UDC 575:635
DOI: 10.2298/GENSR1203663K
Original scientific paper

PHENOTYPIC AND GENETIC CHARACTERIZATION OF *BOTRYTIS CINEREA* ISOLATES FROM TOMATO

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Kuzmanovska B., R Rusevski, Lj. Jankuloski, M.Jankulovska, D.Ivic, and K. Bandzo (2012): *Phenotypic and genetic characterization of Botrytis cinerea isolates from tomato.* - Genetika, Vol 44, No. 3,633 -647.

One hundred and twenty-three isolates of *Botrytis cinerea* were collected from 7 different areas in the Republic of Macedonia, where tomato is mostly grown in greenhouses and high tunnels. Based on the mycelial formation, intensity of sporulation and sclerotial production, 9 different phenotypes were detected: 4 mycelial and 5 sclerotial. One sclerotial morphological type has not been previously reported. The presence or absence of two transposable elements, *boty* and *flipper*, was

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detected by PCR. Out of 123 isolates, 20 had two transposable elements, *boty* and *flipper* (*transposa* genotype), 48 had neither of these elements (*vacuma* genotype) and 55 had only the *flipper* element (*flipper* genotype). Isolates that contain only *boty* element were not detected. No relationship between the phenotypes, origin of isolates and the presence/absence of transposable elements, *boty* and *flipper*, was found.

Key words: boty, *flipper ,* grey mold, phenotypes

INTRODUCTION

Botrytis cinerea Pers.: Fr. (teleomorph *Botryotinia fuckeliana* (de Bary) Whetzel) is a cosmopolitan, phytopathogenic and necrotrophic fungus, which attacks more than 230 plant species, causing the grey mold disease (O'NEILL *et al.*, 1997; MA and MICHAILIDES, 2005; WU *et al.*, 2007). It is particularly important in greenhouse production of vegetables, such as tomato and cucumber (SHTIENBERG *et al.*, 1998; ELAD *et al.*, 2004; KALOGIANNIS *et al.*, 2006).

It is one of the major problems in greenhouse production of tomato in the Republic of Macedonia, with yield losses that may exceed 70%. The pathogen can infect the plant at every stage of its development, and can be found in every part of the plant, including leaves, stems, flowers, fruits and petioles (CHARDONNET *et al.*, 2000; VALIUSKAITE *et al.*, 2010). Moreover, the fungus causes significant losses during storage, shipping and marketing (MICHAILIDES and ELMER, 2000; WU *et al.*, 2007; KARCHANI-BALMA *et al.*, 2008; GONZALES *et al.*, 2009).

Many studies have reported that this fungus exhibits great phenotypic (KERSSIES *et al.*, 1997; CHARDONNET *et al.*, 2000; MARTINEZ *et al.*, 2003; TANOVIC *et al.*, 2009) and genetic variability (DIOLEZ *et al.*, 1995; LEVIS *et al.*, 1997; GIRAUD *et al.*, 1999; MUNOZ *et al.*, 2002; FOURNIER and GIRAUD, 2008). Phenotypic variability is mainly based on differences in morphology of colony, mycelial growth, intensity of sporulation, virulence, enzyme production and fungicide resistance (CHARDONNET *et al.*, 2000; YOURMAN *et al.*, 2001; MARTINEZ *et al.*, 2003; MILICEVIC *et al.*, 2006; VALIUSKAITE *et al.*, 2010).

Somatic variability was mainly attributed to the multinucleate and heterocaryotic nature of hyphae or conidia, aneuploidy state of nucleus (HANSEN and SMITH, 1932) and mutations (CHARDONNET *et al.*, 2000). Part of this variation may also be due to the presence of transposable elements (LEVIS *et al.*, 1997).

Based on the presence/absence of two transposable elements, *boty* and *flipper*, GIRAUD *et al.* (1997) have reported that in Champagne region, *Botrytis cinerea* is a complex species, composed of at least two sibling sympatric subpopulations (genetic groups), *transposa* and *vacuma*. *Transposa* group contains both transposable elements, *boty* and *flipper*, whereas *vacuma* group has neither of these two elements. The presence of these two sibling species was later reported by many other authors from different regions (MUNOZ *et al.*, 2002; TOPOLOVEC-PINTARIC *et al.*, 2004; MA and MICHAILIDES, 2005; VACZY *et al.*, 2008; ISENEGGER *et*

al., 2008; KRETSCHMER and HAHN, 2008; TANOVIC *et al.*, 2009; FEKETE *et al.*, 2010; AUGER *et al.*, 2010). Isolates that contain only TE *boty* (GIRAUD *et al.*, 1999; MUNOZ *et al.*, 2002; MA and MICHAILIDES, 2005; MILICEVIC *et al.*, 2006; ISENEGGER *et al.*, 2008; KRETSCHMER and HAHN, 2008; TANOVIC *et al.*, 2009) or only TE *flipper* (DE MICCOLIS *et al.*, 2003; AHMED and HAMADA, 2005; VACZY *et al.*, 2008; ISENEGGER *et al.*, 2008; FEKETE *et al.*, 2010; AUGER *et al.*, 2010) were also detected.

Many studies have been undertaken recently concerning different aspects of fungal pathogens (IVIĆ *et al*., 2009, IAGO *et al*., 2011, IVIĆ *et al*., 2011a, JEMRIĆ *et al*., 2011, IVIĆ *et al*., 2011b, ĐORĐEVIĆ *et al*., 2012), including *Botrytis cinerea* (TOPOLOVEC-PINTARIC *et al*., 2004, MILICEVIC *et al*., 2006, TANOVIC *et al*., 2009). However, the phenotypic and genetic variability of *Botrytis cinerea* populations from the Republic of Macedonia has not been investigated. As a result, the aims of this study were: a) to determine whether the two sympatric species, *transposa* and *vacuma*, are present in *Botrytis cinerea* populations in tomato in the Republic of Macedonia; b) to determine the morphological variability of *Botrytis cinerea* isolates, based on the morphology of colony, intensity of sporulation and sclerotial production on PDA; c) to determine the relationship between the phenotypic variability and the presence of transposable elements.

MATERIALS AND METHODS

Fungal isolates and sampling.

One hundred and twenty-three isolates of *B. cinerea* were collected during February-June 2009 and 2010, from seven different regions in the Republic of Macedonia: Strumica, Bogdanci, Gradsko, Kochani, Vinica, Shtip and Dojran (Table 1). These regions are main areas of tomato production. In each region, isolates were sampled from cultivated tomato plants, grown under greenhouses and high tunnels. All samples were taken from sporulating necroses on stems, fruits and leaves, using sterilized cotton buds. For each fungal isolate, spores were cultured for 5 days at 20°C in dark on PDA plates (diameter 90 cm). Mycelial plugs (diameter 0.6 cm) were cut from the edge of the colonies and placed with the mycelium underneath, at the centre of new Petri dishes. This procedure ensured pure cultures of *B. cinerea*.

Molecular characterization of *B. cinerea* isolates.

Mycelia and conidia were harvested by scraping a pure culture of *B. cinerea* on PDA plates. Genomic DNA was extracted according to LEE and TAYLOR (1990), with slight modifications. The DNA quality and quantity was checked by using the NanoDrop 2000c spectrophotometer, Thermo Scientific.

The primers used to detect *boty* (LTR98: 5´-AGCCTGTAGAATCACCAACG-3´ and LTR728: 5´-CGGTATTTCTGGTTGGCA-3´) amplify a 648-bp product that spans the region corresponding to the long terminal repeat (LTR) of *boty* at positions 98-745 of the sequence (DIOLEZ *et al*., 1995, GeneBank accesion no.X81790). The primers used

to detect *flipper* (F300: 5´-GCACAAAACCTACAGAAGA-3´ and F1550: 5´-ATTCGTTTCTTGGACTGTA-3´) amplify a 1250-bp product, corresponding to the major part of the flipper element (LEVIS *et al.*, 1997, GeneBank accession no. U74294). Detection of *boty* and *flipper* was performed by using the separate PCR reactions. PCR reactions were conducted in a final volume of 20 µl containing 100 nM of each primer, 65 µl of each dNTP, 1,5 mM $MgCl_2$, 70 ng fungal DNA, 1x PCR buffer and 1U Taq DNA polymerase (Promega, Madison, WI). Reactions were performed in a Eppendorf Mastercycler ProS, performing the following parameters: an initial preheat for 4 min at 95°C, followed by 40 cycles of denaturation at 94°C for 40 sec, annealing at 58°C for the primer pair F300 and F1550, or 60.3°C for the primer pair LTR98 and LTR728, for 40 sec, extension at 72°C for 1 min, and terminated with a final extension at 72°C for 10 min.

PCR products were separated by electrophoresis on 1.5% agarose gel in Tris-acetate (TAE) buffer, stained with ethidium bromide, and photographed under UV-light. The presence or absence of amplified DNA fragments was scored as 1 or 0, respectively. Based on this scoring, three different genetic groups were defined (*flipper*, *transposa* and *vacuma*).

Morphological characterization of *B. cinerea* isolates.

All 123 isolates were cultured on PDA plates (three replications) at 20°C in dark. After three weeks, phenotypic characterization was performed macroscopically on the basis of mycelial aspect, intensity of sporulation and sclerotial production (MARTINEZ *et al.*, 2003). Nine different morphological types were defined (Figure 1): four mycelial (M1, M2, M3, M4) and five sclerotial (S1, S2, S3, S4 and S5). All isolates were distributed into these nine classes (Table 2).

In order to produce the conidia for size estimation, one isolate from each region (total of seven) was randomly selected and grown on a PDA plate (diameter 90 cm) for seven days at 20°C, in three replications. The length and width of conidia was measured in 50 conidia from each isolate and each replication at x40 magnification under light microscope. The color of mycelium was visually determined.

Statistical analyses.

The distribution of frequencies in two main morphological classes (sclerotial and mycelial) were compared in pairs, between different organs and genetic groups, using χ^2 test with Yates' correction for continuity at P=0.05. Based on the mycelial aspect, intensity of sporulation and sclerotial production, the Euclidian distance between all pairs of *B. cinerea* isolates was calculated. This distance was used for designing a dendrogram, using UPGMA method. Mean values for the size of conidia were estimated based on 50 conidia from three replications and LSD test was performed. For all analyses R statistical package was used.

Table 1. Region, organ and year of collection of B. cinerea isolates

Isolate No.	Region	Organ	Year
1,2,3,4,5,6,7,8	Bogdanci	Stem	2009
9,10,11,12,13,14,15,16	Bogdanci	Stem	2010
17,18,19	Dojran	Leaf	2009
20,21,22,23,24,25,26,27,28,29,30,31	Dojran	Stem	2010
32,33,34	Shtip	Leaf	2009
35,36,37	Shtip	Stem	2009
38,39,40,41,42,43,44,45,46,47,48,49,50	Shtip	Fruit	2010
51	Gradsko	Leaf	2009
52,53,54,55	Gradsko	Fruit	2009
56,57,58,59,60,61	Gradsko	Fruit	2010
62,63,64,65,66	Vinica	Leaf	2009
67,68,69,70,71,72,73	Vinica	Stem	2010
74,75,76,77,78,79	Vinica	Fruit	2010
80,81,82,83	Kochani	Leaf	2009
84,85,86,87,88,89,90,91,92	Kochani	Stem	2010
93,94,95,96,97,98	Kochani	Fruit	2009
99,100	Strumica	Leaf	2009
101,102,103,104	Strumica	Stem	2009
105,106,107,108,109	Strumica	Fruit	2009
110,111,112,113, 114,115,116,117,118,119,120,121,122,123	Strumica	Fruit	2010

RESULTS AND DISCUSSION

Molecular characterization of *B. cinerea* isolates.

Based on the presence or absence of transposable elements, *B. cinerea* isolates were classified in three genetic groups: *flipper*, *transposa* and *vacuma* (Table 2). The *flipper* genetic group was prevalent, comprising 55 isolates, while *vacuma* was the second one, with 48 isolates. Only 20 isolates belonged to the genetic group *transposa*. In most of the regions, all three genetic groups were identified. In the region of Bogdanci only two genetic groups were detected (*flipper* and *transposa*). All isolates that originated from Vinica region were grouped in *vacuma*.

Morphological characterization of *B. cinerea* isolates.

B. cinerea colonies from tomato on PDA were visually classified into nine morphological types (Table 3 and Figure 1). Based on formation of sclerotia, two main morphological classes were identified: “mycelial” that are characterized by absence or presence of few sclerotia in Petri plates and ‘‘sclerotial”, which form many sclerotia in Petri plates. Sclerotial types were predominant with 90 isolates grouped into this class. Out of 123 isolates, 62 belong to the morphological type S3.

The new sclerotial type was identified in this study, named as S5 (Figure 1). It is characterized by aerial mycelium and profuse sporulation, which is typical for M2 mycelial phenotype, but considering the sclerotial aspect, it is identical as S4 sclerotial phenotype. According to these characteristics, S5 phenotype can be considered as transient type between mycelial and sclerotial morphological classes.

Table 2. Classification of B. cinerea isolates in genetic groups

Region	Number of isolates	Genetic group		
		flipper	*transposa*	*vacuma*
Bogdanci	16	1, 2, 3, 4, 5, 6, 11, 14, 15	7, 8, 9, 10, 12, 13, 16	/
Gradsko	11	52, 53, 55, 57, 58, 61	54	51, 56, 59, 60
Vinica	18	/	/	62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79
Dojran	15	17, 18, 19, 21, 23, 25, 26, 27, 30	20, 22, 28, 29	24, 31
Strumica	25	99, 100, 104, 107, 108, 109, 113, 115, 120, 121, 122, 123	101, 105, 106, 110, 112	102, 103, 111, 114, 116, 117, 118, 119
Shtip	19	32, 36, 37, 38, 39, 40, 41, 42, 44, 49	34	33, 35, 43, 45, 46, 47, 48, 50
Kochani	19	80, 81, 83, 84, 86, 89,91, 95, 96	85, 92	82, 87, 88, 90, 93, 94, 97, 98
Total	123	55	20	48

Table 3. Phenotypic characterization of B. cinerea isolates on PDA medium

Phenotype	Mycelial type 'M'				Sclerotial type 'S'				
	M1	M2	M3	M4	S1	S2	S3	S4	S5
Mycelium	Short	Aerial	Mycelial masses	Thick and woolly	Rather short	Rather short	Rather short	Rather short	Aerial
Sporulation[1]	−	+	±	−	±	±	±	−	+
Sclerotia[1]	0	−	−	−	In the edge of Petri dish	Often large, in circle	Often large, placed irregularly	Numerous small and scattered	Numerous small and scattered
Number of strains	6	19	2	6	12	3	62	10	3

[1]0: absence; −: absence or very rare; ±: more or less sporulating; +: sporulating profusely

The frequency distributions in nine morphological types are given in Figure 2. Within the *flipper* and *vacuma* strains, the distribution into sclerotial or mycelial classes was significantly different for isolates that originated from the stem ($\chi 2$=11.68 and $\chi 2$=6.93, respectively, df=1) and leaf (χ^2=6.00 and $\chi 2$=6.26, respectively, df=1). Within the *transposa* isolates, considering different organs, no significant differences were detected. As it can be seen from Figure 2, all isolates from leaves that belong to *vacuma* genetic group have been classified into M2 morphological type.

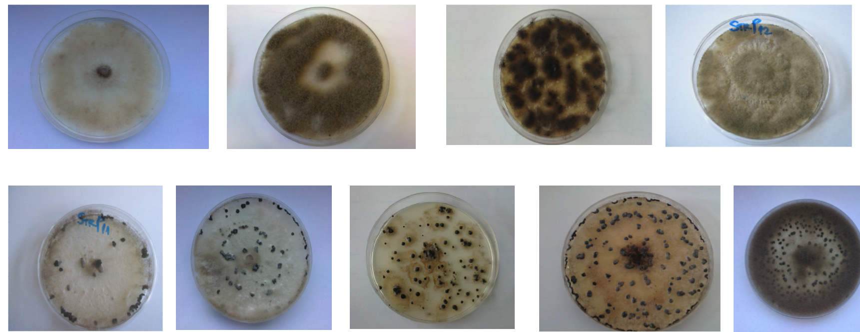
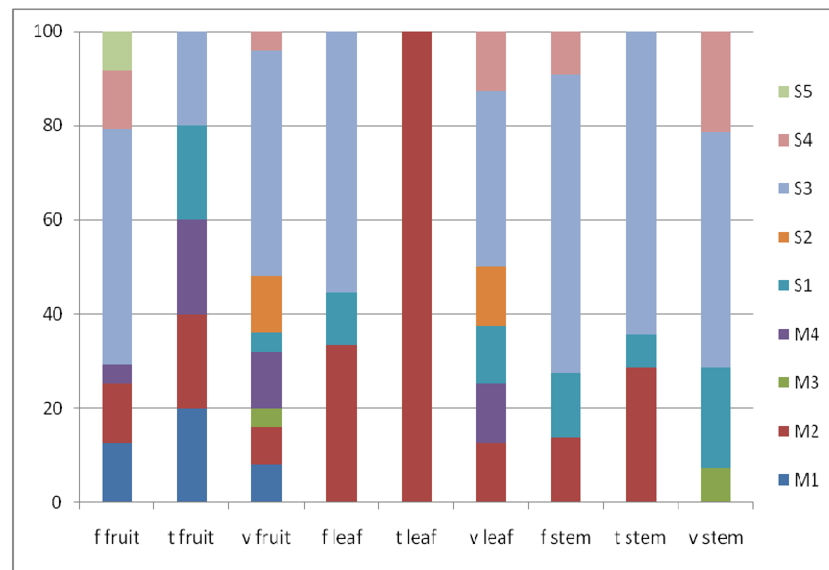


Figure 1. Morphological types of *B. cinerea* isolates: a-M1, b-M2, c-M3, d-M4, e-S1, f-S2, g-S3, h-S4 and i-S5

Considering the mycelial aspect, intensity of sporulation and sclerotial production, the genetic distance between all pairs of isolates is presented in Figure 3. All isolates are classified in 2 main groups. The first main group comprised sclerotial phenotypes and was split in five subclasses: A (S5 phenotypes), B (S1 phenotypes), C (S4 phenotypes), D (S3 phenotypes) and E (S2 phenotypes). All isolates that had S5 phenotypes belonged to *flipper* genetic group and all S2 strains to *vacuma* group. The genetic group *transposa* was not present in isolates with phenotype S4. The isolates from S1 and S3 morphological types belonged to all genetic groups. The second cluster is consisted of mycelial types and is divided in four subgroups. Isolates that have M1 morphological type belonged to group G, M2 in group H, M3 in group I and M4 in group F. Regarding the genetic groups, strains with phenotype M3 were part of the *vacuma* genetic group. Other phenotypes from mycelial morphological class were classified in all three genetic groups.

Phenotypic differences between isolates from different regions were also observed in color of mycelium and size of conidia. The color of mycelium varied from dirty white in isolate from Dojran, to dark grey in isolates from Bogdanci and Gradsko. The highest values for length and width of conidia were detected in isolate from Dojran (13.2 µm and 8 µm, respectively). The shortest conidia had the isolate from Kochani (11.7 µm), while the width was only 7.3 µm in isolates from Gradsko and Strumica.



f-*flipper*, t-*transposa*, v-*vacuma*

Figure 2. Frequency distribution of *B. cinerea* isolates from different genetic groups and organs in morphological classes

Table 4. Color of mycelium and size of conidia of seven B. cinerea isolates from different regions

Isolate	Region	Color of mycelium	Size of conidia (μm)	
			Length	Width
1	Bogdanci	Dark grey	12.7	7.7
61	Gradsko	Dark grey	12.3	7.3
68	Vinica	Light grey	12.8	7.8
18	Dojran	Dirty white	13.2	8
111	Strumica	Light grey	11.8	7.3
37	Shtip	Grey	12.8	7.8
96	Kochani	Grey	11.7	7.5
		$LSD_{0.05}$	0.58	0.27
		$LSD_{0.01}$	0.79	0.37

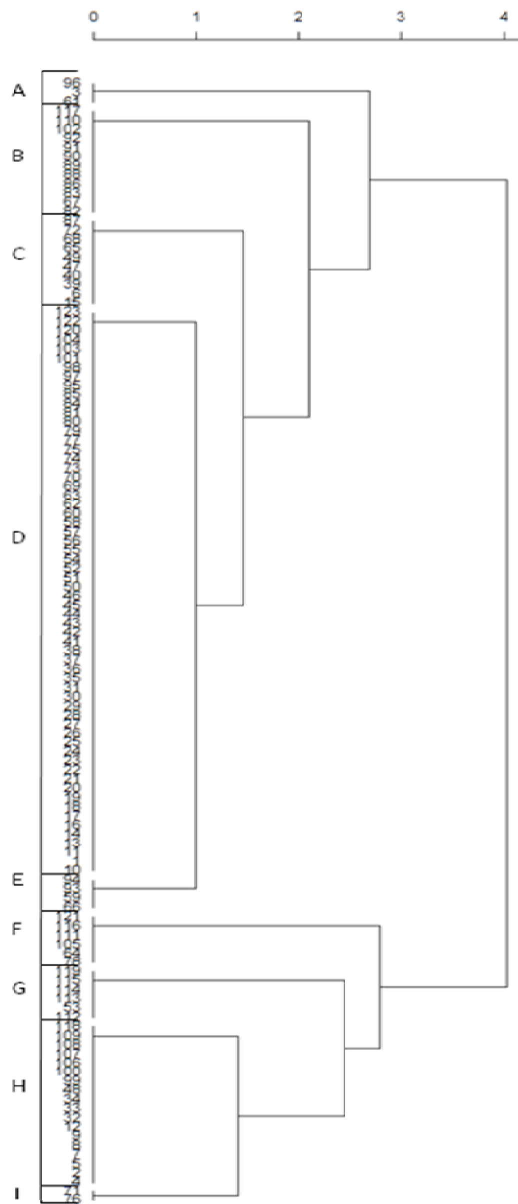


Figure 3. Genetic distance between *B. cinerea* isolates

DISCUSSION

In this study, three genetic groups of *Botrytis cinerea* were detected (*flipper*, *transposa* and *vacuma*), with prevalence of *flipper* genetic group (44.7% of 123 isolates). These results are in agreement with ISENEGGER *et al.* (2008), but are not consistent with the results obtained in many other studies (GIRAUD *et al.*, 1999; MUNOZ *et al.*, 2002; MA and MICHAILIDES, 2005; ISENEGGER *et al.*, 2008; KRETSCHMER and HAHN, 2008; TOPOLOVEC-PINTARIC *et al.*, 2004; TANOVIC *et al.*, 2009), in which *transposa* genotype was the most frequent one. To the contrary, *transposa* was the least present genetic group in this study. Furthermore, many previous studies revealed the fourth group that contains only the transposable element *boty* (GIRAUD *et al.*, 1999; MUNOZ *et al.*, 2002; MA V MICHAILIDES, 2005; ISENEGGER *et al.*, 2008; KRETSCHMER and HAHN, 2008; TOPOLOVEC-PINTARIC *et al.*, 2004; TANOVIC *et al.*, 2009; VACZY *et al.*, 2008; FEKETE *et al.*, 2010; DE MICCOLIS *et al.*, 2003; AHMED and HAMADA, 2005), which was not detected among isolates included in this study.

It should be highlighted that all isolates originating from Vinica region (18) belonged to *vacuma* genetic group. According to GIRAUD *et al.* (1999) and AHMED and HAMADA (2005), the occurrence of *flipper* and *boty* genetic groups is probably due to migration of one transposable element from *transposa* genetic group to the *vacuma* genetic group. As a result of this migration, *transposa* is invading *vacuma* group. Considering this hypothesis, maybe *vacuma* strains are natives in region of Vinica, and they had never been in contact with *transposa* strains, therefore there was no possibility for gene flow between these two groups. However, further investigations with larger number of isolates are needed to determine whether all isolates from this region belong to *vacuma* genetic group or there is more than one genotype present in this region.

Previous studies of phenotypic diversity in *B. cinerea* revealed two main morphological classes (mycelial and sclerotial). MARTINEZ *et al.* (2003) and TANOVIC *et al.* (2009) identified eight morphological types (four mycelial and four sclerotial) within these two main morphological classes. In both studies, sclerotial phenotypes were prevalent. In this study, the same four mycelial (M1, M2, M3 and M4) and four sclerotial (S1, S2, S3 and S4) types were determined. Sclerotial phenotypes were dominant, with prevalence of S3 phenotype which is completely in agreement with previous studies. Beside these eight phenotypes, new sclerotial phenotype of *B. cinerea*, named as S5, was identified. This observation only confirms that phenotypic diversity of *B. cinerea* is even more complex than previously reported.

Transposa isolates derived from the leaves of tomato exhibited only one phenotype-M2, compared with *transposa* isolates from stem and fruit, which belonged to different morphological types. *Flipper* strains from leaves also exhibited less phenotypes (M2, S1 and S3) than *flipper* isolates that originated from stem (M2, S1, S3 and S4) and fruit (M1, M2, M4, S3, S4 and S5). Considering *vacuma* genetic group, the isolates from stems of tomato were the least divergent, belonging to M3, S1, S3 and S4 morphological classes, compared to the isolates that derived from

leaves and fruits. Moreover, no relationship between the presence of transposable elements, *boty* and *flipper*, and morphological types of isolates could be observed. Isolates which belonged to morphological types M3 (*vacuma*), S5 (*flipper*) and S2 (*vacuma*) have been classified in only one genetic group, but at the same time these phenotypes comprised only few isolates. Taking into account the morphological types that comprised higher number of isolates, it can be seen that all three genetic groups are present. Furthermore, isolates from different regions included in this study expressed high variability in color of mycelium and size of conidia as well, which additionally confirmed the great phenotypic diversity of this pathogen. Morphological differences in color of mycelium and size of conidia of *B. cinerea* were also observed in other studies (NIELSEN *et al.*, 2001; PANDE *et al.*, 2010).

As a result, it can be concluded that the relationship between the genetic group, morphological types and organ of origin of *Botrytis cinerea* isolates from tomato cannot be established. Other researchers (GIRAUD *et al.*, 1997; GIRAUD *et al.*, 1999) also indicate that association between organ of origin, region of collection, phenotypes and genetic groups in *B. cinerea* cannot be found.

This study represents the first report of the phenotypic and genetic characterization of *B. cinerea* population from tomato in the Republic of Macedonia. The obtained results confirm the great phenotypic diversity of the analyzed isolates on one side. On the other side, these results incite the need for further investigations, in which populations from different host species will be included. As well, more aspects of phenotypic (evaluation of the fungicide resistance and virulence of the pathogen) and molecular characterization (different DNA markers) should be assessed.

CONCLUSIONS

B. cinerea population from tomato in the Republic of Macedonia that was subject of this study showed great phenotypic diversity. According to the mycelial formation, intensity of sporulation and sclerotial production, analysed *Botrytis cinerea* isolates belonged to 9 different phenotypes: 4 mycelial and 5 sclerotial. Sclerotial phenotype S5 that was observed in this study, has not been previously reported. Based on the presence or absence of transposable elements *boty* and *flipper,* out of 123 isolates, 20 belonged to the *transposa* genotype, 48 to *vacuma* genotype and 55 to the *flipper* genotype. Isolates that contain only *boty* element were not detected. No relationship between the phenotypes, origin of isolates and the presence/absence of transposable elements, *boty* and *flipper*, was found. Further investigations on *B. cinerea* populations from different host species are needed which will include more aspects of phenotypic and molecular characterization.

Received July17th, 2012
Accepted November30th, 2012

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FENOTIPSKA I GENETSKA KARAKTERIZACIJA *BOTRYTIS CINEREA* IZOLATA SA PARADAJZA

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Sto dvadeset i tri izolata *Botrytis cinerea* su kolekcionirani sa sedam lokaliteta u Makedoniji, gde se paradajz gaji u plastenicima i staklenicima. Na osnovi formiranja micelije, intenziteta sporulacije i produkcije sklerocija, utvrđeno je 9 različitih fenotipova: 4 miceliskih i 5 sklerociskih. Jedan sklerociski fenotip utvrđen u ovom istraživanju, do sada nije opisan. Prisutnost dva transpozabilna elemenata (TE), *boty* i *flipper*, određena je PCR metodom. Od ukupno 123 izolata, 20 je imalo oba transpozabilna elemenata, *boty* i *flipper* (*transposa* genotip), 48 nije imalo TE (*vacuma* genotip) i 55 izolata je sadržalo samo *flipper* TE (*flipper* genotip). Nisu detektirani izolati koji sadrže samo *boty* element. U ovom istraživanji nije utvrđena povezanost fenotipa sa poreklom izolata i prisutnosti TE (*boty* i *flipper*).

Primljeno17. VII. 2012.
Odobreno 30. XI. 2012.