

## Morphological variability of *Botrytis cinerea* – causal agent of Japanese quince grey mould

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**Abstract.** *Botrytis cinerea* is a causal agent of grey mould that damages many species of plants including Japanese quince (*Chaenomeles japonica* (Thumb.) Lindley ex Spach). Grey mould has been found on *Chaenomeles* spp. flowers, fruits in all stages as well as on twigs. Morphological variability within *Botrytis* species has been previously reported in the literature, but no information is available about *B. cinerea* isolated from *Chaenomeles* spp. The aim of this study was to describe the symptoms of grey mould and clarify the morphological variability of *B. cinerea* isolates obtained from samples collected in commercial plantations of Japanese quince. Samples of plant parts with different fungal disease symptoms were collected in eight commercial plantations of Japanese quince during vegetation seasons of 2017 and 2018. Some samples were taken in Japanese quince plantations in Lithuania and Estonia. A total of 286 isolates of fungi were isolated from damaged shoots, leaves and fruits of Japanese quince plants, using potato dextrose agar. *Botrytis cinerea* isolates (39) were separated depending on the morphological characteristics and were proved by using methods of molecular biology. *B. cinerea* was isolated from shoots, leaves and fruits. The isolates of *B. cinerea* were described and classified into distinct morphological types depending on the characteristics of mycelia, sclerotia, reverse side of media and the presence or absence of sporulation.

**Key words:** *Chaenomeles* spp., fruit rot, sporulation, morphological type, sclerotia.

### INTRODUCTION

Japanese quince *Chaenomeles japonica* (Thumb.) Lindley ex Spach belongs to the family *Rosaceae* subfamily *Maloidae* together with other wide growing fruit species such as apple, pear, soft berries, etc. (Weber, 1964; Phipps et al., 1990). Fruits of Japanese quince (hereinafter – quince) are an important source of minerals, ascorbic acid, phenols and a lot of other valuable nutrients (Mierina et al., 2011; Baranowska-Bosiacka et al., 2017). The use of quince fruits is confined not only to food production. Quince fruits are a potential source for the medical and cosmetic production (Nahorska et al., 2014; Banaś & Korus, 2016). The first commercial plantation of quince for fruit production in Latvia was established in the 1950s (Kaufmane et al., 2013). Since then, the area of quince plantations in Latvia has repeatedly increased and decreased. Based on the information

provided by the Ministry of Agriculture of the Republic of Latvia (2018), since 2013, the total area of Japanese quince plantations in Latvia has rapidly increased from 102 ha to 200 ha in 2015, and reached 326 ha in 2017.

A significant development of diseases was observed in eight commercial Japanese quince fields in Latvia in the vegetation periods of 2017–2018. During observations, several symptoms of diseases as leaf spots, shoot damages and fruit rot were detected and fungi from damaged plant parts were isolated. An essential proportion of them could be caused by *Botrytis cinerea*.

Only few reports describing symptoms of damages caused by *B. cinerea* on several parts of *Chaenomeles japonica* are available. In most cases, *B. cinerea* is associated with shoot die-back (dead shoots at the beginning of vegetation affected either by unfavourable environmental conditions or by fungal pathogen) and with wilted flowers. The first symptoms – small spots with a dark margin – of fruit rot caused by *B. cinerea* appeared mostly on fruits from the stem or calix side or around a wound. During the development of the disease, spots enlarged and the whole or part of quince fruit became brown (Norin & Rumpunen, 2003). In moisture conditions, grey wooly mycelium appeared on the surface of rotted fruit. Damaged areas can be surrounded by a red margin (Fedulova et al., 2017).

Rumpunen (2002) and Norin & Rumpunen (2003) reported that *B. cinerea* has been found on flowers and fruits at all stages of *Chaenomeles* spp. in Sweden, England, and Romania. Norin & Rumpunen (2003) isolated *B. cinerea* from cankers in shoots. Also, *B. cinerea* has been identified on the fruits of *Chaenomeles japonica* in the Vilnius Botanical Garden (Grigaliūnaitė et al., 2012) and on flowers and fruits in Tambow region of Russia (Fedulova et al., 2017). Jakobija & Bankina (2018) had previously reported the results of research about fruit rot incidence in plantations of Japanese quince in Latvia.

*Botrytis cinerea* is a worldwide distributed and well-described pathogen. *B. cinerea* belongs to the phylum *Ascomycota*, family *Sclerotiniaceae* (Williamson et al., 2007). This pathogen has a necrotrophic lifestyle (Williamson et al., 2007). *B. cinerea* causes grey mould on 589 species of plants (Elad et al., 2016), including Japanese quince (Hennebert, 1973). *B. cinerea* can cause important damages on all above-ground parts of Japanese quince, which results in serious yield losses, especially in fruit growing. The pathogen overwinters as mycelium, conidia, and, for a long-lasting period, with sclerotia in or on host tissues and soil surface (Elmer & Michailides, 2007; Williamson et al., 2007).

Sclerotia (dense formation of mycelium with a reserve of nutrients for survival) are the main source of inoculum in the life cycle of *B. cinerea* (Elmer & Michailides, 2007). Sclerotia develop on infected and dying host tissues. Fully formed sclerotia are black (Rasiukevičiūtė et al., 2017) and can have different shapes and length (Williamson et al., 2007). The apothecia or sexual stage of *B. cinerea* were detected in rare cases in orchards and are not considered an important part of disease life cycle (Beever & Weeds, 2007).

Conidia of *B. cinerea* can be produced on mycelium and sclerotia on the remains of hosts and on soil surface. It is the most important source of infection at the beginning of vegetation period (Elmer & Michailides, 2007). Conidia spread mainly with air flows and rain splashes (Jarvis, 1962), and start to germinate after 6 h of soaking in free water at the temperature of 20 °C (Hawker & Hendy, 1963). Results of the research of Xu et al. (2009) and Mehra et al. (2019) also showed that optimal temperatures for the development of *B. cinerea* conidia are 20°C and 22–25 °C respectively. Mehra et al. (2019) recognized that both air temperature and humidity are important factors for the

development of grey mould. *B. cinerea* can directly penetrate host tissues or infect the plant through wounds and/or develop on senescent and dead plant parts (Elad, 1997). Many repeating generations of conidia from the infected plant parts are observed during vegetation (Elmer & Michailides, 2007).

Morphological variability among *Botrytis* isolates obtained from apple and strawberry in Lithuania (Rasiukevičiūtė et al., 2017), from blackberry, strawberry, grapevine and raspberry in Serbia (Tanović et al., 2009; Tanovic et al., 2014), from several species of ornamentals in South Spain (Martínez et al., 2008) and from many other hosts in Iran (Mirzaei et al., 2009) and India and Nepal (Kumari et al., 2014) has been previously reported in the literature. Chang et al. (2001) have shown that the amount, shape and size of sclerotia of *B. cinerea* on natural or culture media are very variable. Despite the comprehensive investigations in this research area, no information is available about similar studies on *B. cinerea* isolated from *Chaenomeles* spp.

Clarification of the morphological variability of *Botrytis* isolates obtained from different parts of Japanese quince could be used in the identification of the pathogen in Latvia and other countries where quince is cultivated. Moreover, the ability of *Botrytis cinerea* to form mycelium, sclerotium and spores, determine the potential for the spread and infection process of the pathogen (Rasiukevičiūtė et al., 2017). Information about the morphological and genetic diversity could be important for the control and forecast of the disease. According to the results of several studies (Kumari et al., 2014; Zhou et al., 2014; Tanovic et al., 2014; Isaza et al., 2019), the susceptibility of the genetical groups of *B. cinerea* to fungicides could be different.

The aim of this study was to describe the symptoms of Japanese quince diseases caused by *Botrytis cinerea* and clarify the morphological variability of *B. cinerea* isolates obtained from the samples collected in commercial plantations of Japanese quince.

## MATERIALS AND METHODS

### Collection of fungi isolates

Leaf, shoot, inflorescence and fruit samples with visible damages (associated with symptoms caused by fungi) were collected in eight commercial Japanese quince plantations in Latvia during the vegetation periods of 2017 and 2018 and two by two in Lithuania and Estonia in 2018. When necessary, collected samples were stored in humidity chambers until macroscopic structures (for example, mycelium, sclerotium, etc.) of fungi appeared on the damage point. A piece of fungus structure or damaged area of quince part was placed on a Petri dish containing potato dextrose agar (hereinafter – PDA) with streptomycin (100 ppm L<sup>-1</sup>). Fragments of leaves with damages associated with fungal diseases were placed on the PDA directly. Fungi were incubated at 20 °C in the dark. Sample purification was done until a pure culture of fungal isolate was obtained and stored at 5 °C on PDA.

The identification of isolated fungi was performed based on the morphological characteristics of the isolates grown on PDA and using a microscope. Isolates of *Botrytis cinerea* were separated from all obtained isolates for further morphological studies. Isolates were sorted in groups by common morphological features. Representative isolates from each group were selected for the identification by molecular methods.

DNA extraction was done using E.Z.N.A.® HP Fungal DNA Kit (Omega Bio-tek, USA), following the manufacturer's instructions. ITS fragment was amplified using

primers ITS1-F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS-4 (5'-TCCTCCGCTTATTGATATGC-3'). PCR products were sent for sequencing to Latvian Biomedical Research and Study Centre (BMC). Fungal samples were identified using the NCBI BLAST® database.

### **Morphological characterization of *Botrytis cinerea* isolates**

Mycelial pieces (length of square edge approx. 5 mm) from isolates identified as *B. cinerea* were placed on a Petri dish (9 cm diameter) filled with PDA with addition of streptomycin (as described above); each isolate in three replicates for inter-comparison. Samples were grown in dark at the temperature of 20 °C for three weeks.

Morphological traits of *B. cinerea* were recorded 3, 7, 10 days, and three weeks after incubation. Mycelium type, colour, margin and size (diameter in cm), media (reverse side of plate) colour, sclerotia amount (pcs. on plate), diameter (mm), arrangement, colour, development stage and visibility were recorded at each observation.

Classification of *B. cinerea* isolates into distinct morphological types was performed depending on the last assessment after three weeks of incubation of isolates. The number of sclerotia on the plate filled with PDA (hereinafter – plate) was recorded. The classification of sclerotia-forming isolates depending on sclerotia amount on plate was performed, and isolates were separated into five classes: up to 10 sclerotia, 11 to 30, 31 to 50, 51 to 100, and more than 100 sclerotia on plate.

To evaluate the absence or presence of sporulation and sporulation rate on an isolate three weeks after incubation, a microscopic analysis was done using a microscope with 40× magnification (Table 1).

Mycelium type and sclerotia arrangement were described following the methodology previously used by Tanovic et al. (2014) with some modifications. The arrangement of sclerotia on the plate was divided into classes. No scattered arrangement was observed as that in the above-mentioned methodology. Instead of the four types of mycelium used in the mentioned methodology, five types were separated (Table 1).

**Table 1.** Separating of *B. cinerea* isolates depending on mycelium type, arrangement of sclerotia, and sporulation rate

Mycelium type		Arrangement of sclerotia		Sporulation rate	
designation	description	designation	description*	designation	description
M1	short, aerial	S1	at the edge	no	absence of sporulation
M2	short, tight	S2	in circle around centre	weak	less than 20 spores in field of view
M3	medium, aerial	S3	in circle around outer area	medium	20 to 50 spores in field of view
M4	mycelial masses	S4	irregularly	abundant	more than 50 spores in field of view
M5	thick, woolly				

\*Description of basic arrangement of sclerotia. Combinations of characteristics hereinafter were marked, for example, as S1;2 if sclerotia were arranged at the edge and in a circle in one plate etc.

### **Statistical processing of data**

To determine the dependence among qualitative variables, two-way contingency tables were used at a significance level of  $\alpha = 0.05$ . Programs 'R' (version 3.5.2.), 'R Studio' and 'Excel 2016' were used for data processing.

## **RESULTS AND DISCUSSION**

### **Symptoms of damages caused by *B. cinerea* on quince**

*B. cinerea* was isolated from fruits with varied symptoms of the disease. Most often, damage of *B. cinerea* appeared as rot on the fruit. The first grey mould symptoms can be recognized as brown sunken spots, sometimes with showy red halo on fruits at all growth stages. During the development of the disease, all fruit or part of it turned evenly brown or, in some cases, with alternating darker and lighter bands. It is observed that the infection of grey mould on quince fruits most often starts from stem side of fruit, sometimes from calix or other parts of quince fruit. In several cases, infection started at the point of contact between the healthy fully developed and rotted fruit. It was found that fruitlets not fallen in the first or second fruit drop and rotted, is a source of grey mould infection during the latest fruit development stages (Jakobija & Bankina, 2018). In moist conditions, grey mycelium covered the rotted surface of fruit. Similar symptoms of grey mould on fruits have been described and identified by Norin & Rumpunen (2003). The causal agent of grey mould was also isolated from wintered rotted fruits (hereinafter – mummies). Mummies from which *B. cinerea* was isolated were dense and brown, in some cases with a brown or pale rustle paper-like surface.

Taking into consideration the fact that *B. cinerea* was found within isolates obtained from damages on dead shoots of quince, it can be concluded that the pathogen can initiate the dieback on quince shoots, as described by Norin & Rumpunen (2003). The bark at the damage point cracked, became dry and separated from wood, and cambium turned dark brown. Most often, infection started from the fruit branch where wintered mummy was hanging.

The causal agent of grey mould was also isolated from the leaves of quince. Most often, *B. cinerea* was isolated from dark brown spots with concentric rings or with a cream-coloured centre and could reach a size of up to 2 mm. Spots were round or irregular, in some cases, central vein of leaf restricted an enlargement of them, located over all leaf e.g. edge. During disease development, spots merged and induced drying of damaged area or yellowing of leaf and premature leaf fall. In a few cases, *B. cinerea* was isolated from small, not more than 1 mm in diameter, red spots with a pale centre.

However, in general, the symptoms of diseases on the leaves caused by *Botrytis cinerea* are not typical and can be easily confused with those of other diseases; therefore, isolation in pure culture is necessary. Information in the literature about isolation of *B. cinerea* from the leaves of *Chaenomeles* spp. in other countries has not been found so far.

### **Morphological characterization of *B. cinerea* isolates**

Entirely 286 fungal isolates were obtained during the study, of which 39 were *B. cinerea*, which were used for the evaluation of the morphological variability.

Three days after incubation on PDA media, the first morphological differences among isolates were detected. Mycelium differed in colour from colourless, white and cream-colored to light grey and grey with entire or wavy margins. Reverse side of plates

was cream-colored or white in most cases, and colourless or grey in a few cases. The diameter of colonies varied from 2 to 9 (full plate) cm among isolates. Our data are in contradiction with other investigations; whereas in the investigations by Tanovic et al. (2014), on *B. cinerea* isolates obtained from raspberry at the beginning of incubation, all isolates showed white mycelium with an entire edge. Visible differences appeared only after six days of incubation in similar conditions.

Seven days after incubation, the white colour of mycelium changed to grey, some of isolates stayed cream-colored, but the reverse side of plates, white in previous observation, in most of cases became cream-colored. Mycelium, in most cases, filled the plate. Changes in the formation of mycelium type continued till the last assessment after three weeks of incubation.

Sclerotia-forming isolates were detected on 87.2% of all investigated *B. cinerea* isolates. Investigations of Tanovic et al. (2014) conducted in the same conditions on *B. cinerea* isolates from strawberries showed similar results, i.e., sclerotial-type isolates were observed on 81.5% of all studied isolates. Also, Kumari et al. (2014) reported that the presence or absence of sclerotia formation depends on *B. cinerea* isolate.

The first signs of sclerotia appeared as white beginnings on mycelium – after seven days of incubation, on 61.8% of isolates forming sclerotia. On 32.4% of sclerotia-forming isolates, the first visible signs of sclerotia appeared after ten days of incubation and on 5.9% – after three weeks of incubation.

Fully formed sclerotia were black, 1 to 5 mm in diameter depending on an isolate. The time to full formation of sclerotia fluctuated from 10 to more than 21 days depending on an isolate. These results conform to the findings of Mehra et al. (2019), where sclerotia appeared up to 20 days of the incubation of isolates obtained from capsicum. Different results were obtained in the research of Rasiukevičiūtė et al. (2017) where fully developed sclerotia were detected on *Botrytis cinerea* isolates from apple and strawberry already after 6 to 7 days after incubation on PDA at 20 °C in the dark. This is a reason to consider that the development of *B. cinerea* isolates obtained from Japanese quince is different compared to the development of isolates obtained from other fruits, as it was in the case with apples and strawberries. Also Rasiukevičiūtė et al. (2017) claimed that *B. cinerea* isolates obtained from different plants had variable phenotypic characteristics.

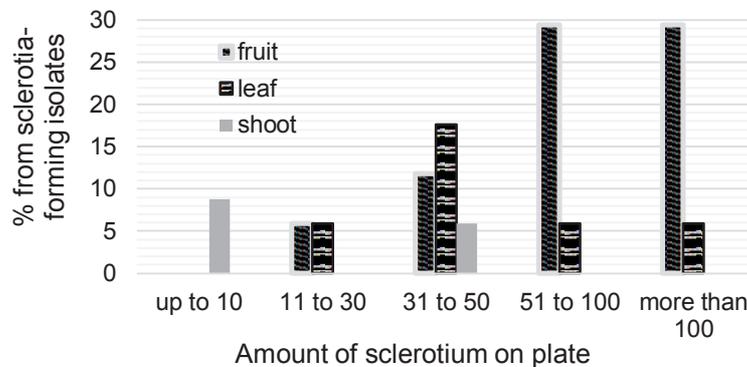
#### **Classification of *B. cinerea* sclerotia-forming isolates depending on sclerotium amount on plate**

The number of sclerotia on the plates varied from 5 to 134. Also, Tanović et al. (2009) reported that the amount of sclerotia on plates significantly differed.

Most frequently, sclerotia-forming isolates with 31 to 50 and 51 to 100 sclerotia on plate were detected – proportionally in 35% of cases for both classes. Isolates with less than 10 sclerotia on plate were found in 12% of cases, and with 11 to 30 and with more than 100 sclerotia on plate were found in 9% of cases for both classes. Also, the amount of sclerotia on plate fluctuated among the isolates obtained from different parts of Japanese quince (Fig. 1).

It was established that the amount of sclerotia on plate was significantly dependent on the part of quince from which an isolate was obtained ( $p = 0.001$ ). The largest amount of sclerotia were found on isolates obtained from the fruits of Japanese quince, and the lowest amount was found on isolates obtained from leaves and shoots. In the light of this fact, it can be assumed that infected fruits are the main source for surviving of *B. cinerea*

with sclerotia and can be an important reason for primary infection at the beginning of vegetation of Japanese quince, compared to infected leaves and shoots.



**Figure 1.** Proportion of sclerotia amount on sclerotia-forming isolates of *B. cinerea* obtained from different parts of Japanese quince.

#### Classification of *B. cinerea* isolates depending on sporulation

Sporulation was observed on 74.4% of *B. cinerea* isolates after three weeks of incubation. Tanović et al. (2009) obtained different results, where sporulation was observed only on 6.4% of *B. cinerea* isolates obtained from soft fruits in Serbia.

Most frequently (46.2% of cases), abundant sporulation was observed. Medium and weak sporulation was detected in 10.3% and 17.9% of cases respectively (Table 3), whereas in similar conditions, most frequently a medium sporulation was detected by Mehra et al. (2019).

Sporulation rate did not significantly depend on the amount of sclerotium on plate ( $p = 0.728$ ) or on the type of mycelium ( $p = 0.289$ ).

Sporulation rate fluctuated among the isolates obtained from different parts of quince (Table 3) but did not significantly depend on the part of quince from which an isolate was obtained ( $p = 0.898$ ).

**Table 3.** Proportion of sporulation rate among *B. cinerea* isolates obtained from different parts of Japanese quince

Sporulation rate	Proportion (%) among isolates obtained from:		
	fruit	leaf	shoot
Abundant	20.5	17.9	7.7
Medium	5.1	5.1	0.0
Weak	10.3	5.1	2.6
No	15.4	5.1	5.1

#### Classification of *B. cinerea* isolates depending on mycelium type and sclerotium arrangement

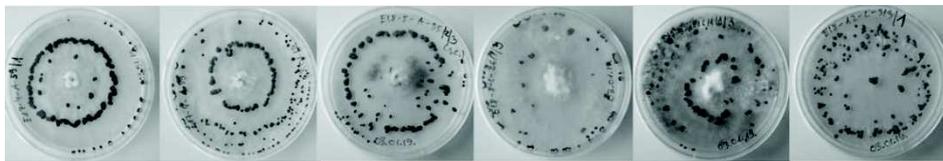


**Figure 2.** Mycelium types on *B. cinerea* isolates obtained from Japanese quince (left to right): M1 – short, aerial; M2 – short, tight; M3 – medium, aerial; M4 – mycelial masses; M5 – thick, woolly.

Five mycelium types (Fig. 2) were recorded on the isolates of *B. cinerea*. Mycelium type M2 was recorded in 26%, M3 – in 23%, M4 – in 20%, M1 – in 18%, and M5 – in 13% of cases. However, the overall proportion among different types of mycelium was equal. Three mycelium types – aerial, short, and cottony (woolly) – were distinguished among investigated isolates of *B. cinerea* obtained from blackberries by Isaza et al. (2019). This was similar to the findings of Martínez et al. (2008), who studied isolates from ornamental plants.

The research results demonstrated that the type of mycelium did not significantly depend on the part of quince from which an isolate was obtained ( $p = 0.153$ ).

Six types or combinations of *B. cinerea* sclerotium arrangement were observed (Fig. 3).



**Figure 3.** Sclerotium arrangement on *B. cinerea* isolates obtained from Japanese quince (left to right): S1;2 – at the edge, and in circle around the centre; S1;2;4 – at the edge, in circle around the centre, and irregularly; S1;3;4 – at the edge, in circle around outer area, and irregularly; S1;4 – at the edge, and irregularly; S2;4 – in circle around the centre, and irregularly; S4 – irregularly.

Irregular arrangement of sclerotia dominated (detected in 53% of cases) among all sclerotia-forming isolates. Similar results were obtained in the studies of Tanovic et al. (2014) and Kumari et al. (2014) where an irregular placement of sclerotia was found on 45% to 65% and on 58% (respectively) of *B. cinerea* isolates. The arrangement of other isolates was formed in combinations of different types.

During the study, it was found that sclerotium arrangement did not significantly depend on the part of quince from which an isolate was obtained ( $p = 0.904$ ). Also, sclerotium arrangement did not depend on the type of mycelium ( $p = 0.689$ ).

## CONCLUSIONS

1. Sclerotium amount significantly depended on the type of mycelium, and sclerotium arrangement did not significantly depend on the part of quince from which an isolate was obtained.

2. The highest amount of sclerotia was formed on isolates of *B. cinerea* obtained from fruits compared to isolates from shoots and leaves. This suggests that infected fruits could be the main source for pathogen surviving and can continuously influence the primary spread of grey mould at the beginning of vegetation period.

3. Among the isolates of *B. cinerea* after three weeks of incubation at 20 °C in the dark, abundant sporulation dominated; however, a considerable part (26%) of isolates did not sporulate under those conditions.

4. Five types of mycelium and six types or combinations of sclerotium arrangement were recorded on the isolates of *B. cinerea*, which is an evidence for a high morphological variability of *B. cinerea*.

5. All mentioned data are a justification to conduct further investigations about the traits of *B. cinerea* in Japanese quince. The results obtained in this research can be used as a visual and informative material for further studies of *B. cinerea*.

ACKNOWLEDGEMENTS. The research was financed by the ERDF project ‘Environment-friendly cultivation of emerging commercial fruit crop Japanese quince (*Chaenomeles japonica*) and waste-free methods of its processing’, Nr.1.1.1.1/16/A/094.

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