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RESEARCH PAPERS

Esca disease complex of grapevine in Montenegro: incidence, economic importance, variety sensitivity, and identification of the pathogens

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Summary. Grapevine cultivation in Montenegro has long historical and economic significance, with wine being a key trade product. However, under Montenegrin agroecological conditions, grapevine cultivation is increasingly threatened by plant diseases, particularly the Esca disease complex (EDC) which is the most economically damaging among grapevine trunk disease. A 4 year survey from 2021 to 2024 confirmed presence of EDC in all Montenegrin winegrowing regions, as Grapevine Leaf Stripe Disease (GLSD) and apoplexy. Symptom severity varied by grapevine variety and vineyard age, with the greatest incidence in vineyards aged 30–35 years. The indigenous varieties Vranac and Kratošija were more susceptible than introduced varieties (Syrah, Merlot, Chardonnay, Cabernet Sauvignon). Laboratory analyses of symptomatic trunks yielded numerous isolates of endophytic microorganisms. Preliminary identification of these, based on morphology, and molecular PCR diagnostics targeting the ITS rRNA genomic region, plus analyses of the partial *tub2* and *tef1-α* genes, identified three key fungi responsible for EDC in Montenegro: *Phaeoacremonium minimum*, *Phaeomoniella chlamydospora*, and *Fomitiporia mediterranea*. In the investigated vineyards, numbers of empty places remaining after removal of dead grapevines varied, depending on vineyard age and grape variety. In a 21-year-old vineyard where dead vines were not replaced, vine loss of 32%, and direct yield reduction of 44%, were recorded, highlighting the substantial economic impact of esca disease.

Keywords. Fungi, grapevine trunk disease, vineyard, autochthonous varieties.

INTRODUCTION

Montenegro is one of the smallest European countries, with an area of 13,888 km^2 (MONSTAT, 2023). Despite its size, the country has diverse biogeographical conditions that support viticulture (Grbić *et al.*, 2017). Grapevine cultivation dates back to the pre-Roman period. Today, four winegrow-

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ing regions are recognized in Montenegro: the Skadar Lake Basin, the Montenegrin Coast, the Nudo region, and the North (Maraš *et al*., 2017). Vranac is the dominant variety, alongside other indigenous varieties including Kratošija, Čubrica, Krstač, and Žižak (Maraš *et al*., 2020). In 2021, vineyards covered 2,825 ha, placing Montenegro 70th globally (OIV). Red grape varieties dominate vineyard area (69.01%), followed by white (23.11%) and table grapes (7.89%). Indigenous varieties account for 86.25% of planted area, while the rest (13.75%) is in introduced varieties, mostly Chardonnay and Cabernet Sauvignon (Maraš *et al*., 2021). Viticulture is a key agricultural sector, with wine exports representing 26.04% of the total value of agricultural exports from Montenegro (MONSTAT, 2023).

Grapevine trunk diseases (GTDs) are among the most devastating and severe grapevine diseases in Mediterranean countries, including Spain, France, Portugal, and Italy (Guerin-Dubrana *et al*., 2019; Raimondo *et al*., 2019). In Europe, incidence of GTDs is similar across countries, with reported rates ranging from 1.8% to 10.5% in Spain, and from 8% to 19% in Italy. In some old vineyards, particularly in Tuscany, Apulia, and Sicily, GTD incidence has reached as high as 60% to 80% (Surico *et al*., 2000; Romanazzi *et al*., 2009; Claverie *et al*., 2020). Outside Europe, an increasing trend in reported GTD cases has occurred since the late 20th century (Rubio and Garzon, 2011; Kraus *et al*., 2019). These diseases are becoming more prevalent in countries such as Chile (Grinbergs *et al*., 2023), Argentina (Paolinelli *et al*., 2022), Jordan (El Samen *et al*., 2023), British Columbia (Úrbez Torres *et al*., 2023), Australia and New Zealand (Baskarathevan *et al*., 2012; De la Fuente *et al*., 2016), and China (Yan *et al*., 2013), where they are causing significant economic losses (Leal *et al*., 2023). In France, the National Institute of Viticulture and Wine estimated that esca, a major GTD, leads to annual losses in wine production valued at approx. €1 billion, equating to approx. $US 1,500 per hectare per year (De la Fuente *et al*., 2016). The cost of replacing dead grapevines has been estimated at more than $US 1.5 billion annually, which is probably a significant underestimate (Ouadi *et al*., 2019).

Esca is one of the most important GTDs, capable of causing severe wilting of vineyard plants. Research on esca etiology began in the late 19th century in France, continued in Italy and California, United States of America, during the early 20th century, and intensified in the 1990s (Mugnai *et al*., 1999). Within the esca complex, five distinct disease or syndrome classes have been identified, based on vine age, symptom type, and the fungi involved: (1) dark wood streaking, and (2) Petri disease, both in young vines (<7-years-old); (3) white rot, (4) esca proper in vines >8-years-old); and (5) Grapevine Leaf Stripe Disease (GLSD), which affects vines of all ages and is often associated with partial or complete vine apoplexy (Calzarano *et al*., 2021; Kraus *et al*., 2022; Berris *et al*., 2022). Esca is a complex disease in which infections can remain latent within grapevine trunks, showing no significant external symptoms until affected plants experience unfavourable or stressful growth conditions (Fischer *et al*., 2019). Expression of foliar symptoms is influenced by several factors, including grapevine variety, age, rootstock, and environmental conditions, including soil water retention capacity and summer climate (Laveau and Meri, 2015; Latinović and Latinović, 2017; Kovács *et al*., 2017; Calvo-Garrido *et al*., 2021; Dewasme *et al*., 2022).

Apoplexy is characterized by sudden partial or complete wilting of grapevine shoots (Goufo *et al*., 2019). During apoplexy, parts of leaf interveinal tissues rapidly discolour, turning pale green to gray, becoming necrotic, and wilting. Affected shoots typically lose all leaves within a few days (Lecomte *et al*., 2012; Magnin Robert *et al*., 2017). GLSD symptoms of esca, include necroses of interveinal leaf tissues, that are often accompanied by variable red pigmentation along leaf edges, and formation of characteristic “tiger stripe” patterns (Gramaje *et al*., 2018; Del Frari *et al*., 2022, Del Frari *et al*., 2025). GLSD symptoms most commonly occur in mid-summer, but appear sporadically and can vary from year to year. They may affect entire vine canopies or only parts of each grapevine (Serra *et al*., 2018; Del Frari *et al*., 2019). Occurrence and severity of GLSD symptoms are influenced by numerous variables, including a complex interplay of biotic and abiotic factors, as well as agricultural practices (Claverie *et al*., 2020; Del Frari *et al*., 2021). Although the mechanisms underlying GLSD development remain under debate, the prevailing hypothesis is that phytotoxic compounds produced by fungi in grapevine tracheids are translocated to leaves, where they disrupt photosynthesis and trigger plant defense responses (Calzarano *et al*., 2018). In addition, foliar symptoms may result from disruptions in sap flow caused by pathogen-induced changes in wood structure, such as non-gaseous embolism, vascular occlusion by gels and/or tyloses, or pathogen infections of annual shoots (Larignon, 2017; Bortolami *et al*., 2019; Pouzoulet *et al*., 2019).

Internal symptoms of esca typically manifest as dark brown to black spots visible in leaf cross-sections, or black necrotic lesions along longitudinal sections of grapevine trunks (Gramaje *et al*., 2009). In old vines, white rot is often observed in the central parts of trunks, as white to light brown decayed wood, clearly demarcated from healthy tissues by dark lines (Mugnai *et al.*, 1999). Although grapevines affected by esca host diverse microflora, the pathogens most commonly associated with this disease are: *Phaeomoniella chlamydospora* (Crous and Gams, 2000), more than 30 *Phaeoacremonium* species (Spies *et al.*, 2018), several species of *Cadophora*, especially *Cadophora luteo olivacea* (Fontaine *et al.*, 2025), and *Fomitiporia mediterranea* (Fischer, 2002). Mugnai *et al.* (1999) proposed two hypotheses regarding infection processes. The first was that *Pa. chlamydospora* and *Pm. minimum* initially colonize grapevines, followed by *F. mediterranea* invasion. The second hypothesis assumes that *Pa. chlamydospora*, *Pm. minimum*, and *F. mediterranea* act simultaneously, and contribute collectively to disease development. Spore release by these fungi is generally associated with rainfall, although optimum periods for inoculum dissemination vary depending on the fungal species. *Phaeomoniella chlamydospora* can spread throughout each year, but most commonly infects grapevines through wounds created during pruning periods. In contrast, *Pm. minimum* is more active during vine vegetative phases, and infections often occur after bud break (Larignon and Gramaje, 2015). Fresh wounds are considered necessary for GTD pathogens to infect grapevine tissues, as most pathogens are unable to colonize healthy plants, due to their periderm and rhytidome protective layers (Claverie *et al.*, 2020). Grapevines usually undergo heavy annual pruning, and winter pruning wounds are the primary entry points for GTD pathogens. Several studies have examined the susceptibility of pruning wounds to fungal infections, and have indicated variations depending on the pathogen species, pruning time (early or late winter), and climatic conditions. Pruning wounds remain susceptible to infections for several weeks and up to 4 months (Gramaje *et al.*, 2018; Mondello *et al.*, 2019). In production of planting material, injuries may occur that are entry points for GTD pathogens. These pathogens are often present on rootstocks and scions prior to grafting, while nursery practices, including rehydration, grafting, and field cultivation, can contribute to spread of these pathogens in planting material (Gramaje *et al.*, 2018). Grapevines from asymptomatic planting material that is infected by GTD pathogens can remain symptomless for several years (Akgul *et al.*, 2023).

Latinović (2010) showed that GTD pathogens occur every year in Montenegro, but a comprehensive analysis of their presence has not been conducted. Therefore, the main objectives of the present study were: a) to survey the presence of esca disease in all vineyard regions across of Montenegro; b) to isolate and molecularly identify microorganisms from symptomatic grapevines; and c) to obtain reliable data on the distribution and incidence of GTDs, susceptibility of local grapevine varieties, and make a preliminary assessment of the economic impacts of esca disease in this country.

MATERIAL AND METHODS

Field surveys

A four year field study was carried out from 2021 to 2024, encompassing 30 vineyards across Montenegro. To obtain the most representative data possible, vineyards were selected from each of the four distinct Montenegrin winegrowing regions. The surveyed vineyards varied in age and layout, and their distribution across the country is shown in Figure 1. The main criteria for vineyard selection were: (1) the vineyard was officially registered in the Ministry of Agriculture and Rural Development of Montenegro; (2) it contained more than 10,000 vines; and (3) the grower was willing to allow on-site evaluation of vine health, estimation of disease symptom incidence, and sampling of potentially symptomatic plants. Of the 30 vineyards included in the study, six were classified as young (≤8 years old), while the remaining 24 were categorized as mature (>8 years). Some young blocks (≤8 years) were present within mature vineyards. Only six vineyards were uniform for grapevine variety and age; the remaining 24 comprised multiple grapevine varieties and ages, which were clearly separated into distinct plots. Table 2 outlines details of the surveyed vineyards.

To assess incidence of esca disease symptoms, a total of 500 vines per vineyard (ten rows × 50 vines) were visually inspected. Vines of different age and variety compositions were evaluated for the presence of the characteristic leaf symptoms associated with esca disease (GLSD and the apoplexy). To assess susceptibility of different grapevine varieties, a total of 9,500 vines were evaluated in young vineyards, and 34,500 vines were evaluated in mature vineyards. All observed symptoms were photographed and described. Depending on the numbers of symptomatic vines and diversity of affected grapevine varieties within each vineyard, a variable number of whole plant samples was randomly collected. In total, 40 whole-plant samples were collected: four from young vines, and 36 from mature vines. Of these, seven samples were taken from vines exhibiting the apoplexy, while 33 exhibited symptoms characteristic of GLSD. Samples were collected from the following grapevine varieties: Vranac (18 samples), Kratošija (six), Afus Ali (three), Ribijer (two), Cardinal (four), Merlot (two), Cabernet Sauvignon (two), Krstač

Figure 1. Map indicating inspected and tested vineyards in Montenegro that were assessed for presence of esca disease.

(one), Čubrica (one), and Moldava (one sample). All collected samples underwent further laboratory analyses at the Phytopathology Laboratory of the Biotechnical Faculty in Podgorica.

In parallel with field assessments, a survey was conducted among 70 grape growers. To ensure the objectivity and reliability of the survey results, no visual inspections or disease assessments were carried out in the vineyards of the surveyed growers. All participants and their vineyards were selected in an unbiased manner, using the criteria applied in the field study, representing different viticultural regions, a broad range of grapevine varieties, and different vineyard ages. To facilitate accurate symptom recognition, colour photographs of esca symptoms were presented to respondents. The survey consisted of the following items:

a) Do the presented symptoms occur in your vineyard?
b) Is the appearance of symptoms related to vine age?
c) Is the appearance of symptoms associated with the grapevine variety?
d) Estimate the level of disease incidence in your vineyard, using the following numerical scale (1 = a few symptomatic vines scattered across the vineyard; 2 = a few symptomatic vines within a single row; 3 = a large number of symptomatic vines in a single row).

Isolation of pathogens

The collected samples were transported to the laboratory, where presence of fungal fruiting bodies (perithecia and pycnidia) was initially examined using a stereomicroscope. The bark of plants in each sample was carefully removed, and transverse and longitudinal sections were cut from the wood to assess alterations in the vascular tissues. Grapevine trunks were each sectioned transversely at 10 cm intervals down to the plant grafting point, to observe for vascular necroses of the conductive tissues. Following the protocol of Armengol *et al.* (2001), small wood fragments (3 to 5 mm) were excised from the transition zone between healthy and diseased tissue. To eliminate epiphytic fungi, the wood fragments were surface sterilized by immersion in 1.5% sodium hypochlorite solution for 2 min, followed by 30 sec in 70% ethanol, and were then rinsed twice in sterile distilled water. Under aseptic conditions in a laminar flow cabinet, sterilized wood fragments were placed in Petri dishes containing potato dextrose agar (PDA; Biolife, Ref. 4019352), containing streptomycin sulfate (0.5 mg mL^{-1}) to suppress bacterial contamination. A total of 20 Petri dishes were inoculated per sample, corresponding to five dishes per symptom type, and each dish contained four plant tissue fragments. The Petri dishes were then incubated in the dark at 25°C. After 2 weeks, emerging fungal colonies were individually transferred to fresh PDA in Petri plates for further cultivation and identification.

Morphological characterizations of isolated fungi

Identification and selection of colonies of isolated microorganisms for further molecular diagnostics was based on the morphological and cultural characteristics of their fruiting structures and spores as determined using a microscope, and comparing these with previously identified isolates of *Pa. chlamydospora*, *Pm. minimum*, *F. mediterranea*. These examinations were carried out at the Agricultural University of Athens, Greece.

Molecular characterizations of isolated fungi

Thirteen representative isolates were selected for molecular diagnoses. DNA was extracted from each isolate following the PMI Zipfer Lab protocol. Total genomic DNA was extracted from 50 to 100 mg of mycelium scraped from 15-d-old colonies grown on PDA. For preliminary species identification of each isolate, the ITS region was amplified using the primer pair ITS5 (5' GGAAGTAAAAGTCGT AACAAGG 3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3') (White *et al*., 1990). Depending on the preliminary species identification, additional gene fragments of β-tubulin (*tub2*) and *tef1a* were amplified and sequenced. The primer pairs used were Bt1a (5' TTCCCCCGTCTCCACTTCTTCATG 3') and Bt1b (5' GACGAGATCGTTCATGTTGAACTC 3') (Glass and Donaldson, 1995), and EF1-728F (5' CATCGAGAAGTTCGAGAAGG 3') and EF1-986R (5' TACTTGAAGGAACCCTTACC 3') (Carbone and Kohn, 1999). The PCR conditions for the ITS regions were as follows: initial DNA denaturation at 95°C for 3 min, followed by 40 cycles each of denaturation at 95°C for 30 sec, primer annealing at 55°C for 30 sec, and DNA chain elongation at 72°C for 1 min. PCR products were separated on 2% agarose gels at 80 V in TAE buffer. For *tub2* amplification, PCR conditions were: initial denaturation at 95°C for 5 min, followed by 35 cycles each of denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec, elongation at 72°C for 60 sec, and a final elongation at 72°C for 10 min. PCR products were separated on 1.5% agarose gels, each run for 20 min at 300 V, and visualized under UV light. For *tef1a* amplification, PCR conditions were: initial denaturation at 94°C for 5 min, followed by 30 cycles each of denaturation at 94°C for 60 seconds, annealing at 55°C for 60 sec, elongation at 72°C for 60 sec, and final elongation at 72°C for 8 min. PCR products were separated on 1.5% agarose gels, each run for 20 min at 300 V, and visualized under UV light. Preliminary fungal identifications based on ITS sequencing were carried out by the CEMIA company (Larissa, Greece). Additional sequencing of *tub2* and *tef1a* genes was carried out by Macrogen Europe B.V. (Netherlands). Sequence quality was verified using BioEdit software. Sequence comparisons with GenBank data were carried out using BLASTn.

Nucleotide and phylogenetic analyses

All gene sequences were manually checked for quality, and compared against sequences available in the National Center for Biotechnology Information (NCBI) database using the nucleotide BLAST (BLASTn) tool. Partial coding sequences of the ITS, *tub2*, and *tef1α* genes from thirteen representative isolates obtained in this study, along with reference strains retrieved from the NCBI database (Table 1), were aligned using the MUSCLE algorithm.

Phylogenetic trees based on the analyzed genomic regions of the studied microorganisms were constructed using the Maximum Likelihood method, with 1000 bootstrap replicates, in MEGA software version 11. Evolutionary distances for the phylogenetic analyses were calculated according to the Tamura-Nei model, and were expressed as the number of base differences per site (Tamura *et al*., 2021).

Impacts of esca disease on grape yield quantity and quality

The dynamics of esca disease spread were monitored over a four year period (2021 to 2024) in a vineyard plot belonging to the Biotechnical Faculty in Podgorica. The vineyard was established in 2003, with 3,615 grapevines of the autochthonous variety Vranac, that were planted over an area of 1.1 ha. All the vines were trained using the double Guyot Poussard pruning system. Since planting, no young vines have been used to replace dead or symptomatic plants. During the four year monitoring period, the dates of first appearance of esca disease symptoms and evaluations of the symptom types apoplexy and GLSD, were carried out annually. Disease incidence assessments were carried out as performed in mid to late summer (July–August) each year, using the methods of Guerin-Dubrana *et al*. (2013). Observations were recorded in excel spreadsheets using a numerical scoring system: where 1 = asymptomatic vine, 2 = symptomatic vine, and 0 = empty vine site in the vineyard. Throughout the study period, no foliar symptoms of other GTDs were observed in the vineyard.

Table 1. Montenegrin and reference fungus isolates (NCBI) used in the phylogenetic analysis of this study.

Strain/Isolate	Host	Country	Year	Acc. No. (ITS)
Pm. minimum				
MN001	*Vitis vinifera*	Montenegro	2023	PQ191303
MN002	*Vitis vinifera*	Montenegro	2023	PQ191304
MN003	*Vitis vinifera*	Montenegro	2023	PQ191305
MN004	*Vitis vinifera*	Montenegro	2023	PQ191306
MN005	*Vitis vinifera*	Montenegro	2023	PQ191307
MN007	*Vitis vinifera*	Montenegro	2023	PQ191309
MN009	*Vitis vinifera*	Montenegro	2023	PQ191310
Y161-19-2z	*Vitis vinifera*	Spain	2016	JF275867
Y038-05-3z (a)	*Vitis vinifera*	Spain	2016	JF275866
CBS 860.73	*Vitis vinifera*	South Africa	2000	AF197980
Y038-05-3z (b)	*Vitis vinifera*	Spain	2011	JF275866
Bent 720	*Vitis vinifera*	USA	2022	OP038029
Kern 725	*Vitis vinifera*	California	2022	OP038082
MEND-F-0825	*Prunus domestica*	Czech	2023	OQ358090
Pa. chlamydospora				
MN012	*Vitis vinifera*	Montenegro	2023	PQ191313
MN013	*Vitis vinifera*	Montenegro	2023	PQ191314
MN014	*Vitis vinifera*	Montenegro	2023	PQ191315
MN015	*Vitis vinifera*	Montenegro	2023	PQ191316
MN010	*Vitis vinifera*	Montenegro	2023	PQ191311
IBVDSP08	*Vitis vinifera*	Brazil	2019	MK903785
Y112-42-3	*Vitis vinifera*	Spain	2007	EU018415
JKI-Ap04	*Vitis vinifera*	Germany	2018	MH999525
Kern717	*Vitis vinifera*	California	2022	OP038080
Bent710	*Vitis vinifera*	California	2022	OP038023
JKI-May05	*Vitis vinifera*	Germany	2018	MH999527
P. pinifoliarum	*Vitis vinifera*	South Korea	2015	MH862974
P. tardicola	*Prunus armeniaca*	South Africa	2015	NR_132006
F. mediterranea				
MN008	*Vitis vinifera*	Montenegro	2023	PQ200513
Fmed2395	*Vitis vinifera*	Austria	2023	OQ534545
AFTOL-ID 688	*Vitis vinifera*	USA	2005	AY854080
MF3/22	*Vitis vinifera*	USA	2012	XM_007267815
MUCL 38514	*Vitis vinifera*	Belgium	2010	GU461953
MUCL34101	Unknown	Germany	2010	GU461947
MUCL53548	Unknown	Estonia	2012	JX093790
MUCL49406	Unknown	Australia	2010	GU462001

The annual incidence of esca symptoms was calculated as the proportion of symptomatic vines in a given year relative to the total number of vines in the vineyard:

Incidence (%) = {Number of symptomatic vines/
Total number of vines in vineyard} × 100.

Similarly, the percentage of empty places was calculated by dividing the number of missing vines by the total number of originally planted vines in the vineyard:

Empty places (%) = {Number of missing vines/
Total number of vines planted} × 100.

To assess cumulative progression of esca, the Cumulative Incidence (CI) for the year 2024 was calculated using the following formula:

CI (%) =(SP1+SP2)/TNbP × 100,

where: SP1 = number of vines with foliar symptoms observed in 2024, SP2 = number of vines without current symptoms but previously symptomatic in earlier years, and TNbP = total number of grapevines in the vineyard in the year of assessment.

To preliminarily assess the impacts of esca disease on grape yield during the 2024 harvest, on the date determined by the vineyard manager, grape clusters were separately collected from each symptomatic vine exhibiting apoplexy and GLSD, as well as from asymptomatic vines, throughout the experimental vineyard. Grape yield for each diseased vine was determined by weighing the harvested clusters, and the yields from healthy vines were also measured. Throughout 2024, regular chemical treatments were applied to control other grapevine diseases, including downy mildew, powdery mildew, and gray mold, thus excluding potential impacts these diseases on yield reductions. By comparing the measured yields of healthy and esca symptomatic vines, the percentage of yield loss attributable to esca disease was determined. To determine the effect of esca disease on grape must quality, sugar contents (Brix), total acidity, and pH were determined. At harvest, grape samples were collected separately from vines with clearly visible esca foliar symptoms and from healthy vines. Grapes were processed at the Biotechnical Faculty's enology laboratory. Two grape must samples were prepared: one obtained from symptomatic vines and the other from asymptomatic vines. Their sugar contents (expressed in Brix) were determined using an Exl refractometer, pH was measured using a calibrated pH meter, and total acidity was determined by titrations.

RESULTS

Symptoms in the field

Typical symptoms of esca disease were observed in 22 vineyards (73% of those surveyed), while no symptoms were detected in the remaining eight vineyards (27%). Among the vineyards without symptoms, three were younger than five years, while one was between 80 and 100 years old, and contained the Vranac grapevine variety. In the other asymptomatic vineyards, various introduced grape varieties were growing. In all vineyards where esca symptoms were identified, apoplectic events were recorded (Figure 2a), as well as GLSD symptoms [clear "tiger stripe" patterns on the leaves (Figure 2b)]. Symptomatic leaves had characteristic interveinal necroses of leaf tissues, with lesions bordered by purple margins. In addition to leaf wilting, gradual shriveling, drying, and eventual decay of grape clusters on affected shoots were observed. In some affected plants, regardless of variety, the clusters did not dry completely, but had uneven and incomplete ripening. In white grape varieties Krstač, Victoria, and Afus Ali with GLSD had characteristic dark brown spots on the grape berries. These spots were irregular in shape and unevenly distributed on the berry skins. In rare cases, the spots covered almost entire berries, which then became dark to purplish brown. During the four year study period, fluctuations in GLSD were observed in the 22 affected vineyards.

For symptoms on grapevine trunks, cross sectional and longitudinal examinations of the trunks revealed several characteristic internal alterations. These were: a) necrotic lesions as black spots, distributed individually or in clusters (Figure 3 a); b) dark to reddish brown necrotic zones, located either in trunk centres or along the edges of cross sections (Figure 3 b); c) brown areas of varying shades and textures, often blending with the types of necroses in Figure 3, a and b) (Figure 3c); or d) white rot, characterized by progressive decay of hardwood tissues, transforming it into spongy masses. Decayed tissues were creamy yellow and were each distinctly bordered by a black or brown demarcation line separating healthy from diseased tissues (Figure 3 d). Longitudinal sections of the trunks showed that white rot typically originated from pruning wounds greater than 2 cm diam. In many examined samples, white rot was found to extend toward or reach trunk sur-

Figure 2. (a) Symptoms of apoplexy of grapevine. (b) The"tiger stripe" pattern symptom on grapevine leaves

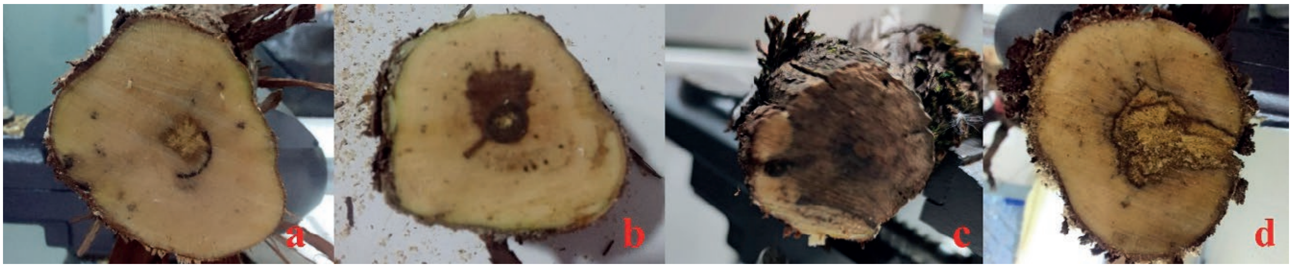

Figure 3. Disease symptoms in grapevine trunks. a) Necrosis in the form of black spots. b) Dark to red-brown necrotic zone in the central part of the trunk. c) Brown necrotic trunk surfaces of various shades and textures. d) White rot bordered by a black line.

faces, leading to visible cracks along affected trunks. The decay tended to spread downwards, but was not observed below graft unions. In some samples, separate initiations of white rot were identified in the lower third of the affected trunks, where damage caused by insects was found and were probably the entry points for pathogens. Unlike white rot, black necrotic spots were also observed at the graft unions, as well as on the rootstocks onto which the plants were grafted. Symptoms of white rot on transverse and longitudinal trunk sections were observed only in grapevines older than 15 years. In trunks younger than 15 years, only black necrotic spots or dark brown necrotic zones were recorded, with no presence of white rot.

Inspections of the vineyards revealed that esca disease was most prevalent in vineyards containing plants of Montenegrin grape varieties, that were older than five years, and incidence of esca increased with the vineyard age. The GLSD were most pronounced in vineyards aged between 15 and 25 years. In vineyards older than 35 years, large numbers of missing plants were recorded, but presence and severity of disease symptoms were low. Data of presence of apoplexy and GLSD in vineyards of different ages are presented in Table 2. No symptoms of esca were recorded in vineyards aged between 80 and 100 years. However, more than 50% of missing plants were observed in these vineyards. Surveys among grape producers could not provide clear information on whether plant decline in the past was due to esca disease or to other causes. Plants in these old vineyards were most often established on their own roots, and are some of the few vineyards that survived the phylloxera (*Phylloxera vastatrix*) infestation in Montenegro.

Grapevine variety sensitivity

During the four-year monitoring of apoplexy and GLSD symptoms, these symptoms were not observed in the following introduced grapevine cultivars: Syrah, Cabernet Franc, Malvasia, Probus, Pinot Noir, Grenache, Marselan, Sauvignon Blanc, and Chardonnay. The only introduced cultivars that exhibited these symptoms were Cabernet Sauvignon and Merlot, and only in vineyards aged between 15 and 30 years. These results that the autochthonous Montenegrin grapevine cultivars Vranac and Kratošija have greater esca susceptibility than introduced cultivars, at least under Montenegrin agro-climatic conditions. Analysis of variance (ANOVA) and Bonferroni *post hoc* tests confirmed that Vranac was more susceptibile than Cabernet Sauvignon ($P = 8.25\times10^{-17}$) or Merlot ($P = 3.68 \times 10^{-14}$), in vineyards of comparable age. Likewise, the Kratošija cultivar was more susceptible esca than Cabernet Sauvignon ($P = 2.03 \times 10^{-21}$) or Merlot ($P = 1.79\times10^{-18}$). No statistically significant difference in susceptibility to esca disease ($P = 0.36$) was observed between Cabernet Sauvignon and Merlot. Compared to Vranac, the old autochthonous Montenegrin cultivars Krstač ($P = 1.0 \times 10^{-5}$) and Čubrica ($P = 4.01 \times 10^{-24}$), which are sporadically cultivated in Montenegro, had greater tolerance to esca disease. Furthermore, Krstač ($P = 1.52 \times 10^{-8}$) and Čubrica ($P = 2.57 \times 10^{-29}$) had greater esca tolerance than Kratošija. In addition to wine grape cultivars, the table grape cultivars Cardinal ($P = 2.18 \times 10^{-15}$; $P = 3.97 \times 10^{-18}$), Ribier ($P = 9.71 \times 10^{-19}$; $P = 1.06 \times 10^{-21}$), and Afus Ali ($P = 9.16\times10^{-9}$; $P = 6.17\times10^{-11}$) had high susceptibility to esca disease than the introduced cultivars Merlot and Cabernet Sauvignon.

No statistically significant differences in esca susceptibility were detected between the table grape cultivars Cardinal ($P = 0.70$), Ribier ($P = 0.17$), and Afus Ali ($P = 0.05$) when compared to Vranac. Cardinal ($P = 0.37$) and Ribier ($P = 0.94$) also did not differ in susceptibility from Kratošija, while the Afus Ali showed statistically significant different susceptibility compared to Kratošija ($P = 0.003$). A detailed overview of the data on the presence and incidence of apoplexy and GLSD symptoms in different grapevine cultivars, across vineyards of varying ages, is presented in Table 2.

Table 2. Presence and incidence of GLSD and apoplexy symptoms in inspected Montenegrin vineyards and grapevine cultivars during 2021 to 2024.

Plot No.	Locality	Cultivar	Age (years)	GLSD/ apoplexy symptoms	Incidence (%)
1	Vuksanlekić	Vranac	9	Yes	1-5
		Vranac	10	Yes	1-5
		Cardinal	14	Yes	10-15
		Vranac	14	Yes	10-15
2	Vuksanlekić	Cabernet Franc	10	No	0
		Syrah	10	No	0
		Marselan	12	No	0
		Skadarka	12	No	0
		Cabernet Sauvignon	12	No	0
		Chardonnay	10	No	0
		Sauvignon Blanc	10	No	0
3	Sige	Vranac	35	Yes	50-55
		Krstač	35	Yes	10-15
		Cardinal	35	Yes	20-25
		Kratošija	35	Yes	20-25
		Ribier	35	Yes	20-25
		Afus ali	35	Yes	15-20
4	Sutomore	Vranac	17	Yes	15-20
		Kratošija	17	Yes	15-20
5	Gluhi do	Probus	3	No	0
		Vranac	100	No	0
		Vranac	80	No	0
		Merlot	10	No	0
		Marselan	10	No	0
6	Godinje	Vranac	10	Yes	1-5
7	Mataguži	Vranac	14	Yes	10-15
		Kratošija	14	Yes	15-20
8	Beri	Vranac	13	Yes	10-15
	Beri	Kratošija	13	Yes	10-15
		Smederevka	13	No	0
		Župljanka	13	No	0
		Malvasia	13	No	0
		Muscat Hamburg	13	No	0
		Chardonnay	13	No	0
9	Savina	Grenache	30	No	0
		Merlot	20	Yes	5-10
		Syrah	20	No	0
		Cabernet Sauvignon	30	Yes	1-5
		Chardonnay	30	No	0
10	Nudo	Vranac	15	Yes	10-15
		Žilavka	15	No	0
11	Dobrsko selo	Bijela Tamjanika	8	No	0
		Crvena Tamjanika	8	No	0
		Pinot Noir	8	No	0
12	Briska gora	Vranac	13	Yes	10-15
		Vranac	53	Yes	1-5
13	Beri	Vranac	11	Yes	5-10
		Kratošija	15	Yes	5-10
		Cabernet Sauvignon	15	Yes	1-5
		Syrah	11	No	0
14	Golubovci	Vranac	12	Yes	5-10
		Ribijer	12	Yes	1-5
15	Golubovci	Cardinal	22	Yes	10-15
		Viktoria	17	Yes	5-10
		Cardinal	17	Yes	10-15
16	Golubovci	Marselan	4	No	0
		Chardonnay	4	No	0
		Cabernet Sauvignon	4	No	0
		Lisičina	4	No	0
		Malvasia	3	No	0
17	Balabani	Vranac	20	Yes	15-20
		Kratošija	20	Yes	20-25
18	Piperi	Merlot	8	No	0
		Cabernet Sauvignon	8	No	0
19	Straševina	Moldava	30	Yes	1-5
20	Orja Luka	Cardinal	5	No	0
		Moldava	5	No	0
		Rozaklija	5	No	0
		Black Magic	5	No	0
		Frankovka	5	No	0
21	Donja Gorica	Vranac	25	Yes	15-20
		Kratošija	25	Yes	15-20
		Ribier	25	Yes	5-10
22	Ubli	Čubrica	50	Yes	1-5
		Čubrica	70	No	0
23	Tološi	Vranac	35	Yes	20-25
24	Lješkopolje	Vranac	21	Yes	25-30
25	Crmnica	Vranac	18	Yes	10-15
26	Drušići	Kratošija	27	Yes	5-10
		Vranac	27	Yes	15-20
27	Reževići	Rozaklija	10	No	0
		Demir Kapija	10	No	0
		Muscat Hamburg	10	No	0
28	Piperi	Vranac	8	Yes	1-5
29	Kuće Rakića	Vranac	10	Yes	5-10
		Kratošija	10	Yes	1-5
30	Martinići	Muscat Hamburg	4	No	0
		Afus ali	4	No	0

Grape producer survey

Results of the survey showed that 17% of grape producers reported never having observed esca disease symptoms in their vineyards. Furthermore, only 31% of respondents were familiar with the disease, while 69% stated they were unaware that the symptoms they had observed were related to esca. For disease severity, esca was recorded at level 1 in 35% of the surveyed vineyards, level 2 in 55%, and level 3 in 10% of the vineyards. Only one respondent reported sudden dieback of young vines during their second or third year of growth. According to the surveyed producers, incidence of esca symptoms reached peak levels in vineyards aged between 15 and 25 years. The results also showed that esca symptoms occur rarely in introduced grapevine cultivars.

Molecular identification of obtained fungal isolates

Many different fungal isolates were obtained from the collected samples. Isolates exhibiting morphological characteristics of the known esca-associated genera (*Phaeomoniella*, *Phaeoacremonium*, *Fomitiporia*), including colony colour, texture, and growth pattern, were selected for further molecular identifications. The remaining isolates were stored at the Phytopathology Laboratory of the Biotechnical Faculty and will be further assessed. Fifty-nine isolates were selected based on colony morphology as potential esca-associated pathogens, of which 13 were chosen for molecular identification. Preliminary PCR analysis using universal ITS5/ITS4 primers amplified a specific fragment of 360–400 bp for these 13 selected isolates. Additionally, fragments were obtained of approx. 550 bp for the partial *tub2* gene, and 290 bp for the partial *tef1α* gene. BLASTn analysis of the sequences of part *tub2* or *tef1a* genes from Montenegrin isolates showed a high level of similarities (98.65–100%) with *Phaeomoniella chlamydospora*, *Phaeoacremonium minimum* and *Fomitiporia mediterranea*. The homology of the 13 isolates for ITS, *tub 2* and *tef1a* is shown in Table 3.

Nucleotide and phylogenetic analyses

The constructed Maximum Likelihood phylogenetic trees for *Pa. chlamydospora*, *Pm. minimum* and *F. mediterranea*, based on concatenated sequences (ITS and *tub2*) and (ITS and *tef1a*), are shown in, respectively, Figure 4a, Figure 4 b and Figure 5. The five Montenegrin *Pa. chlamydospora* isolates clustered into subgroups and were most closely related to isolates from Spain (Y112-42-3) and California (Kern717 and Bent710). Seven Montenegrin isolates of *Pm. minimum* were most closely related to isolates originating from Spain (Y038-05-3z[a] and Y038-05-3z[b]). One isolate *F. mediterranea* formed a separate subgroup, but was phylogenetically most closely related to isolates from Austria (Fmed35) and Belgium (MUCL38514).

Effects of esca disease on grape yield quantity and quality

During the four year monitoring period conducted in the studied vineyard, incidence of esca disease incidence ranged from 9.76% in 2021 to 29.82% in 2024. Data on the number of healthy and symptomatic vines, the number of empty vine positions in the vineyard, and

Table 3. Percent similarities of the Montenigrin *Fomitiporia, Phaeomoniella* or *Phaeoacremonium* isolates assessed in this study with the reference strains deposited in the NCBI database, as shown by the Nucleotide BLAST (BLASTn).

Isolate ID number	Gene	Reference strain from NCBI	Accession number	Percent similarity (%)
MN008	ITS	*F. mediterranea* FOMI3	KR909222	82.64
	tef1a	*F. mediterranea* Fmed2395	OQ541847	98.65
MN002	ITS	*Pm. minimum* TBC0157.1	PQ555856	99.47
	tub2	*Pm. minimum* Y161-19-2z	JQ691672	99.80
MN003	ITS	*Pm. minimum* MBAi155AG	KP083220	99.10
	tub2	*Pm. minimum* Y108-02-2z	JQ691670	100.00
MN001	ITS	*Pm. minimum* TBC0157.1	PQ555856	99.82
	tub2	*Pm. minimum* Y038-05-3a	JQ691665	99.61
MN005	ITS	*Pm. minimum* AFP57	OP412815	98.55
	tub2	*Pm. minimum* Y235-04-1a	JQ691673	99.61
MN004	ITS	*Pm. minimum* TBC0017.7	PQ555844	99.47
	tub2	*Pm. minimum* Y038-05-3z	JF275884	100.00
MN007	ITS	*Pm. minimum* TBC0157.1	PQ555856	97.85
	tub2	*Pm. minimum* Y161-19-2z	JQ691672	100.00
MN009	ITS	*Pm. minimum* MBAi155AG	KP083220	99.11
	tub2	*Pm. minimum* Y235-04-1a	JQ691673	100.00
MN010	ITS	*Pa. chlamydospora* PBH18	MF818059	100.00
	tub2	*Pa. chlamydospora* Pch-07	MG745811	100.00
MN013	ITS	*Pa. chlamydospora* TBC0076.6	PQ555788	100.00
	tub2	*Pa. chlamydospora* 9	OQ459855	99.32
MN015	ITS	*Pa. chlamydospora* TBC0076.6	PQ555788	100.00
	tub2	*Pa. chlamydospora* 2	OQ459851	99.77
MN014	ITS	*Pa. chlamydospora* TBC0284.2	PQ555839	100.00
	tub2	*Pa. chlamydospora* Bent708	OP079854	100.00
MN012	ITS	*Pa. chlamydospora* Pach-59	JQ822220	100.00
	tub2	*Pa. chlamydospora* Pch-07	MG745811	99.77

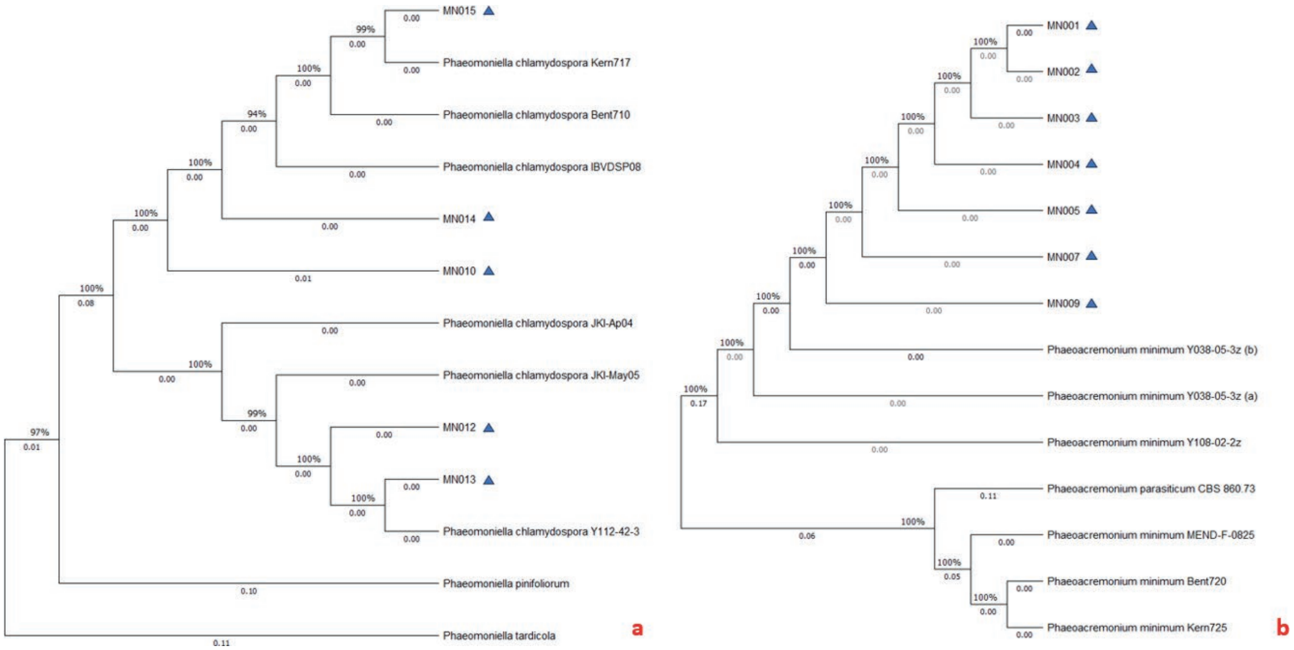

Figure 4. (a). Maximum likehold phylogenetic tree based on concatenated sequences (ITS and *tub2*) of five Montenegrin isolates (MN015, MN014, MN010, MN012 and MN013), and reference strains of *Pa. chlamydospora*, *Pa. pinifoliorum*, and *Pa. tardicola* retrieved from NCBI shown in Table 1. (b). Maximum likehold phylogenetic tree based on concatenated sequences (ITS and *tub2*) of seven Montenegrin isolates (MN001, MN002, MN003, MN004, MN005, MN007 and MN009), and reference strains of *Pm. minimum* and *Pm. parasiticum* retrieved from NCBI shown in Table 1.

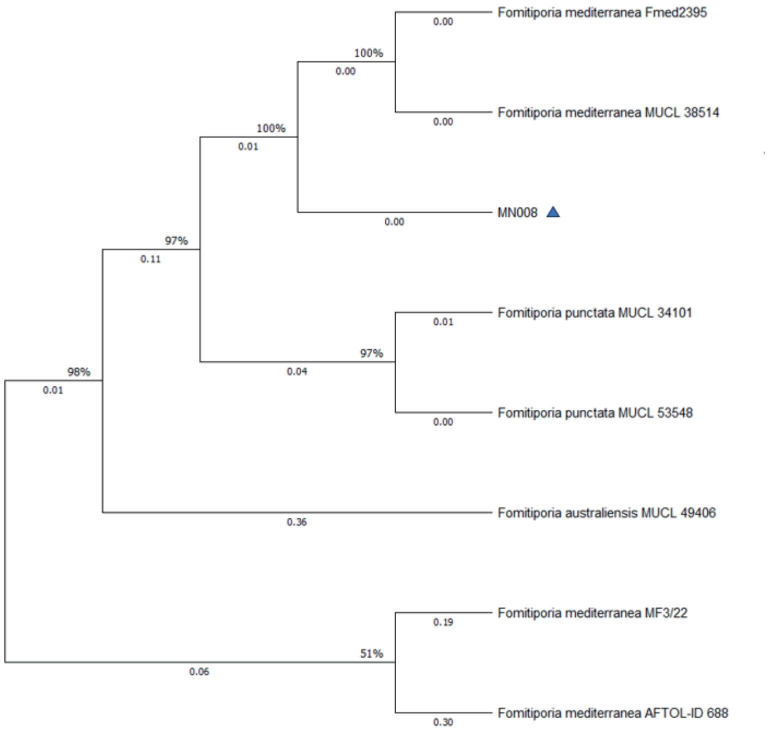

Figure 5. Maximum likehold phylogenetic tree based on the concatenated sequences (ITS and *tef1a*) of one Montenegrin isolates (MN008), and reference strains of *F. mediterranea*, *F. punctata* and *F. australiensis* retrieved from NCBI shown in Table 1.

Table 4. Numbers and percentages of grapevines without or with esca disease symptoms in a vineyard at Lješkopolje, Montenegro (Table 2, Plot no. 24), as assessed in each year from 2021 to 2024.

Year	Plants without symptoms	%	Plants with symptoms	%	Empty places	%	Incidence[a]	Assessment dates
2021	2729	75.49%	295	8.16%	591	16.35%	9.76%	17.06.2021
2022	2206	61.02%	718	19.86%	691	19.11%	24.54%	25.06.2022
2023	2034	56.27%	865	23.93%	716	19.81%	29.82%	14.06.2023
2024	1934	53.50%	536	14.83%	1145	31.67%	21.70%	20.05.2024

[a] Incidence – percentage of plants showing disease symptoms in relation to the total number of plants currently present in the vineyard.

the annual incidence of esca symptoms are presented in Table 4. The four year monitoring and analysed collected data indicated that in this 21-year-old vineyard, approx one third of the vineyard, equivalent to 1,145 plants, had died, primarily due to progression and destructive impact of esca disease. The onset of GLSD occurred in mid June during the study period, except in 2024, when the first symptoms were observed on May 20. Detailed dates of symptom appearance across the years are shown in Table 4.

In 2024, the cumulative incidence esca symptoms in the studied vineyard reached 48.06%, indicating that nearly half of the grapevines were infected with esca-associated pathogens. During the 2024 grape harvest, grape yield analysis showed a total of 43.5% reduction from symptomatic grapevines compared to healthy ones. The average grape yield per healthy vine was 5.24 kg, whereas vines exhibiting severe esca foliar symptoms produced an average of 1.07 kg, corresponding to a yield reduction of 79.6%. Laboratory analyses of must obtained from grapes harvested from healthy and diseased vines showed differences in grape quality. Must from symptomatic vines in 2024 had sugar content of 13.22%, pH of 3.41, and total acidity of 6.75 g L^{-1}. In contrast, must from healthy vines contained 19.61% sugar, had pH of 3.52, and total acidity of 4.87 g L^{-1}.

DISCUSSION

According to data from the Ministry of Agriculture, Forestry and Water Management of Montenegro (https://www.gov.me/clanak/vinogradarsko-vinarska-proizvodnja) and the study on new zoning of viticulture (A.A., 2017) in this country, 312 grape producers and 43 grapevine varieties are registered in Montenegro. The present study included a survey of 70 grape producers as well as a field evaluation of the phytosanitary status of 30 vineyards, encompassing a total of 32 grapevine varieties of different ages and geographic locations across all the viticultural regions of Montenegro. The number of surveyed vineyards and grape producers represents a substantial sample, enabling provision of a representative and realistic overview of the phytosanitary conditions of viticulture in this country, particularly for presence and significance of esca disease. Previous studies in Montenegro (Latinović *et al*., 2005; Latinović and Latinović, 2017) indicated that esca is one of the most important GTDs, occurring almost annually. Therefore, the results from the present study are a significant additional contribution to understanding of the current status of esca disease in this country.

Analysis showed that vineyards aged between 15 and 35 years were the most affected by esca disease. The greatest recorded disease incidences, from 50% to 55%, were observed in vineyards of the indigenous Vranac variety aged 35 years. Incidence recorded in this study is similar to results from Italy (Pollastro *et al*., 2000), but was greater than reported in Canada (Urbez Torres *et al*., 2014). The incidences of apoplexy and GLSD in Montenegro varied depending on the grapevine variety and vineyard age, with fluctuations of symptoms from year to year being a typical and previously described feature of this disease (Surico *et al*., 2006). Maher *et al*. (2012) reported that symptom variability complicated accurate estimation of esca prevalence.

The described external and internal symptoms of esca disease observed in Montenegrin vineyards, including GLSD and apoplexy, as well as brown to black and white necrosis of the xylem vessels, correspond to previously reported symptoms (Mugnai *et al*., 1999; Gubler *et al*., 2015; Cloete, 2015; Mondello *et al*., 2018). However, it is important to note that the first of these symptoms in 2024 were observed early, at May 20, which is probably the earliest recorded annual occurrence of esca symptoms.

In the present study, morphological characterization combined with molecular and phylogenetic analyses showed that fungal isolates obtained from symptomatic grapevines belonged to *Phaeoacremonium minimum*, *Phaemoniella chlamydospora*, and *Fomitiporia mediterranea*. *Phaeoacremonium minimum* and *Pa. chlamydospora* were mainly isolated from black spots and vascular necroses, whereas *F. mediterranea* was isolated from white rot symptoms, consistent with most previous reports. Latinović *et al.* (2005) identified *Phaeoacremonium aleophilum* as the sole causal agent of this disease, but without species confirmation through molecular diagnostics. The present paper is the first to report *Pm. minimum*, *Pa. chlamydospora*, and *F. mediterranea* in Montenegro, with these fungi identified and confirmed using molecular methods.

The present study results on the susceptibility of indigenous Montenegrin grapevine varieties has indicated greater esca sensitivity of the grapevine varieties Vranac and Kratošija compared for introduced cultivars. This study did not investigate the underlying causes of increased susceptibility in the indigenous varieties, so future research should focus on potential host anatomical traits, particularly xylem vessel diameter in Vranac and Kratošija. Pouzoulet *et al.* (2017) indicated that xylem vessel diameter may be a key factor in host plant varietal tolerance to esca disease.

To date, no studies in Montenegro have assessed the direct impacts of esca disease on grape yield and must quality. The present study has provided the first preliminary data of effects on some parameters of economic impacts of esca disease, including effects of empty places (from plant death) and yield reductions. Contrary to the findings of Dewasme *et al.* (2022), who reported lower yield losses per individual vine than those reported here. The present results indicate substantial yield reductions, particularly from vines exhibiting GLSD symptoms for several consecutive years. Yield losses reported here probably represent a worst-case scenario, as the study focused on Vranac, one of the most susceptible grapevine varieties in Montenegro, and was in a vineyard of optimal age for the manifestation of esca symptoms. Therefore, the economic impact analysis presented in this study had limitations that should be addressed in future research. Detailed assessment is required to distinguish how many vines died specifically from esca disease versus other causes. Additionally, a comprehensive economic analysis should be carried out that reflects costs of replacing dead vines and market price fluctuations.

Beyond yield, the value of grapes for winemaking largely depends on wine quality. The present study results indicate that esca disease affects basic grape quality parameters, particularly sugar content and total must acidity. At the 2024 harvest date (determined by the vineyard manager), a delay in grape ripening was observed on vines exhibiting esca foliar symptoms, accompanied for Vranac by decreased sugar levels and increased total must acidity. These results were similar to those of Calzarano *et al.* (2004) in Italy and Bruez *et al.* (2021) in France. The present study did not aim to assess differences in finished wine quality, so future research should explore the impacts of esca disease on sensory and chemical properties of red wines in Montenegro.

Although preliminary, the present study results on the susceptibility of the Vranac and Kratošija grapevine varieties to esca disease are of importance for Montenegrin viticulture and for grape production across the Balkans. This is because these varieties are also grown in Serbia and North Macedonia (Dimovska *et al.*, 2024), and because that Kratošija is genetically identical to several internationally important varieties, including Primitivo, Zinfandel, and Crljenak Kaštelanski (Maraš *et al.*, 2014).

As viticulture is important Montenegrin agriculture, and particularly because wine as a national brand and as an important export product, there an urgent need to direct further research toward developing effective strategies to mitigate the negative impacts of esca disease in Montenegrin vineyards.

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