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Pathogenicity on grapevine and sporulation of *E. lata* isolates originating from Serbia

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SANJA ŽIVKOVIĆ[1], TANJA VASIĆ[2], VOJISLAV TRKULJA[3], VESNA KRNJAJA[4], JORDAN MARKOVIĆ[2]
[1]*Faculty of Agriculture, University of Belgrade, Nemanjina 6, Belgrade, Republic of Serbia*
[2]*Institute for Forage Crops, Globoder37251, Kruševac, Republic of Serbia*
[3]*Agricultural Institute of Republic of Srpska, Knjaza Miloša 17, Republic of Srpska, Bosnia and Herzegovina*
[4]*Institute for Animal Husbandry, Autoput 16, 11080 Zemun, Republic of Serbia*
**Correspondence to: gajicsanja43@gmail.com tel/fax: + 381 37 441 295*

Abstract

Pathogenicity of four isolates of Eutypa lata originating from Serbia (VL 17, VL 27, VL 29, VL 30) and two French reference isolates (8F and BX 1.10), were examined and compared on unrooted cuttings of Cabernet Sauvignon. Additionally, susceptibility to infection by all six isolates of E. lata was tested on unrooted cuttings of six different grapevine cultivars including Riesling white, Opuzenska rana, Sauvignon, Radmilovacki muskat, Beogradska besemena and Negotinski rubin in greenhouse experiments. All tested isolates proved to be pathogenic on Cabernet Sauvignon. Moreover, each grapevine cultivar showed different degrees of susceptibility to infection depending on the E. lata isolate.

The effect of different culture media (PDA, MA, GWA, YA, WA and TA) on both sporulation of E. lata isolates and germination of conidia was also studied. Sporulation occurred after 30 days in three media (PDA, MA, GWA) and after 2 months on WA. No sporulation was observed on YA and TA. The ability of conidia to germinate was assessed on PDA, MA, GWA and WA under continuous UV light. Calculation revealed that the average percentage germination of conidia was very low 0,15%.

Keywords: conidia, Eutypa dieback, germination, *Vitis vinifera*

Introduction

The ascomycete fungus *Eutypa lata* (Pers.: Fr.) Tul. & C. Tul. (syn. *Eutypa armeniacae* Hansf. & Carter), anamorph *Libertella blepharis* A.L. Smith [syn. *Cytosporina* sp.]) causes perennial cankers and consequent dieback in many perennial woody hosts including grapevines worldwide. *Eutypa lata* is responsible for Eutypa dieback of grapevines (*Vitis vinifera* L.), an important disease that reduces considerably the life span of vineyards worldwide [5, 7, 24, 37, 44]. Ultimately, the fungus kills infected cordons or entire vines. The external symptoms of Eutypa dieback are most conspicuous during the first months of the annual growth cycle and include dwarfed shoots with smaller and necrotic leaves [3, 6, 11, 15].

Eutypa dieback is a complicated disease because the length of time between infection and the appearance of the first symptoms, which may be up to more than 4 years in the vineyard. The earliest symptoms are generally the formation of cankers around pruning wounds in older wood and the necrosis of vascular tissue, which impedes water and nutrient movement within the plant. Cankers are difficult to detect because they are covered with bark. Removal of bark around the canker reveals a characteristic region of darkened or discoloured wood. If a cross-section of an infected trunk is made, the canker appears as a wedge-shaped area of discoloured wood spreading to the centre of the trunk. Eutypa dieback produces toxic metabolites causing shoot symptoms to appear at distance of colonized wood parts.

Characteristic foliar symptoms include the dwarfing of internodes, tattering, and necrosis of leaves as well as the death of infected cordons. The pathogen may also cause the failure of berry set, uneven rates of berry maturation and shrivelling of bunches following flowering. This can lead to the failure of bunches to mature, or premature dropping of berries from the vine. Foliar symptoms are initially confined to one arm of infected vines, however, as the disease progresses symptoms may spread throughout the entire vine [5, 6, 7, 18, 15, 17, 20, 21, 37, 38, 40, 42].

Many studies showed that by inoculating healthy cuttings with mycelium of *E. lata* high incidence of foliar symptoms can be induced [7, 8, 18, 21, 22, 27, 38, 39, 40, 42]. The anamorph of *E. lata* has been described by various authors [4, 14, 9, 11, 2, 23, 13, 19, 26, 28, 43], but only few authors reported germination of conidia of *E. lata* [2, 13, 28].

The aim of this research was to confirm the pathogenicity of some isolates of *E. lata* on different cultivars and to determine whether the anamorph of *E. lata* was capable to germinate on artificial media. Thus, two separate studies were carried out i) to establish the pathogenicity of *E. lata* isolates in different cultivars representative of the local vineyards and ii) to assess the germination potential of conidia on six artificial media.

Materials and methods

Isolates. Four *E. lata* isolates originated from Central Serbia (VL 17 from cv Cabernet Sauvignon, VL 27 from cv Rkaciteli, VL 29 from cv Prokupac, VL 30 from cv Reisling) were obtained by isolation from parts of necrotic wood of grapevine. Two reference isolates of *E. lata* (BX 1.10 and 8F) were obtained from the Institute National de la Recherche Agronomique (INRA), France provided by P. Lecomte. Identification was done by morpohology [5, 6 ,11, 14] and PCR methods [33, 34].

Pathogenicity test. Pathogenicity test was performed following standard methods [21]. Unrooted cuttings of Cabernet Sauvignon inoculated with mycelium of six isolates: VL 17, VL 27, VL 29, VL 30, 8F and BX 1.10. The day before the inoculation, 2-node cuttings with the basal bud eliminated were prepared and soaked in water overnight. A whole (5 mm diameter, 5 mm deep) was drilled 4 cm below the upper bud and a plug (5 mm diameter) of agar and mycelium, cut from a fresh culture on PDA medium inserted into the hole with the mycelium towards the wood. Plugs of PDA medium used for the control. Cuttings were inserted just after inoculation into pieces of rock wool. The pieces were then put into drained plastic containers and finally placed on benches in a greenhouse.

The effect of the position of the inoculation site studied. Cuttings of cv Cabernet Sauvignon were inoculated with six above mentioned isolates in April 2005. The inoculum placed 4 cm either under the bud or on the opposite side. For the cultivar experiments, a total of six cultivars including Riesling white, Opuzenska rana, Sauvignon, Radmilovački muskat, Beogradska besemena and Negotinski rubin were inoculated with isolate VL 17 in April 2006. Experiments were arranged in a complete randomized design. There were nine replicates of eight cuttings per treatment.

Sixteen weeks after bud break, nine plants were observed in the second cycle of vegetative growth cycle and sampled for each treatment of the isolate experiment. The part of the cutting between the inoculation point and the upper node selected. Three thin chips cut, and cross-sections made at 5 and 20 mm from the inoculation point. After incubation for one week at 25 ºC, the plates examined for the presence of the mycelium of *E. lata*. Re-isolated cultures from the six isolates were propagated and inoculated to unrooted cuttings of cultivar Cabernet Sauvignon, Reisling white, Opuzenska rana, Sauvignon, Radmilovacki muskat,

Beogradska besemena and Negotinski rubin in a randomized complete block design with four replicates of eight cuttings.

Sporulation and conidial germination in *E. lata.* The sporulation of the six different isolates were performed on six artificial media as follows : potato dextrose agar (PDA), Maltose agar (MA), Grape wood agar (GWA), Yeast agar (YA), Water agar (WA), and Tomato agar (TA) following a standard microbiological procedure [30]. Grape wood agar contained 18 g of agar and 300 g of 1 year old Cabernet Sauvignon which were ground in a mill and boiled with 1000 ml boiling RO water and clarified by filtration. Tomato agar contained 250 g of tomato pulp, 18 g of agar and 1000 ml of RO water.

The level of sporulation presented according to the following scale: + (weak sporulation <5000 spores per ml), + + (medium sporulation, 5000-10000 spores per ml) and + + + (heavy sporulation, > 10,000 spores per ml) [25].

Using six isolates of *Eutypa lata* plated on four different medium (PDA, MA, GWA and WA) the capability of conidia for germination was assessed under continuous UV light. As soon as sporulation was observed, conidial masses were removed from cultures with a sterile loop and placed in the small amount of sterile distilled water into the glass plate. After 2-3 weeks in a humid chamber under continuous UV light, there was a germination of conidia. A minimum of 100 plates and 100 conidie were observed per isolate and per medium [2]. Germinating conidia were located by microscope using 10X objective of Olympus BX 51/BX52 and microscope digital camera Olympus DP 71.

Statistical analysis. Statistical analysis performed in order to determine the relationship between *Eutypa lata* isolate and grapevine cultivar, different media and sporulation [12]. Data were analyzed by variance analysis (ANOVA) using a 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

Pathogenicity study. Results from all pathogenicity experiments showed that *E. lata* produced foliar symptoms when inoculated on unrooted grapevine cuttings. Dwarfed and necrotic leaves appeared during the development of the shoots. It was possible to observe the characteristic symptoms of the disease as soon as 3-4 weeks after the inoculation when the shoots were at the F stage. Symptom severity varied within the same treatment. Some shoots rapidly showed smaller, chlorosed and necrotic leaves and they generally died. For other shoots, the necrosis appeared firstly on leaves of almost normal size leaves and the severity gradually increased on new leaves. Shoots sometimes stayed alive. In other cases, a few leaves became necrotic and the new leaves, which appeared, were normal, of reduced size or chlorosed. In addition, the cuttings showed other abnormalities: 1) a shoot that died rapidly without producing necrotic leaves; or 2) a shoot with a necrosed tip after emission of a few normal leaves. These abnormalities appeared with a higher incidence for the cuttings inoculated with *E. lata* than for the control cuttings. Therefore, the number of cuttings with these abnormalities added to the number of cuttings with foliar symptoms for the analysis of data. The number of abnormal cuttings recorded five, seven and ten weeks after the inoculation. The effect of the inoculum position was marked five weeks after inoculation.

The percentage of affected cuttings was indeed considerably larger when the inoculum positioned under the bud than on the opposite side. However, no difference between the treatments was noticed seven weeks after inoculation. At that time, the percentage reached

67% and found unchanged three weeks later, which is in accordance with the literature data [21, 27, 38, 39, 40, 42].

For the isolate experiment, the number of Cabernet Sauvignon cuttings showing foliar symptoms and other abnormalities was recorded eight weeks after inoculation in the first growth cycle (Fig. 1) and eight weeks after bud break in the second vegetation growth cycle. The plants rated with symptoms or other abnormalities in the second year were those rated as healthy in the first year. The analysis of the percentage of total abnormal plants after the first and second years indicated a highly significant effect of the treatment (Table 1).

Fig. 1. Foliar symptoms on cv Cabernet Sauvignon in the first (left) and second growth cycle (right)

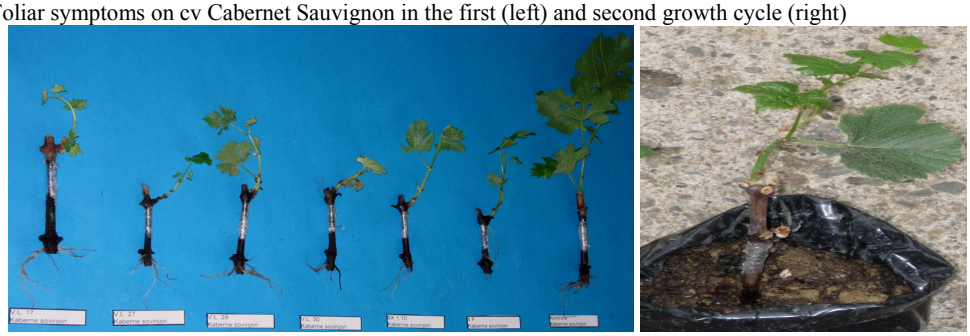

Table 1. Effect of the *E. lata* isolates on the number of Cabernet Sauvignon cuttings showing foliar symptoms during 2 consecutive vegetative seasons in the greenhouse.

Isolates	Cultivar Cabernet Sauvignon	
	Year n	Year n+1
	Foliar symptoms (%)	
VL 17	75.00[a]	50.00[ab]
VL 27	72.22[a]	58.33[a]
VL 29	77.78[a]	55.56[a]
VL30	75.00[a]	44.44[ab]
8 F	83.33[a]	38.89[b]
B X 1.10	80.56[a]	47.22[ab]

[a,b]: Means in a column followed by the same letter are not significantly different according to Duncan's multiple range test at the P=0.05.

Inoculated cuttings produced foliar symptoms during the first vegetative growth cycle. The analysis of the percentage of total abnormal plants after the first and second years indicated a highly significant effect of the treatment. Disease expression mainly occurred in the first year for isolate 8 F. For the six tested isolates, the appearance of leaf symptoms after the first year varied from 72.22% to 83.33% (Table 1). The most aggressive isolate in experiment was 8F, while studies in France [21] showed that the most aggressive isolate was BX 1.10. It was also observed that grapevine cuttings infected with isolate 8F rooted in a very small percentage and it was likely the cause of the death of a large number of vine cuttings. For these reasons in the second cycle of vegetation there were a small number of plants that survived and showed foliar symptoms. This is true for the other isolates, except that in the other isolates was lower percentage of poor or underdeveloped root system, and those plants withered in the second vegetative cycle. It happened that the plants had well developed root system but withered shoots that recovered during the second vegetation cycle and developed shoots with small, chlorotic, deformed leaflets, with scattered necrotic spots. The differences between isolates confirmed in further experiments. For reisolation, we used semi-selective medium. When the fungus was reisolated from a cutting, the samples taken at 5 and 20 mm were generally

positive. For all isolates, re-isolation was positive in some of the cuttings that had a normal growth. The number of available cuttings showing symptoms was low in the second vegetative growth cycle due to high mortality (8F) or the low incidence of symptoms. All the isolates tested were again re-isolated but the percentage of positive cuttings (65.38%) was higher than for normal plants.

Table 2. Effect of grapevine cultivar on percentage of plants showing foliar symptoms and abnormalities after inoculation with *E. lata* isolates VL 17.

Cultivars	**Isolate VL 17**			
	Year n		**Year n +1**	
	5th week	**10th week**	**5th week**	**10th week**
	Foliar symptoms (%)			
Reisling	82.82^{b}	76.57^{b}	80.21^{b}	65.63^{bc}
Opuzenska rana	83.60^{b}	83.39^{ab}	82.64^{b}	74.31^{ab}
Sauvignon	69.54^{c}	84.38^{ab}	81.25^{b}	87.15^{a}
Radmilovački muskat	96.10^{a}	93.75^{a}	95.49^{a}	89.58^{a}
Beogradska besemena	86.72^{ab}	80.47^{b}	68.75^{c}	62.50^{c}
Negotinski rubin	94.53^{a}	94.53^{a}	59.38^{c}	61.81^{c}

$^{a-c}$: Means in a colum followed by the same letter are not significantly different according to Duncan's multiple range test at the P=0.05.

The foliar expression of Eutypa dieback was compared on six cultivars of grapevine following inoculation by the *E. lata* isolate VL 17 (Table 2). The numbers of cuttings showing foliar symptoms and others abnormalities were record five and ten weeks after inoculation in cultivar experiments. All the cultivars inoculated exhibited foliar symptoms of the disease and the other abnormalities. In both experiments, the effect of cultivar was highly significant. Some of the shoots showing necrotic leaves five weeks after inoculation showed normal growth ten weeks after inoculation and this phenomenon explained the decrease in some percentage. The differences between cultivars were less acute ten weeks after inoculation (Table 3) indicating that the effects of the fungus appeared faster for some cultivars than for others.

Inoculated unrooted cuttings produced foliar symptoms during the first vegetative growth cycle. The smaller and necrotic leaves were similar to that observed in shoots of infected vines in the vineyard. Moreover, the necrosis of lamina was identical to that reproduced on leaves by the fungal culture filtrate or by eutypine [21, 29, 31, 32, 35, 41].

Table 3. Grapevine cultivar effect and mean squares (MS) for percentage of abnormal plants after inoculation with *E. lata* isolate VL 17 in cultivar experiment.

Source of variation	**df**	**Number of weeks after inoculation**			
		5		**10**	
		MS	**F**	**MS**	**F**
Experiment 1					
Replication	3	114.35	2.45	23.22	0.37
Cultivar	5	368.83	7.91*	208.40	3.29*
Error	15	46.64		63.23	
Experiment 2					
Replication	2	133.97	3.78	28.25	0.55
Cultivar	5	465.o5	13.13*	459.23	9.01*
Error	10	35.42		50.98	

* Test significant at P=0.05 Duncan's multiple range test randomized complete blocks

Therefore, the rapid appearance of symptoms seems to be consequence of the transport of toxic products from the inoculation site to the growing shoot. The effects of the position of the inoculum site sustain this conclusion. The symptoms appeared faster when the fungus first colonized the vessels in direct connection with the sprouting shoot. The late appearance of symptoms during the second vegetative growth [22, 20, 21, 27, 35, 38, 39] was probably due to difference in the method or the isolate characteristics. The cultivars had different responses in the test indicating that cultivars differ in their susceptibility to fungal toxins and to fungal invasion. The high susceptibility of cultivars "Radmilovacki muskat", "Reisling white", "Opuzenska rana", "Beogradska besemena" was confirmed. Cultivar "Radmilovacki muskat" was considerably more affected than the cultivar "Sauvignon" (white cv.) and the inoculation of cuttings caused a faster appearance of abnormal plants for Radmilovacki muskat" than for the cultivar "Sauvignon". The cultivars had different responses in the test indicating that cultivars may differ in their susceptibility to fungal toxins and to fungal invasion.

Observations of *E. lata* anamorphs. The anamorph has been described by various author [2, 6, 9, 10, 11, 13, 16, 19, 23, 24, 26, 28]. Mycelia occurred after ten days in five media (PDA, MA, GWA, YA and WA). On tomato agar medium (TA) the growth of isolates VL 27, VL 29 and 8F was very weak, while for the isolates VL 17, VL 30 and BX1.10 mycelium did not developed (Table 4).

Table 4. Influence of different media on the mycelial growth of the six isolates of *E. lata tested in this study*.

Isolates	Media					
	PDA	**MA**	**GWA**	**YA**	**WA**	**TA**
Diameter of colony (mm)						
VL 17	89.20^{a}	87.00^{ab}	90.00^{a}	66.80^{a}	74.20^{bc}	1.00^{b}
VL 27	88.80^{a}	87.80^{ab}	90.00^{a}	59.80^{ab}	64.20^{d}	3.30^{a}
VL 29	88.60^{a}	90.00^{a}	90.00^{a}	61.20^{ab}	78.00^{ab}	1.74^{ab}
VL 30	88.40^{a}	88.00^{ab}	71.40^{b}	56.40^{b}	69.00^{cd}	1.00^{b}
8 F	89.40^{a}	88.60^{ab}	88.00^{a}	54.20^{b}	81.80^{a}	1.71^{ab}
BX 1.10	88.80^{a}	90.00^{a}	89.00^{a}	64.40^{a}	82.00^{a}	1.00^{b}
Daily growth (mm) **						
VL 17	6.64^{a}	5.56^{b}	5.76^{a}	4.24^{a}	5.80^{ab}	0.00^{b}
VL 27	5.80^{b}	5.32^{b}	5.48^{ab}	3.10^{b}	3.16^{c}	1.88^{a}
VL 29	6.60^{a}	6.80^{a}	5.84^{a}	4.64^{a}	6.00^{ab}	0.00^{b}
VL 30	5.44^{b}	5.80^{b}	4.00^{d}	4.44^{a}	5.12^{b}	0.00^{b}
8 F	4.72^{c}	5.12^{b}	4.96^{c}	3.92^{a}	5.28^{ab}	0.52^{b}
BX 1.10	6.92^{a}	6.72^{a}	5.28^{bc}	4.64^{a}	6.52^{a}	0.00^{b}

** Average daily increase calculated per formula $(D_2-D_1)/(T_2-T_1)$; **$^{a-d}$:** Means in a column followed by the same letter are not significantly different according to Duncan's multiple range test at the P=0.05.

Colonies were initially hyaline with cottony aerial growth and diffuse margins. After 2-4 week, regions of dark gray to black aerial hyphe developed. Pale yellow conidial masses began exuding from black, subconical pycnidia. Conidia were 17.50-33.25 μm x 1.28-2.94 μm, single celled, moderately curved, filiform, and hyaline [2, 6, 9, 10, 11, 13, 19].

Table 5. Influence different media on sporulation tested isolates of *E. lata.*

Isolates	Media					
	PDA	MA	GWA	YA	WA	TA
Sporulation after 2 month ***						
VL 17	+++	+++	+++	-	++	-
VL 27	+++	+++	+++	-	+++	-
VL 29	+++	+++	+++	-	+++	-
VL 30	+++	+++	+++	-	+++	-
8 F	+++	+++	+++	-	++	-
BX 1.10	+++	+++	+++	-	+++	-
Number of conidia after two months per mm^2						
VL 17	28.50^{a}	41.44^{a}	31.13^{b}	-	1.80^{b}	-
VL 27	21.56^{b}	43.06^{a}	38.13^{a}	-	2.50^{ab}	-
VL 29	21.75^{b}	35.94^{ab}	31.19^{b}	-	3.00^{a}	-
VL 30	21.88^{b}	36.25^{ab}	31.25^{b}	-	2.50^{ab}	-
8 F	19.63^{b}	30.00^{b}	32.19^{b}	-	1.75^{b}	-
BX 1.10	20.31^{b}	35.00^{ab}	34.88^{ab}	-	2.31^{ab}	-

*** Sporulation: + = low, ++ = medium, +++ = abundant ; ^{a,,b}: Means in a colum followed by the same letter are not significantly different according to Duncan's multiple range test at the P=0.05.

Past attempts to germinate conidia by stretching them on agar frequently resulted in the formation of mycelia, but such mycelia not traced to identifiable germinating conidia and therefore regarded as likely resulting from contaminating hyphal fragments. Possibly, such mycelia did result from conidial germination but the germinating conidia were not recognized [1, 9, 13, 16, 43].

Table 6. Number of germinated conidia

Isolates	Media							
	PDA	%	MA	%	GWA	%	WA	%
Number of germinated conidia								
VL 17	15	0.15	17	0.17	18	0.18	5	0.05
VL 27	17	0.17	20	0.20	28	0.28	7	0.07
VL 29	13	0.13	21	0.21	24	0.24	7	0.07
VL 30	15	0.15	18	0.18	20	0.20	3	0.03
8 F	12	0.12	14	0.14	20	0.20	3	0.03
BX 1.10	14	0.14	16	0.16	18	0.18	5	0.05
Σ	**86**	0.14	**106**	0.18	**128**	0.21	**30**	0.05

Conidia of *E. lata* germinated in this study at low rates. A total of 350 conidia of *Eutypa lata* were found to germinate in our experiment. Calculation revealed that the average percentage germination of conidia was very low 0,15% (Tab. 6).

Conidia that originated from the GWA medium germinated in the largest number (128), 0.21% compared to conidia from other media. On the WA medium the number of germinated conidia was the lowest (30), 0.05%. As for the isolates, isolate VL 27 had the most germinated conidia (72), 0.18% on all mediums, while the 8F isolate had the least germinated conidia 49 or 0.12%.

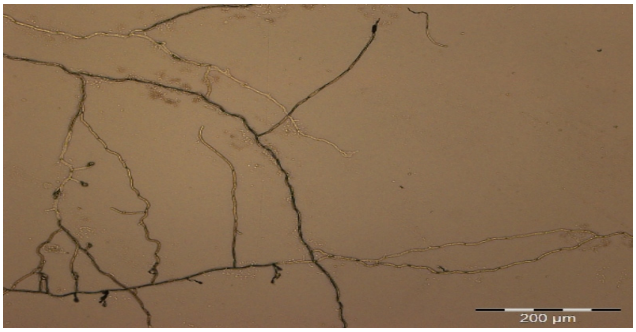

Figure 2. Germination conidia of isolate VL 27 *E. lata* from GWA

Most conidia germinated within two weeks after dispersing them on drop of sterile water, while some additional conidia found germinating at period up to three weeks from the time they placed on sterile water. Most conidia germinated by producing a germ tube at each end while others germinated by forming a medial, lateral germ tube (Fg. 2). Germ tubes were usually of greater diameter than the conidia from which they originated [1, 2, 9, 10, 11, 13, 16, 36, 43]. Results indicate that *E. lata* is able to germinate in very low rate under suitable conditions.

Conidia might be most effective in dispersal over relatively short distances, since they typically are produced in miculage and presumably are splash-dispersed. Even if germination rates of conidia are similarly low in nature, the huge numbers of conidia produced by each pycnidium may include germinating conidia sufficient to serve as a significant source of inoculum.

Conclusion

The results of this study indicate that isolates originating from Serbia as well as the reference isolates were pathogenic to all cultivars of grapevine, but there were differences in susceptibility of cultivars depending on some isolates. It was also determined that studied isolates formed anamorphic stage on artificial nutrient media and that led to germination of conidia in a very small percentage. Further studies to determine the role of the asexual state and the effectiveness of conidial inoculum are needed to provide a clearer picture of the epidemiology of *Eutypa* disease on grapevine.

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