмирания винограда – эутипоз – eutipa dieback) и *Botryosphaeria dothidea* (возбудитель отмирания – botrioshaeria dieback). **Выводы.** ДНК-идентификация грибных патогенов показала различие в видовом составе между бессимптомными растениями винограда и растениями, пораженными эской. Образцы с симптомами эски выявили наличие видов *Eutypa lata* и *Botryosphaeria dothidea*, которые отсутствовали в бессимптомных растениях.

Ключевые слова: болезни многолетней древесины винограда, эска, *Cadophora luteo-olivacea*, ПЦР, секвенирование ДНК.

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