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SUSCEPTIBILITY OF GRAPEVINE CULTIVARS TO *Eutypa lata* IN SERBIA

Sanja P. Živković[1] ✉, Tanja P. Vasić[1], Jordan P. Marković[2], Darko Jevremović[3]

[1] Faculty of Agriculture, Kruševac, University of Niš, Serbia
[2] Fruit Research Institute, Čačak, Serbia
[3] Institute for Forage Crops, Kruševac, Serbia

ABSTRACT

The species *Eutypa lata* is one of the most critical phytopathogenic fungi that presents a threat to vineyards by causing significant economic damage. This grapevine disease is hazardous because there is no adequate chemical protection, so after its progression, there is a decrease in yield and dieback of parts or whole infected shoots. There are also no grapevine cultivars resistant to this disease. For these reasons, in this paper, testing of the susceptibility of a pair of grapevine cultivars to this disease was carried out. The testing was performed under semicontrolled conditions and included six of the most economically essential grapevine cultivars in Serbia (Riesling italico, Cabernet Sauvignon, Cardinal, Rkaziteli, Pinot noir, and Prokupac). Testing was performed using 14 isolates of *E. lata* isolated in Serbia and two control isolates obtained from the INRA. For specific detection of isolates of *Eutypa* spp. included in the experiment, a specific pair of primers, Lata 1/ Lata 2.2, was applied, and an amplicon of the expected size of approximately 385 bp was established in all isolates. In the conditions of the set experiment, of all of the domestic and foreign-tested grapevine cultivars, the local cultivar Prokupac showed greater resistance to the isolates. In contrast, the cultivars Riesling italico, Cabernet Sauvignon and Cardinal, Pinot noir showed sensitivity to the isolates of *E. lata*, but not to the same extent as the cultivar Rkaziteli, which proved to be susceptible.

Key words: cultivars, *Eutypa lata*, grapevine, molecular detection, susceptibility, virulence

INTRODUCTION

Grapevine trunk diseases (ESCA disease complex, Botryosphaeria dieback, and Eutypa dieback) comprise multiple fungal pathogens that act individually or in association and can infect a grapevine plant at all stages of growth [Fontaine et al. 2016, Sosnowski and McCarthy 2017, Gramaje et al. 2018]. These fungi cause similar or identical symptoms on the leaves, shoots, and the grapevine's trunk. This makes it difficult to separate the symptoms in the vineyard and accurately identify the causal agent of the disease [Gramaje et al. 2018, Hrycan et al. 2020]. Apart from preventive measures, there is currently no effective fungicide to control this disease [Mondello et al. 2017]. The intensity of symptoms on grapevine varies among varieties [Sosnowski et al. 2007a, b, Travadon et al. 2013, Cardot 2019]. Additionally, resistance genes have not yet been identified in the grapevine. One of the causes of grapevine trunk dieback is the fungus *Eutypa lata*. This fungus is a vascular pathogen that infects the grapevine through wounds caused by pruning. The pathogen spreads by ascospores formed by the fungus [Carter 1994, Rolshausen et al. 2015,

✉ zivkovic.sanja@junis.ni.ac.rs

Hrycan et al. 2020]. Infection occurs when ascospores reach fresh tissue sections caused by pruning, followed by penetration into the vascular tissue in the presence of water droplets. Wounds on the vine caused by pruning wounds only after two weeks. Raindrops are necessary for the release of ascospores, and after being transmitted by the wind and reaching the sections created by pruning, ascospores germinate in conducting vessels. The mycelium of the fungus in the stem and branches first infects the xylem and then spreads to the cambium and phloem. In the dormant season, infection of xylem conducting tissue occurs, followed by weakening of plants due to toxin production and causing wood rot by secretion of enzymes for cell degradation [Carter 1994, Rolshausen et al. 2008, Gramaje et al. 2018, Hrycan et al. 2020].

Symptoms of grapevine dieback, caused by *E. lata*, usually appear on infested grape plants only a few years after the infection. They are manifested by trunk necrosis and reduced growth of shoots, often with small, chlorotic leaves. Due to the growth of healthy tissue on the vine's trunk, cracking occurs along the necrotized tissue, leading to the premature death of the grapevine within 3 to 5 years. The fungus was isolated only from the trunk of the grapevine. Symptoms on leaves and shoots are caused by toxins, such as eutypine, glycoproteins, or enzymes that break down the cell wall [Rolshausen et al. 2008, Cardot et al. 2019].

According to Munkvold et al. [1994], grapevine dieback is a hazardous grapevine disease, as there is no adequate chemical protection. After the progression of this disease comes a decrease in yield and dieback of parts or whole infected grapevines, leading to significant production losses. Chemical protection against this disease is ineffective [Rolshausen et al. 2015, Cardot et al. 2019, Hrycan et al. 2020]. The application of preventive combat measures is reflected in the selection of genotypes, the selection of the growing form of grapevine, the time of pruning, and the removal and burning of diseased plant parts. In regions with multiple hosts of *E. lata*, disease control cannot be done solely by sanitary measures [Munkvold et al. 1994, Rolshausen et al. 2015]. For vineyard establishment, considering that there are no E. lata-resistant grapevine cultivars, it is recommended to use more resistant cultivars and avoid sensitive cultivars. Based on studies performed thus far, it was determined that cultivars Cabernet Souvignon and Chardonnay are more susceptible to this pathogen than Merlot, Semllon, and Zinfandel [Rolshausen et al., 2015].

The main aim of this study was to determine the susceptibility of grapevine cultivars to isolates of *E. lata*. Six grapevine cultivars (Riesling italico, Cabernet Saovignon, Cardinal, Rkaziteli, Pinot noir, and Prokupac) were selected for the investigation.

MATERIAL AND METHODS

Symptoms, samples, and fungal isolation. Dieback symptoms were noticed on grapevines in 14 locations in Serbia from 2004 to 2019. Plant sampling was conducted in the second half of May and early June in vineyards 11 to 22 years old. Vine trunks and cordon fragments were taken from the plant parts between diseased and healthy tissues during the sampling. The samples were 10 to 20 cm in size.

Pathogen isolation was performed by surface sterilizing diseased parts of the grapevine trunk with 5% sodium hypochlorite for 2 min, then rinsing thrice with sterile distilled water. After sterilization, fragments of approximately 1 cm were cut from the junction of healthy and diseased grapevine tissue. They were placed on sterile filter paper to remove excess fluid and then on potato dextrose agar (PDA) containing streptomycin. Samples were then incubated at 24°C in 24 h UV light for 30 days. Individual conidia were sampled and transferred directly to the PDA plate by the procedure described by Choi et al. [1999] and stored on PDA in tubes at 4°C. Every obtained pathogen isolate was characterized at the pathogenic, morphological, and molecular levels [Lecomte et al. 2000, Živković et al. 2012a, 2012b, Živković 2019].

Molecular detection. Polymerase chain reaction (PCR) was used to detect fourteen isolates of *Eutypa* spp. from Serbia and two reference isolates of this fungus obtained from the INRA (Tab. 1). PCR was performed using one pair of specific primers. Visualization of the obtained products was performed by separation using agarose gel electrophoresis.

DNA extraction. Colonies of the tested isolates were grown on PDA in the dark at a temperature of 25°C for 7 days. DNA extraction was performed according to the method described by Day and Shattock [1997]. First, the colonies of the tested isolates were scraped from the surface of the substrate using a steri-

Table 1. Isolates chosen for further research

No.	Isolate	District	Site	Cultivar
1	VL117	Pomoravski, Serbia	Dobričevo, Ćuprija	Cabernet Sauvignon
2	VL217	Nišavski, Serbia	Praskovče, Ražanj	Rkaziteli
3	VL219	Rasinski, Serbia	Kobilje, Kruševac	Prokupac
4	VL249	Pomoravski, Serbia	Drenovac, Paraćin	Riesling early
5	VL250	Rasinski, Serbia	Tulež, Aleksandrovac	Pinot noir
6	VL251	Rasinski, Serbia	Bela voda, Kruševac	Riesling italico
7	VL252	Rasinski, Serbia	Krvavica, Kruševac	Pinot noir
8	VL253	Rasinski, Serbia	Suvaja, Varvarin	Riesling italico
9	VL254	Južnobački, Serbia	SremskiKarlovci	Sauvignon blanc
10	VL255	Južnobanatski, Serbia	Gudurica, Vršac	Chardonnay
11	VL256	Rasinski, Serbia	Trnavci, Aleksandrovac	Franconia nero
12	VL257	Borski, Serbia	Karbulovo, Negotin	Chardonnay
13	VL258	Nišavski, Serbia	Lipovac, Ražanj	Cardinal
14	VL299	Pčinjski, Serbia	Strezovce, Preševo	Riesling italico
15	8F	Verona, Italy	Verona, Italy	Unknown
16	BX1.10	Bordeaux, France	Bordeaux, France	Cabernet Sauvignon

le spatula and then placed into a 2 ml tube filled with liquid nitrogen. After the liquid nitrogen evaporated, 800 µl of 2% CTAB buffer was poured into a tube and incubated at 65°C for 1 h, and the contents of the tube were shaken vigorously every 15 min during incubation. After incubation, 800 µl of chloroform was added to each microtube, mixed using a vortex mixer, and then centrifuged for 10 min at 11000 rpm and 4°C (Eppendorf 5804 R, Germany). The resulting supernatant (approximately 700 µl) was pipetted into a new 1.5 ml tube, and approximately 420 µl of isopropanol was added and centrifuged for 15 min at 11000 rpm at 4°C. Afterwards, the supernatant was carefully drained and 1 ml of ice-cold 70% ethanol was added to the tubes, which were then carefully drained again. The open microtubes were left for 10–15 min at room temperature. After drying, the DNA pellet was suspended in 100 µl TE buffer.

Chain amplification of nucleic acid fragments. PCR using the specific primer pair Lata 1 and Lata 2.2 was used to detect the studied *Eutypa* spp. isolates (Tab. 2). PCR amplification of the samples was performed using a thermocycler (Mastercycler® Eppendorf, Germany).

Lata 1/Lata 2.2 primers enable fragment amplification in the ITS1 and ITS2 regions. This primer pair serves for the specific detection of *Eutypa lata* species by amplifying a fragment of approximately 385 bp located in the ITS1 and ITS2 regions [Lecomte et al. 2000] (Tab. 2). The 25 µl PCR mix contained 12.5 µl 2 × Master mix, 9 µl sterile distilled water, 1 µl 10 µM Lata 1 and Lata 2.2 primers, and 1.5 µl DNA. PCR amplification of the samples was performed according to the same program as with ITS1-ITS4 primers. Sterile water (PCR grade) was used as a negative control in all PCRs instead of the target DNA sample (PCR mixture with RNAse-free water). Reference isolates of *E. lata* from international collections were also used in PCRs as positive controls.

Visualization and analysis of PCR products. Analysis of PCR products was performed after electrophoretic separation of the obtained products in 1.5% agarose gel and 0.5 × TBE buffer. The agarose gel was prepared by dissolving 3.6 g of agarose (Merck,

Table 2. Specific primer pair for detection of *Eutypa lata*

Targeted sequence	Primer name	Sequence 5′-3′	Fragment size	Literal source
ITS1-ITS2	Lata 1	GAGCTACCCTGTAGCCCGCTG	~385 bp	Lecomte et al. (2000)
	Lata 2.2	GACGTCAGCCGTGACACACC		

Germany) in 240 ml of 0.5 × TBE buffer and heating to boiling point in a microwave oven. After cooling, the gel was poured into the molds of an electrophoresis apparatus (Serva, Germany). A comb from the appropriate mold trays was immersed in the gel. After polymerization of the gel, the comb was removed, the gel mold was placed in an electrophoresis apparatus, and 0.5 x TBE buffer was added to the apparatus to a point where the gel was completely immersed in the buffer.

Before being pipetted into the wells, 5 µl of the PCR product of each sample was mixed with 1.5 µl of infusion dye (loading dye, MBI fermentas, Lithuania). A 50 bp ladder marker (Sigma Aldrich, Germany) was used in electrophoresis to determine the size of the product by comparison with the expected size of the DNA fragments of the marker.

Electrophoresis was performed at 100 V for 30 minutes. Staining was performed by immersing the agarose gel in 0.5 µg/ml ethidium bromide solution for 15 min. Amplified fragments in the gel were observed under UV light using a transilluminator (Biometer, UK).

Cultivar susceptibility. A test of the susceptibility of investigated grapevine cultivars (Riesling italico, Cabernet Sauvignon, Cardinal, Rkaziteli, Pinot noir, and Prokupac) to the E. lata isolate was carried out by inoculation of plants according to the methods of Sosnowski et al. [2007a].

Cuttings were planted a year earlier in the substrate (Class 1) in 12 × 12 × 30 cm black plastic bags. In the spring of the first and second years, the plants were fertilized with artificial NPK (15 : 15 : 15) at 2 g per plant. They were then pruned to two buds to reduce the number of new shoots in both growing seasons to facilitate plant maintenance and assess the shoots' symptoms more quickly.

Before the beginning of the vegetation, grapevine plants were inoculated by cutting a 5 mm long trunk section with a scalpel, into which a piece of 10-day-old culture mycelium of E. lata was inserted. The used E. lata isolates were grown on PDA. The inoculation site was then wrapped with parafilm tape. Four plants were inoculated with each isolate in 2 replicates. As a control, four plants per cultivar were used in 2 replicates. Control plants were inoculated with fragments of sterile PDA medium. The experiments included 14 isolates of E. lata originating from grapevine from Serbia (Tab. 1), as well as two reference isolates of E. lata 8F and BX1.10 from the collection of phytopathogenic fungi (Institute National de la Recherche Agronomique, INRA, France).

Inoculated plants were watered as needed. The plants were stored in a greenhouse until the first higher temperatures when they were taken outside. The results were evaluated 8 and 27 months after inoculation by monitoring the appearance of symptoms on leaves and shoots.

The assessment of the susceptibility of grapevine cultivars to E. lata isolates was performed according to a scale containing five categories, as follows:

– category 0: plants with healthy shoots;
– category 1: plants with shortened shoots and a combination of small and large leaves;
– category 2: plants with shortened shoots and only small leaves;
– category 3: plants with drastically reduced shoots and barely present leaves;
– category 4: plants with dried leaves and shoots.

The symptom intensity was calculated based on the difference in the length of the shoots on the inoculated plants and the length of the shoots on the control plants.

After 27 months, necrosis on the trunk of inoculated grapevine plants was monitored by removing the plants from the substrate, removing the bark from the trunk, and measuring the length of tissue necrosis on inoculated grapevine plants.

Statistical analysis. The relationship between *E. lata* isolates and grapevine genotypes was determined by statistical analysis. Analysis of variance

(ANOVA) using computerized software (PROC GLM, SAS, System, version 8.1; SAS Institute, Cary, NC) was used for data analysis. The arcsine transformation of the proportion (Y = 2 xarcsine √p) was used to satisfy the assumptions of the ANOVA. The experiment was set up by the random block system method. The homogeneity of groups was assessed using Duncan's test with p = 0.05.

RESULTS

Symptoms, samples, and fungal isolation. Based on our observations, in the agro-ecological conditions of Serbia, the symptoms of the disease are most noticeable in the second half of May on the shoots of plants. During the vegetation, the diseased parts of the grapevine are covered with new leaf mass, so the symptoms are more difficult to notice. Symptoms are manifested in the form of small, chlorotic spots distributed around the rim of the leaves. Due to the slow growth, the shoots are significantly shortened and light green. More or less pronounced necrosis is manifested on the cross-section of the diseased trunk, which is in the initial phase in the shape of the letter "V.".

There is no difference in the manifestation of symptoms on grapevine cultivars in vineyards. In the last decade, in Serbia, grapevine dieback caused by *E. lata* has caused significant economic losses in grapevines.

From 2004 to 2019, 150 grapevine samples were collected and analyzed from 14 localities in Serbia. Based on the color and appearance of the fungal mycelium on the PDA medium, as well as the anamorphic stage, isolates were identified up to the genus level.

Molecular detection. The specific primer pair Lata 1/Lata 2.2 for determining *E. lata* species enables amplification of the ITS region of ribosomal DNA and 5.8 rRNA (ITS1/ITS2 region). The amplicons of the expected size of approximately 385 bp were found in all isolates by comparing the amplified fragments of the tested isolates with the used marker (M). It confirms that all tested isolates belong to the E. lata species. There was no amplification in the negative control (Fig. 1).

Cultivar susceptibility. Eight months after inoculation, not all isolates caused symptoms on the tested cultivars (Fig. 2, Tab. 3). On the Riesling italico cultivar, eight months after inoculation, isolates VL253, VL256, VL257, and VL258 did not cause symptoms. Similarly, the Cabernet Sauvignon cultivar did not develop symptoms when inoculated with VL255, VL256, and VL258 isolates. The isolates VL253, VL254, and VL257 did not show symptoms on the cultivar Cardinal. Considering the cultivar Rkaziteli, only isolate VL257 did not cause symptoms on leaves and shoots. The most significant number of isolates (VL253, VL254, VL256, VL257, VL258, and VL299) did not cause the appearance of symptoms on the Prokupac

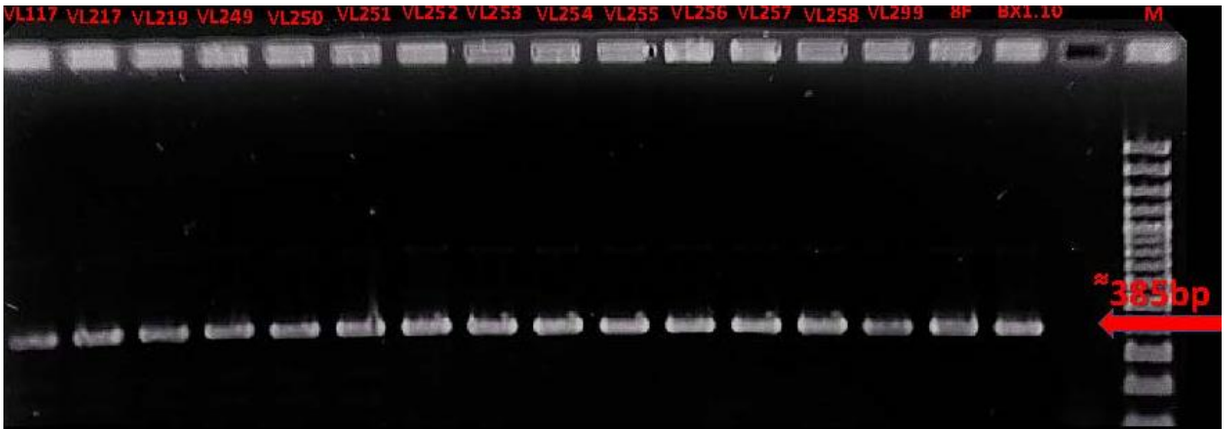

Fig. 1. Amplified DNA fragments of *Eutypa lata* approximately 385 bp in size were obtained using Lata 1/Lata 2.2 primer pairs. Columns: VL117, VL217, VL219, VL249, VL250, VL251, VL252, VL253, VL254, VL255, VL256, VL257, VL258, VL299 – studied isolates; 8F and BX1.10 – reference isolates; – negative control; M-50 bp DNA Step Ladder

Table 3. Parameters used in the assessment of the susceptibility of inoculated rooted cuttings of 6 grapevine cultivars to the tested isolates of *E. lata* 8 and 27 months after inoculation, using Duncan's significance test $p = 0.05$

Isolates	Difference in shoot length of inoculated and control plants (cm)											
	8 months						27 months					
	Riesling italico	Cabernet Sauvignon	Cardinal	Rkaziteli	Prokupac	Pinot noir	Riesling italico	Cabernet Sauvignon	Cardinal	Rkaziteli	Prokupac	Pinot noir
VL 117	14.30 a	8.80 a	21.30 ab	21.30 b	8.00 bcd	15.00 ab	23.80 a	16.30 ab	21.30 ab	36.30 a	13.90 b	27.60 a
VL 217	14.70 a	8.90 a	15.00 abc	26.30 ab	10.00 b	13.80 ab	21.30 a	22.50 a	26.30 a	35.00 a	17.60 ab	27.60 a
VL 219	14.00 a	6.40 ab	16.50 ab	31.30 a	15.30 a	21.30 a	22.50 a	17.60 a	22.50 ab	38.80 a	16.30 ab	26.30 a
VL 249	12.40 ab	3.90 ab	15.00 abc	26.30 ab	8.90 cde	12.40 ab	23.50 a	12.60 ab	26.30 a	31.30 a	16.30 b	25.00 a
VL 250	2.60 bcd	1.30 b	7.50 cde	6.30 d	5.00 def	2.60 de	3.80 b	2.60cd	5.00 c	13.90 bc	8.90 cd	3.90 c
VL 251	2.8 bcd	2.60 ab	9.00 bcd	13.80 c	7.90 bc	13.90 ab	10.00 b	6.30bcd	15.00 b	19.00 b	5.20 de	13.60 b
VL 252	1.50 cd	1.50 b	1.30 de	2..50 d	4.90 bc	13.90 ab	2.60 b	2.50cd	5.00 c	5.00 de	5.00 de	1.30 c
VL 253	0.00 d	2.60 ab	0.00 e	1.30 d	0.00 g	2.70 de	3.70 b	6.30bcd	1.30 c	2.60 de	3.00 de	4.00 c
VL 254	2.70 bcd	0.60 ab	0.00 e	6.30 d	0.00 g	2.70 de	3.70 b	6.30bcd	1.30 c	5.00 de	4.00 de	4.30 c
VL 255	2.70 bcd	0.00 b	3.00 de	3.90 d	5.00 def	3.90 cde	11.70 b	2.60cd	0.30 c	5.00 de	5.00 de	13.90 b
VL 256	0.00 d	0.00 b	2.60 de	5.00 d	0.00 g	3.20 cde	6.50b	6.30bcd	0.40 c	3.90 de	3.60 de	4.00 c
VL 257	0.00 d	0.60 ab	0.00 e	0.00 e	0.00 g	0.00 e	13.60 b	2.60cd	1.30 c	6.00 de	3.00 de	2.00 c
VL 258	0.00 d	0.00 b	1.30 de	13.80 c	0.00 g	0.00 e	4.30 b	2.60cd	5.60 c	6.30 de	2.00 de	5.00 c
VL 299	11.60 abc	2.60 ab	1.30 de	6.30 d	0.00 g	3.90 cde	9.50 b	2.60cd	0.40 c	8.80 de	4.00 de	3.80 c
8F	12.90 ab	0.60 ab	13.80 abc	27.70 ab	8.00 cde	15.00 ab	21.30 a	13.90 ab	27.50 a	32.50 a	16.30 b	25.00 a
BX1.10	11.90 abc	3.80 ab	16.30 ab	22.60 b	8.80 cde	16.30 a	20.00 a	5.90 bcd	25.00 a	31.50 a	16.30 b	23.80 a
Control	0.00 d	0.00 b	0.00 e	0.00 c	0.00 g	0.00 e	0.00 b	0.00 d	0.00 e	0.00 e	0.00 e	0.00 c

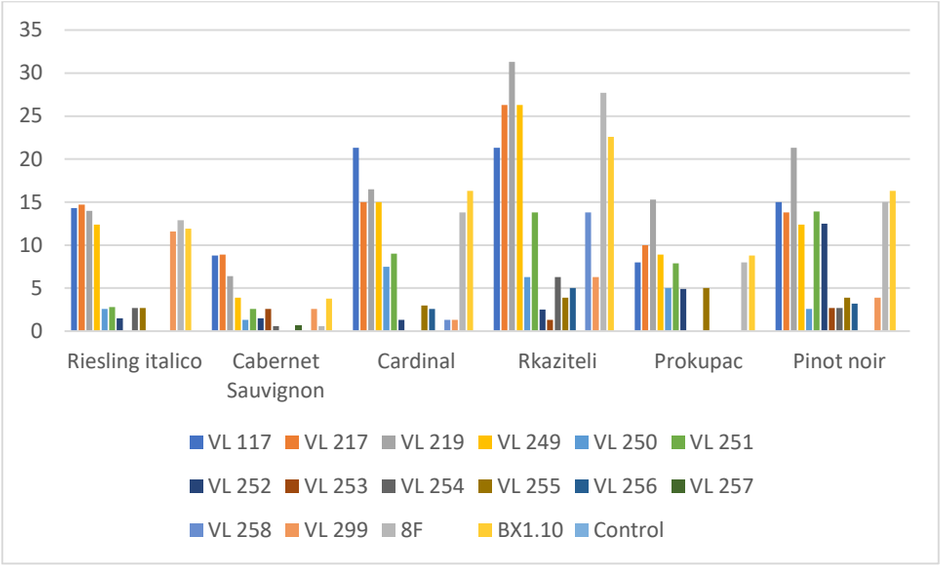

Fig. 2. Evaluation of the susceptibility of tested grapevine cultivars based on the difference in shoot length of inoculated and control plants 8 months after inoculation. X-axis: cultivar, Y-axis: intensity of symptoms

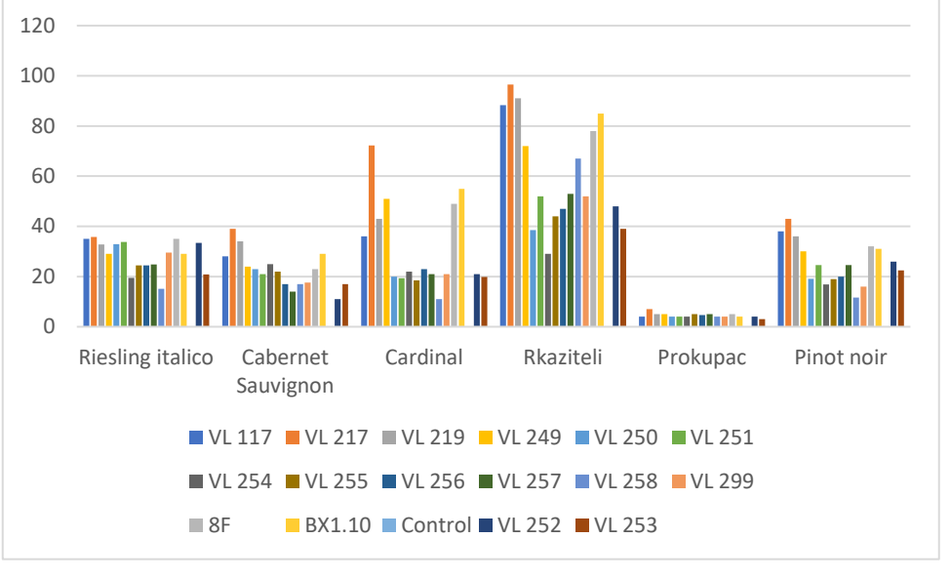

Fig. 3. Evaluation of the susceptibility of tested grape cultivars based on the difference in shoot length of inoculated and control plants 27 months after inoculation. X-axis: cultivar, Y-axis: intensity of symptoms.

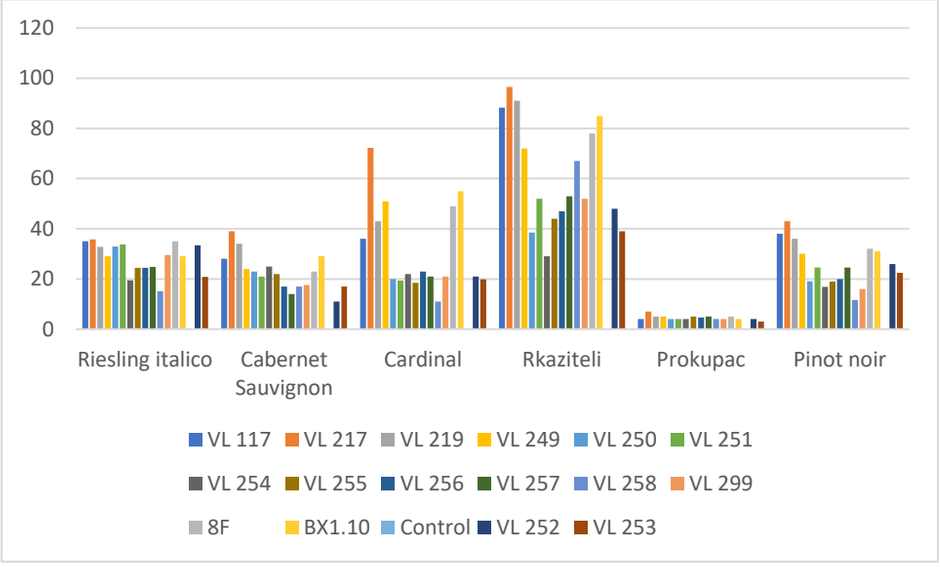

Fig. 4. Evaluation of the susceptibility of tested grapevine cultivars based on the length of necrosis of the inoculation site after 27 months. X-axis: cultivar, Y-axis: length of necrosis (mm).

cultivar eight months after inoculation. Pinot noir variety did not develop symptoms with only two isolates, namely, VL257 and VL258 (Fig. 2, Tab. 3).

A second evaluation of the onset of symptoms was performed twenty-seven months after inoculation. All studied isolates caused symptoms on leaves and shoots (Fig. 3, Tab. 3). The symptoms were more significant on the Rkaziteli, Pinot noir, and Cardinal cultivars than on the Cabernet Sauvignon and Riesling italico cultivars. Considering the Prokupac cultivar, symptoms were expressed to the lowest degree, as shown in Figure 3 and Table 3.

Additionally, twenty-seven months after inoculation, necrosis around the inoculation site was observed. All studied isolates caused tissue necrosis around the inoculation site on the tested grapevine cultivars (Fig. 4, Tab. 4). Depending on the isolate, the average length of necrosis on grapevine stems ranged from 3 mm (Prokupac cultivar, isolate VL253) to 96.5 mm (Rkaziteli cultivar, isolate VL217). After statistical analysis of the average length of necrotic tissue on inoculated plants of all tested cultivars, it was determined that the Rkaziteli cultivar is more sensitive than the Cardinal cultivar. The Cardinal cultivar proved more sensitive than the Riesling italico, Cabernet Sauvignon, and Pinot noir cultivars. The Prokupac cultivar proved to be the most resistant of all tested cultivars (Fig. 4, Tab. 4). The results revealed that the most aggressive isolates were VL117, VL217, VL219, VL249, 8F, and BX1.10 (Figs 2–4, Tabs 3 and 4). It was also observed that symptoms on leaves and shoots are not correlated with the appearance of tissue necrosis around the inoculation site, which can be seen from Figures 2, 3 and 4 and Tables 3 and 4.

DISCUSSION

Eutypa lata is one of the most critical phytopathogenic fungi that threaten grapevine plantations, causing significant economic damage. This grapevine disease is hazardous because there is no adequate chemical protection, so after its advance, there is a decrease in yield and the dieback of parts or whole infected shoots [Pitt et al. 2013, Travadon et al. 2013, Cardot et al. 2019, Hrycan et al. 2020]. These studies aimed to determine the virulence of isolates based on symptoms in inoculated plants, meaning the susceptibility of different grapevine genotypes to the tested isolates. The testing was performed according to the method by Sosnowski et al. [2007a].

Table 4. Parameters used in the assessment of susceptibility (necrosis length in mm) of inoculated rooted cuttings of 6 grapevine cultivars to the tested *E. lata* isolates 27 months after inoculation, using Duncan's significance test *p = 0.05*

Isolate	Length of necrosis (mm)					
	Riesling italico	Caberne Sauvignon	Cardinal	Rkaziteli	Prokupac	Pinot noir
VL 117	35.00 a	28.00 ab	36.00 bcde	88.30 bc	4.00 b	38.00 ab
VL 217	35.80 a	39.00 a	72.30 a	96.50 a	7.00 a	43.00 a
VL 219	32.80 ab	34.00 a	43.00 bcd	91.00 b	5.00 ab	36.00 ab
VL 249	29.00 ab	24.00 ab	51.00 ab	72.00 bc	5.00 ab	30.00 bcd
VL 250	32.90 ab	23.00 bc	20.00 de	38.50 g	4.00 b	19.10 defgh
VL 251	33.80 ab	21.00 bc	19.30 de	52.00 def	4.00 b	24.60 defg
VL 252	33.40 ab	11.00 cd	21.00 cde	48.00 def	4.00 b	26.00 cdef
VL 253	20.90 bcd	17.00 bcd	19.80 de	39.00 efg	3.00 bc	22.4 defg
VL 254	19.50 bcd	25.00 ab	22.00 cde	29.00 fg	4.00 b	16.90 fgh
VL 255	24.40 bc	22.00 bc	18.50 de	44.00 efg	5.00 ab	19.00 efgh
VL 256	24.40 bc	17.00 bcd	23.00 cde	47.00 def	4.6 ab	20.00 defgh
VL 257	24.80 bc	14.00 cd	21.00 cde	53.00 def	5.00 ab	24.60 defg
VL 258	15.10 cd	17.00 bcd	11.00 e	67.00 cd	4.00 b	11.60 h
VL 299	29.50 ab	17.60 bcd	21.00 sde	52.00 de	4.00 b	16.00 gh
8F	35.00 a	23.00 bc	49.00 ab	78.00 bc	5.00 ab	32.00 abc
BX1.10	29.00 ab	29.00 ab	55.00 ab	85.00 bc	4.00 b	31.10 abc
kontrola	0.00 e	0.00 d	0.00 f	0.00 h	0.00 c	0.00 i

***** The data in the columns marked with the same letters are not significantly different based on Duncan's test *p = 0.05*

For this reason, six grapevine genotypes were included in this experiment. A comparison of the obtained results considering the symptoms from the experiment with the symptoms caused by natural infections in Serbia, as well as with the descriptions of this phenomenon from the literature, indicates a remarkable similarity in the appearance and dynamics of the development of the symptoms.

Identification of the fungus *E. lata* is difficult due to the considerable morphological similarities of related species of the genus *Eutypa* [Rolshausen et al. 2014]. Therefore, many authors have developed molecular methods for the detection of *Eutypa lata* species [Lecomte et al. 2000, Rolshausen et al. 2014, Trouillas et al. 2010, Trouillas and Gubler 2010, Urbez-Torres et al. 2012, Moisy et al. 2017].

Molecular detection of the studied isolates of Eutypa spp. was performed after the isolation of total DNA. The extracted DNA was intact and suitable for further PCR amplification, enabling the successful detection of all isolates used in this work. A fragment of approximately 385 bp in length was successfully amplified in all studied isolates using the specific primer pair Lata 1/Lata 2-2 [Lecomte et al. 2000].

Lecomte et al. [2000] used DNA sequences to identify *E. lata* species. Based on the ITS region rDNA, they synthesized three pairs of primers, Lata1/Lata 2-1, Lata1/Lata 2-2, and Lata3/Lata 2-1. Using those primer pairs, they tested 60 isolates of *E. lata* of different geographical origins isolated from grapevines. The Lata1/Lata 2-2 primer pair was the most specific for detecting *E. lata* species.

Trouillas and Gubler [2010] successfully determined 35 isolates of *E. lata* and seven isolates of other fungi originating from different plant hosts using universal primers ITS1-ITS4. They used sequencing of products of approximately 566 bp in all tested isolates and sequence analysis. Namely, these authors confirmed the morphological identification of these species as well as genetic variations within the *E. lata* species based on phylogenetic analysis of DNA sequences of the ITS region, genes for β-tubulin, and DNA-dependent RNA polymerase (RPB2), which was also confirmed by Rolshausen et al. [2014].

Likewise, Úrbez-Torres et al. [2012] used the ITS region, EFI-α, and β-tubulin for sequence analysis to separate the grapevine dieback fungus complex. The authors identified as many as 17 different species from the family Diatrypaceae, fungi associated with causing cancer, including species *E. lata*., using these tests.

Moisy et al. [2017] developed a real-time PCR method that efficiently measures fungal biomass, which was found to correlate with isolate aggressiveness based on foliar symptom severity.

Using the method according to Sosnowski et al. [2007a], symptoms of dieback were noted on grapevine plants 8 and 27 months after inoculation. The results were confirmed by Cardot et al. [2019], who found that different cultivars exhibit different intensities of symptoms after artificial inoculation with E. lata. In this paper, 8 months after inoculation, isolate VL257 on six examined cultivars only caused weak symptoms on shoots of the Cabernet Sauvignon cultivar. There were no symptoms on the leaves and shoots of the other tested cultivars. Only after twenty-seven months do symptoms appear on the leaves and shoots of the other cultivars. Regardless of the appearance and intensity of symptoms, after twenty-seven months, the VL257 isolate caused tissue necrosis around the inoculation site on all tested cultivars.

In the experiments by Sosnowski et al. [2007a], symptoms were reproduced after 8 months. Of the 28 isolates of E. lata, 24 isolates caused the appearance of symptoms on shoots of the cultivar Grenache. According to Sosnowski et al. [2007a], isolates have significant differences. It is manifested in the intensity of symptoms on the shoots and the length of the necrotic part of the tissue above and below the inoculation site. After analyzing the results, Sosnowski et al. [2007a] concluded that there was no relationship between the appearance of symptoms on the shoots and tissue necrosis around the inoculation site. However, after 24 months, all studied isolates caused necrosis on the tissue around the inoculation site [Sosnowski et al. 2007a]. In this experiment, the first symptoms on shoots appeared 8 months after inoculation of plants of all tested grapevine cultivars, which agrees with the statements of Sosnowski et al. [2007a, 2007b]. However, the second reading was performed 7 months later concerning the experiment set up by Sosnowski et al. [2007a]. Namely, our research showed no symptoms on the shoots 20 months after inoculation. According to Sosnowski et al. [2007b], this phenomenon can be explained by the correlation between the intensity of symptoms of inoculated grapevine plants and the origin of isolates. Namely, the origin of the isolate can affect the degree of aggressiveness of the isolate. For example, isolates originating from grapevines from areas where there is a long tradition of growing apricots and grapevines, and thus the presence of grapevine dieback, are better adapted to climatic conditions in the greenhouse [Sosnowski et al. 2007 b, Pitt et al. 2013, Travadon et al. 2013]. Additionally, climate change can affect the appearance and intensity of symptoms on shoots. Specifically, the climate causes the host to become more sensitive to specific isolates that cause symptoms in greenhouse conditions [Sosnowski et al. 2007b, Trouillas et al. 2010, Travadon et al. 2013, Rolshausen et al. 2015, Cardot et al. 2019]. Since symptoms did not appear in our experiment 20 months after inoculation, the examination was followed up until the appearance of symptoms on the shoots.

After 27 months, when the second assessment of symptoms was performed, all tested isolates caused symptoms on these plant organs on six tested cultivars. The most aggressive isolates in this experiment were VL117, VL217, VL219, VL249, 8F, and BX1.10. Additionally, the highest degree of sensitivity to the studied isolates of E. lata was shown by the cultivar Rkaziteli. The cultivar Prokupac proved to be more resistant. Other tested cultivars, Riesling italico, Cabernet Sauvignon, Cardinal, and Pinot noir, also proved to be susceptible to infection by the fungus *E. lata*, but unlike the cultivar Rkaziteli, showed a slightly lower degree of susceptibility.

The length of necrosis around the inoculation site was measured after 27 months. All studied isolates on all six tested grapevine cultivars caused necrosis around

the inoculation site. It was also observed that different isolates caused the appearance of different necrosis surfaces on the tested cultivars. Thus, all studied isolates developed the smallest area of necrosis on the cultivar Prokupac. Considering the cultivar Rkaziteli, they caused the most extensive length of necrosis. It confirms that the cultivar Rkaziteli is susceptible to the pathogen *E. lata*. In other tested cultivars, including Riesling italico, Cabernet Sauvignon, Cardinal, and Pinot, a significant area of necrotic tissue developed around the inoculation site, which confirms the high susceptibility of these cultivars to the pathogen E. lata. The results follow data from the literature [Sosnowski et al. 2007a, Travadon et al. 2013, Cardot et al. 2019].

According to Sosnowski et al. [2007a], Merlot, Gamay, Grenache, and Semillon proved to be more resistant in vineyard trials than Cabernet Sauvignon, Shirah, and Pinot noir cultivars, which also proved to be very sensitive. Travadon et al. [2013] found no significant difference in susceptibility between the Merlot and Cabernet Sauvignon cultivars. Based on the results of Rolshausen et al. [2008], differences in the expressed sensitivity to *E. lata* in the cultivars Merlot and Cabernet Sauvignon may be more pronounced. It is due to wood aging as the lignin content and the composition of the cell wall change with age. Merlot has been shown to have a higher lignin content than the Cabernet Sauvignon cultivar [Rolshausen et al. 2008].

Cardot et al. [2019] inoculated rooted grapevine cuttings of Cabernet Sauvignon, Ugni Blanc, and Merlot. Inoculation was performed with isolate BX1.10, which served as the reference isolate in this paper. Under the conditions of this experiment, the Merlot cultivar was the most tolerant to the *E. lata* fungus, as 38% of the inoculated plants were asymptomatic. In contrast, the cultivar Ugni Blanc showed a very high susceptibility to *E. lata*. The Cabernet Sauvignon cultivar is deemed sensitive because only 14% of the plants were asymptomatic. Compared to the Ugni Blanc cultivar, it can be considered less sensitive. The results are generally consistent with the results of this study.

CONCLUSIONS

A fragment of 385 bp was multiplied using Lata 1/Lata 2.2 primers in all studied isolates and reference isolates from international collections. This confirmed that the isolated fungi belong to species *E. lata*.

The results in these studies indicate different levels of susceptibility of the studied grape cultivars to *E. lata*. From the tested domestic and foreign grapevine cultivars, in the conditions of the set experiments, it can be concluded that the most significant resistance to the studied isolates was shown by the local cultivar Prokupac. In contrast, the cultivar Rkaziteli showed great sensitivity.

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Faculty of Agriculture, Kruševac, University of Niš, Serbia

REFERENCES

Cardot, C., Mappa, G., La Camera, S., Gaillard, C., Vriet, C., Lecomte, P., Ferrari, G., Coutos-Thévenot., P. (2019). Comparison of the molecular responses of tolerant, susceptible and highly susceptible grapevine cultivars during interaction with the pathogenic fungus *Eutypa lata*. Front. Plant Sci., 10, 991. https://doi.org/10.3389/fpls.2019.00991

Carter, M.V. (1994). Wood and root diseases caused by fungi. Eutypa dieback. In: Compendium of grape diseases, Pearson, R.C., Goheen, A.C. (eds.). APS Press, The American Phytopathological Society, St. Paul, Minnesota, USA, 32–34.

Choi, Y.W., Hyde, K.D., Ho, W.H. (1999). Single spore isolation of fungi. Fungal Divers., 3, 29–38.

Day, J.P., Shattock, R.C. (1997). Aggressiveness and other factors relating to displacement of populations of Phytophthora infestans in England and Wales. Eur. J. Plant Pathol., 103, 379–91.

Fontaine, F., Pinto, C., Vallet, J., Clément, C., Gomes, A.C., Spagnolo, A. (2016). The effects of grape vine trunk diseases (GTDs) on vine physiology. Eur. J. Plant Pathol., 144, 707–721. https://doi.org/10.1007/s10658-015-0770-0

Gramaje, D., Úrbez-Torres, J.R., Sosnowski, M.R. (2018). Managing grapevine trunk diseases with respect to etiology and epidemiology: current strategies and future prospects. Plant Dis., 102, 12–39. https://doi.org/10.1094/PDIS-04-17-0512-FE

Hrycan, J., Hart, M., Bowen, P., Forge, T., Úrbez-Torres, J.R. (2020). Grapevine trunk disease fungi: their roles as latent pathogens and stress factors that favour dis-e-ase development and symptom expression. Phytopathol. Mediterr., 59(3), 395–424. https://doi.org/10.14601/Phyto-11275

Lecomte, P., Péros, J.P., Blancard, D., Bastien, N., Délye, C. (2000). PCR Assays that identify the grapevine dieback fungus *Eutypa lata*. Appl. Environ. Microbiol., 4475–4480. https://doi.org/10.1128/aem.66.10.4475-4480

Moisy, C., Berger, G. Flutre, T. Lee Cunffandand, L., Péros, J.P. (2017). Quantitative assessment of grape vine wood colonization by the dieback fungus *Eutypa lata*. J. Fungi, 3(2), 21, 1–15. https://doi.org/10.3390/jof3020021

Mondello, V., Songy, A., Battiston, E., Pinto, C., Coppin, C., Trotel-Aziz, P., Clément, C., Mugnai, L., Fontaine, F. (2017). Grapevine trunk diseases: a review of fifteen years of trials for their control with chemicals and biocontrol agents. Plant Dis., 102, 1189–1217. https://doi.org/10.1094/PDIS-08-17-1181-FE

Munkvold, G.P., Duthie, J.A., Marois, J.J. (1994). Reductions in yield and vegetative growth of grapevines due to Eutypa dieback. Phytopathology, 84, 186–192. https://doi.org/10.1094/Phyto-84-186

Pitt, W.M., Trouillas, F.P., Gubler, W.D., Savocchia, S., Sosnowski, M.R. (2013). Pathogenicity of Diatrypaceous fungi on grapevine in Australia. Plant Dis., 97, 749–756. https://doi.org/10.1094/PDIS-10-12-0954-RE

Rolshausen, P.E., Greve, L.C., Labavitch, J.M., Mahoney, N.E., Molyneux, R.J., Gubler, W.D. (2008). Pathogenesis of *Eutyapa lata* in grapevine: Identification of virulence factors and biochemical characterization of cordon dieback. Phyto-pathology, 98, 222–229. https://doi.org/10.1094/PHYTO-98-2-0222

Rolshausen, P.E., Baumgartner, K., Travadon, R., Pouzoulet, J. (2014). Identification of *Eutypa* spp. causing Eutypa dieback of grapevine in Eastern North America. Plant Dis., 98, 483–491. http://dx.doi.org/10.1094/PDIS-08-13-0883-RE

Rolshausen, P.E., Sosnowski, M., Trouillas, F.P. Gubler, W.D. (2015). Diseases caused by fungi and Oomycetes. Eutypa dieback. In: Compendium of grape diseases, disorders, and pests, Wilcox, W.F., Gubler, W.D., Uyemoto, J.K. (eds.). APS Press, The American Phytopathological Society, St. Paul, Minnesota, USA, 57–61.

Sosnowski, M.R., Lardner, R., Wicks, T.J., Scott, E.S. (2007a). The influence of grapevine cultivar and isolate of Eutypa lata on wood and foliar symptoms. Plant Dis., 91, 924–931. https://doi.org/10.1094/PDIS-91-8-0924

Sosnowski, M.R., Shtienberg, D., Creaser, M.L., Wicks, T.J., Lardner, R., Scott, E.S. (2007b). The influence of climate on foliar symptoms of Eutypa dieback in grapevines. Phytopathology 97, 1284–1289. https://doi.org/10.1094/PHYTO-97-10-1284

Sosnowski, M.R., McCarthy, G. (2017). Economic impact of grapevine trunk disease management in Sauvignon Blanc vineyards of New Zealand. Wine Vitic. J., 32, 42–48.

Travadon, R., Rolshausen, P.E., Gubler, W.D., Cadle-Davidson, L., Baumgartner, K. (2013). Susceptibility of cultivated and wild Vitis spp. to wood infection by fungal trunk pathogens. Plant Dis., 97, 1529–1536. https://doi.org/10.1094/PDIS-05-13-0525-RE

Trouillas, F.P., Gubler, W.D. (2010). Host range, biological variation, and phylogenetic diversity of *Eutypa lata* in California. Phytopathology, 100(10), 1048–1056. https://doi.org/10.1094/PHYTO-02-10-0040

Trouillas, F.P., Úrbez-Torres, J.R., Gubler, W.D. (2010). Diversity of diatrypaceous fungi associated with grapevine canker diseases in California. Mycologia, 102(2), 319–336. https://doi.org/10.3852/08-185

Úrbez-Torres, J.R., Peduto, F., Striegler, R.K., Urrea-Romero, K.E., Rupe, J.C., Cartwright, R.D, Gubler, W.D. (2012). Characterization of fungal pathogens associated with grapevine trunk diseases in Arkansas and Missouri. J. Fungal Div., 52 (1), 169–189. https://doi.org/10.1007/s13225-011-0110-4

Živković, S., Vasić, T., Anđelković, S., Jevremović D., Trkulja, V. (2012a). Identification and characterization of *Eutypa lata* on grapevine in Serbia. Plant Dis., 96, 913. https://doi.org/10.1094/PDIS-11-11-0990-PDN

Živković, S., Vasić, T., Trkulja, V., Krnjaja V., Marković, J. (2012b). Pathogenicity on grapevine and sporulation of *Eutypa lata* isolates originating from Serbia. Rom. Biotechnol. Lett., 3(17), 7379–7388.

Živković, S. (2019). Characterization of *Eutypa lata*, causal agent of grapevine dieback in Serbia and susceptibility of varieties. (Dissertation). Faculty of Agriculture. University of Belgrade, 1–171.