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Study of *Eutypa lata* isolates originating from Serbia

Original Article

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Abstract:
The potential influence of different types of light on the growth and sporulation of *Eutypa lata* isolates was assessed, and detection at the molecular level was also performed. By applying *in vitro* observations, the influence of different types of light was monitored, (24 h exposure to UV light; alternating 12 h UV and 12 h darkness; alternating 12 h artificial fluorescent light and 12 h darkness), on the radial growth and sporulation of the anamorphic stage of three isolates of *Eutypa lata* fungus (EL117, EL153 and EL199) by comparison with two reference isolates of *Eutypa lata,* BX1.10 and 8F, obtained from the INRA, France. After the molecular detection using of the specific primer pair Lata 1/ Lata 2.2, all the studied isolates were found to belong to the species *Eutypa lata* (syn. *Eutypa armeniacae*), anamorph *Libertella blepharis*. It was also found that the most suitable type of light for radial growth and sporulation of the studied isolates is exposure to 24 h UV light for 30 days.

Key words:
isolates, molecular detection, breeding traits, light types, *Eutypa lata*

Apstrakt:
Izučavanje izolata *Eutypa lata* poreklom iz Srbije
U ovom radu je proučavan jedan od uzročnika odumiranja čokota vinove loze gljiva *Eutypa lata*. Praćen je uticaj različitih tipova svetlosti na porast i sporulaciju izolata *Eutipa lata*, a izvršena je i detekcija na molekularnom nivou. Naime, u *in vitro* uslovima praćen je uticaj različitih tipova svetlosti (24 h izlaganje uticaju UV svetla (UV); alternativno, 12 h UV i 12 h tama (UV-T); alternativno, 12 h veštačko fluorescentno svetlo i 12 h tama (S-T)) na radijalni porast i sporulaciju anamorfnog stadijuma tri izolata gljive *Eutypa lata* (EL117, EL153 and EL199) upoređivanjem sa dva referentna izolata *Eutypa lata* BX1.10 i 8F dobijenih sa Institute National de la Recherche Agronomique, INRA, France. Takođe je urađena detekcija izolata na molekularnom nivou primenom specifičnog para prajmera Lata 1/Lata 2.2. Nakon sprovedene molekularne detekcije, utvrđeno je da svi proučavani izolati pripadaju vrsti *Eutypa lata* (syn. *Eutypa armeniacae*), anamorf *Libertella blepharis.* Takođe je ustanovljeno da je najpogodniji tip svetlosti za radijalni porast i sporulaciju, proučavanih izolata izlaganje, 24 h UV svetlu u trajanju od 30 dana.

Ključne reči:
izolati, molekularna detekcija, odgajivačke odlike, tipovi svetlosti, *Eutypa lata*

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Introduction

Eutypa dieback, ESCA complex and Botryosphaeria dieback (Sosnowski & McCarthi, 2017) cause the greatest economic damage to vines as the cause of grapevine death (GTD), affecting plant growth, reducing yield and quality of grapes and premature decay of grapevines (Gramaje et al., 2018). Grapevine dieback disease is caused by multiple fungal pathogens that act individually or in combination and can infect a grapevine plant at all stages of growth (Fontaine et al., 2016). It must be noted that these fungi cause similar and even the same symptoms on the leaves and shoots, as well as on the trunk of the vine. This complicates the separation of symptoms and the precise identification of disease causes in vineyards (Gubler et al., 2005; Gramaje et al., 2018). The dieback of the grapevine occurs in almost all countries of the world where the grapevine is grown commercially. One of the causes of grapevine dieback (GTD), which will be discussed in this text, is the fungus *Eutypa lata*. This fungus is a vascular pathogen that infects the vine through fresh pruning wounds. The disease is spread by ascospores (Carter, 1994; Rolshausen et al., 2015).

The fungus *E. lata* is very difficult to identify because this fungus does not form a teleomorphic stage (ascospores) in regions where annual rainfall is less than 330 mm (Ju et al., 1991), as well as on artificial nutrient media (Carter, 1994; Munkvold, 2001; Živković, 2019). Adam (1938) caused an infection on the apricot tree, for the first time, by using a suspension of conidia of this fungus. Glawe et al. (1982) determined this fungus based on host symptoms, pathogenicity tests on apricot and identification of anamorphs in culture (loc cit. Ju et al., 1991). Fungus *Eutypa lata* can be easily grown on conventional laboratory media, but not all isolates sporulate (Ju et al., 1991; McKemy et al., 1993; Munkvold, 2001). For these reasons, a number of authors have studied the anamorphic stage of the fungus *E. lata* (Rolshusen et al., 2006; Trouillas & Gubler, 2010).

Lecomte et al. (2000) were the first to design specific primers for the determination of *E. lata* species. The Lata 1/Lata 2.2 primer pair was particularly successful in separating *E. lata* isolates from isolates of over fifty different species of grapevine microorganisms.

The aim of this study was to determine the affiliation of three studied isolates of *Eutypa lata* (syn. *Eutypa armeniacae*), anamorph *Libertella blepharis*, with the help of molecular detection, using a specific pair of primers Lata 1/Lata 2.2 for the species *Eutypa lata*. Also for this purpose, sporulation and radial increase of the anamorphic stage of three isolates (EL117, EL153 and EL199) on the nutrient medium of PDA under the influence of different types of light were monitored.

Materials and Methods

Samples and fungal isolation

Examination of vineyards in several sites of grape growing in our country in the period from 2004 to 2021, revealed symptoms of dieback of grapevine plants. Symptoms on the leaves of diseased grapevine plants are manifested in the form of small, chlorotic spots, distributed along the edge of the leaves, while the central part of the leaf blade has a wrinkled appearance. The edges of the leaves are worn and bent downwards, and in severe infections, the surface of the leaves is mostly covered with necrotic spots. The shoots are light green in color, have a shortened appearance and the so-called zigzag growing of internodes. In the later stages, the disease is manifested by the appearance of a large number of very short shoots that grow from the same place on the branch of the vine, very close to each other. The first signs of the disease first develop around larger pruning wounds.

By removing the dead bark, necrotic parts can be seen that extend along the stem and can be several tens of centimeters long. Necrosis first affects the surface, and then penetrates into the interior, affecting the central part of the vine. Dead tissue is dry, light or dark brown in color. On the cross-section of the diseased tree, more or less pronounced necrosis is manifested, which is in the initial phase in the shape of the letter „V".

For isolation, 1 cm fragments of grapevine stem and cordons were used, from the junction between the healthy and diseased parts. To isolate the pathogen, diseased parts of grapevine plants were surface-sterilized with 5% sodium hypochlorite for 2 min, followed by three washes with sterile distilled water. Surface sterilized tissue was transferred to sterile filter paper and placed on potato dextrose agar (PDA) containing streptomycin, and incubated at 24 °C. After incubation for 3 to 5 days, colonies of fungi developed around the fragments, which were sieved on potato dextrose agar (PDA) medium and served to obtain monosporial isolates. Based on the color and appearance of the fungal mycelium on potato dextrose agar (PDA) medium, as well as the anamorphic stage, isolates were determined to the genus level.

Individual conidia were selected and transferred directly to the PDA plate according to the procedures described by Dhingra & Sinclair (1995), and stored on PDA in tubes at 4 °C.

In order to check the pathogenicity of the obtained monosporial isolates of the fungus, artificial inoculations of the vine were performed. The pathogenicity test of the isolates was performed by inoculation of non-rooted grapevine cuttings (Peros & Berger, 1994). The Cabernet Sauvignon variety was used for the inoculation of cuttings in the experiment.

Breeding traits (radial growth and sporulation under the influence of three types of light) of 3 isolates of *Eutypa* spp. (EL117, EL153 and EL199) isolated from Serbia and two control isolates from international collections were studied after obtaining monosporial cultures and confirming the pathogenicity of fungi isolates.

Influence of light on the development and sporulation of the studied anamorphic phase isolates of Eutypa spp.

To study the influence of different variants of artificial fluorescent light and darkness on colony growth and sporulation of the studied isolates of *Eutypa* spp. pure cultures of 3 isolates and 2 reference isolates of *E. lata* grown on PDA at 25 °C were used. The seeded Petri plates were then exposed to 3 variants of artificial fluorescent light and darkness, as follows:

1. 24 h exposure to UV light (UV);
2. alternatively, 12 h UV and 12 h darkness (UV-T);
3. alternating 12 x artificial fluorescent light and 12 x darkness (S-T).

Three 40 W bulbs served as a source of fluorescent light, and two UV bulbs, with a wavelength of 366 nm, which were about 50 cm away from the Petri plate, served as a source of ultra-violet light.
Mycelial growth was monitored by measuring the diameter of the colony for 10 days until the first isolate completely covered the Petri plate.

The first measurement of colony growth was performed after three days, and then, during the next ten days, until the first isolate completely filled the Petri plate, the linear growth of colonies was monitored by linear diameter measurement.

The average daily colony growth was calculated according to the formula (Brasier and Weber, 1987):

$$(D_2-D_1)/(T_2-T_1)=\text{daily growth level}$$

where $\mathbf{D_1}$ and $\mathbf{D_2}$ are the diameters of the colony after the first and second measurements, and $\mathbf{T_1}$ and $\mathbf{T_2}$ days elapsed from seeding to the first and second measurements (loc. cit. Živković, 2019).

Determination of sporulation levels, i.e. the number of conidia per ml of suspension, was performed using a hemocytometer by Thom with 16 fields, whose size is 1 mm^2 and depth is 0.2 mm. The number of conidia was determined in order to determine the differences in the intensity of sporulation of 3 isolates of *Eutypa* spp. studied in this paper.

For this purpose, a suspension of spores was prepared by adding 5 ml of distilled water to a Petri plate with a culture of the studied isolates of *Eutypa* spp. The resulting conidia suspension together with the mycelial fragments was poured into a clean tube and then squeezed through a polyethylene mesh to remove the mycelial fragments. The counting was performed in 2 x 16 repetitions (32 fields), and from that, the average number of conidia per 1 mm^2 was calculated. The number of conidia thus obtained was taken to calculate the density of spores in 1 cm^3 (1 ml) of the suspension according to the formula:

$$N=nx5x1000$$

where: **N** - number of spores in 1 ml of suspension (spore density/1ml), **n** – the average number of spores per 1 mm^2. The density of the spores is obtained by multiplying the average number of spores by the number 5 (because the depth of the pits is 0.2 mm). To obtain the number of spores in 1 mm^3, and then multiplying the obtained number by 1000, since 1 cm^3 contains 1000 mm^3, to obtain a spore density in a volume of 1 cm^3 (1 ml). The number of conidia per 1 mm^2 of the colony was determined based on the number of conidia/ml and the area of the colony, which was calculated from the proportion of paper weight on which the contours of colonies were traced and a paper weight of 1 dm^2 (Živković, 2019).

The level of sporulation is expressed according to the scale by Quesad and Lopez (1980), where: +=weak sporulation (<5.000 spores/ml), ++=medium sporulation (5.000-10.000 spores/ml) and +++=abundant sporulation (>10.000 spores/ml) (loc. cit. Živković, 2019).

Photographing the test results was performed 30 days after sowing the substrate. Two reference isolates, 8F and BX1.10, were used as controls.

Statistical analysis

Statistical analysis was performed in order to determine the relationship between *Eutypa* spp. isolate and two reference isolates 8F and BX1.10. Data were analyzed by variance analysis (ANOVA) using computer software (PROC GLM, SAS, System, version 8.1; SAS Institute, Cary, NC). To satisfy the assumptions of the ANOVA, the arcsine transformation of the proportion was used ($Y=2x\arcsin\sqrt{p}$). The homogeneity of groups was assessed using Duncan's test with p=0.05.

Molecular detection

Detection of three isolates of *Eutypa* spp. from Serbia and 2 reference isolates of *E. lata* was performed using the Polymerase Chain Reaction (PCR) method. PCR was performed using 1 pair of specific primers. Visualization of the obtained products was performed by electrophoretic separation in an agarose gel.

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 & Shattock (1997). In the first step, the colonies of the tested isolates were scraped from the surface of the substrate with a sterile spatula and transferred to a 2 ml tube with liquid nitrogen. After evaporation of the liquid nitrogen, 800 µl of 2% CTAB buffer was poured into a tube and incubated at 65 °C for 1 h. During incubation, the content of the tube was shaken vigorously every 15 min. After incubation, 800 µl of chloroform was added to each microtube and vortexed, then centrifuged for 10 min at 11000 rpm and 4 °C (Eppendorf 5804 R, Germany). The resulting supernatant (about 700 µl) was pipetted into a new 1.5 ml tube, about 420 µl of isopropanol was added and centrifuged for 15 min at 11000 rpm and 4 °C. After centrifugation,

the supernatant was carefully drained, and 1 ml of ice-cold 70% ethanol was added to the tubes, which was then carefully drained. The open microtubes were left for 10-15 min at room temperature. After drying, the DNA pellet was resuspended in 100 µl TE buffer.

Chain amplification of nucleic acid fragments

For the detection of the studied isolates of *Eutypa* spp. The PCR reaction was applied using a specific primer pair Lata 1 and Lata 2.2 (**Tab. 1**). PCR amplification of the samples was performed in 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 about 385 bp located in the ITS1 and ITS2 regions (Lecomte et al., 2000) (**Tab. 1**). The 25 µl PCR mix contained: 12.5 µl 2 x Master mix, 9 µl sterile distilled H_2O, 1 µl 10 µM Lata 1 and Lata 2.2 primer and 1.5 µl DNA. PCR amplification of the samples was performed according to the same program as with primers ITS1-ITS4. As a negative control in all PCR reactions, sterile water (PCR grade) was used instead of the target DNA sample (PCR mixture with RNAse-free water).

Reference isolates of *E. lata* from international collections were also used in PCR reactions as positive controls.

Visualization and analysis of PCR reaction products

Analysis of PCR products was performed after electrophoretic separation of the obtained products in 1.5% agarose gel and 0.5 x TBE buffer. The agarose gel was prepared by dissolving 3.6 g of agarose (Merck, Germany) in 240 ml of 0.5 x 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 immersed in the appropriate mold trays is immersed in the gel. After the polymerization of the gel, the comb was removed and the gel mold was placed in an electrophoresis apparatus. 0.5 x TBE buffer was added to the apparatus to a level where the gel was completely immersed in the buffer.

Before pipetting into the wells, 5 µl of the PCR reaction product of each sample was mixed with 1.5 µl of infusion dye (Loading dye, MBI fermentas, Lithuania). In electrophoresis, a 50 bp ladder marker (Sigma Aldrich, Germany) was used 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 min. 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).

Results

Samples and fungal isolation

Examination of vineyards in several localities where grapevine is grown in our country, in the period from 2004 to 2019, showed symptoms of dying of grapevine vines. Symptoms on the leaves of diseased vines are manifested in the form of small, chlorotic spots, distributed along the edge of the leaves, while the central part of the leaf blade has a wrinkled appearance. The edges of the leaves are worn and bent downwards, and in severe infections, the surface of the leaves is mostly covered with necrotic spots. The shoots are light green in color, have a shortened appearance and the so-called zigzag growth of internodes. In the later stages, the disease is manifested by the appearance of a large number of very short shoots that grow from the same place on the branch of the vine, very close to each other. The first signs of the disease first develop around larger pruning wounds.

By removing the dead bark from the vine, necrosis is noticed, which develops around older pruning wounds. Necrotic parts extend along the stem and can be several tens of centimeters long. Necrosis first affects the surface, and then penetrates into the interior, affecting the central part of the grapevine. Dead tissue is dry, light or dark brown in color.

Due to the growth of a healthy part of the wood around the infected one, the cracks appear on the stem surface along the necrotic belt, whereby outside, on the dead part of the stem tissue, smaller or larger longitudinal cracks appear (**Fig. 1**). On the cross-section of the diseased stem, more or less pronounced necrosis is manifested, which is in the initial phase in the shape of the letter „V” On

Table 1. Specific primer pair for detection of *Eutypa lata*

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

Fig. 1. *Eutypa* spp.: Tissue cracking and deformations due to the growth of healthy tissue

partially or completely dried limbs and sleeves, the bark peels and falls off over time. On a stem without bark, especially around older sections from pruning, slight surface depressions of dead tissue can be noticed.

Samples were taken from diseased vines by cutting fragments 10 to 20 cm long. From these fragments, pieces about 1 cm long were cut from the junction of healthy and diseased tissue and seeded on a sterile PDA medium. On potato-dextrose (PDA) medium, 24 h after sowing, the studied isolates of *Eutypa* spp. form the beginning of white mycelium. The mycelium is hyaline, milky white in color, more or less airy. Over time, the mycelium becomes light gray-white in color with a thick, cottony, airy mycelium adhering to the substrate, reminiscent of down feathers.

All studied isolates showed pathogenic properties and caused changes in the inoculated tissue by inoculation of unrooted cuttings of Cabernet Sauvignon grapevine.

Influence of light on the development and sporulation of the studied isolates of Eutypa spp.

During these studies, the radial growth of colonies and sporulation of all examined isolates under the influence of 12 h UV and 12 h darkness, under the influence of 12 h light and 12 h darkness and under the influence of 24 h UV (**Fig. 2**) in a period of 30 days were studied.

To study the influence of different variants of artificial light and darkness on colony growth and sporulation of the five studied isolates, three isolates *Eutypa* spp., from grapevine, originating from Serbia, and two determined control isolates of *E. lata*, originating from Italy and France, were used. The radial growth of colonies and sporulation of five selected isolates was studied on a potato-dextrose medium by exposing seeded Petri dishes at 25 °C to three variants of artificial light and darkness, as follows: 1) 24 h exposure to UV light (UV), 2) alternating 12 h UV and 12 h darkness (UV-T); 3) alternating 12 h of artificial fluorescent light and 12 h of darkness (S-T). The aim of this experiment was to examine which isolate has the largest radial increase, daily increase, and degree of sporulation.

Fig. 2. *Eutypa* spp.: Appearance of a colony of EL199 isolates on a PDA medium exposed to 24 h of ultraviolet light (UV) for a period of 30 days

Radial colony growth

The radial growth of colonies of all five studied isolates was statistically significantly influenced by different variants of artificial light and darkness to which the seeded cultures were exposed (**Tab. 2**). Thus, during these studies, it was found that the highest increase in colonies of all studied isolates was under the influence of alternating 12 h UV and 12 h darkness, a slightly smaller increase was under the influence of 24 h UV over a period of 30 days, and the weakest increase was under the influence of 12 h light and 12 h darkness (**Tab. 2**). It is also observed that isolates EL153, EL199 and reference isolate BX1.10, under the influence of 12 h UV and 12 h darkness have a statistically significantly higher increase compared to other tested isolates. While these isolates under the influence of 12 h of light and 12 h of darkness achieve a statistically significantly smaller rise compared to other tested isolates. (**Tab. 2**).

Average daily growth

The average daily colony growth in all five studied isolates of different variants of artificial fluorescent light and darkness was statistically significantly

affected. Considering this trait, some isolates differed significantly. It was found that all tested isolates (EL117, EL153 and EL199, 8F and BX1.10) achieved a statistically significantly higher daily gain when exposed to 12 light and 12 h of darkness compared to other types of light (**Tab. 2**). After statistical analysis of the data, it can be concluded that isolate EL117 has a statistically significantly higher daily yield compared to other tested isolates at all types of light, except isolate 8F, which, under the influence of treatment with light 12 h UV and 12 h darkness, achieved statistically significantly higher growth compared to isolate EL117 (**Tab. 2**).

Sporulation (Number of conidia per mm² of colony)

The type of light greatly affects the ability of the studied *Eutypa* spp. isolates to form conidia in greater or lesser numbers. Thus, different types of light in all tested isolates influenced the level of sporulation, which can be seen from **Tab. 3**.

The number of conidia formed per mm² of colony was statistically significantly conditioned by the variant of artificial light and darkness in all five studied isolates of *Eutypa* spp. In terms of this trait, some isolates differed significantly. Thus, under the influence of 12 h of light and 12 h of darkness after 30 days of exposure, no sporulation of the EL153 and EL199 isolates occurred, while in isolates EL117, as well as two reference isolates 8F and BX1.10, sporulation was abundant (**Tab. 3**). When all five studied isolates were exposed to 24 h UV light for 30 days and a combination of 12 h UV and 12 h darkness, sporulation occurred in all studied isolates of *Eutypa* spp., as shown in **Tab. 3**. Under the influence of both types of light, the EL117 isolate exhibited statistically significantly higher extent of sporulation compared to other tested isolates, as well as two reference isolates.

Molecular detection and identification of the studied isolates

The molecular method of polymerase chain reaction has been successfully applied for the detection of the studied isolates of *Eutypa* spp. After extraction of the total DNA of the tested isolates, PCR reaction was performed with one pair of specific primers (Lata 1/Lata 2.2) in order to detect the tested isolates to the level of the species.

The specific primer pair Lata 1/Lata 2.2 for the determination of *E. lata* species enables amplification of the ITS region of ribosomal DNA and 5.8 rRNA (ITS1/ITS2 region). By comparing the amplified fragments of the tested isolates, with the marker (M) used, the presence of amplicons of the expected size of about 385 bp was found in all isolates, which confirms that all three tested isolates belong to the *E. lata* species. No amplification occurred with the negative control (**Fig. 3**).

Discussion

During the multi-year period (2004-2021) monitoring in vineyards in 14 localities, but also on individual vines in yards in Serbia, it was found that

Table 2. *Eutypa* spp.: Influence of light on colony growth and sporulation of studied isolates of this parasite

Isolates	Different light types		
	24 h UV 30 days	12 h UV+12 h darkness	12 h light+12 h darkness
	Colony diameter (mm)		
EL117	88.00 a	89.00 ab	88.00 a
EL153	86.60 ab	90.00 a	85.40 ab
EL 199	88.40 a	90.00 a	87.40 a
8F	87.80 a	89.00 ab	86.00 ab
BX1.10	87.00 a	90.00 a	85.40 ab
	Daily growth (mm)**		
EL117	5.80 a	5.80 ab	7.16 a
EL153	5.16 a	5.72 ab	6.48 ab
EL199	4.76 ab	5.70 ab	6.52 ab
8F	5.12 a	6.08 a	6.08 ab
BX1.10	5.32 a	4.68 b	6.24 ab

*Data in columns labeled with the same letters were not statistically significantly different based on the Duncan test (p=0.05);
**Average daily increment calculated by formula $(D_2-D_1)/(T_2-T_1)$

Table 3. *Eutypa* spp.: Influence of light on colony growth and sporulation of studied isolates of this parasite

Isolates	Different light types		
	24 h UV 30 days	12 h UV+12 h darkness	12 h light + 12 h darkness
	Sporulation after 30 days ***		
EL117	+++	+++	+++
EL153	+++	+++	-
EL199	+++	+++	-
8F	+++	+++	+++
BX1.10	+++	+++	+++
	Number of conidia after 30 days per mm²		
EL117	40.94 a	40.75 a	33.75 a
EL153	12.13 bc	16.19 b	0.00 b
EL199	15.00 b	15.38 b	0.00 b
8F	33.94 ab	34.44 ab	33.31 a
BX1.10	33.31 ab	38.12 ab	32.44 ab

*Data in columns labeled with the same letters were not statistically significantly different based on the Duncan test (p=0.05);
***Sporulation: +=weak, ++=medium, and +++=abundant

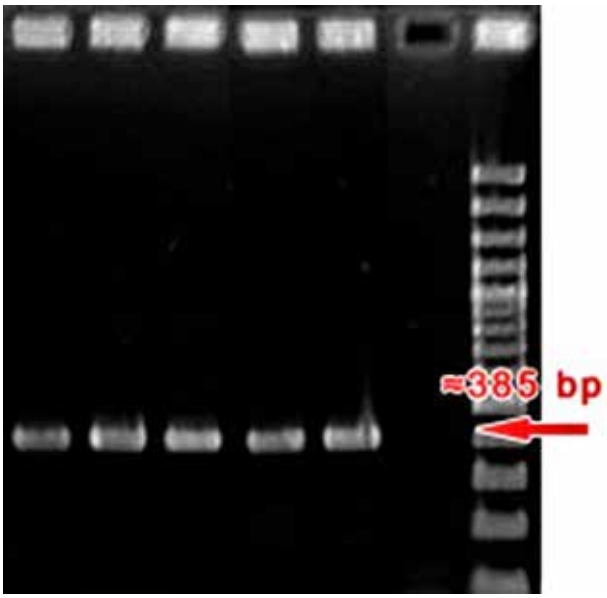

Fig. 3. Amplified DNA fragments of *Eutypa lata* about 385 bp in size obtained using Lata 1/Lata 2.2 primer pairs. EL117, EL153, EL199 - studied isolates; 8F and BX1.10 - reference isolates; - negative control; M-50 bp DNA Step Ladder

the fungus *Eutypa lata* causes symptoms on shoots 25 to 50 cm long, and later as the disease progresses, on the stem and branches of diseased plants. This has been proven by the isolation of pathogens, and then by artificial inoculations on the cuttings of a large number of grapevine varieties. During the mentioned period of monitoring in vineyards in 14 localities in Serbia, the presence of symptoms that the species *Eutypa lata* causes both on inflorescences and berries of grapes.

According to Day and Carter (1976), symptoms on shoots can be observed when shoots have 3 or 4 internodes. The symptoms are very difficult to notice and detect later, during the vegetation, because the diseased leaves are sheltered by healthy shoots and leaves. Diseased leaves stop growing, are chlorotic and bend towards the back. The internodes are shortened and are often zigzagged, and the clusters are poorly developed and sparse (Munkvold et al., 1993, 1994). Cancerous wounds are caused by the growth of healthy tissue at the junction of healthy and diseased tissue, they are brown in color and are usually covered with tree bark. The grapevine dieback when the necrosis spreads to the entire stem (Péros and Berger 1994; Sosonowski et al. 2007; 2013; Sosnowski, 2016).

Symptoms on grapevine shoots may indicate the presence of this fungus in the stem or branches of the vine, and as the disease progresses, the symptoms spread to the entire plant. The growth of the fungus in the stem is slow, 10-20 cm per year, which is why after infection, the symptoms of dying on herbaceous organs do not appear in the first 2 to 3 vegetation seasons (Munkvold et al., 1993). The first symptoms are noticed only in the third or fourth year after the infection. Such a long incubation of the fungus in the stem and branches makes this disease „insidious", and due to the slow development of the disease, economic losses are manifested in vineyards older than eight years. Also, the fungus does not infect

new vines younger than 5 years, and symptoms are rarely seen on a vine younger than 8 years (Gubler et al., 2005; Sosnowski et al. 2007; 2013; Živković et al., 2012 a, b; Sosnowski, 2016; Živković, 2019).

Tissue necrosis spreads in a circle over time, covering an increasing part of the cross-section, and at the same time, it spreads along the stem, up and down, and reaches the root neck. Drying of the limbs, vines and sleeves, which occurs as a consequence of disease progression, is best observed during the winter (Munkvold et al., 1993; Sosnowski et al., 2013; Živković, 2019).

Fungus *Eutypa lata* can be easily grown on conventional laboratory media, but not all isolates sporulate. To encourage sporulation, the cultures are exposed to a light regime of 12 x UV light - 12 x darkness, 12 x light - 12 x darkness or 24 h UV radiation and sporulation occurs after 30 days. McKemy et al. (1993) stimulated sporulation by exposing *E. lata* colonies to a shift of 12 h of light - 12 h of darkness. In this regime, sporulation occurs after 30 days. Ju et al. (1991) stimulated the sporulation of *E. lata* isolates by re-seeding a 2% PDA medium with 5 g/l of yeast extract. The re-seeded cultures of *E. lata* were kept for a period of three weeks at 20 °C and exposed to 12 h of fluorescent light - 12 h of darkness. Glawe & Rogers (1982) and Glawe et al. (1982**)**, in order to accelerate sporulation, exposed *E. lata* isolates on a PDA substrate to shifting of 12 h of fluorescent light - 12 h of darkness for 30 days. Carter (1994) stimulated sporulation of *E. lata* isolates on a PDA medium by exposing it to 24 h of UV light for 30 days or a combination of 12 h of UV light - 12 h of darkness, as well as a combination of 12 h of light - 12 h of darkness. Munkvold (2001) stated that sporulation of conidia of *E. lata* isolates on PDA medium is stimulated by constant exposure to UV rays for 30 days.

Dye & Carter (1976), Glawe et al. (1982) and McKemy et al. (1993) described cotton-white colonies of *E. lata* isolates on a PDA medium. Over time, the color of the colony turns to cream. Under the influence of a shift of 12 h of light - 12 h of darkness and at alternating temperatures of 19 °C and 15 °C, sporulation occurs after 30 days, which agrees with the statements of Carter (1994) and Munkvold (2001). The same authors state that in some cultures a gray pigment is formed after 15 days and the back turns black. Three to four weeks after seeding, small black pycnidia appear in the culture under the influence of constant UV light. Conidia are excreted from the pycnidia in the form of a cream to an orange gelatinous mass (Rolshusen et al., 2006; Trouillas & Gubler, 2010).

The test results show that not all studied light types have a statistically significant effect on the radial growth of the tested isolates, but they do cause a significant statistical difference in their sporulation. Thus, different types of light, in all tested isolates, influenced the level of sporulation. Isolates EL117, 8F, and BX1.10 form a statistically significantly higher number of conidia at 24 h UV light and then under the influence of 12 h UV and 12 h darkness (Glawe & Rogers, 1982; Glawe et al., 1982; Munkvold, 2001).

Identification of the fungus *E. lata* is difficult due to the large 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 (DeScenzo et al., 1999; Lecomte et al., 2000; Rolshausen et al., 2004, 2006, 2014; Catal et al., 2007; Trouillas & Gubler, 2010; Urbez-Torres et al., 2012). Thus DeScenzo et al. (1999) for the first time used sequences of the ITS region to study 115 isolates of *E. lata*, isolated from ten plant hosts, of different geographical origin. These authors found that in all studied *E. lata* isolates, the 5.8 S rDNA sequences were identical, regardless of the host and origin of the isolates.

Then, Lecomte et al. (2000) use DNA sequences to identify *E. lata* species. Based on the ITS region rDNA, they synthesized 3 pairs of primers Lata1/Lata2-1, Lata1/Lata2-2 and Lata3/Lata2-1, as well as 3 pairs of primers based on the fragment of the RAPD sequence SCA 10A/SCA 10B, SCB 02A/SCB 02B and SCD 18 A/SCD 18 B. Using the above primer pairs, they tested 60 isolates of *E. lata* of different geographical origin, isolated from vines. However, the Lata1/Lata2-2 primer pair was found to be the most specific for the detection of *E. lata* species.

Rolshausen et al. (2004) using a universal primer pair ITS1-ITS4 and the restriction enzyme AluI, by analysis of RFLP products in an agarose gel, separated over 30 isolates of *E. lata* from different hosts and different geographical origins, from other species from the family Diatrypaceae.

Rolshausen et al. (2006) analyzed the sequences of 46 fungal isolates representing 15 different species from the Diatrypaceae family, including 25 *E. lata* isolates collected from different plant hosts and different geographical origins, using ITS rDNA and the protein part of the ß-tubulin gene. Catal et al. (2007) based on the ITS1-ITS2 region designed a specific pair of EL1/EL4 primers, the application of which proved successful for the detection of American isolates of *E. lata*, while in European and Australian isolates there was no amplification.

Trouillas & Gubler (2010) successfully determined 35 isolates of *E. lata* as well as 7 isolates of other fungi, originating from different plant hosts,

using universal primers ITS1-ITS4, sequencing of products of about 566 bp in all tested isolates, and sequence analysis

Likewise, Úrbez-Torres et al. (2012) used ITS region, EFI-α and β-tubulin for sequence analysis in order to separate the grapevine dieback fungus complex. In this way, as many as 17 different species from the family Diatrypaceae have been identified, which contribute to the formation of cancer, including the species *E. lata*.

Due to the above, this paper approaches, among other things, the application of molecular detection for the determination of the studied isolates to species levels.

Molecular methods, as a modern approach to the study of plant pathogens, have a great advantage in the precise identification and characterization of various pathogens. Due to its simplicity and reliability, the PCR method has become one of the most commonly used methods in the detection of phytopathogenic fungi. Molecular detection of the studied isolates of *Eutypa* spp. it was performed after isolation of total DNA. The extracted DNA was intact and suitable for further successful amplification in the PCR reaction, enabling successful detection of all isolates used in this work. Using a specific primer pair Lata 1/Lata 2.2 (Lecomte et al., 2000), a fragment of about 385 bp in length was successfully amplified in all three studied isolates. These primers were selected based on the recommendation given by Lecomte et al. (2000) in a protocol for rapid and efficient detection of this pathogen. In the studied isolates, profiles corresponding to the *E. lata* species were obtained, which fully corresponds to the identification using the conventional and molecular methods. 8F isolates originating from Italy and BX1.10 originating from France, previously identified as *E. lata*, were also used as reference isolates in these studies.

Based on the breeding and molecular characteristics of the three isolates of *Eutypa* spp. originating from parts of the grapevine with symptoms of extinction, collected from Serbia, and comparing them with two reference isolates of *E. lata* originating from Italy and France, it was determined that grapevine dieback in our country is caused by species of the genus *Eutypa,Eutypa lata* (syn. *Eutypa armeniacae*), anamorph *Libertella blepharis*.

Conclusions

Based on many years of research, the results were obtained from which the following can be concluded: examination of vineyards in several localities in Serbia, in the period from 2004 to 2021, showed the appearance of symptoms of death of grapevine vines.

From the breeding traits to the development of colonies of the studied isolates of *Eutypa lata* the type of light had the most significant influence. Under the effect of light type 12 h of light and 12 h of darkness, the isolates EL153 and EL199 did not sporulate, while under the action of 24 h of UV light, and at 12 h of UV and 12 h of darkness these isolates, as well as other tested isolates, achieved abundant sporulation.

Molecular detection of isolates was performed using a specific primer pair Lata 1/Lata 2.2, which allows amplification of the ITS region of ribosomal DNA and 5.8 rRNA (ITS1/ITS2 region). Using these primers in all studied isolates, as well as reference isolates from international collections, a fragment of 385 bp was multiplied, which confirmed that it belongs to *E. lata* species. In the studied isolates, profiles corresponding to *E. lata* species were obtained, which fully corresponds to the identification using the conventional method.

References

Carter, M.V. 1994: Wood and root diseases caused by fungi. *Eutypa dieback.* In: Pearson R.C. and Goheen A.C. (ed.), *Compendium of grape diseases*. 3rd ed: 32-34, APS Press. St. Paul, Minnesota.

Catal, M., Jordan, S.A., Butterworth, S.C., Schilder, A.M.C. 2007: Detection of *Eutypa lata* and *Eutypella vitis* in Grapevine by Nested Multiplex Polymerase Chain Reaction. *Phytopathology*, 97: 737-747.

Day, J.P., Shattock, R.C. 1997: Aggressiveness and other factors relating to displacement of populations of *Phytophthora infestans* in England and Wales. *European Journal of Plant Pathology*, 103: 379–91.

DeScenzo, R.A., Engel, S.R., Gomez, G., Jackson, E.L., Munkvod, G.P., Weller, J., Irelan, N.A. 1999: Genetic analysis of *Eutypa* strains from California supports the presence of two pathogenic species. *Phytophtapogy*, 89: 884-893.

Dhingra, O.D., Sinclair, J.B. 1995: *Basic Plant Pathology Methods*. CRC Press, Boca Raton, Florida.

Dye, M.H., Carter, M.V. 1976: Association of *Eutypa armeniacae* and *Phomopsisviticola* with a dieback disease of grapevines in New Zealand. *Australasian Plant Pathology Society Newsletter*, 5: 6-7.

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. *European Journal of Plant Pathology*, 144: 707–721.

Glawe, D.A., Rogers, J.D. 1982: Observations on the anamorphs of six species of *Eutyp*a and *Eutypella*. *Mycotaxon*, 14: 334-346.

Glawe, D.A., Skotland, C.B., Moller, W.J. 1982: Isolation and identification of *Eutypa armeniacae* from diseased grapevines in Washington state. *Mycotaxon*, 16: 123-132.

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 Disease*, 102: 12-39.

Gubler, W.D., Rolshausen, P.E., Trouillas, J.R., Urbez, J.R., Voegel, T. 2005: Grapevine trunk disease in California. http:www.practicalwinery.com. SAD.

Ju, Y.M., Glawe, D.A., Rogers, J.D. 1991: Conidial germination in *Eutypa armeniacae* and selected other species of *Diatrypaceae*: Implications for the systematics and biology of Diatrypaceous fungi. *Mycotaxon*, XLI(1): 311-320.

Lecomte, P., Péros, J.P., Blancard, D., Bastien, N., Délye, C. 2000: PCR Assays That Identify the Grapevine Dieback Fungus *Eutypa lata. Applied and Environmental Microbiology*: 4475-4480.

McKemy, J.M., Glawe, D.A., Munkvold, G.P. 1993: A hyphomycetous synanamorph of *Eutypa armeniacae* in artificial culture. *Mycologia*, 85(6): 941-944.

Munkvold, G.P., Duthie, J.A., Marois, J.J. 1993: Spatial patterns of grapevines with Eutypa dieback in vineyards with or without perithecia. *Phytopathology*, 83: 1440-1448.

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.

Munkvold, G.P. 2001: Eutypa dieback of grapevine and apricot. Online. *Plant Health Progress*, doi: 10.1094/PHP-2001-0219-01-DG.

Peros, J.P., Berger G. 1994: A rapid method to assess the aggressiveness of *E. lata* isolates and the susceptibility of grapevine cultivars to Eutypa dieback. *Agronomie*, 14: 515-523.

Rolshausen, P.E., Trouillas, F., Gubler, W.D. 2004: Identification of *Eutypa lata* by PCR-RFLP. *Plant Disease* 88: 925-929.

Rolshausen, P.E., Mahoney, N.E., Molyneux, R.J., Gubler, W.D. 2006: A Reassessment of the Species Concept in *Eutypa lata*, the Causal Agemt of Eutypa Dieback of Grapevine. *Phytopathology*, 96: 369-377.

Rolshausen, P.E., Baumgartner, K., Travadon, R., Pouzoulet, J. 2014: Identification of *Eutypa* spp. causing Eutypa dieback of grapevine in Eastern North America. *Plant Disease*, 98: 483-491.

Rolshausen, P.E., Sosnowski, M., Trouillas, F.P., Gubler, W.D. 2015: Diseases Caused by Fungi and Oomycetes. *Eutypa dieback.* In: Wilcox W.F., Gubler W.D, Uyemoto J.K. (Ed.) *Compendium of Grape Diseases, Disorders, and Pests*, 2nd ed: 57-61, APS Press, St. Paul, Minnesota.

Sosnowski, M.R., Lardner, R., Wicks, T.J., Scott, E.S. 2007: The influence of grapevine cultivar and isolate of *Eutypa lata* on wood and foliar symptoms. *Plant Disease*, 91: 924-931.

Sosnowski, M.R., Loschiavo, A. P., Wicks, T.J., Scott, E.S. 2013: Evaluating treatments and spray application for the protection of grapevine pruning wounds from infection by *Eutypa lata*. *Plant Disease*, 97: 1599-1604.

Sosnowski, M.R. 2016: Best practices management guide. Eutypa dieback. Available at: http: www.Barossa.com/uploads/214/20160621 eutypa – dieback – best – practice – management.guide.pdf [Accessed on 5 June 2017].

Sosnowski, M., McCarthy, G. 2017: Economic impact of grapevine trunk disease management in Sauvignon Blanc vineyards of New Zealand. *Wine & Viticulture Journal,* 32: 42–48.

Trouillas, F.P., Gubler, W.D. 2010: Host range, biological variation, and phylogenetic diversity of *Eutypa lata* in California. *Phytopathology*, 100(10): 1048-1056.

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. *Journal Fungal Diversity*, 52(1): 169-189.

Živković, S., Vasić, T., Anđelković, S., Jevremović, D., Trkulja, V. 2012a: Identification and Characterization of *Eutypa lata* on Grapevine in Serbia. *Plant Disease*, 96(6): 913.

Živković, S., Vasić, T., Trkulja, V., Krnjaja, V., Marković, J. 2012b: Pathogenicity on grapevine and sporulation of *Eutypa lata* isolates originating from Serbia. *Romanian Biotechnological Letters*, 17(3): 7379-7388.

Živković, S. 2019: *Karakterizacija Eutypa lata, prouzrokovača odumiranja čokota vinove loze u Srbiji i osetljivost sorti.* PhD thesis. Univerzitet u Beogradu, Poljoprivredni fakultet, Beograd.