

CHARACTERISATION OF GROWTH VARIABILITY AND MYCELIAL COMPATIBILITY OF *BOTRYTIS CINEREA* ISOLATES ORIGINATED FROM APPLE AND STRAWBERRY IN LITHUANIA

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Botrytis cinerea Pers.:Fr. is a widespread necrotrophic pathogen causing grey mould on many economically important horticultural crops. The variability in various *B. cinerea* populations is known to be very high. Despite the economic importance, the variability of *B. cinerea* has not been investigated previously on fruit crops in Lithuania. The aim of the study was to characterise the variability of *B. cinerea* strains isolated from strawberry and apple in different growth conditions on various agar media and to assess mycelial compatibility among the isolates. Larger colony diameter after four days of incubation was observed for isolates from strawberry on potato dextrose and beer universal agars in 24 h dark or light regime, followed by pectin agar in 24 h light. Similarly, the maximum radial growth of the isolates from apple was on potato dextrose agar (dark), followed by beer universal agar (dark and light), after four days of incubation at 20 °C. In the mycelial compatibility tests, barrage formation was evident in mycelial contacts between several isolates, indicating their vegetative incompatibility. The tests revealed that 76% were compatible and 24% were incompatible among investigated strains.

Key words: growth rate, barrage, dark/light, *Fragaria x ananassa*, *Malus domestica*, sclerotia, temperature.

INTRODUCTION

Strawberry (*Fragaria x ananassa* Duch.) is the most popular soft fruit in the world, and apple (*Malus domestica* Borkh.) is one of the most widely cultivated fruit crop in Lithuania (Uselis *et al.*, 2009; Liaudanskas *et al.*, 2015).

The main reasons for strawberry and apple yield losses in Lithuania include fungal and bacterial infections (Valiuškaitė *et al.*, 2006, 2008). *Botrytis cinerea* is an important pathogenic and highly variable fungus with wide host range. Plant diseases caused by *Botrytis* species depend on a complex of biological events involving the plant, environment, chemical and physical interactions between the pathogen and the host (Fillinger and Elad, 2016). Geographically, *Botrytis* species occur wherever their hosts are grown, ranging from the northern to the southern regions, including areas with extremely harsh weather and in the deserts where agriculture is practiced (Fillinger and Elad, 2016). *Botrytis cinerea* cause qualitative (fruit appearance, taste) and quantitative (yield) damage on strawberry and apple. In straw-

berry, *B. cinerea* overwinters mainly as sclerotia, but also as mycelium in leaves, crop debris, other parts of the plant, and in mulches. Although *B. cinerea* is not the primary pathogen on apple, it does significant post-harvest damage (Valiuškaitė *et al.*, 2006, 2008; Fillinger and Elad, 2016). The yield losses of horticultural crops in Lithuania caused by *B. cinerea* in favourable years are up to 50% (Rasiukevičiūtė *et al.*, 2015; 2016; Rasiukevičiūtė, 2016).

The information about pathways of spread, development, and diversity of the pathogen is essential for efficient disease control. Genetic variability in *B. cinerea* populations is high (Martinez *et al.*, 2003; Kumari *et al.*, 2014; Fillinger and Elad, 2016). Detection of vegetative compatibility groups (VCGs) and mycelial compatibility groups (MCGs) are useful tools for characterising diversity and substructuring of the populations of fungal species (Korolev *et al.*, 2008; Fillinger and Elad, 2016). Barrage formation or its absence traditionally is used for the assessment of mycelial compatibility or incompatibility in fungal isolates (Papaioannou and Typas, 2015; Fillinger and Elad, 2016).

VCG inter-strain pairings showed that 93% of the tested *B. cinerea* strains were incompatible and only 7% were compatible (Korolev *et al.*, 2008). Two genetically different groups are known for *B. cinerea*, which are considered as cryptic species (Fournier *et al.*, 2005). In the study by Fournier *et al.* (2005) *B. cinerea* isolates from group I formed unique single VCG while isolates from group II formed several VCGs. MCGs are used for *B. cinerea* characterisation and indicate incompatibility or compatibility in *B. cinerea*, although direct correlation with VCGs has not been found (Fournier *et al.*, 2005; Korolev *et al.*, 2008; Fillinger and Elad, 2016).

Environmental conditions are one of the most important factors for pathogen development and reproduction (Fillinger and Elad, 2016). The favourable combination of temperature and humidity greatly influence the spread of the pathogenic fungi, including *B. cinerea* on strawberry (Valiuškaitė *et al.*, 2008; Rasiukevičiūtė *et al.*, 2013; Survilienė-Radzevičė *et al.*, 2013; Fillinger and Elad, 2016). Photoperiod is an important environmental factor for fungi and affects mycelial pigmentation, growth, and development of sexual and asexual morphs (Fillinger and Elad, 2016). Near UV-light increases *B. cinerea* sporulation (Fillinger and Elad, 2016). Martinez *et al.* (2003) found that *B. cinerea* development *in vitro* depends on the host where from isolates are isolated and agar media. Fernandez *et al.* (2014) showed that growth of *B. cinerea* from grape was reduced at 4 °C compared with growth at 12 °C and 28 °C. Growth of *B. cinerea* on nutrient agar medium can be correlated to an indirect assessment of the colonisation potential of each strain and the ability to spread on the particular host (Fillinger and Elad, 2016).

Despite the economic importance of grey mould caused by *B. cinerea*, the variability of the pathogen has not been investigated previously on fruit crops in Lithuania. The aim of the study was to characterise the diversity of *B. cinerea* isolates originated from strawberry and apple fruits in different growth conditions on various agar media, and to assess their MCGs.

MATERIALS AND METHODS

Isolates. To evaluate growth variability of *B. cinerea*, strawberry and apple fruits with grey mould symptoms were collected randomly in 2012–2013 from the experimental fields of the Institute of Horticulture, Lithuanian Research Centre for Agriculture and Forestry (LAMMC) and commercial farms in Lithuania. Pieces of symptomatic plant tissues with diameter 2 cm were sterilised in 1% sodium hypochlorite and placed on potato dextrose agar medium (PDA). Plates were incubated at 20 °C for seven days and then single-conidial isolates were obtained. Single-spore isolates were selected based on colony morphology and microscopic observations for further molecular identification using PCR and species-specific primers described by Rigotti *et al.* (2006). Molecular identification, DNA extraction are described by Rasiukevičiūtė (2016).

Growth variability and sclerotia development. The growth variability and development of sclerotia were observed for 18 *B. cinerea* isolates originated from strawberry and apple fruits (Table 1). Potato dextrose agar (PDA), beer universal agar (BUA), Czapek-Dox agar (CDA), pectin agar (PA), plate count agar (PCA) and onion leaf agar (LA) were used for studying the effect of media on growth, develop-

Table 1

DESCRIPTION OF *BOTRYTIS CINEREA* ISOLATES FROM LITHUANIA USED IN THIS STUDY

Isolate	Year of isolation	Location	Type of plantation	Host	
				Cultivar	Organ
12BF27	2012	Babtai, Kaunas dist.	Experimental orchard 1	Strawberry, Elkat	Fruit
12KF5	2012	Kaunas city	Home garden	Strawberry, Elkat	Fruit
13KDF7	2013	Klebiškis, Kaunas dist.	Commercial orchard	Strawberry, unknown	Leaf
13BF18	2013	Babtai, Kaunas dist.	Experimental orchard 2	Strawberry, Darselect	Fruit
13BF19	2013	Babtai, Kaunas dist.	Experimental orchard 2	Strawberry, Darselect	Fruit
13BF16	2013	Babtai, Kaunas dist.	Experimental orchard 2	Strawberry, Darselect	Fruit
13BF17	2013	Babtai, Kaunas dist.	Experimental orchard 2	Strawberry, Darselect	Fruit
13BF20	2013	Babtai, Kaunas dist.	Experimental orchard 2	Strawberry, Darselect	Fruit
13BF31	2013	Babtai, Kaunas dist.	Experimental orchard 2	Strawberry, Darselect	Fruit
13BF26	2013	Babtai, Kaunas dist.	Experimental orchard 3	Strawberry, Elkat	Fruit
12BM8	2012	Babtai, Kaunas dist.	Experimental orchard 4	Apple, Auksis	Fruit
12BM9	2012	Babtai, Kaunas dist.	Experimental orchard 4	Apple, Spartan	Fruit
12BM28	2012	Babtai, Kaunas dist.	Experimental orchard 4	Apple, Spartan	Fruit
12BM29	2012	Babtai, Kaunas dist.	Experimental orchard 4	Apple, Jonagold King	Fruit
12BM21	2012	Babtai, Kaunas dist.	Experimental orchard 5	Apple, Connell Red	Fruit
12BM22	2012	Babtai, Kaunas dist.	Experimental orchard 5	Apple, Auksis	Fruit
12BM24	2012	Babtai, Kaunas dist.	Experimental orchard 5	Apple, Auksis	Fruit
12BM25	2012	Babtai, Kaunas dist.	Experimental orchard 5	Apple, Auksis	Fruit

ment and formation of sclerotia of the fungus. Mycelial plugs about 10-mm in diameter of the purified isolates were placed in the centre of a sterile Petri dish with each agar media. Each isolate was grown at 10, 15, and 20 °C temperature. The plates were incubated in 24 h dark or light period for 30 days and colony growth, development, appearance (days) and arrangement of sclerotia were recorded. The growth rates were determined by daily measuring the diameter of fungal colonies and counted according to Målama *et al.* (1985). The sclerotia formation types at 15 °C temperature were evaluated according to Kumari *et al.* (2014). A five-point scale was used to assess sclerotia distribution: A — single sclerotia, hardly visible; B — sclerotia located in the center; C — sclerotia arranged in concentric circles; D — sclerotia located at the margins of Petri dish; E — sclerotia arranged irregularly. Five replicates for each isolate were tested.

Mycelial compatibility. Eighteen isolates of *B. cinerea* from apple and strawberry (Table 1) were tested for their mycelial compatibility grouping (MCG's). The isolates were grown on malt extract agar (MEA) + NaCl (40 g/liter) according to Beever and Parkes (2003). For MCGs determination six isolates were paired in all possible combinations on a single Petri dish according to template suggested by Burgess *et al.* (2009). The plates were incubated for two weeks at 22 ± 0.5 °C temperature in the dark. The compatibility or incompatibility was evaluated observing the interaction zone between paired isolates. Isolates that formed an inhibi-

tion zone, dark pigmentation or barrage were considered incompatible and as compatible those that overgrew without any reactions of mycelium. Each isolate was scored at least in two replicates, and self-pairings of the isolates were included as controls in all experiments.

Statistical analysis. The data were analysed with ANOVA module of STATISTICA 7.0 software. Standard error (SE) marked in the figures as an error bar was estimated for growth rates of the isolates and sclerotia development.

RESULTS

Molecular identification. The *B. cinerea* isolates (Table 1) used in this study were molecularly confirmed by specific primers. Isolates were analysed using polymerase chain reaction (PCR). The *Botrytis* spp. isolates were identified as *B. cinerea* (Rasiukevičiūtė, 2016).

Growth variability and sclerotia development. Morphological differences among the isolates on six agar media were evaluated after 4, 8, and 11 days of incubation. The isolates from apple and strawberry showed different mycelial growth rates measured as radial growth on various media. Colonies of *B. cinerea* isolates changed colour from white to grey with age. Distribution of sclerotia varied among the isolates originated from both hosts (Fig. 1). Black, fully developed sclerotia appeared after 6–7 days at 20 °C, regardless of host species of the isolates (Fig. 2). The

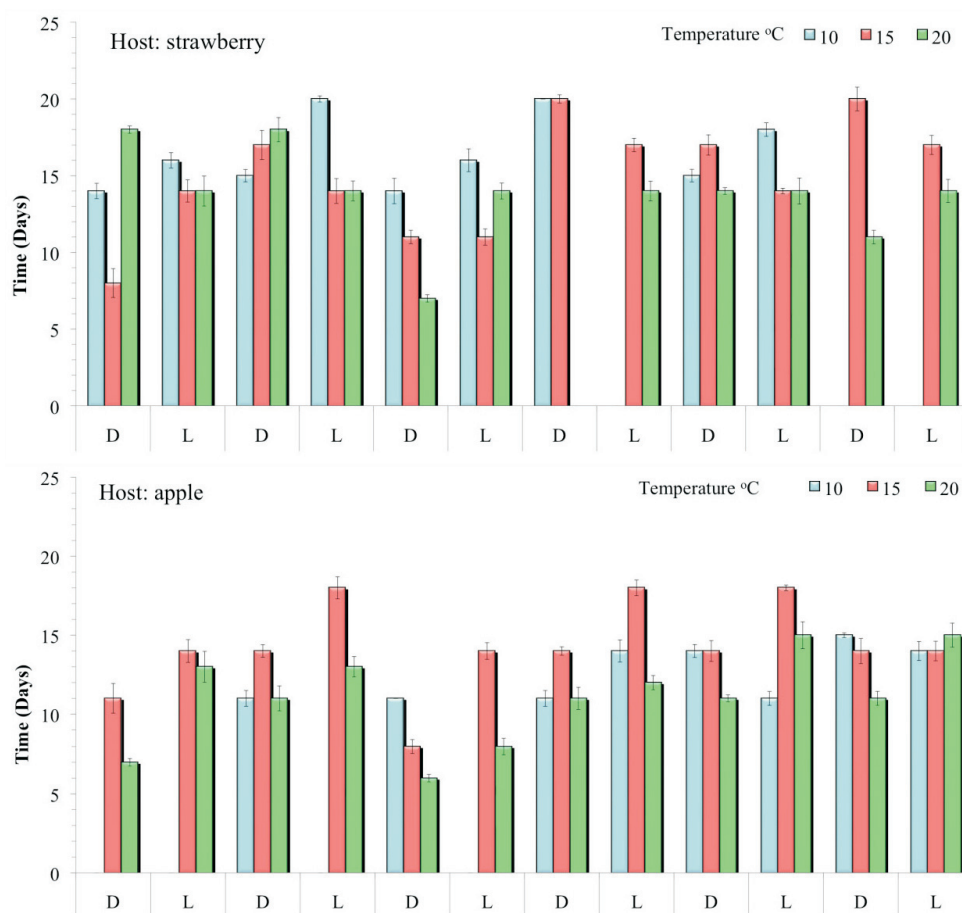
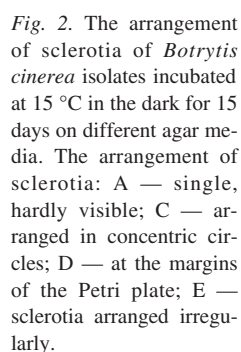


Fig. 1. The mean time (days) of sclerotia appearance for the studied *Botrytis cinerea* isolates originated from strawberry and apple on different agar media and incubation temperatures. D – dark, L – light.



The temperature, incubation at light or dark, and medium influenced cultural and morphological characteristics of *B. cinerea* originated from both hosts (Fig. 3) According to our analysis the average growth rate of *B. cinerea* on agar media depended on the host, temperature, and incubation in light or dark (Fig. 3). However, isolates from both hosts grew slowest at 10 °C regardless of incubation at light or dark and agar media. Colonies of isolates from strawberry were still not visible on CDA after incubation at 10 °C for four days. The highest average colony growth rate of isolates from strawberry was on PDA, BUA, and PA followed by LA and PCA after eight days of incubation at 15 °C. A similar trend of average colony growth was observed for isolates from apple. For the isolates from apple, the highest colony growth rate was observed on PDA, followed by



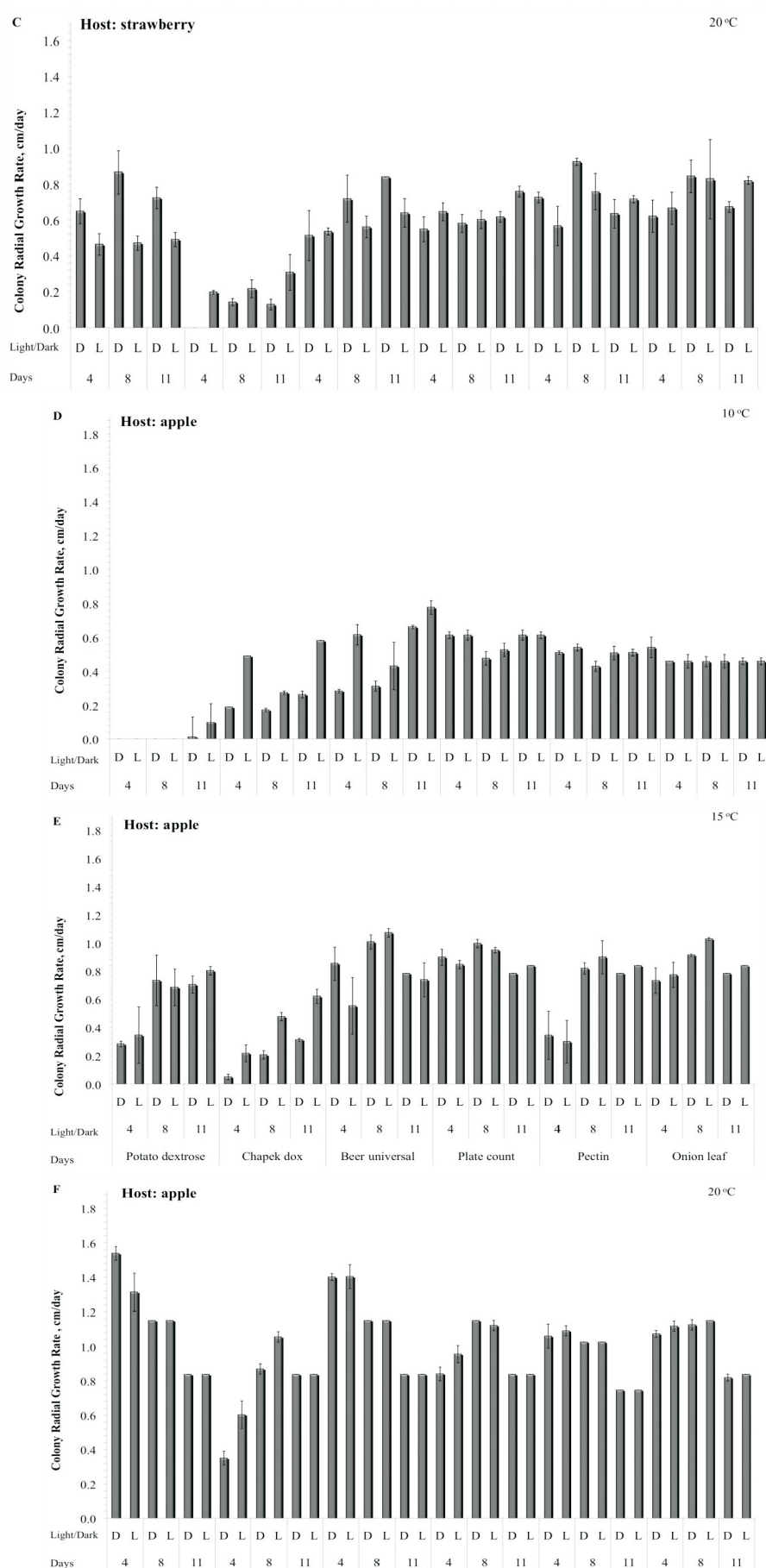


Fig. 3. The average radial growth of *Botrytis cinerea* isolates originated from strawberry (A–C) and apple (D–E) on various culture media at different temperatures.

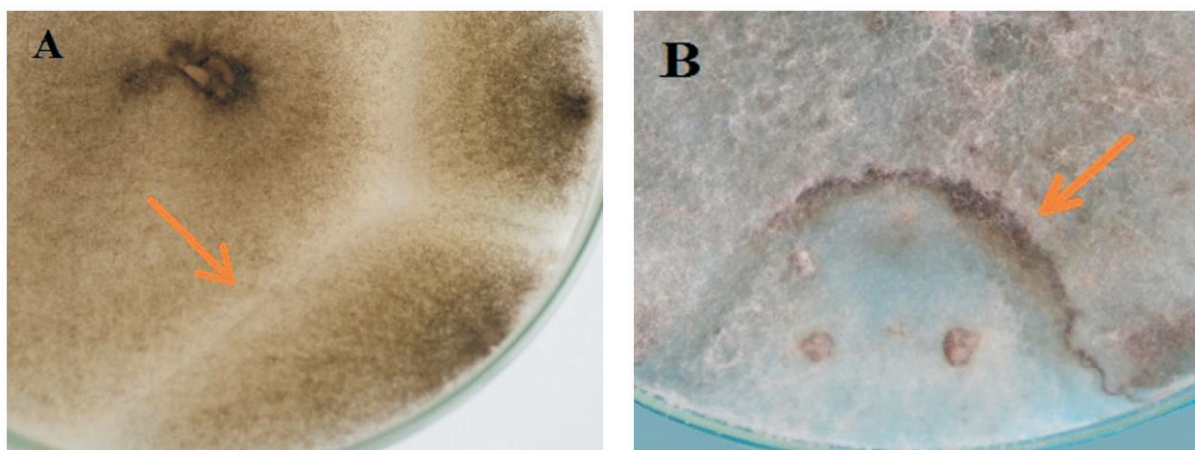


Fig. 4. Mycelial growth compatibility (A) and incompatibility (B) of *Botrytis cinerea* isolates on Malt extract agar amended with NaCl 40 g per liter.

12K F5	13K DF7	12B M8	12B M9	13B F16	13B F17	13B F18	13B F19	13B F20	12B M21	12B M22	12B M24	12B M25	13B F26	12B F27	12B M28	12B M29	13B F31	
1	2	2	1	0	0	1	1	1	1	0	2	0	0	1	0	1	1	12K F5
	1	1	1	1	2	1	0	1	2	2	1	2	1	2	1	1	1	13K DF7
		1	2	2	2	2	1	1	1	2	1	1	1	1	2	1	1	12B M8
			1	2	1	2	1	1	1	1	1	0	1	0	1	1	1	12B M9
				1	0	0	0	1	0	2	1	0	2	2	1	0	1	13B F16
					1	1	1	1	1	0	0	2	1	0	1	0	0	13B F17
						1	1	1	1	1	0	0	1	2	1	1	1	13B F18
							1	1	1	2	1	0	1	1	1	1	0	13B F19
								1	1	2	1	2	1	1	1	1	1	13B F20
									1	1	1	1	1	2	1	1	1	12B M21
										1	2	1	1	2	1	1	1	12B M22
											1	2	2	1	1	1	0	12B M24
												1	2	2	1	1	0	12B M25
													1	1	1	1	1	13B F26
														1	2	0	2	12B F27
															1	0	0	12B M28
																1	2	12B M29
																	1	13B F31

Fig. 5. Mycelial compatibility of *Botrytis cinerea* isolates originated from strawberry and apple. 0 – not evaluated, 1 – compatible, 2 – incompatible.

BUA after four days of incubation in the dark at 20 °C. For strawberry isolates the largest colony diameter was on PDA (dark) followed by PA, BUA, and LA after eight days at 15 °C. Substantial differences for growth at different temperatures were noted between isolates from different hosts. The average radial growth of *B. cinerea* strains isolated from apple was maximum on PDA (dark) and BUA (dark and light) at 20 °C, but for isolates from strawberry maximal growth was on PDA, BUA (dark and light), and PA (light) at 15 °C.

Mycelial compatibility. The mycelial compatibility tests among 18 isolates showed two types of reaction for the tested strains. Formation of a barrage and dark pigmentation

of the mycelium at the contact zones indicated an incompatible interaction between the isolates. Overgrowing of the colonies without any reaction of mycelium was a characteristic of compatible strains (Figs. 4, 5). The most frequent (76%) reaction within the tested population was no formation of barrages (Fig. 5). The pairings revealed that 84% and 85% of the combinations tested among isolates from strawberry and apple, respectively, were compatible. Between isolates from strawberry and apple compatible were only 65% of the tested combinations. The formation of barrages was mostly among the isolates originating from different hosts, locations and obtained in different years (Table 1, Fig. 5).

DISCUSSION

To obtain insight into the variability of *B. cinerea* populations on horticultural crops in Lithuania, isolates from apple and strawberry were studied. Phenotypic characteristics that could be related to the diversity and ability of the pathogen to spread and infect plant tissues, such as sclerotia production, mycelial growth rate, and various nutrient media, and mycelial compatibility, were assessed.

In this study, we found that *B. cinerea* isolates from different hosts have different phenotypic characteristics. Differences in the growth rate on various agar media and sclerotia formation were noted between isolates originated from strawberry and apple. The range of favourable temperatures for *B. cinerea* strains from strawberry was between 10–15 °C, compared to 15–20 °C for isolates from apple. According to colony morphology on PDA medium, strains were sorted into eight groups as described by Martinez *et al.* (2003). For isolates from apple the optimal mycelial growth was at 20 °C and for strawberry isolates at 15 °C. Our findings contrasted with the study by Fernandez *et al.* (2014), who reported that for *B. cinerea* strains from apple optimal mycelial growth was at 12 °C and the slowest growth was at 4 °C. Sehajpal and Singh (2014) found that 20 °C was optimal for mycelial growth of *B. gladiolorum*. The pathogen growth on agar media can be correlated with colonisation potential of each strain and the ability of the pathogen to spread on a host (Fernandez *et al.*, 2014). The temperature influences the ability of the pathogen to spread (Kumari *et al.*, 2014; Fillinger and Elad, 2016). The growth of *B. cinerea* *in vitro* on agar media is significantly influenced by concentration of ammonium nitrate in the medium (Lecompte *et al.*, 2010). Formation of sclerotia can be related to spread pathways of the pathogen. Our study revealed that isolates from strawberry and apple form sclerotia differently on the tested media. Kumari *et al.* (2014) showed that 30 strains among 79 studied isolates did not form sclerotia, confirming great diversity of *B. cinerea*. Martínez *et al.* (2003) found that temperature has an effect on sclerotia formation of *B. cinerea*. In this study, we found that sclerotia formation increased at 20 °C in the dark, regardless of the host of isolates and optimal agar medium for sclerotia formation was BUA, confirming the previous findings.

The vegetative compatibility or incompatibility reactions show the genetic differences, structure, dynamics between the fungal individuals and gene-to-gene interactions (Fillinger and Elad, 2016). Although direct correlation between VCGs and MCGs has not been demonstrated in *B. cinerea*, MCG can reveal the potential genetic diversity in the particular population of the pathogen (Fillinger and Elad, 2016). In general, VCGs and MCGs in *B. cinerea* populations are not sufficiently studied (Korolev *et al.*, 2008; Burgess *et al.*, 2009; Fernandez *et al.*, 2014; Kumari *et al.*, 2014; Fillinger and Elad, 2016). Korolev *et al.* (2008) found that inter-strain incompatibility was widespread among tested *B. cinerea* strains and the dark pigmentation zone along the line of confrontation was observed in 36% of the tested combinations of strains. In this study, mycelial compatibil-

ity and incompatibility among *B. cinerea* isolates from strawberry and apple in Lithuania were analysed for the first time. Among the analysed isolates, the most frequent were compatible strains (76%). Papaioannou and Typas (2015) found that about 75% of *Verticillium dahliae* isolates were incompatible. Burgess *et al.* (2001) in studies of vegetative compatibility in *Sphaeropsis sapinea* revealed that barrage formation indicating incompatibility occurred between strains originating from different locations. Similarly, in our study on the phenotypic diversity of *B. cinerea* isolates, incompatible isolates originated either from different sites, hosts or were isolated in different years. Our findings revealed that the studied *B. cinerea* isolates from strawberry and apple are variable and incompatible isolates exist in the studied group of the isolates; they differ in their requirements to growth media, temperature, and light, and these differences are related to the host where from they originated.

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NO ĀBOLIEM UN ZEMENĒM IZDALĪTU *BOTRYTIS CINEREA* IZOLĀTU AUGŠANAS ĪPATNĪBU UN VEĢETATĪVĀS SADERĪBAS RAKSTUROJUMS

Botrytis cinerea Pers.:Fr. ir plaši izplatīta nekrotrofiska patogēnā sēne, kura izraisa pelēko puvi daudzām ekonomiski nozīmīgam dārzkopības kultūrām. *Botrytis cinerea* populācijām raksturīga augsta daudzveidība. Zemenes un ābeles ir ekonomiski nozīmīgas augļaugu kultūras Lietuvā, bet to audzēšanu negatīvi ietekmē pelēkās puves radīti bojājumi. Neraugoties uz ekonomisko nozīmīgumu, *B. cinerea* populāciju daudzveidība un mainība Lietuvā līdz šim nav pētīta. Veiktā pētījuma mērķis bija raksturot no āboliem un zemenēm izdalīto *B. cinerea* izolātu daudzveidību atšķirīgos audzēšanas apstākļos uz dažādām barotnēm un novērtēt izolātu veģetatīvo saderību. Lielāko koloniju diametru sasniedza izolāti no zemenēm uz kartupeļu dekstrozes agara un uz alus universālā agara barotnes pēc četrus dienu inkubācijas 24 h tumsas vai gaismas režīmā, pēc tam sekojot uz pektīna agara 24 h gaismas režīmā. Līdzīgi, izolāti no āboliem maksimālo augšanu sasniedza uz kartupeļu dekstrozes agara (tumsā), tālāk uz alus universālā agara (gaismā un tumsā) pēc četrus dienu inkubācijas 20 °C. Izolātu savstarpējās veģetatīvās saderības testos tika novērota barjeras veidošanās micēliju kontaktu vietās, kas liecināja par izolātu savstarpēju veģetatīvo nesaderību. Testi parādīja, ka kopumā no testētajiem izolātiem 76% izolātu bija savstarpēji veģetatīvi nesaderīgi, bet 24% izolātu bija saderīgi.