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Genetic diversity and pathogenicity traits of *Botrytis* spp. isolated from horticultural hosts

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Abstract

Botrytis cinerea is an economically important pathogen that can infect more than 200 plant species. It has been demonstrated that *B. cinerea* species has a complex genetic structure with partially isolated genetic groups. The high degree of genotypic diversity results in high variability of biological traits among different genetic groups, including traits important for pathogenicity of the fungus. This study assessed the variation of genetic structure and pathogenicity traits of *Botrytis* spp. isolates originating from different horticultural plants and different geographic locations. 18 isolates with distinct morphological-cultural properties specific to *B. cinerea*, *B. squamosa* and *B. aclada* were prepared. Assessment of genetic polymorphism using amplified fragment length polymorphism (AFLP) method revealed high genetic diversity among the selected *Botrytis* spp. isolates. There was no obvious specificity for plant host or geographic location within the *B. cinerea* population represented by the selected isolates. Investigation of pathogenicity traits of *B. cinerea* isolates originating from strawberry fruits showed variations in respect of their cultural, morphological characteristics. Different degree of virulence on different strawberry plant parts was observed for the three isolates.

Key words: AFLP, *Botrytis*, conidia, cultural traits, morphology, geographic location.

Introduction

The genus *Botrytis* contains 22 species and a large number of host-specific pathogens. *Botrytis cinerea* Pers.: Fr is an economically important plant pathogen that can attack more than 200 species in the field, greenhouse and storage (Jarvis, 1980; Brandenburger, 1985; Holz et al., 2004). This fungus can infect the plant at every stage of its development and has been found in every part of the plant, including leaves, fruits, flowers, petioles.

Traditionally, identification of *Botrytis* species is based on morphological traits (Maude, Presly, 1977; Presly, 1985). Detection of *Botrytis* spp. at early stages is difficult because of a latent character of the infection (Xu et al., 2000; Boff et al., 2001). Infected tissues usually remain symptomless until they ripen, senesce or die. Epidemiological studies are also problematical because of the genetic variability within this species.

Genetic polymorphism of *B. cinerea* is the most studied among the *Botrytis* spp., and the species represents a good example of high genetic diversity within the genus. For a long time, *B. cinerea* has been thought to be a homogeneous species with several morphological traits and plant hosts. However, studies on genetic polymorphism have demonstrated that the species has a complex genetic structure with limited gene flow between different genetic groups (Giraud et al., 1997; Albertini et al., 2002; Fournier et al., 2002). The high degree of genotypic diversity explains high variability in several biological traits among isolates. The complexity within species might be linked to differences in reproduction types (Kumar et al., 1999), phenology, host specificity (Poulin, 1997) or resistance to fungicides (Albertini et al., 2002; Fournier et al., 2002). Therefore assessment of a genetic complexity of populations of the plant pathogen is important for development of efficient plant protection strategies.

Recently, molecular biology methods have been often used to study genetic diversity of fungi. Amplified fragment lenght polymorphism (AFLP) is a common choice for organisms with unknown ge-

nomic sequence. The method combines advantages of DNA restriction and PCR amplification methods, such as sensitivity and reproducibility (Blears et al., 1998).

The aim of the present study is to assess the variation of genetic structure and pathogenicity traits of *Botrytis* spp. isolates originating from different horticultural plant hosts and different agroecosystems.

Materials and methods

Isolate preparation and identification. Specimens were collected from Kaunas, Pasvalys, Panevėžys, Šiaulai, and Širvintai regions. A total of 206 samples were collected from fruits and vegetables with characteristic symptoms of *Botrytis* spp. infection grown at different locations, average 10 specimens per region. *Botrytis* spp. specimens displaying characteristic fungal disease symptoms were collected from fruits of *Malus* x *domestica*, *Fragaria ananassa*, and vegetables *Allium cepa*, *Brassicae oleracea* var. *capitata*, *Lycopersicon esculentum*. Fungal cultures were established using wet chamber and growth on solid medium methods and assessed microscopicaly. Isolates were grown on potato dextrose agar pH-5.0 (PDA) (Merck KGaA) at 20 ± 2°C in the dark for 2–3 weeks, and identified according to fungal identification manuals (Domsch et al., 1980; Brandenburger, 1985; Ellis, Ellis, 1987; Билай и др., 1988; Lugauskas et al., 2002).

DNA preparation and AFLP analysis. AFLP samples were prepared using AFLP Microbial fingerprinting kit ("Applied Biosystems" Ltd.) using manufacturer instructions based on method described by Vos et al. (1995). 100 mg of hyphae was collected from fungal isolates grown on PDA medium, and genomic DNA was isolated using DNeasy Plant Mini kit ("Qiagen" Ltd.) using manufacturer instructions. AFLP samples were prepared using AFLP Microbial fingerprinting kit ("Applied Biosystems" Ltd.) using manufacturer instructions. 10 ng of isolated DNA was digested using EcoRI and Tru1I (MseI) restrictases ("Fermentas" Ltd.) and specific adapters were ligated using T4 DNR ligase ("Fermentas" Ltd). After nonselective amplification step, fragments were amplified using primers containing one selective nucleotide: A/G, C/A, and G/T for EcoRI/Tru1I adapters, respectively. The amplified fragments were analyzed on ABI 3130 genetic analyzer ("Applied Biosystems" Ltd.) using 36 cm capillary array and POP-7 polymer.

Allele scoring was performed using Gene Mapper v.4.0 ("Applied Biosystems" Ltd.). The scoring parameters of 50 to 500 bp range and higher than 50 rfu signal intensity were used. To assess genetic distance of different isolates, cluster analysis was performed on *Systat v.13* software ("Systat Software" Ltd.) using hierarchical analysis algorithm of χ^2 distance.

Pathogenicity test. Investigation was based on methods described by Jarvis (1980) and Holz et al. (2004). The isolates were grown on PDA plates at 20 ± 2°C temperature in the dark for 2–3 weeks. Plugs (0.6 cm diameter) of fully developed hyphae were placed at the centre of a PDA plate. Three replicates of each isolate were analyzed. Cultural, morphological and pathogenic traits of the isolates were characterized. Size of conidia of *B. cinerea* isolates was calculated based on the average values obtained from 20 samples.

Strawberry root core, petiole and leaves were taken from visually healthy plants, washed under tap water and rinsed twice in distilled water and dried with blotting paper. The plant parts were placed on moist blotting paper covered with plastic net in the polystyrene boxes. 3 mm diameter mycelia disks, taken from edge of colonies were applied on leaf petioles, base of leaves and root core. Boxes were covered with foil and incubated at 20 ± 2°C, 80% humidity in climate chamber in light. Length of necrotic lesions was measured after 10 days of incubation. Experimental design was completely randomized with three replication and 5 plant parts in each replication. The trial was repeated twice at 3 weeks' interval.

Anova was applied for the statistical processing of data. The significance of data was determined by the Fisher's criterion with a significance level of $P \leq 0.01$ and 0.05. Averages for the other data were calculated (Tarakanovas, Raudonius, 2003).

Results and discussion

A total of 206 samples were collected from fruits and vegetables with characteristic symptoms of *Botrytis* spp. infection grown at different locations. Based on morphological characteristics of fungal growth on culture medium, 18 isolates were selected for further studies that displayed distinct morphological-cultural properties specific to *Botrytis cinerea* Pers.:Fr. from *Brassicae oleracea* var. *capitata*, *Lycopersicon esculentum*, *Fragaria ananassa*, *Malus* x *domestica*; *Botrytis squamosa* J. C. Walker and *Botrytis aclada* (Fresen.) Yohalem from *Allium cepa* (Table 1).

Previously published data demonstrated that a high genetic polymorphism was characteristic of fungi of *Botrytis* spp. *B. cinerea* that had been defined as a single species with variation of morphological traits and polyphagy was shown to present a genetically complex population including groups with partial genetic isolation (Giraud et al., 1997; Albertini et al., 2002; Fournier et al., 2002).

Table 1. List of *Botrytis* spp. isolates selected based on distinct morphological-cultural properties

Isolates	Plants	Locations
OB-09-01	*M.* x *domestica*	Kaunas reg.
OB-09-02	*M.* x *domestica*	Kaunas reg.
BB 1.1	*F. ananasa*	Panevėžys reg.
BB 1.3	*F. ananasa*	Kaunas reg.
BB 2.3	*F. ananasa*	Šiauliai reg.
BB 3.1	*F. ananasa*	Kaunas reg.
BB 4.1	*F. ananasa*	Kaunas reg.
29-5-0	*B. oleracea* var. *capitata*	Kaunas reg.
29-11-I	*B. oleracea* var. *capitata*	Širvintai reg.
29-15-II	*A. cepa*	Šiauliai reg.
20-20-II	*L. esculentum*	Kaunas reg.
29-23-I	*L. esculentum*	Kaunas reg.
29-24-II	*L. esculentum*	Kaunas reg.
29-30-II	*B. oleracea* var. *capitata*	Širvintai reg.
29-34-Ia	*A. cepa*	Kaunas reg.
29-35-IIa	*B. oleracea* var. *capitata*	Kaunas reg.
29-X-II	*A. cepa*	Šiauliai reg.
35-I	*B. oleracea* var. *capitata*	Kaunas reg.

Consistent to these findings, our study revealed a large genetic diversity within the population of fungal pathogen. An assessment of genetic polimorphism of the 18 selected *Botrytis* spp. isolates using AFLP method with three pairs of primers with one selective nucleotide resulted in identification of 819 polymorphic alleles (Table 2). Cluster analysis of genetic similarity of the isolates revealed eight distinct groups with genetic distance value larger than 0.5 (Fig. 1). Five isolates, including 29-11-I (*B. oleracea* var. *capitata*, irvintai reg.), BB1.1 (*F. ananassa*, Panevėžys reg.), BB1.3 (*F. ananassa*, Kaunas reg.), 29-34-Ia (*A. cepa*, Kaunas reg.), 29-X-II (*A. cepa*, Šiauliai reg.), were identified as unique genetic branches representing different genetic groups. The first genetic cluster of more than one isolate included 29-24-II (isolated from *L. esculentum* in Kaunas reg.) and 35-I (*B. oleracea* var. *capitata*, Kaunas reg.). The second group consisted of eight isolates including 29-35-IIa, 29-30-II (isolated from *B. oleracea* var. *capitata* in Kaunas reg. and Širvintai reg.), 29-23-I, 20-20-II (*L. esculentum*, Kaunas reg.), OB-09-02 (*M.* x *domestica*, Kaunas reg.), BB3.1, BB4.1 and BB2.3 (*F. ananassa*, Kaunas reg. and Šiauliai reg.). The third group included OB-09-01 (isolated from *M.* x *domestica*, Kaunas reg.), 29-5-0 (*B. oleracea* var. *capitata*, Kaunas reg.), and 29-15-II (*A. cepa*, Šiauliai reg.). The results demonstrated no explicit specificity for plant host or geographic location within the *B. cinerea* population represented by the selected isolates. All of the plant hosts and geographic locations contained a high genetic diversity of the pathogen represented by isolates from two or three distinct genetic groups. Isolates from the similarity clusters including more than one isolate were found on different plant hosts and at various locations. Isolates from the largest cluster including eight isolates originated from four different plants and three different locations. Isolates assigned to the first similarity cluster were found only in Kaunas reg., but were isolated from different plant hosts (*L. esculentum* and *B. oleracea*).

Table 2. Results of AFLP analysis of the 18 selected *Botrytis* spp. isolates

EcoRI/MseI selective primers	Number of alleles	
	Total	Polymorphic
A/G	276	249
C/A	303	290
G/T	296	280
Total:	875	819

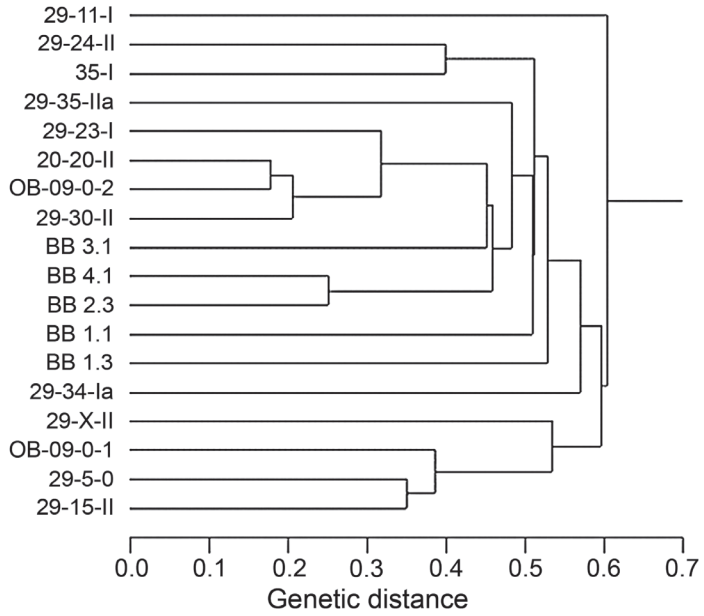

Figure. Dendrogram of genetic similarity of the selected isolates of *Botrytis* spp.

Pathogenicity assessment. Our study revealed variation in cultural, morphological and pathogenic characteristics among the three isolates of *B. cinerea* BB 2.3, BB 4.1, BB 1.1. Assessment of cultural and sclerotial characteristics demonstrated variation in colony, colour, shape, margin, texture, and also sclerotial features among the isolates (Table 3).

The conidia release rate of *B. cinerea* depends on conidia size – small conidia dried faster and consequently released faster (Holz et al., 2004). Therefore conidia size is an important factor in pathogenicity of the fungus, and the trait was assessed for the *B. cinerea* isolates (Table 4). The length of conidia varied from 7.50 to 15.00 μm. Maximum (12.00 μm) and minimum (8.00 μm) of mean length of conidia was found in isolate BB 4.1 and BB 1.1, respectively. The width of conidia ranged from 5.00 to 10.00 μm. The highest (8.25 μm) and the lowest (6.00 μm) mean of width was observed in BB 4.1 and BB 1.1, respectively.

Table 3. Cultural* and sclerotial characteristics of *B. cinerea* isolates

Isolate	Colony characteristics				Sclerotial features
	colour	texture	shape	margin	
BB 2.3	ashy white	fluffy	irregular	irregular	few, moderate to large size, scattered in entire plates
BB 4.1	light ash	fluffy	regular with sector	entire	high, large size, spread all over the plate preferably peripheral region
BB 1.1	off white	velvet	regular without sector	entire	few, moderate to large size, scattered in entire plates

Note. * – cultural characteristics recorded after 5 days of incubation on PDA medium.

Table 4. Size of conidia of *B. cinerea* isolates after 15 days of incubation on PDA at 20°C

Isolate	Dimension (μm) of conidia			
	conidia length		conidia width	
	range	mean*	range	mean
BB 2.3	7.50–11.25	10.00	6.25–7.50	7.25
BB 4.1	7.50–15.00	12.00	5.00–10.00	8.25
BB 1.1	7.50–11.25	8.00	6.25–7.50	6.00

Note. * – mean of 20 replicates for each isolate.

Three isolates of *B. cinerea* exhibited different reaction to set of different strawberry parts after 10 days of inoculation in a climate chamber. Among them BB 2.3 was found the most virulent isolate on all of the investigated strawberry parts. The isolate BB 4.1 showed high virulence on leaves and root core, but the virulence was low on petioles. The isolate BB 1.1 showed relatively low virulence on all strawberry parts (Table 5).

Table 5. Pathogenicity of three isolates of *B. cinerea* on different strawberry parts 10 days after inoculation

Isolate	Diameter of necrosis								
	root core			petioles			leaves		
	mm	difference from mean		mm	difference from mean		mm	difference from mean	
		±	%		±	%		±	%
BB 2.3	5.93c	+0.844	117	10.33b	+5.533	215	3.7abc	+0.68	122
BB 4.1	5.25c	+0.111	102	2.93ab	−1.866	61	4.78c	+1.753	158
BB 1.1	4.1a	−0.955	81	1.13ab	−3.666	24	0.59a	−2.433	20
Mean		5.089			4.8			3.027	

Considering the importance of *Botrytis cinerea* as pathogen and its significant damage to agricultural products, its management is necessary. The first step in the management of a pathogen is its recognition, and taxonomy is one of the best tools that helps us to distinguish pathogens. *Botrytis* taxonomy has traditionally been based on morphological and cultural characteristics such as dimension and shape of conidia also conidia size, form and colony characters coupled with host specificity (Domsch et al., 1980; Jarvis, 1980; Ellis, Ellis, 1987; Lugauskas et al., 2002). In addition, morphological characters have been used in *Botrytis* taxonomy, and just in recent years molecular markers have been used in identification of *Botrytis* species. Therefore, despite the fact that the morphological structure of 335 *Botrytis* isolates varies in a wide range, all of the isolates belong to morphospecies of *Botrytis cinerea* (Mirzaei et al., 2007).

Conclusions

1. Isolates of *Botrytis* spp. with distinct macroscopic and microscopic traits were prepared and identified as *B. cinerea*, *B. squamosa* and *B. aclada*.

2. Assessment of genetic polymorphism using AFLP method revealed high genetic diversity among the selected *Botrytis* spp. isolates. The isolates clustered into eight genetic groups with a genetic distance value larger than 0.5. There was no obvious specificity for plant host or geographic location within the *B. cinerea* population represented by the selected isolates.

3. Isolates BB 2.3, BB 4.1, BB 1.1 of *B. cinerea* showed variations in respect of their cultural and morphological characteristics. Different degree of virulence on different strawberry plant parts was observed for the three isolates.

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Botrytis spp. izoliatų, iškirtų iš sodo ir daržo augalų, genetinė įvairovė ir patogeniškumo savybės

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Santrauka

Botrytis cinerea yra ūkiniu atžvilgiu reikšmingas patogenas, galintis užkrėsti daugiau kaip 200 augalų rūšių. Įrodyta, kad *B. cinerea* rūšies grybai turi sudėtingą genetinę struktūrą, kurioje išskiriamos kelios genetinės grupės. Genotipinį įvairumą lemia didelis biologinių savybių kintamumas tarp skirtingų genetinių grupių, taip pat ir grybo patogeniškumo savybės. Šių tyrimų metu vertintas *Botrytis* spp. izoliatų iš įvairių sodo bei daržo augalų ir skirtingų vietovių genetinės struktūros kitimas bei patogeniškumas. *Botrytis* 18 izoliatų pagal būdingas morfologines ir kultūrines savybes buvo priskirti *B. cinerea*, *B. squamosa* bei *B. aclada* rūšims. Genetinio polimorfizmo tyrimas, taikant pagausintų fragmentų ilgio polimorfizmo (AFLP) metodą, atskleidė didelį genetinį *Botrytis* spp. izoliatų kintamumą. Tirti izoliatai *Botrytis cinerea* populiacijoje neatskleidė akivaizdaus specifiškumo tarp augalų šeimininkų ir geografinės vietovės. Patogeniškumo tyrimų metu iš braškių vaisių išskirti *B. cinerea* izoliatai pasižymėjo skirtingais kultūriniais morfologiniais požymiais. Trys *B. cinerea* izoliatai pasižymėjo įvairaus laipsnio virulentiškumu ant skirtingų braškių augalo dalių.

Reikšminiai žodžiai: AFLP, augimo ypatybės, *Botrytis*, konidija, morfologija, vietovė.