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MONITORING OF GERMINATION ABILITY OF CONIDIA OF *EUTYPA LATA* FUNGUS ANAMORPHIC STAGE ISOLATED FROM GRAPEVINE IN SERBIA

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ABSTRACT

The germination of conidia of anamorphic stage of four Eutypa lata isolates (EL117, EL219, EL227, EL310), previously determined at the molecular level, and two reference isolates BX1.10 and 8F obtained from the Institute National de la Recherche Agronomique, INRA, France was monitored in an in vitro experiment. Conidia germination was performed in two methods. The first method of conidia germination was performed by transferring the conidia to YPDA (Yeast Potato Dexstrose Agar), and the second method of germination of conidia, originating from PDA, MA, GWA and WA media, was performed in a drop of sterile water under UV lights for 24 h. By comparing the germination results using the above methods, it was found that the percentage of conidia germination in both cases was very low. Calculation revealed that the average percentage of conidia germination was very low, 0.12%, when germinated on YPDA medium, while the average percentage of conidia germination by exposure to UV light for 15 days was 0.15%.

Keywords: *Isolates, conidia, germination, anamorphic stage, Eutypa lata.*

INTRODUCTION

Fungi of the genus *Eutypa* are widespread in the world and include numerous pathogens of cultivated woody plants, forest and ornamental trees. These fungi mainly specialize in living on the dried tissue of infected plants. Several members of the genus *Eutypa* are known to be significant pathogens on apricot, grapevine, apple, and other cultivated and forest trees in the United States and Europe (Trouillas and Gubler, 2010; Trouillas et al., 2010; Rolshausen et al., 2015). The species *E. lata* causes great economic damage to grapevines and apricots, and has therefore been studied in detail in areas where these crops are intensively grown (Carter, 1994; Rolshausen et al., 2015). Studies conducted in California show the possibility of a correlation between grapevine infection with *Eutypa* species and the presence of fruiting bodies of these fungi on surrounding hosts, often in the immediate vicinity of vineyards (Trouillas et al., 2010; Travadon et al, 2012; Pitt et al, 2013). Trouillas and Gubler (2010), based on phylogenetic analysis of DNA sequences of the ITS region, β-tubulin, DNA-dependent RNA polymerase (RPB2) genes, as well as morphological traits of teleomorphic and anamorphic stage, pathogenicity test performed on Sauvignon White and Chardonnay distinguished closely related species: *E. lata*, *E. lata* var. *aceri*, *E. laevata* and *E. petrakii* var. *petrakii*, which were previously described only on the basis of morphology. Afterwards, the

authors confirmed the morphological identification of these species as well as genetic variations within the *E. lata* species, which was also confirmed by Trouillas et al. (2010).

The grapevine dieback occurs in almost all countries of the world where vines are grown commercially. In Serbia, this disease was first detected by Živković et al. (2012a, b).

The fungus *Eutypa lata*, which causes grapevine dieback, in addition to the teleomorphic stage, also forms the anamorphic stage *Libertella blepharis* (Glawe et al., 1982; Yu et al., 1991; Rolshausen et al., 2015). Today, *Libertella blepharis* Desmis accepted as a generic name for the anamorph of the fungus *E. lata* (Carter, 1994). The fungus *E. lata* is very difficult to identify because this fungus does not form a teleomorphic stage (ascospores) in regions where the annual rainfall is less than 330 mm (Ju et al., 1991), as well as on artificial nutrient media (Carter, 1994; Munkvold, 2001; Živković, 2019).

Black stromatic structures, pycnidia, form on the dead bark or tree in infected tissue. Conidiophores are formed from pycnidia, on which numerous single-celled conidia 18–45 μm long and 0.8–1.5 μm wide are formed (Rolshausen et al., 2015). Conidia of this parasite are released from pycnidia in culture in a sticky, gelatinous mass, from cream to pale orange in color. Conidia are moderately curved with a flattened base, hyaline and unseptated. The spread of conidia from young pycnidia takes place in water droplets, while the wind can spread dry scaly masses of conidia from older pycnidia (Glawe and Rogers, 1982; Munkvold, 2001; Travadon et al., 2012). Grapevines younger than five years are not susceptible to infection, while the onset of symptoms is rare in grapevine plants aged eight to ten years (Glawe et al., 1982; Gubler et al., 2005; Sosnowski et al., 2007; Rolshausen et al., 2014, 2015).

The aim of this study was to monitor the emergence of conidia germination of anamorphic stage of four isolates of *Eutypa lata*. Isolates EL117, EL219, EL227, EL310 and 2 reference isolates BX1.10 and 8F obtained by the Institute National de la Recherche Agronomique, INRA, France, were studied, using two methods.

MATERIALS AND METHODS

The tested isolates in this paper were obtained from grapevine plants with grapevine dieback symptoms collected in the period 2015–2022. Sampling was performed in the main vine production areas on the territory of the Republic of Serbia from 14 sites: Dobričevo, Drenovac (Pomoravlje district), Praskovče, Lipovac (Niš district), Kobilje, Bela Voda, Krvavica, Suvaja, Trnavci, Tulež (Rasina district), Gudurica (South Banat district), Karbulovo (Bor district), Strezovac (Pčinja district), Sremski Karlovci (South Banat district). A total of 150 samples were taken and analyzed. After being brought to the laboratory, the samples were first washed with running water. Fungal isolation was afterwards performed using standard phytopathological methods. Isolation of the pathogen was performed from the grapevine stem and cordon. In order to remove surface impurities, parts of the stem and cordon were washed with running water for 2 hours, and then cut into 1 cm long fragments. Fragments of the stem and cordon were cut at the junction of necrotic and healthy tissue, superficially disinfected for 5 minutes in 5% sodium hypochlorite solution (NaOCl) (14% NaOCl, Superlab, Belgrade) and washed 3 times for 5 minutes in sterile distilled water. The fragments were transferred to sterile filter paper to remove excess liquid and then placed on a nutrient medium. Potato dextrose agar (PDA) with the addition of 300 μl/L gentamicin sulfate was used to isolate the pathogen. This medium was prepared from 200 g of potatoes, 20 g of dextrose (Torlak, Institute of Immunology and Virology, Belgrade), 20 g of agar (Torlak, Institute of Immunology and Virology, Belgrade), and 1 L of distilled water (Dhingra and Sinclair, 1995).

Petri dishes with fragments were incubated in a thermostat at a temperature of 24±2°C, in the dark until the development of fungal colonies around the fragments, and incubated at 24°C in the 24 h UV light for 30 days. Individual conidia were selected and transferred directly to the PDA plate according to the procedures described by (Dhingra and Sinclair, 1995), and stored on PDA in tubes at 4°C.

Conidia germination of anamorphic stage of 4 isolates (EL117, EL219, EL227, EL310) isolated and 2 reference isolates BX1.10 and 8F obtained by the Institute National de la Recherche Agronomique, INRA, France, was studied in this paper. Identification was done using pathogenicity test (Peros and Berger, 1994), morphology (Glave and Rogers, 1982; Glave et al., 1982) and PCR methods (Lecomte et al., 2000).

Germination of conidia of the studied isolates (EL117, EL219, EL227, EL310) and two reference isolates BX1.10 and 8F, was monitored using 2 methods, according to Ju et al. (1991) and Belarby and Mur (1983). These isolates were chosen because they proved to be the most aggressive in pathogenicity tests. The method according to Ju et al. (1991) involves growing *Eutypa lata* colonies on 2% PDA medium with the addition of 5 g/L yeast extract (Yeast Potato Dextrose Agar, YPDA). Sown cultures of the tested isolates were grown under a light regime of 12 h darkness and 12 h UV light for a period of 30 days at a temperature of about 20°C. After 30 days, the first conidia appeared. The minimal conidial mass was re-seeded with a sterile bacteriological needle into the center of a Petri dish with YPDA substrate. By adding a small amount of sterile water and gently stirring, the conidia were placed on the surface of the Petri dish. The results were read after 2, and no later than 4 days after sowing. 100 randomly selected conidia were observed using a microscope. The method according to Belarbi and Mur (1983) involved growing the tested isolates (EL117, EL219, EL227, EL310, 8F and BX1.10) on four media (PDA, MA) (Maltose Agar-30 g maltose, 18 g agar and 1 L distilled water), GWA (Grape Wood Agar-300 g of chopped woody vine shoots, 18 g of agar and 1 L of distilled water) and WA (Water Agar-17 g of agar and 1 L of distilled water) (Dhingra and Sinclair, 1995) under the continuous UV light.

A few drops of conidia suspension from each of the studied isolates were taken with sterile needle and placed in drops of sterile water on microscopic plates. The preparations were then kept for 15 days in humid chamber conditions, under the influence of continuous UV light. In order to prevent drying, the tiles were regularly moistened with sterile water, 100 randomly selected conidia were observed. The study of conidia germination was performed by observing 100 randomly selected conidia of the studied isolate, using a microscope at a magnification of 400 times (Olympus BX51/BX52, Japan) and a digital camera (Olympus DP71, Japan).

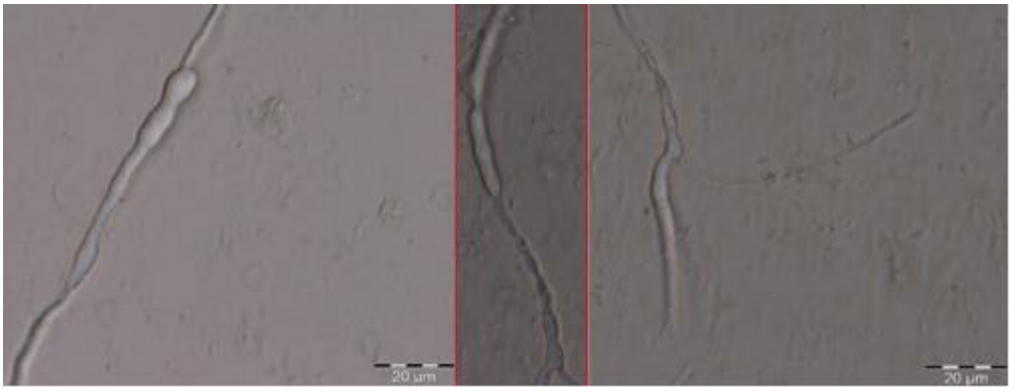
Statistical analysis performed in order to determine the relationship conidia germination percentage between *E. lata* isolate and two reference isolates 8F and BX1.10. Data were analyzed by variance analysis (ANOVA) using the computerized software (PROC GLM, SAS, System, version 8.1; SAS Institute,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

RESULTS AND DISCUSSION

Examination of vineyards in several sites of grapevine cultivation in our country in the period from 2015 to 2022 revealed symptoms of grapevine dieback. Identification was done by pathogenicity test (Peros and Berger, 1994), morphology (Glave and Rogers, 1982; Glave et al., 1982) and PCR methods (Lecomte et al., 2000). 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 rise of internodes. On potato dextrose medium (PDA) 24 h after seeding, the studied *E. lata* isolates form the beginning of white mycelium. After 10 days, the mycelium has a cottony appearance, white color with a weak air growth. Sporulation, formation of conidia, occurred 30 days after seeding under the influence of 24 h UV light (Munkvold, 2001; Rolshausen et al., 2015; Živković, 2019). The dimensions of the conidia of the studied *Eutypa lata* isolates on the PDA were as follows: conidia length 17.82–(32.00)–35.52 μm and width 1.39–(1.45)–2.74 μm, which is within the values cited by other authors for *E. lata* isolated from grapevines and other hosts (Ju et al., 1991; McKemy et al., 1993; Gubler et al., 2005; Rolshausen et al., 2014, 2015; Živković, 2019).

During germination, conidia undergo certain morphological changes (Figure 1). During germination, conidia form a germinal tube at both ends. Conidia germination was performed in two ways: conidia were transferred to YPDA medium (Yeast Potato Dexstrose Agar), and another method of conidia germination, originating from PDA, MA, GWA and WA media, was performed in a drop of sterile water under by the action of 24 h UV light.



a **b** **c**

Figure 1. *Eutypa lata*: Conidia germination on YPDA medium according to the method of Ju et al. (1991) – isolate EL219 (a) and isolate EL310 (b, c)

Conidia germination is a parameter that was individually statistically analyzed in order to determine its suitability as a criterion for distinguishing the examined *Eutypa lata* isolates. The results obtained from the first method of conidia germination showed in Table 1.

In the first conidia germination procedure, in all studied isolates, conidia germinated in a very small percentage, 0.12%, which is in accordance with data from the literature (Belarbi and Mur, 1983; Ju et al., 1991; Živković, 2019). Conidia of isolates EL227 and EL219 germinated in the largest number, 0.19% and 0.17%, respectively. In isolates of EL117 and EL310, germination conidia were 0.10% and 0.08%, respectively, on YPDA medium (Table 1). Most conidia germinated after transfer to YPDA medium after three days of setting up the experiment.

Table 1. Number of germinating conidia of studied *Eutypa lata* isolates and two control isolates on YPDA medium according to the method of Ju et al. (1991)

Medium	No. of conidia	Isolates					
		EL117	**EL219**	**EL227**	**EL310**	**8F**	**BX1.10**
YPDA	Average	0.10	0.17	0.19	0.08	0.12	0.07
	Minimum	0	0	0	0	0	0
	Maximum	7	5	8	6	5	7

The number of germinating conidia is one of the measurement parameters and the obtained results were processed by analysis of variance as a two-factorial experiment, where one factor is the number of germinating conidia of the tested isolates and the other the substrate (Table 2). Belarbi and Mur (1983) kept the conidia of the investigated isolates of the fungus *E. lata* in a hanging drop under the influence of continuous UV light. After 2–3 weeks, conidia germinated. The results obtained in this paper are in accordance with the results obtained by Belarbi and Mur (1983), and Živković (2019).

Table 2. Results of a two-factor analysis of the variance considering the number of germinating conidia of the examined *Eutypa lata* isolates according to the method of Belarbi and Mur (1983), using the Duncan test of significance p = 0.05

Variation source	Sum of squares	Degrees of freedom	Variance	F quotient	*p* values
Isolates	0.880	5	0.176	0.227	0.951
Medium	9.819	3	3.273	4.216	0.006
Isolate + medium	0.875	15	0.058	0.075	1.000
Error	1862.947	2400	0.776		
Total	1874.535	2423			

After calculating the analysis of variance, a pairwise comparison of isolates was performed using the Duncan test 0.05 (Table 2). Thus, conidia of all studied isolates originating from different substrates showed a statistically significant difference in germination after pairwise comparison of isolates. It should also be noted that conidia derived from GWA medium in all tested isolates had a statistically significantly higher percentage of conidia germination compared to all other media, especially compared to conidia of all studied isolates derived from WA medium. By comparing the F quotient, it was determined that there is no significant statistical difference between the isolates in the number of germinating conidia. However, based on the F quotient for substrates, it can be seen that there is a statistical difference between substrates in conidia germination. Namely, the *p* value is 0.006, which is less than 0.05 (Table 2). After statistical processing of the data, it was found that the percentage of conidia germination on four nutrient media was very low, only 0.15%. Thus, conidia originating from the GWA nutrient medium, isolate EL227 after pairwise comparison of isolates was the largest, i.e. 0.29%. Conidia of isolates EL219 and EL227 originating from WA medium had the highest percentage of germinating conidia on this medium 0.07%, while on this medium isolates EL310 and 8F had the lowest number of germinating conidia, only 0.03%. Also, after a pairwise comparison of isolates, it was determined that isolate EL227 had the highest percentage of germinating conidia on all tested nutrient media, in contrast to other tested isolates. Most conidia germinated after being placed in a drop of sterile water after 2 weeks, while some conidia germinated 3 weeks after the experiment had been set. Most conidia germinated by producing a germ tube at each end while others germinated by forming a

medial, lateral germ tube. The germ tube is usually larger in diameter than the conidia from which it originates (Figure 1). The number of germinating conidia, as a separate morphological criterion, in both experiments, did not group the examined isolates, which means that there is no statistically significant difference in the germination of conidia of the studied isolates. These two methods of germination differ in the speed of the experiment. Namely, in the first procedure, conidia germinate after 2 to 4 days, and in the second, it takes 15 days for conidia to germinate. In the first process of conidia germination, it often happens that elongated conidia are difficult to distinguish from the mycelium they create.

In second method of conidia germination, it was observed that germinating conidia continue to germinate in a row, connecting with each other. The obtained results are in accordance with the data cited in the literature (Moller and Kasamatis, 1978; Glawe and Rogers, 1982; Belarbi and Mur, 1983; Ju et al., 1991; Živković, 2019). Older attempts to germinate conidia on agar have usually resulted in the formation of mycelium. It is possible that such mycelium is formed as a result of conidia germination, but germinated conidia cannot be recognized in mycelial mass (Ju et al., 1991). Conidia can be very effective in spreading the disease over short distances, as they form in the mucous mass and spread with water droplets. Although the degree of conidia germination in nature is very small, similar to experimental conditions, the formation of a huge number of conidia from each pycnidia may contain enough germinating conidia to serve as a significant source of inoculum (Ju et al., 1991; McKemy et al., 1993; Gubler et al., 2005; Rolshausen et al., 2014, 2015; Živković, 2019).

CONCLUSION

The results of this study indicate that the anamorphic stage of the fungus isolates *E. lata* originating from Serbia as well as the reference isolates were able to germinate, albeit in a small percentage. Calculation revealed that the average percentage of conidia germination was very low, 0.12%, when germinated on YPDA medium, while the average percentage of conidia germination by exposure to UV light for 15 days was 0.15%. This study was a successful attempt to germinate the conidia of the fungus *E. lata* and to determine the role of the asexual stage in the spread of this disease in vineyards.

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