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Original scientific paper

Botrytis cinerea in raspberry in Serbia II: Growth rate and virulence of isolates

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SUMMARY

Growth rate and virulence of 130 isolates of *Botrytis cinerea*, derived from raspberry fruit originating from six commercial fields in a raspberry growing region of Serbia and classified in two morphological and four genetic groups were studied. The results showed significant differences in mycelial growth rate among the isolates. The highest and lowest recorded growth rates were 24.5 mm/day and 8.4 mm/day, respectively, while the growth rate of most isolates ranged from 15.8 to 21.8 mm/day. The growth rate of isolates that belong to different morphological and genetic subgroups varied similarly. Furthermore, growth rate intervals of all subgroups overlapped, suggesting that the groups cannot be distinguished based on growth rates of the isolates contained. The studied *B. cinerea* isolates exibited different levels of virulence towards vine, sunflower and raspberry leaves, while an analysis of variance revealed that both the isolates and the inoculated host species were significant sources of variation ($P<0.01$). Sunflower and raspberry leaves were significantly more sensitive than vine leaves. However, correlation between isolate virulence and different hosts was not found.

Keywords: *Botrytis cinerea*; Raspberries; Serbia

INTRODUCTION

Botrytis cinerea is a necrotrophic polyphagous plant pathogen well-known for its great phenotypic and genetic variability. Differences among isolates in colony morphology, sporulation and sclerotia production are usually attributed to the multinucleate and heterocaryotic nature of hyphae or conidia and the aneuploid state of nuclei (Hansen & Smith, 1932; Büttner et al., 1994; Chardonnet et al., 2000; Yourman et al., 2001). It is also believed that the presence of *Boty* and *Flipper* transposon elements in the genom (Giraud et al., 1999) could contribute to isolate phenotypic diversity. Thus, Martinez et al. (2003, 2005) found *vacuma* isolates (without transposons) mostly to belong to the mycelial type and have a higher growth rate than *transposa* isolates (containing both transposons), while Giraud et al. (1999) reported a difference in fungicide

resistance frequencies in *transposa* and *vacuma* isolates. Our previous study (Tanović et al., 2014) revealed that all *B. cinerea* isolates originating from raspberry fields in Serbia belonged exclusively to the Group II genetic entity of *B. cinerea* described by Fournier et al. (2003). The isolates were divided into two main morphological (mycelial and sclerotial) and four genetic groups (*transposa*, *vacuma*, *boty* and *flipper*). In order to improve our understanding of *B. cinerea* populations, growth rates and virulence of isolates from different subgroups were determined and analyzed in this paper.

MATERIAL AND METHODS

Fungal isolates

A total of 130 *B. cinerea* isolates, derived from diseased raspberry fruit collected at six locations in a major raspberry growing region in Serbia, identified based on their pathogenic and morphological characteristics and classified in two morphological and four genetic groups (Tanović et al., 2014), were used in this study (Tables 1 and 2).

Maintenance

The isolates were cultured on potato dextrose agar (PDA) medium at 20°C and stored on slants at 4°C for short-term or in 20% glycerol at –80°C for long-term storage.

Mycelial growth *in vitro*

In vitro growth rate of the isolates was determined by transferring mycelial plugs (Ø 10 mm) from the edge of 4-day-old colonies on PDA plates. Their growth was recorded after 3-day incubation at 20°C in the dark by measuring two diameters of each colony at right angles. Three replicates per isolate were used and the experiment was repeated twice. All data were pooled together and subjected to analysis of variance and Duncan's multiple range test.

Table 1. A list of *Botrytis cinerea* isolates originating from different locations in Serbia and their classification as *vacuma* (without transposable elements), *transposa* (containing both *Boty* and *Flipper* elements), *flipper* (containing only *Flipper*) and boty (containing only *Boty* element)

Location	Number of isolates	Codes of isolates	Number of isolates			
			vacuma	*boty*	*flipper*	*transposa*
Valjevo	30	V1-30	3	14	1	12
Požega	20	Po1-20	2	9	0	9
Šabac	20	S1-20	8	5	1	6
Arilje	20	A1-20	1	9	0	10
Ivanjica	20	I1-20	0	11	0	9
Prilike	20	Pr1-20	0	10	0	10
Total	130		14	58	2	56

Table 2. Morphological features and sporulation ability of *Botrytis cinerea* isolates originating from different locations in Serbia

Location	Number of isolates	Codes of isolates	Number of isolates			
			Type of isolates		Sporulation	
			sclerotial	mycelial	present	absent
Valjevo	30	V1-30	23	7	1	29
Požega	20	Po1-20	17	3	0	20
Šabac	20	S1-20	13	7	6	14
Arilje	20	A1-20	16	4	0	20
Ivanjica	20	I1-20	17	3	0	20
Prilike	20	Pr1-20	20	0	4	16
Total	130		106	24	11	119

Virulence test

A virulence test was performed on leaf discs of three host species (*Vitis vinifera* L., cv. Reisling Italico, *Helianthus annus* L., cv Kolos, and *Rubus idaeus* L., cv. Willamette) using a method described by Martinez et al. (2003). Primary leaves of sunflower, grown in a greenhouse, or fully developed leaves of vine and raspberry collected from a vineyard and an orchard, respectively, were detached, rinsed in distilled water and air dried. Five leaf discs per host per isolate (total 1950), 25 mm in diameter, were cut using a cork borer and placed on moist filter paper in 90 mm Petri dishes. Mycelial plugs 10 mm in diameter, cut from the edge of 4-day-old colonies grown on PDA, were placed centrally on the upper side of each leaf disc. The inoculated discs were incubated in moist chambers at 20°C for 3 days. Lesion development on leaf discs was assessed visually on a semi-quantitative graded scale (**0** = healthy, **1** = 10% rotten, **3** = 20%, **5** = 40%, **7** = 60%, **9** = 80%, **11** = 90% and **13** = totally rotten) as proposed by Martinez et al. (2003). A mean disease severity index (DSI) was calculated. To assess the effects of the inoculated host plants and the isolates from each location separately the data were analyzed by ANOVA at 5% probability level with individual pairwise comparisons made using Duncan's multiple range test (Statistika Inc, 2001). In addition, correlation coefficients were calculated using the mean values of virulence for each of 130 isolates.

RESULTS

Mycelial growth rate

The isolates showed significant differences in mycelial growth rate at 20°C on PDA medium (Figure 1). The highest and lowest growth rates were 24.5 mm/day and 8.4 mm/day, respectively, while the growth rate of most isolates ranged from 15.8 to 21.8 mm/day. These values, as well as the average growth rates of the isolates from each location are shown in Table 3

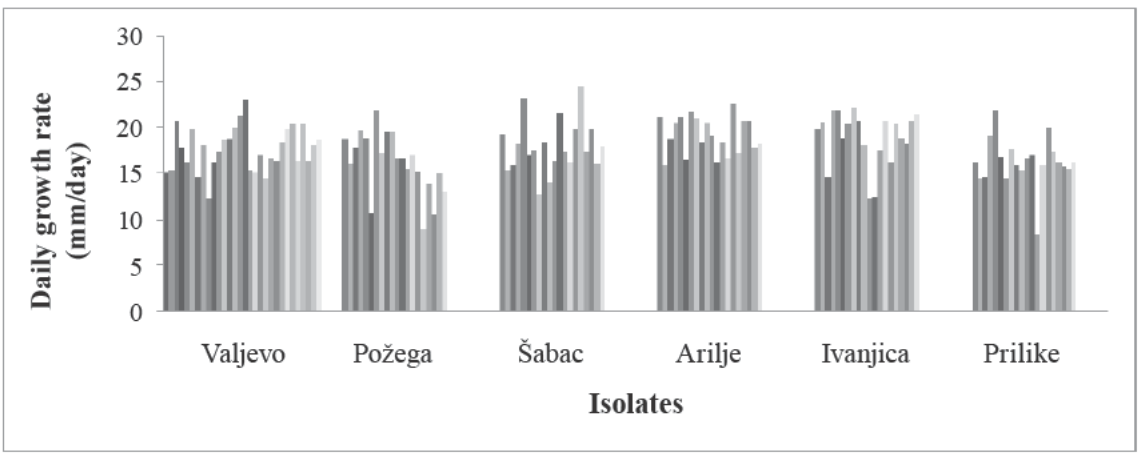

Figure 1. Growth rate of *Botrytis cinerea* isolates originating from raspberry fields at different locations in Serbia

Table 3. Growth rates of *Botrytis cinerea* isolates from different locations

Locations	Lowest growth rate (mm/day)	Highest growth rate (mm/day)	Average growth rate (mm/day)[1,2]
Valjevo	12.3±4.1	23.1±0.5	17.6±2.4 a
Požega	8.9±1.2	21.8±0.4	16.1±3.4 b
Šabac	12.7±0.7	24.5±0.5	17.9±2.9 a
Arilje	15.8±0.6	22.6±0.9	19.2±2.1c
Ivanjica	12.3±0.6	22.1±0.1	18.9±3.0 c
Prilike	8.4±2.8	21.8±0.8	16.2±2.6 b

[1]Calculated as average growth rate of all isolates from a location.
[2]The same letter in column indicates non-significant difference

The results of Duncan's multiple range test showed that both the isolates and isolate locations were statistically significant sources of variation ($p<0.01$). However, the isolates from a single location were not grouped together, each group rather contained several subgroups of isolates characterized by different growth rates (data not shown).

Growth rates of the isolates from different genetic, morphological and sporulation ability groups are presented in Figure 2. The highest and lowest recorded growth rates of the isolates from each subgroup are summarized in Table 4.

The lowest and highest growth rates, 8.4±2.8 mm/day and 24.5±0.6 mm/day respectively, were both recorded for sclerotial *transposa* isolates. Among sporulating isolates, the highest growth rate (23.2±1.2 mm/day) was found in a sclerotial isolate belonging to the *vacuma* genetic group.

Virulence

The studied *B. cinerea* isolates originating from each location exibited different levels of virulence to each host plant. Furthermore, the analysis of variance revealed that both the isolates and inoculated host species were significant sources of variation ($P<0.01$).

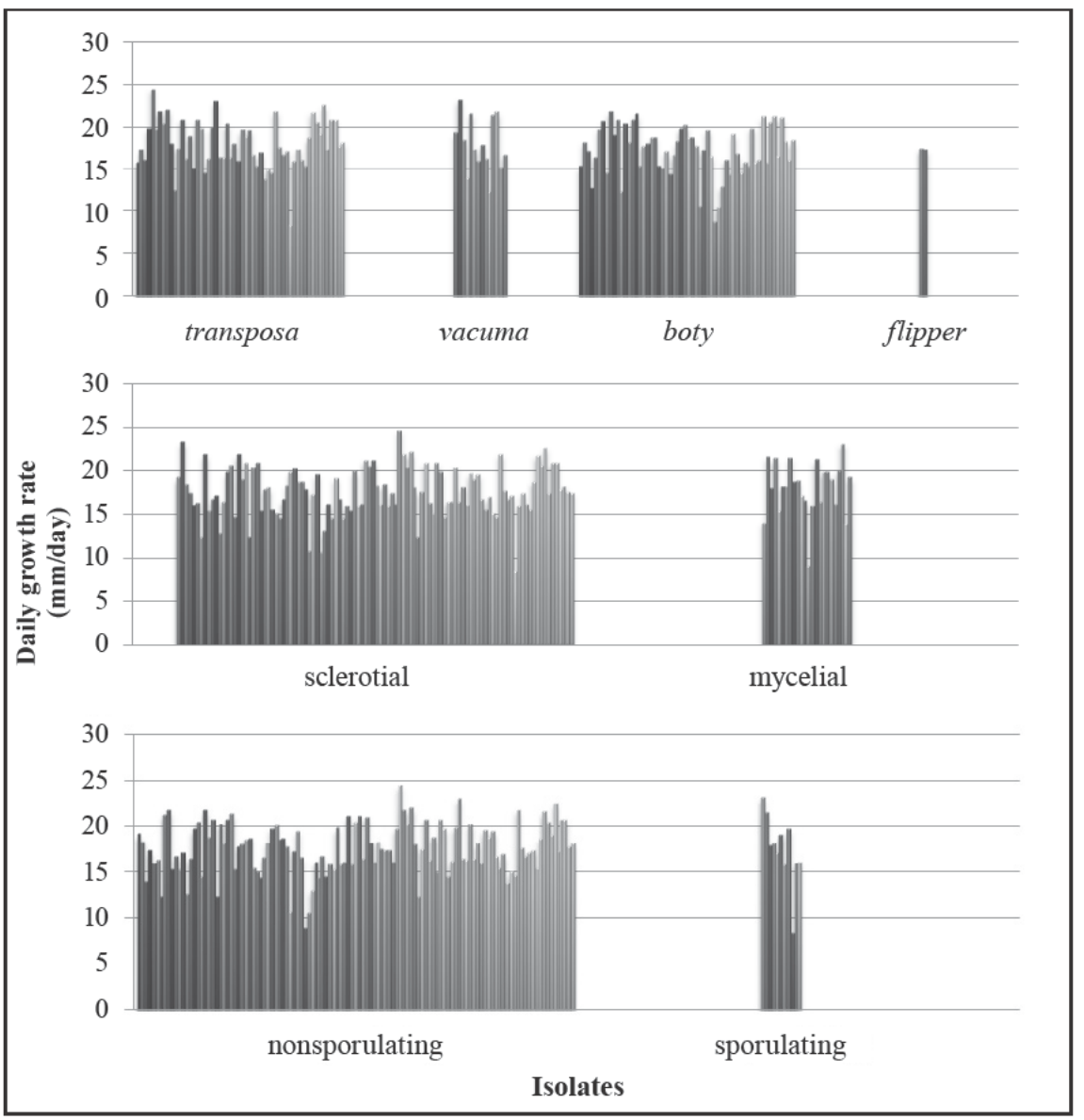

Figure 2. Growth rate of *Botrytis cinerea* isolates belonging to different genetic, morphological and sporulation ability groups

Table 4. The lowest and highest growth rates of *Botrytis cinerea* isolates belonging to different genetic, morphological and sporulation ability groups

Groups	Type of isolates	Lowest growth rate (mm/day)	Highest growth rate (mm/day)
Genetic groups[1]	*transposa*	8.4±2.8	24.5±0.6
	vacuma	12.3±4.1	23.2±1.2
	boty	8.9±1.2	21.8±0.4
	flipper	17.3±0.3	17.4±1.1
Morphological groups	sclerotial	8.4±2.8	24.5±0.6
	mycelial	8.9±1.2	23.1±0.5
Sporulation ability groups	sporulating	8.4±2.8	23.2±1.2
	nonsporulating	8.9±1.2	24.5±0.6

[1]Based on all available isolates from each genetic, morphological and sporulation ability subgroup: 14 *vacuma*, 58 *boty*, 56 *transposa* and 2 *flipper*; 106 sclerotial and 24 mycelial; 119 nonsporulating and 11 sporulating isolates

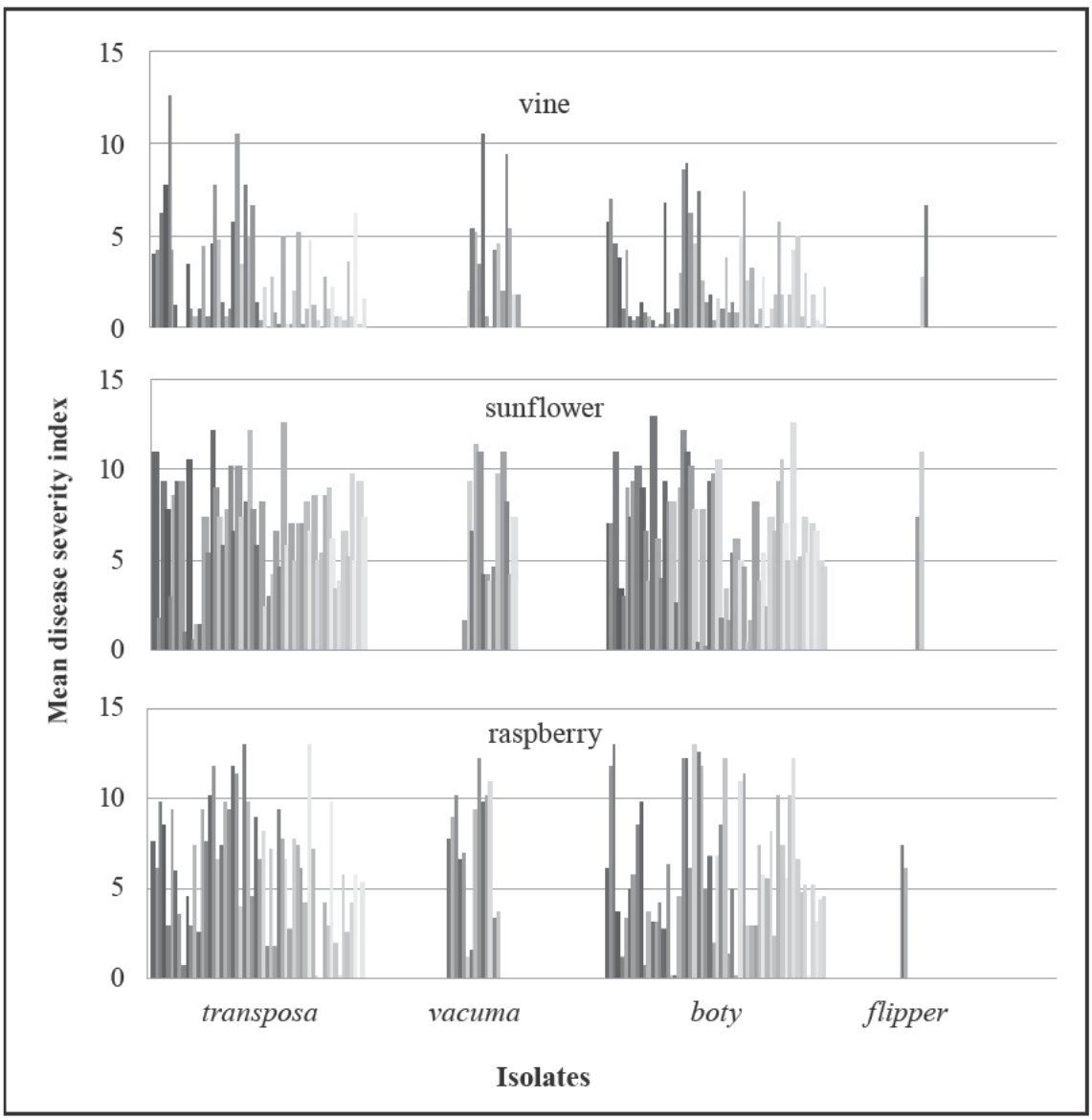

Figure 3. Virulence of *vacuma*, *transposa*, *boty*, and *flipper* isolates of *Botrytis cinerea* to detached vine, sunflower and raspberry leaves

Sunflower and raspberry leaf discs were significantly more sensitive than vine leaf discs (F=60.34; df= 2; F_{crit} =3.02; P<0.0001). The average DSI was 6.2 for both sunflower and raspberry leaves and 2.8 for vine leaves (Table 5).

Table 5. The average disease severity index (DSI) for vine, sunflower and raspberry leaves inoculated with *Botrytis cinerea* isolates from different locations

Location	DSI[1,2]		
	vine	sunflower	raspberry
Valjevo	4.4±3.1 a	8.3±3.1 b	8.3±3.8 b
Požega	2.3±2.1 a	4.9±3.3 b	6.4±3.8 c
Sabac	4.8±3.1 a	6.6±3.3 b	7.5±3.5 c
Arilje	1.8±1.8 a	6.6±2.3 c	4.5±3.0 b
Ivanjica	1.4±1.8 a	6.7±3.6 c	4.8±2.6 b
Prilike	1.8±1.7 a	6.7±2.0 c	5.9±3.3 b
Total[3]	2.8±1.5 a	6.2±2.0 b	6.2±1.5 b

[1]Calculated as average for all isolates from a location
[2]The same letter in a row indicates non-significant difference
[3] The average for all isolates from all locations

Differences in virulence were also detected within all four genetic subgroups of *B. cinerea* as presented in Figure 3. The highest recorded value of DSI for all hosts was 12.6, while the lowest ranged from 0 for vine and raspberry to 0.2 for sunflower. Isolates unable to infect raspberry or vine leaves (DSI=0) were found within *transposa*, *vacuma* and *boty* subgroups (Figure 3). The lowest and highest values of DSI for the isolates belonging to four different genetic subgroups were summarized in Table 6.

Table 6. The lowest and highest values of disease severity index (DSI) for vine, sunflower and raspberry leaves inoculated with *Botrytis cinerea* isolates

Host plant	Type of isolates[1]	Lowest DSI	Highest DSI
Vine	*transposa*	0.0±0.0	12.6±0.9
	vacuma	0.0±0.0	10.6±0.9
	boty	0.0±0.0	8.6±3.0
	flipper	2.8±2.3	6.6±1.7
Sunflower	*transposa*	0.6±0.5	12.6±0.9
	vacuma	1.6±1.9	11.4±1.7
	boty	0.2±0.4	12.6±0.9
	flipper	7.4±2.6	11.0±0.0
Raspberry	*transposa*	0.0±0.0	11.8±1.1
	vacuma	1.2±1.1	12.2±1.8
	boty	0.2±0.4	12.6±0.9
	flipper	6.2±1.8	7.4±1.7

[1]Based on all available isolates from each genetic subgroup: 14 *vacuma*, 58 *boty*, 56 *transposa* and 2 *flipper* isolates

The calculated correlation coefficients of isolate virulence to the three host plants were as follows:

$r_{vine,\ raspberry} = 0.61$, $r_{vine,\ sunflower} = 0.27$ and $r_{sunflower\text{-}raspberry} = 0.\ 26$.

Neither of these values was statistically significant at 5% probability level.

DISCUSSION

Our examination of growth rates and virulence of 130 isolates of *B. cinerea*, originating from six raspberry fields, revealed a great diversity among the isolates concerning both features. The difference between the highest and lowest growth rates was 16.1 mm/day, suggesting different abilities of the isolates to live and spread saprotrophically, regardless of their morphological or genetic grouping. The results of Martinez et al. (2003) and Samuel et al. (2012) showed that the growth rate of *vacuma* isolates was higher than that of *transposa*. However, according to our results, mycelial growth rates of the isolates belonging to *vacuma*, *transposa* or *boty* subgroups varied similarly (*flipper* group contained only two isolates and was excluded from the analysis). Although we did not have enough individuals in each subgroup to perform a regular statistical analysis, we noticed that the growth rate intervals of all genetic subgroups overlapped, suggesting that groups cannot be distinguished based on the growth rate of the isolates contained. Therefore, *vacuma* isolates did not grow faster than the isolates containing any of the transposons, which had been suggested earlier (Martinez et al., 2003).

The growth rate, virulence, fungicide sensitivity, and genetic variability of *B. cinerea* have been studied extensively over the past decades (Giraud et al., 1997, 1999; Martinez et al., 2003, 2005; Pollastro et al., 2007). However, search for relationships between genetic and biological or ecological features of its isolates was generally unsuccessful (Kerssies et al., 1997; Alfonso et al., 2000; Martinez et al., 2003). To investigate the hypothesis that isolates from different genetic subgroups differ in their ability to establish infection, we performed *in vitro* sensitivity tests using detached leaves of vine, sunflower and raspberry. The results showed similar differences among the isolates in each genetic subgroup, suggesting that *vacuma* isolates were neither more nor less pathogenic to any of the investigated hosts than the other *B. cinerea* genetic subgroups. Furthermore, there was no correlation in

isolate virulence to different hosts, which supports the idea that hosts may shape the pathogen's population structure (Fournier & Giraud, 2008). This finding confirmed the importance of knowing the pathogen population structure on each attacked host for developing an effective control strategy (Samuel et al., 2012). However, further research is needed to improve our understanding of the genetic structure of the pathogen using more powerful molecular techniques.

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Botrytis cinerea na malini II: Brzina rasta i virulentnost izolata

REZIME

U radu su predstavljeni rezultati proučavanja brzine rasta i virulentnosti 130 izolata *Botrytis cinerea*, dobijenih iz obolelih plodova maline poreklom sa šest lokaliteta iz područja komercijalnog gajenja maline u Srbiji i razvrstanih u dve morfološke i četiri genetičke grupe. Rezultati su pokazali da je razlika između izolata u brzini rasta statistički značajna. Najveći zabeleženi porast bio je 24,5 mm/dan, dok je najmanji porast iznosio 8,4 mm/dan. Utvrđena su slična variranja u porastu izolata koji pripadaju različitim morfološkim ili genetičkim grupama. Drugim rečima, rasponi brzine rasta izolata iz različitih morfoloških i genetičkih grupa međusobno se preklapaju, što ukazuje da ovaj parametar nije pogodan za razvrstavanje izolata u grupe. Proučavani izolati su ispoljili različit nivo virulentnosti za listove vinove loze, suncokreta i maline, dok je analiza varijanse pokazala da su i izolati i domaćini statistički značajan izvor variranja ($P<0,01$). Listovi suncokreta i maline bili su značajno osetljiviji od listova vinove loze. Međutim, korelacija u virulentnosti izolata za različite domaćine nije ustanovljena.

Ključne reči: *Botrytis cinerea*; malina; Srbija