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Botrytis cinerea in raspberry in Serbia I: Morphological and molecular characterization

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SUMMARY

Morphological and molecular characterisation of 130 isolates of *Botrytis cinerea*, derived from raspberry fruit originating from six commercial fields in a raspberry growing region of Serbia (locations: Požega, Prilike, Arilje, Ivanjica, Šabac and Valjevo) was performed. The results showed that all isolates formed white, uniform, aerial mycelia with entire margin on PDA medium. First morphological differences among the isolates appeared after six days of incubation. Three-week old isolates were grouped into eight distinct morphological types – four mycelial and four sclerotial. Mostly, they were of sclerotial type (81.5%) and the most frequently found was an S3 type, which formed large irregularly placed sclerotia. This type was dominant in five of six investigated locations and represented 45-65% of the isolates. The least frequent was the mycelial type M3 (0.7% of the isolates) characterized by mycelial masses.

The presence of *Boty* and/or *Flipper* transposons was detected in isolates originating from all investigated locations. It was discovered that the *B. cinerea* population in raspberry in Serbia, besides the well-described genetically isolated sympatric species *transposa* (43.1%) and *vacuma* (10.8%), contains also another two, *boty* (44.6%) and *flipper* (1.5%) species with only one transposon (either *Boty* or *Flipper*) in the genome. In addition, it was revealed that all isolates from raspberry collected in Serbia, *transposa*, *vacuma*, *boty* or *flipper*, are sensitive or weakly resistant to fenhexamid and therefore belong to the *B. cinerea* genetical Group II.

Keywords: *Botrytis cinerea*; Raspberries; Serbia

INTRODUCTION

Over the last decade, Serbia has been one of the top world producers and exporters of raspberry (*Rubus idaeus* L.) (Nikolić & Tanović, 2012). The average production over a 10-year period (2002-2012) was 89.476 tons/year (FAOSTAT, 2014) from an area of about 11.041 ha (Anonymous, 2013), mostly concentrated in the western and southwestern parts of the country. Botrytis fruit rot, caused by the phytopatogenic fungus *Botrytis cinerea* Pers. Fr., is one of major factors limiting raspberry production. Yield losses in commercial fields have been found to exceed 50%, especially during periods of rainy or humid weather right before harvest. In addition, the fungus causes significant losses during shipping and marketing, which makes it one of the most important pathogens of raspberry worldwide (Nikolić et al., 2008). *B. cinerea* (anamorph of *Botryotinia fuckeliana*), a pathogen that causes grey mould on a wide variety of plants worldwide, has been extensively studied on many major crops, including grapes, strawberry, kiwifruit, tomato and bulb crops, while its characterization on raspberry is still limited and based on a small number of isolates (Tanović et al., 2009). Well-documented phenotypic diversity of the pathogen is usually explained by the multinucleate and heterocaryotic nature of hyphae or conidia and aneuploid state of nuclei (Hansen & Smith, 1932; Büttner et al., 1994; Chardonnet et al., 2000; Yourman et al., 2001). Initial molecular investigation of French and Chilean populations of *B. cinerea* had shown that the species is composed of two sympatric species, *transposa* and *vacuma*, characterized by the presence of two transposable elements, *Boty* and *Flipper*, or by absence of both of them (Giraud et al., 1997, 1999; Muñoz et al., 2002). Afterwards, *boty* (containing only *Boty*) (Giraud et al., 1999; Muñoz et al., 2002; Vaczy, 2009; Fekete et al., 2012) and *flipper* (containing only *Flipper*) (Albertini et al., 2002; Beever & Weeds, 2004; Isenegger et al., 2008; Vaczy, 2009; Fekete et al., 2012) isolates were found, suggesting a more complex population structure of *B. cinerea* than it was previously recognized. Additional molecular studies of different nuclear genes have shown that *B. cinerea* population is grouped in two genetic entities – Group I and Group II. The described Group I isolates are exclusively *vacuma* type, belong to one vegetative compatibility group (VCG) and are naturally resistant to fenhexamid, while Group II contains *transposa*, *vacuma*, *boty,* and *flipper* isolates that belong to several VCGs and are sensitive to fenhexamid (Fournier et al. 2003; Leroux, 2004; Fournier et al., 2005).

Giraud et al. (1997) hypothesized that a certain degree of host specialization exists within *B. cinerea* population. Further studies have revealed significant differences regarding the prevalence of *vacuma* and *transposa* isolates on different host plants (Giraud et al., 1999). In addition, Martinez et al. (2003, 2005) found that *vacuma* isolates were mostly of mycelial type and with higher growth rate than *transposa* isolates, while Giraud et al. (1999) reported difference in fungicide resistance frequencies in *transposa* and *vacuma* isolates. Since successful management of grey mould disease is based on the depth of our knowledge of the pathogen, information on the genetic structure of its population could be a useful tool for developing effective control strategies. Therefore, the aims of this study were: a) to determine the presence and distribution of *transposa*, *vacuma*, *boty*, and *flipper* isolates of *B. cinerea* on raspberry fruit in Serbia; b) to characterize some biological features of the isolates from all described subpopulations in terms of colony morphology, virulence, growth rate, sporulation, sclerotia production and pigment release; c) to evaluate the sensitivity of isolates to fenhexamid using a qualitative sensitivity test. The first part of the study focusing on pathogen isolation, morphological and molecular characterization, and sensitivity to fenhexamid is presented in this paper, while the growth rate and virulence, as important fitness parameters of the isolates, will be investigated afterwards and presented in a follow-up paper.

MATERIAL AND METHODS

Fungal isolates

Ripe raspberry fruits expressing grey mould symptoms were randomly collected from commercial orchards from six locations in the raspberry growing region in Serbia. In order to potentiate overgrowth of the fungi the diseased fruits were incubated individually on two layers of moist paper towels in plastic containers for seven days at 20°C, 97% RH (relative humidity). The isolates were derived by placing a small fragment of developed mycelia on Potato Dextrose Agar (peeled potato – 200 g, dextrose – 20 g, agar – 17 g, distilled water – 1 L, PDA) and allowing a 48 h incubation at 20°C. The obtained isolates were then purified by monospore isolation and cultured on PDA medium at 20°C.

Control strains 397 (*transposa*) and 412 (*vacuma*) were kindly provided by Dr. E. Fournier from INRA Centre de Versailles, France.

Maintenance

The isolates were stored on slants at 4°C for short-term or in 20% glycerol at -80°C for long-term storage.

Pathogenicity test

A pathogenicity test was performed as described by Vignutelli et al. (2002) with slight modifications. Apples cv. Golden Delicious were surface sterilized with ethanol (70%) and wounded using sterile nail (4-mm diameter and 3-mm depth) at three position. Mycelial plugs on PDA (Ø 3 mm) were placed into the wounds, while sterile PDA disks were used for inoculation in the control. The isolates were considered pathogenic if fruits showed soft rotting around wounds after two to four days of incubation at 20°C in the dark.

Identification

The isolates were identified based on pathogenic characteristics, morphology of the colony and microscopic observations of conidiophores and conidia and their comparison with the available literature data (Ellis & Waller, 1974).

Morphological characterization

The isolates were grown on PDA medium at 20°C in darkness for three weeks. Afterwards, phenotypic observations were performed based on mycelial aspect, sporulation and sclerotia production. The isolates were classified into eight morphological types described by Martinez et al. (2003) and used earlier by Tanović et al. (2009) (Figure 1).

Molecular characterization

DNA isolation: DNA was obtained directly by scraping mycelia with a pipette tip from 4-day-old culture on PDA. The mycelia was transferred into 50 µl of PrepMan Ultra Sample Preparation Reagent (Applied Biosystems, CA, USA), vortexed briefly, incubated for 30 min at 56°C, followed by 10 min incubation at 100°C, and stored at -20°C until use (Harrington & Wingfield, 1995). The DNA quality of each isolate was confirmed to be suitable for polymerase chain reaction (PCR) by generation of a single band with the universal primers ITS1 and ITS4 (White et al., 1990).

Detection of transposable elements *Flipper* and *Boty*: The presence of the transposable elements *Flipper*, a 1872-bp class II element (Levis et al., 1997), and *Boty,* a 6-kb retrotransposon (Diolez et al., 1995), previously

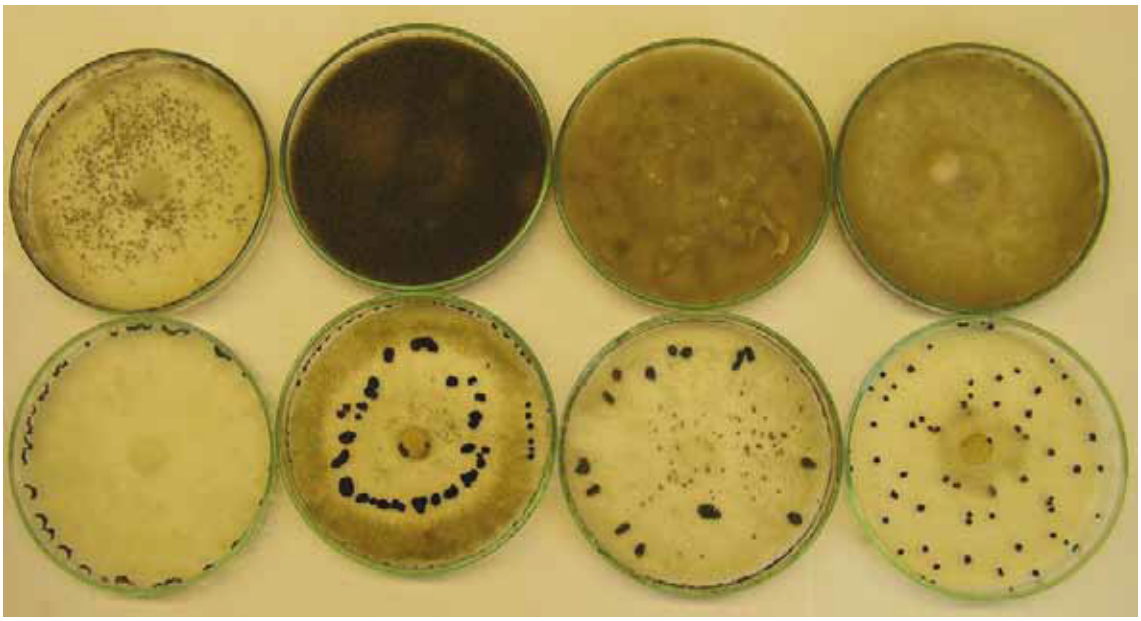

Figure 1. *Botrytis cinerea:* morphological types of colonies: mycelial (upper row - left to right: M1 - short mycelium without sporulation, M2 - aerial mycelium with sporulation, M3 – mycelial masses, and M4 - thick and woolly mycelium) and sclerotial (row below - left to right: S1- sclerotia at the edge of Petri dish, S2 - sclerotia large and arranged in a circle, S3 - sclerotia large, placed irregularly, and S4 - sclerotia small and scattered)

identified in the *B. cinerea* genome, was tested in all obtained isolates using PCR methods described by Levis et al. (1997) and Ma & Michailides (2005). The primers used for detection of the *Flipper* element (F300: 5'-GCA CAA AAC CTA CAG AAG A-3' and F1550: 5'-ATT CGT TTC TTG GAC TGT A-3') amplify a 1250-bp product, corresponding to a major part of the *Flipper* element. The presence of the *Boty* element was tested using BotyF4: 5'-CAG CTG CAG TAT ACT GGG GGA-3' and BotyR4: 5'-GGT GCT CAA AGT GTT ACG GGA G-3' primers that amplify a 510-bp product (Ma & Michailides, 2005). The primers were synthesized by Fermentas (Lithuania). PCR reactions were conducted in final volume of 25 µl containing: 1 X Master mix (Fermentas, Lithuania) (0.625 U Taq polymerase, 2 mM $MgCl_2$, 0.2 mM of each dNTPs), 1 µl of each primer (20 µM), and 1 µl of fungal DNA. Thermal cycler was programmed as follows: an initial preheat at 95°C for 3 min, followed by 40 cycles of denaturation at 94°C for 40 s, annealing at 60°C for primers F300 and F1550, or 68°C for BotyF4 and BotyR4, for 40 s, extension at 72°C for 1 min, and a final extension at 72°C for 10 min. Products were separated on 1% agarose gel and stained with ethidium bromide. All negative PCR reactions were performed three times. DNAs from the strains 397 (*transposa*) and 412 (*vacuma*) were used as templates in each PCR reaction as a positive and negative control, respectively.

Sensitivity of the isolates to fenhexamid

Sensitivity of the isolates to fenhexamid was determined on PDA medium amended with discriminating concentrations of 1, 5 and 10 mg/L of fenhexamid, allowing the growth of resistant but fully inhibiting the growth of sensitive isolates (Stehman & De Waard, 1996). Fenhexamid (Teldor SC, 500 g/l, Bayer CropScience) was suspended in sterile distilled water and added to autoclaved media that had cooled to 50°C. Petri plates were inoculated with inverted mycelial plugs (10 mm), cut at the edge of 4-day-old colonies on PDA, and incubated for 48 hours at 20°C. The experiment was conducted in four replicates and repeated twice. Isolates that did not grow at the lowest concentration (1 mg/L) were designated as sensitive, while those able to grow at the higest fungicide concetration (10 mg/L) were considered as highly resistant. The remaining two groups, those that grew at 1 mg/L but not at 5 mg/L, and those that grew at 5 mg/L but not at 10 mg/L, were considered as weakly or moderately resistant, respectively.

RESULTS

Disease simptoms

The most common disease symptom was fruit decay in the form of spreading lesions, typically at the stem end of ripening fruit, accompained by profuse sporulation of the pathogen (Figure 2).

Figure 2. *Botrytis cinerea*. Decay of raspberry fruits accompained by profuse sporulation of the pathogen

After a 7-day incubation of diseased fruits under moist conditions at 20°C, thick and woolly micelium developed. In total, 130 isolates were derived by transfering the developed mycelium onto PDA medium. The isolates were marked by a combination of letters indicating the origin of isolates and seriatim numbers as presented in Table 1.

Pathogenicity of the isolates

All tested isolates caused soft rotting of apple fruits after two to four days of incubation (Figure 3). Pathogenicity of the isolates was confirmed by reisolation of the pathogen from inoculated fruits.

Morphological characterization

At the beginning of mycelial development on PDA medium at 20°C, all isolates formed white uniform aerial mycelium with entire margin (Figure 3).

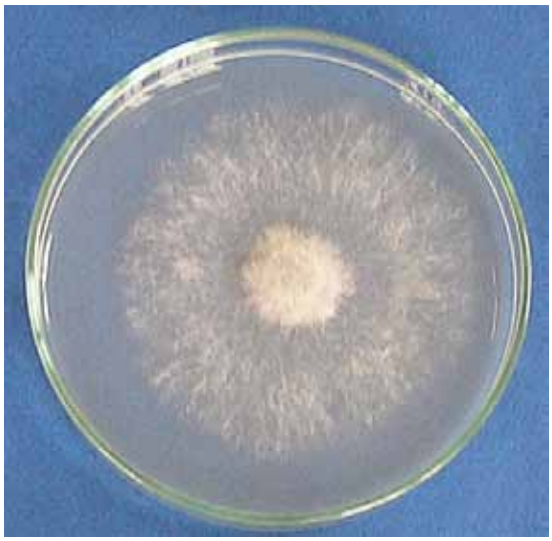

Figure 3. *Botrytis cinerea*. White uniform aerial mycelium with entire margin after incubation at 20°C for 3 days

Table 1. List of *Botrytis cinerea* isolates, collected from different locations in Serbia and their classification as *vacuma, transposa, flipper* and *boty*

Location	Number of isolates	Codes of isolates	Number of isolates			
			vacuma[1]	*boty*[2]	*flipper*[3]	*transposa*[4]
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

[1]Isolates without transposable elements
[2]Isolates containing only the transposable element *Boty*
[3]Isolates containing only the transposable element *Flipper*
[4]Isolates containing both transposable elements *Boty* and *Flipper*

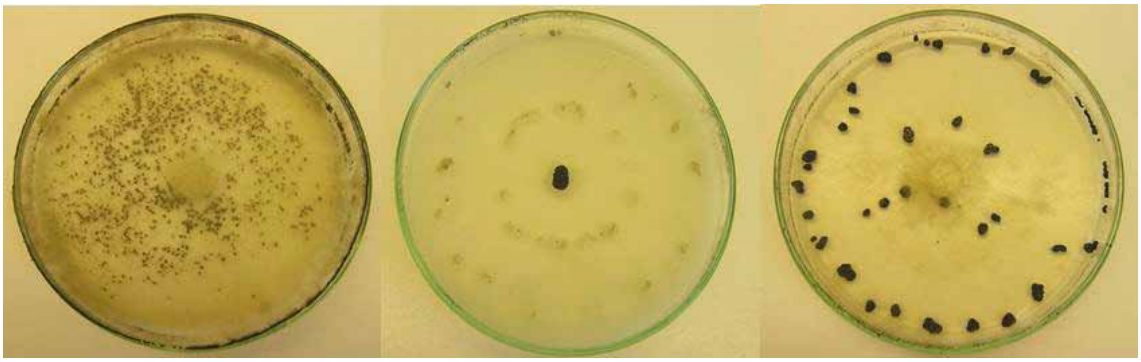

Figure 4. *Botrytis cinerea* - differences in morphology of the isolates: 10-day old mycelial isolate with homogeneous mycelium (left); 7-day old sclerotial isolate with zones of compact areal mycelium and white beginnings of sclerotia (middle); and 10-day old sclerotial isolate containing fully developed black sclerotia (right)

Morphological differences among the isolates were noticable after six-day incubation. Mycelia of the isolates producing no sclerotia remained homogeneous, aerial or substrate, while sclerotia-producing isolates formed zones of compact areal mycelium containing white beginnings of sclerotia. Fully developed sclerotia were black, hitched to the medium and appeared in cultures after incubation of 10 days (Figure 4).

Final observation of the morphological characteristics of the isolates was performed after three-week incubation and the isolates were classified into eight morphological types (Figure 1).

Under the given experimental conditions, most isolates formed colonies of the sclerotial type (81.5%) (Figure 5). Among them, the most frequently found were isolates forming large irregularly placed sclerotia, corresponding to the S3 type of isolates. That type was dominant in five of the six investigated locations and represented 45-65% of isolates (Table 2).

Grown on PDA medium in darkness, a vast majority of the isolates did not sporulate (91.5%). Sporulation of the remaining 8.5% of the isolates was mostly sparse and started after incubation for six or seven days. Only three isolates (2.3% of all) sporulated abundantly. Pigment release was also rarely observed and was found in 16 isolates (12.5%) (Table 3).

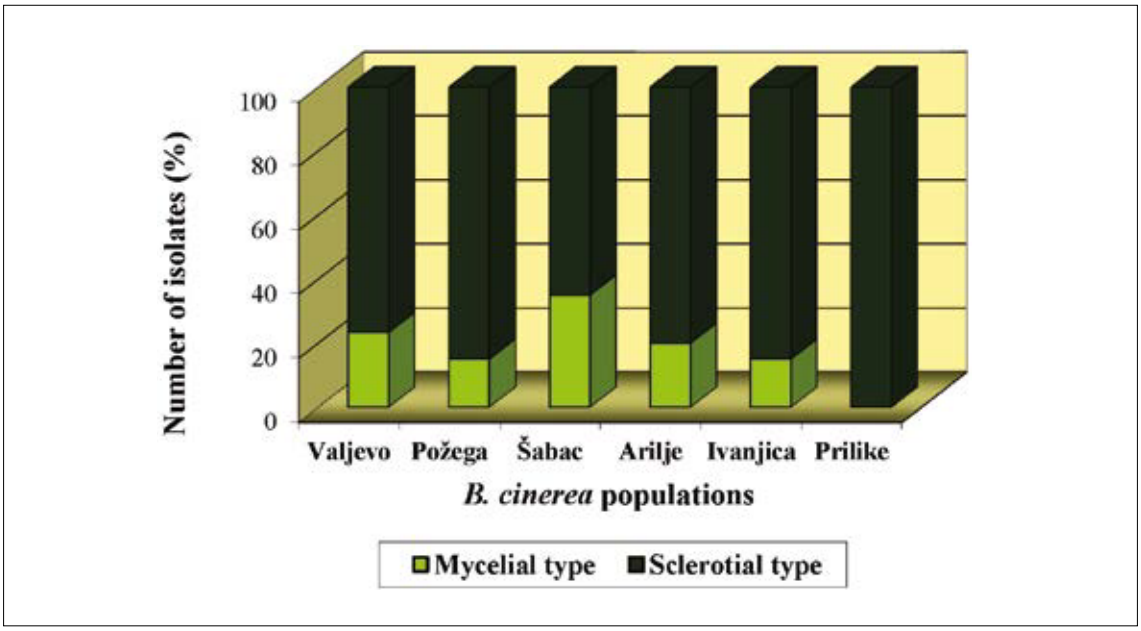

Figure 5. Distribution of sclerotial and mycelial morphological types of *Botrytis cinerea* isolates at different locations

Table 2. Morphological types of *Botrytis cinerea* isolates originating from different locations in Serbia - mycelial (M1 - short mycelium without sporulation, M2 - aerial mycelium with sporulation, M3 – mycelial masses, and M4 - thick and woolly mycelium) and sclerotial (S1- sclerotia at the edge of Petri dish, S2 - sclerotia large and arranged in a circle, S3 - sclerotia large, placed irregularly, and S4 - sclerotia small and scattered)

Location	Number of isolates	Number of isolates							
		Mycelial type				Sclerotial type			
		M1	M2	M3	M4	S1	S2	S3	S4
Valjevo	30	2	0	1	4	7	5	9	2
Požega	20	3	0	0	0	5	3	6	3
Šabac	20	3	2	0	2	0	1	7	5
Arilje	20	3	1	0	0	4	3	6	3
Ivanjica	20	1	0	0	2	10	2	5	0
Prilike	20	0	0	0	0	4	1	13	2
Total	130	12	3	1	8	30	15	46	15

Detection of transposable elements *Flipper* and *Boty*

Transposable elements were detected in 89.2% of the isolates (Figure 6). Besides *transposa* (featuring both transposons) and *vacuma* (without either transposon), subpopulations, i.e. two additional types of isolates containing only one transposable element, either *Boty* or *Flipper,* were found. These isolates were designated as *boty* and *flipper*, respectively. The resulting molecular determination of the elements at each location is presented in Table 1.

Sensitivity of the isolates to fenhexamid

The results of the sensitivity test revealed a high level of sensitivity to fenhexamid. Moderately or highly resistant isolates were not found in any of the populations, while the presence of weakly resistant isolates varied depending on population. In two of six populations all the isolates were sensitive to fenhexamid. The highest precentage of weakly resistant isolates was recorded in the population originating from Šabac (Figure 7).

Table 3. Sporulation and pigment release of *Botrytis cinerea* isolates originating from different locations in Serbia

Location	Number of isolates	Number of isolates	
		Sporulation	Pigment release
Valjevo	30	1	3
Požega	20	0	1
Šabac	20	6	6
Arilje	20	0	5
Ivanjica	20	0	1
Prilike	20	4	0
Total	130	11	16

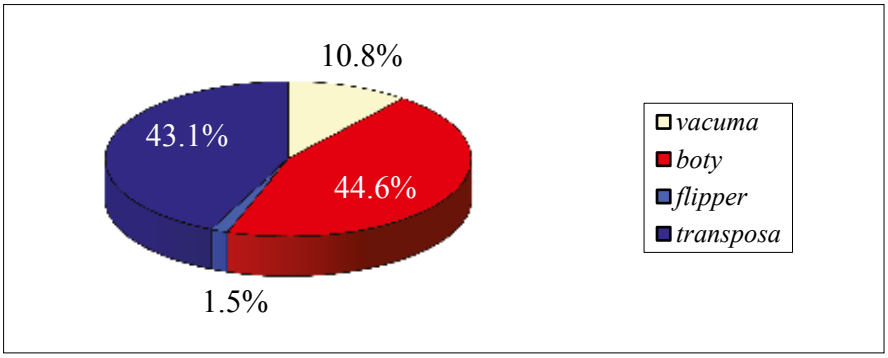

Figure 6. *Botrytis cinerea.* Frequency of *transposa, vacuma, boty* and *flipper* isolates

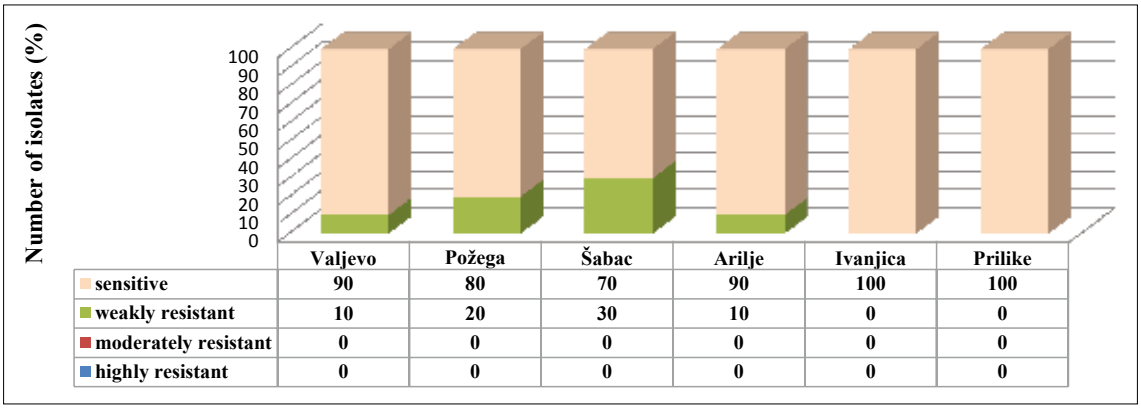

Figure 7. Sensitivity of *Botrytis cinerea* isolates to fenhexamid

DISCUSSION

Our study of the biological traits of 130 isolates of *B. cinerea*, originating from six raspberry fields, revealed a great phenotypic variability of this species in raspberry, confirming previous findings in other host plants (Grindle, 1979; Di Lena et al., 1981; Faretra et al., 1988; Leone, 1990; Kerssies et al., 1997; Alfonso et al., 2000; Chardonnet et al., 2000; Yourman et al. 2001; Baraldi et al., 2002; Vaczy et al., 2006; Decognet et al., 2007). Based on colony morphology on PDA medium, the isolates were sorted into eight groups, described by Martinez et al. (2003). Among the isolates from all locations, those forming large, irregularly placed sclerotia were prevalent, while the mycelial type isolates with mycelial masses were the least frequent. However, Paul (1929, cited by Lorbeer, 1980) had described only three morphological types of *B. cinerea* isolates – sclerotial, sporulating and mycelial. Van der Spek (1965, cited by Lorbeer, 1980) investigated isolates from different host plants including: flax, strawberry, raspberry, pea, and tomato and found the same three types that had been recognised by Paul (1929, cited by Lorbeer, 1980). In addition, he noticed that different types of isolates had not occurred on different host plants to equal extent. For example, the mycelial type isolates were the most frequent isolates on flax, while sclerotial isolates were found only occasionally, whereas the sporulating type was not detected at all. On the contrary, high frequency of sclerotial isolates was found in vine, tomato, strawberry, blueberry and rose in Uruguay (Gepp et al., 2007), as well as in vine in Austria (Achleitner, 2008).

In the present study, transposable elements were detected in 89.2% of the isolates originating from raspberry from Serbia. The most frequent were those containing only the *Boty* element (44.6%), followed by *transposa* isolates (43.1%), while *vacuma* was less present (10.8%). The least frequent were *flipper* isolates with a share of 1.5%. The highest percentage of *transposa* isolates was detected in Prilike and Arilje locations (50%). Such distribution of subpopulations had not been usually observed in *B. cinerea*. In most studies only two subpopulations, *transposa* and *vacuma*, have been found (McDonald, 1993; Levis et al., 1997; Giraud et al., 1997; 1998; 1999; Coarer, 2003; Martinez et al., 2003; Ben Ahmed & Hamada, 2005). The most comprehensive study of *B. cinerea* population structure so far, which included 840 isolates from 23 hosts originating from 15 countries, has revealed that *transposa* isolates prevailed (69.2%) in all countries and all hosts, followed by *vacuma* (14.4%) and *boty* (13.8%) isolates (Pollastro et al., 2007). *Transposa* isolates were also dominant in vineyards in France (Giraud et al., 1997; Martinez et al., 2005), Austria (Achleitner, 2008), and Croatia (Topalovec-Pintarić et al., 2004), as well as in strawberry in Croatia (Milićević et al., 2006).

Besides *vacuma* and *transposa* isolates, those containing only the *Boty* element have been found in *B. cinerea* populations in Chile (Muñoz et al., 2002), California (Ma & Michailides, 2005), Croatia (Topalovec-Pintarić et al., 2004) Bangladesh, India, and Australia (Isenegger et al., 2008). In addition, populations from Europe, Bangladesh and India have been shown to contain isolates with the *Flipper* transposon only (Albertini et al., 2002; Beever & Weeds, 2004; Isenegger et al., 2008). However, none of these populations had a high percentage of *boty* isolates as observed in our present study. In addition, unexpectedly high percentages of *flipper* isolates have been found in Bangladesh (70%) and Nepal (22%) (Isenegger et al., 2008). Our results showed that the percentage of *vacuma* isolates in raspberry in Serbia ranged from a complete abscence in Ivanjica and Prilike to 40% detected in Šabac. A fact that deserves some attention is that the sampling at the locations Ivanjica and Prilike was conducted at the end of July, while samples were collected in Šabac a month earlier, i.e. at the end of June. This may support an observation of Giraud et al. (1997) that the share of *vacuma* isolates decreases during the growing season. Significant decreases in the frequency of *vacuma* isolates during the vegetation period have also been recorded in France, Italy and Austria (Martinez et al., 2005; De Miccolis Angelini et al., 2006; Achleitner, 2008). However, further investigation with a more appropriate sampling design is needed in order to make a final conclusion about the dynamics of frequency of *vacuma* isolates over the growing season.

In order to determine whether the derived *vacuma* isolates belonged to the described genetic Group I *B. cinerea*, they were tested for sensitivity to fenhexamid. The results showed that all examined isolates of *B. cinerea* from raspberry fields in Serbia were sensitive or weakly resistant to fenhexamid and consequently belonged to the genetic Group II of the species.

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Botrytis cinerea na malini u Srbiji I: Morfološka i molekularna karakterizacija

REZIME

U radu su predstavljeni rezultati morfološke i molekularne karakterizacije 130 izolata *Botrytis cinerea*, dobijenih iz obolelih plodova maline poreklom sa šest lokaliteta iz područja komercijalnog gajenja maline u Srbiji (Požega, Prilike, Arilje, Ivanjica, Šabac i Valjevo). Utvrđeno je da u početnim fazama razvoja na KDA podlozi svi izolati *B. cinerea* formiraju belu, uniformnu, rastresitu, vazdušnu miceliju ravnog oboda. Razlike među izolatima počinju da se javljaju posle inkubacije od šest dana. Na osnovu izgleda kolonije tri nedelje od zasejavanja, izolati su razvrstani u osam morfoloških tipova – četiri micelijska i četiri sklerocijska. Većina izolata je formirala kolonije sklerocijskog tipa (81,5%), a najzastupljeniji je bio tip S3 sa krupnim, nepravilno raspoređenim sklerocijama, koji je dominirao u pet od šest proučavanih populacija patogena i predstavljao 45-65% izolata. Najređi je bio micelijski tip M3 (0,7% izolata) koji se odlikuje nakupinama vazdušne micelije.

Prisustvo transpozona *Boty* i/ili *Flipper* otkriveno je u genomu izolata sa svih lokaliteta. Utvrđeno je da u populaciji patogena na malini u Srbiji, osim genetički izolovanih subpopulacija *transposa* (43,1%) i *vacuma* (10,8%), postoje još dve – *boty* (44,6%) i *flipper* (1,5%) sa izolatima koji sadrže samo jednu vrstu transpozona u genomu. Istraživanje je takođe pokazalo da su svi izolati *B. cinerea* na malini u Srbiji, bilo da su *transposa*, *vacuma*, *boty* ili *flipper*, osetljivi ili slabo rezistentni na fenheksamid i da, prema tome, pripadaju genetičkoj Grupi II *B. cinerea*.

Ključne reči: *Botrytis cinerea*; malina; Srbija