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Susceptibility of apple fruits from selected cultivars to the *Colletotrichum acutatum* species complex

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Abstract: The pathogenicity of five isolates of *Colletotrichum acutatum* species complex (CASC) was tested on artificially injured apple fruits of the cultivar 'Golden Delicious' under laboratory conditions. Then, ten apple cultivars were tested for their relative susceptibility to CASC. Differences in the pathogenicity of the CASC isolates were confirmed in the test. The highest degree of pathogenicity was showed by the isolates from the strawberry and DCSBPM J1 from the apple. No cultivars were resistant to the pathogen. The disease incidence ranged from 57.5% to 100%. The individual cultivars showed different susceptibility to the tested isolates. The cultivars 'Braeburn', 'Jonagold' and 'Rubinola' showed the least susceptibility to the pathogen, while the cultivars 'Jonagored' and 'Otava' were recorded as the most susceptible to CASC. All the tested isolates were confirmed, by the conventional PCR method, to belong to CASC. The sequences of isolates DCSBPM J1 and DCSBPM 1209 were deposited in the GenBank.

Keywords: bitter rot; *Malus domestica*; cross-infection; resistance test; virulence test

Bitter rot in apple (*Malus domestica* Borkh.) is one of the most important apple diseases and it causes significant economic losses in apple production worldwide. The disease can be generated by several different clades of the genus *Colletotrichum*. The most common clades are *Colletotrichum acutatum* species complex (CASC) and *Colletotrichum gloeosporioides* species complex (CGSC). *C. fioriniae* and *C. nymphaea* from CASC and *C. siamense*, *C. theobromicola* and *C. fructicola* from CGSC, are also known, thus far, to cause bitter rot on apple worldwide (Munir et al. 2016; Oo et al. 2018; Khodadadi et al. 2020). Problems with apple bitter rot have been encountered in the USA and Canada (Biggs & Miller 2001), New Zealand (Everett et al. 2018), Japan (Nekoduka et al. 2018), South Korea (Oo et al. 2018) and Norway (Børve & Stensvand 2015). Mari et al. (2012) reported the first occurrence of *Colletotrichum acutatum* on apple fruits in Italy. In the Czech Republic, the post-harvest infection of apple fruits by *C. acutatum* was observed in 2010 (Víchová et al. 2012) and symptoms of the disease were detected on the immature apple fruit the following year (Víchová, unpublished data).

CASC was described on the basis of numerous studies and phylogenetic analyses reporting on the phenotypic diversity within the *C. acutatum* species. Some of the species appear to have preferences to specific hosts or geographical regions. Others appear to be polyphagous and are present in multiple regions (Damm et al. 2012). Currently, CASC includes 34 species. However, the number of species is expected to increase due to the high genetic variability of the system and to the increasing number of population studies (Baroncelli et al. 2017). In general, the patho-

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gens *C. acutatum sensu lato* are polyphagous fungal pathogens and cause considerable economic losses not only in crops, but also in ornamental plants and coniferous woods throughout the world. Symptoms on apple fruit begin as small, dark brown spots 1 to 2 mm in diameter and usually on the sun-exposed side of the fruit. Sometimes, the spots are surrounded by a red halo, which can expand to cover the entire fruit surface with one to several orange, sporulating lesions. The infection eventually results in the fruit drop. Fruits that appear asymptomatic on the apple tree can nevertheless develop symptoms during post-harvest storage (Everett et al. 2015). During the growing season, mummified fruits, infected leaves, twigs, and buds are sources of infection (Børve & Stensvand 2007), with the buds being a more important source of the inoculum than the twigs. Asymptomatic infections of the vegetative and reproductive buds and leaves have been detected, with the pathogen occurring in the conidia formed on asymptomatic parts (Børve & Stensvand 2017; Everett et al. 2018). By the time the fruits begin to mature, the pathogen enters the cells through the hyphae that grow from the appressoria (Prusky et al. 2000).

The CASC species can become an increasing pre- and post-harvest problem for Czech apple growers because apples are the most cultivated fruit with a production of around 200 000 tonnes per year (Buchtová 2020). The aim of this study was to determine the pathogenicity levels of the CASC isolates and the subsequent susceptibility levels of apple fruits from selected cultivars.

MATERIAL AND METHODS

Laboratory cultivation conditions of isolates. All the isolates used in the tests were cultivated on potato dextrose agar (PDA) (HiMedia Lab, India) at a temperature of 25 °C under a 12 h photoperiod and 70% air humidity for 10 days.

Molecular identification of isolates. Monosporic isolates (Table 1) were prepared from all the pathogen isolates. The species identity of the isolates was verified by conventional polymerase chain reaction (PCR) using specific primers designed for the ITS1 a ITS2 regions of gen 5.8S rRNA. Primer CaInt2 (5'-GGG GAA GCC TCT CGC GG-3'), specific for *C. acutatum,* and primer CgInt (5'-GGC CTC CCG CCT CCG GGC GG-3') specific for *Cg* were each used in conjunction with the conserved primer ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') Garrido et al. (2009). The isolates were cultivated for 10–14 days and their DNA was extracted from the freeze-dry mycelium with a DNeasy Plant Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. The PCR procedures were conducted according to Garrido et al. (2008) and adjusted for the reaction conditions of the Taq PCR Master Mix Kit (Qiagen, Germany). The amplification process for both species-specific primers involved an initial denaturation of 3 min at 94 °C, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 2 min, a final extension at 72 °C for a 10 minutes. The amplified PCR products were electrophoresed on 1% agarose gel with a 1× TBE running buffer stained with GelRed® Nucleic Acid Gel Stain (Biotium, Inc., USA). The DNA bands were visualised in an ultraviolet (UV) transilluminator. The PCR products from the isolates were purified with a MinElute Gel Extraction Kit (Qiagen, Germany) according to the manufacturer's instructions and sequenced with the same primers. The homology searches for the resulting sequences were performed by comparing with those deposited in the GenBank Nucleotide database using BLAST.

Table 1. Isolates of the *Colletotrichum acutatum* species complex and their origins

Isolate	Received as	Host	Plant tissue	Geographic origin
CBS 786.86	*C. acutatum* J.H. Simmonds	*Malus sylvestris*	fruit	Italy
DCSBPM J1	*C. acutatum* J.H. Simmonds	*Malus domestica* 'James Grieve'	fruit	Czech Republic
PCF 231	*C. acutatum* J.H. Simmonds	*Fragaria* × *ananassa*	fruit	–
PCF 437	*C. acutatum* J.H. Simmonds	*Lupinus alba*	stem	–
DCSBPM 1209 – standard	*C. acutatum* J.H. Simmonds	*Carthamus tinctorius*	stem	Czech Republic

CBS – culture collection of the Centraalbureau voor Schimmelcultures, Fungal Biodiversity Centre, Utrecht, The Netherlands; DCSBPM – Department of Crop Science, Breeding and Plant Medicine, Mendel University in Brno, Brno, Czech Republic; PCF – Proefcentrum Fruitteelt vzw, Sint-Truiden, Belgium

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Evaluating the pathogenicity level. The pathogenicity of five isolates from CASC (Table 1) was tested on apple fruits of the cultivar 'Golden Delicious'. The isolates were cultured on PDA under conditions suitable for sporulation, at a temperature of 25 °C. The fruits were thoroughly washed to remove any post-harvest superficial infection. The surfaces were disinfected with 1% sodium hypochlorite for 2 min, then rinsed in sterile distilled water. The fruits were injured with a sterile needle and were inoculated by pipetting 10 μL of the spore suspension into the wound. Sterile distilled water was pipetted into the injuries as the control. The number of spores in the suspension was established by a Bürker chamber, and all the samples were adjusted to a uniform concentration 4.2×10^4 conidia/mL. Fruits were placed on plastic trays that had been covered with a sterile textile fabric and then with filter paper that was moistened with sterile distilled water. In this manner, a heightened humidity was subsequently maintained in the vicinity of the sample. The plastic trays with the fruits were transferred to a cultivation room and were carefully wrapped in plastic wrap for 48 hours. The incubation temperature was set at 25 °C. The experiment was established on 10 fruits in four replications. The fruits were visually evaluated at 10 days after inoculation (DAI). The pathogen was reisolated to confirm Koch's postulate. The fruits were rated using the following criteria: disease incidence (%) and disease severity (mm^2) that was measured as the diameter of the lesions and expressed as the surface of the necrotic lesions on all the apple fruits. The results were statistically processed using Unistat, using a one-way analysis of variance (ANOVA) (factor: isolate) and evaluated by Tukey's honestly significant difference test (Tukey-HSD test).

Determining the susceptibility level of the apple cultivars. The level of susceptibility was tested on apple fruits of ten cultivars ('Angold', 'Braeburn', 'Florina', 'Golden Delicious', 'Jonagold', 'Jonagored', 'Otava', 'Rubín', 'Rubinola' and 'Topaz'). The fruits of the cultivars were collected in their optimal harvest window – from mid-September to October. The testing of the susceptibility level was carried out after two months of fruit storage under optimal conditions, a temperature of 4 °C and 85% humidity. Two pathogen isolates, DCSBPM 1209 and DCSBPM J1, were chosen as the inoculum source. Isolate DCSBPM 1209 from the safflower is used as a standard in all our experiments and isolate DCSBPM J1 was a new isolate from the apple. The inoculation of the fruits, incubation conditions and replications were similar to those used in the pathogenicity test. The only differences were that all the samples were tested at a uniform concentration of 8.2×10^4 conidia/mL and the fruits were visually evaluated after seven and 14 DAI. The results were statistically processed using Statistica 12 software to perform the ANOVA and Tukey-HSD test.

RESULTS

Molecular identification of isolates. All the tested isolates were confirmed to belong to the CASC by the conventional PCR method. The sequences of the isolates DCSBPM J1 and DCSBPM 1209 were deposited in the GenBank and assigned accession numbers JN676198 for isolate DCSBPM J1 and JX876549 for isolate DCSBPM 1209. This study was focused only on CASC, without any phylogenetic breakdown by species. However, species matches were found comparing these sequences with other sequences by the BLAST program. The sequence of isolate DCSBPM J1 had 100% similarity to species *C. godetiae* whereas the sequence of isolate DCSBPM 1209 had 100% similarity to two species – *C. chrysanthemi* and *C. laticiphilum*. The sequences of isolates CBS 786.86 and PCF 231 were identical to the sequence of *C. fioriniae* and PCF 437 was matched to *C. lupini*.

Evaluating the pathogenicity level. Differences in the pathogenicity of the CASC isolates were demonstrated in the test. Significant differences were found among the individual isolates in both the disease incidence and disease severity (Table 2). The lowest disease incidence of the apple fruit was observed for the isolate from lupine. Likewise, the lowest disease severity was observed for this isolate together with the isolate from the apple (CBS 786.86). The highest disease severity was confirmed in the isolates from the strawberry (PCF 231) and apple (DCSBPM J1). In comparing the apple isolates (CBS 786.86 × DCSBPM J1), no differences in the disease incidence were found, but significant differences were detected in the disease severity. The greatest disease severity was caused by isolate DCSBPM J1.

Determining the susceptibility level of the apple cultivars. Although the isolates from both the apple and safflower attacked the fruits of the tested apple cultivars with a high disease incidence

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Table 2. Pathogenicity test for the selected *Colletotrichum acutatum* species complex isolates

Isolate	Disease incidence (%)	Disease severity (mm^2)
PCF 437	35.00^A	27.18^A
DCSBPM 1209	82.50^B	236.15^{AB}
CBS 786.86	82.50^B	196.47^A
DCSBPM J1	90.00^B	734.76^C
PCF 231	91.63^B	481.79^{BC}
Mean square	2 205.1	305 071.9

$^{A–C}$Different capital letters within a column indicate significant differences ($P \leq 0.05$) according to the Tukey-HSD test
New identification of the isolates by the PCR method: CBS 786.86 – *Colletotrichum fioriniae*; DCSBPM 1209 – *Colletotrichum chrysanthemi* or *Colletotrichum laticiphilum*; DCSBPM J1 – *Colletotrichum godetiae*; PCF 231 – *Colletotrichum fioriniae*; PCF 437 – *Colletotrichum lupine*

(Table 3), a higher susceptibility of the apple cultivars was recorded at seven DAI for the isolate DCSBPM J1 from the apple. At 14 DAI, the disease incidence was 100% in both isolates. At seven DAI, fruits inoculated with isolate DCSBPM J1 reached almost 2.5 times greater disease severity than did those inoculated with isolate DCSBPM 1209.

Due to the significant differences between the tested isolates, the evaluation of the susceptibility of the apple cultivars was performed for each isolate separately.

Table 3. Significant differences between the sources of inoculum in both evaluation terms

Isolate	7 DAI		14 DAI
	disease incidence (%)	disease severity (mm^2)	disease severity (mm^2)
DCSBPM 1209	92.25^A	86.55^A	387.91^A
DCSBPM J1	99.75^B	223.52^B	910.41^B
Mean square	1 125	375 222.5	5 460 078

DAI – days after inoculation
^{A,B}Different capital letters within a column indicate significant differences ($P \leq 0.05$) according to the Tukey-HSD test
Multi-factor ANOVA (factors: isolate, cultivar). At 14 DAI, the disease incidence was 100% in both isolates. New identification of the isolates by the PCR method: CBS 786.86 – *Colletotrichum fioriniae*; DCSBPM 1209 – *Colletotrichum chrysanthemi* or *Colletotrichum laticiphilum*; DCSBPM J1 – *Colletotrichum godetiae*; PCF 231 – *Colletotrichum fioriniae*; PCF 437 – *Colletotrichum lupine*

Significant differences in the disease incidence and disease severity were found between the tested apple cultivars after inoculation by isolate DCSBPM 1209 (Table 4). At seven DAI, the disease incidence ranged from 57.5% (cultivar 'Braeburn') to 100% (cultivars 'Angold', 'Jonagored', 'Otava', 'Rubín', and 'Topaz'). At 14 DAI, the disease incidence was 100% in all the samples. At seven DAI, the lowest disease severity was demonstrated for the cultivar 'Braeburn', but at 14 DAI, the lowest disease severity was shown for the cultivar 'Jonagold'. At seven DAI, the highest disease severity was observed for the cultivars 'Otava' and 'Jonagored'. At 14 DAI, the highest disease severity was confirmed for the cultivar 'Otava'.

In both evaluation terms, and with the exception for cultivar 'Jonagold' at seven DAI, 100% disease incidence was found after inoculation by isolate DCSBPM J1. Significant differences in the disease severity by the apple isolate were confirmed between the tested apple cultivars (Table 4). At seven DAI, the lowest disease severity was observed for the cultivar 'Jonagold', but at 14 DAI, the lowest disease severity was found for the cultivar 'Rubinola'. At seven DAI, the highest disease severity was reported for the cultivars 'Otava' and 'Jonagored'. At 14 DAI, the greatest disease severity was confirmed for the cultivar 'Otava'.

DISCUSSION

The comparison of the sequences of the isolates in the GenBank revealed similarity to other sequences belonging to specific species CASC. Damm et al. (2012) also confirmed the affiliation of isolate CBS 786.86 to the species *C. fioriniae*. We found a multi-species match of the isolate from the safflower – *C. laticiphilum* and *C. chrysanthemi*. Damm et al. (2012) stated that the Internal transcribed spacer (ITS) sequence of strain CBS 112989 belonging to *C. laticiphilum* matched 100% with AB042306 and AB042307 from the isolates from the safflower and with AJ749675 belonging to *C. chrysanthemi*. Likewise, they claimed, the ITS sequence of strain CBS 126518 belonging to *C. chrysanthemi* matched with 100% identity to AB042306 and AB042307 from the isolates from the safflower and *Chrysanthemum* spp. and with AJ749675 and AY376508 belonging to *C. laticiphilum*.

Although all the tested isolates caused bitter rot on the apple fruits, the degree of pathogenicity

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Table 4. Susceptibility of apple cultivars inoculated by the *Colletotrichum acutatum* species complex isolates

Cultivar	Isolate DCSBPM 1209			Isolate DCSBPM J1		
	7 DAI		14 DAI	7 DAI		14 DAI
	disease incidence (%)	disease severity (mm^2)	disease severity (mm^2)	disease incidence (%)	disease severity (mm^2)	disease severity (mm^2)
'Braeburn'	57.50^{A}	5.75^{A}	245.35^{AB}	100.00^{A}	168.63^{AB}	861.34^{BC}
'Jonagold'	85.00^{B}	20.822^{AB}	175.66^{A}	97.50^{A}	138.28^{A}	712.74^{AB}
'Golden Delicious'	90.00^{B}	36.83^{BC}	267.90^{AB}	100.00^{A}	198.90^{BC}	808.15^{AB}
'Florina'	95.00^{BC}	47.54^{BC}	279.87^{AB}	100.00^{A}	212.60^{C}	910.62^{BC}
'Rubinola'	95.00^{BC}	49.97^{C}	314.84^{B}	100.00^{A}	184.61^{BC}	608.02^{A}
'Rubín'	100.00^{C}	98.67^{D}	430.57^{C}	100.00^{A}	217.03^{C}	936.82^{BCD}
'Topaz'	100.00^{C}	112.33^{D}	467.15^{C}	100.00^{A}	186.24^{BC}	850.04^{BC}
'Angold'	100.00^{C}	117.46^{D}	527.80^{C}	100.00^{A}	266.39^{D}	1 072.78^{CDE}
'Otava'	100.00^{C}	181.10^{E}	643.97^{D}	100.00^{A}	322.69^{E}	1 192.36^{E}
'Jonagored'	100.00^{C}	195.03^{E}	525.80^{C}	100.00^{A}	339.85^{E}	1 151.18^{DE}
Mean square	725.3	17 180.4	92 921.9	8.5	17 402.2	138 466.1

DAI – days after inoculation

$^{A–E}$Different capital letters within a column indicate significant differences ($P \leq 0.05$) according to the Tukey-HSD test One-way ANOVA (factor: cultivar). At 14 DAI, the disease incidence was 100% in both isolates. New identification of the isolates by the PCR method: PCF 437 – *Colletotrichum lupine*; DCSBPM 1209 – *Colletotrichum chrysanthemi* or *Colletotrichum laticiphilum*; CBS 786.86 – *Colletotrichum fioriniae*; DCSBPM J1 – *Colletotrichum godetiae*; PCF 231 – *Colletotrichum fioriniae*

differed among them. As a result, the cross-infection and polyphagous character of the pathogen were confirmed. Oo et al. (2018) showed, in their pathogenicity testing, that all the tested isolates from the *Colletotrichum* species were pathogenic to wounded apples. The authors found that isolates belonging to the CGSC produced larger lesions than did the isolates belonging to the CASC. Munir et al. (2016) came to similar results. The pathogenicity tests of Grammen et al. (2019) also showed that isolates coming from strawberries and the cherry, are also pathogenic on apple fruits. The pathogenicity tests in their study were performed by wounding fruits while they said the removal of the physical barrier might result in a higher virulence of the fungal isolates, might affect the fungal growth rates and can result in differences in susceptibility compared to unwounded fruits. Staňková et al. (2011), in their virulence tests, found that CASC isolates from the safflower were able to infect strawberry and pepper fruits and confirmed the cross-infection ability of other CASC isolates – CBS 786.86, PCF 231 and PCF 437.

In commercial apple cultivars, there are still no varieties resistant to CASC. Jurick et al. (2011) studied resistance in wild apples (working with an apple germplasm collection from a centre of origin in Kazakhstan, maintained in Geneva, New York) and they found that of 271 Kazakh accessions, 94.4% were susceptible to CASC, 4% moderately resistant and only 1.4% were resistant. These data are important for conventional breeding programmes. We also found that none of our tested commercial apple cultivars are resistant to the pathogen isolates. There nevertheless exist different degrees of susceptibility between them. The disease incidence in the individual cultivars ranged from 57.5% to 100%.

The most cultivated apple cultivar in the world – 'Golden Delicious' – showed high disease incidence in our test. Regarding the infection severity, however, it was one of the cultivars with low to medium-high values relative to the others. Biggs and Miller (2001) had classified cultivar 'Golden Delicious' into a moderately susceptible group. Their values for the laboratory severity are close to our values for the disease severity in the second evaluation term. Yoder and Biggs (2019), in their compiled list of apple cultivar susceptibility to bitter rot, stated that the cultivar 'Golden Delicious' is highly susceptible and the cultivars 'Braeburn' and 'Jonagold' are susceptible.

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The following may be concluded, based on our findings: all ten of the tested apple cultivars were susceptible to pathogens from CASC. However, a different level of susceptibility was confirmed for the tested apple cultivars. The cultivars 'Braeburn', 'Jonagold' and 'Rubinola', as the least susceptible to the tested isolates of the pathogen, could be recommended for further studies on a similar topic. On the other hand, more severe occurrences of bitter rot could be recorded in the cultivars 'Jonagored' and 'Otava' that were classified as the most susceptible to the isolates in our study. These results could help not only growers for better orientation in the susceptibility of apple cultivars to pathogens of bitter rot, but also breeders. Breeding for resistance is one of the possible ways of controlling bitter rot.

As part of the testing, a study of the pathogenicity of the isolates from CASC was also included. Isolates were obtained from different host tissues of a wider range of host plants. It was proven that the isolates were shown to be capable of cross-infection, thus confirming the polyphagous ability of the tested isolates despite the genetic differences between the individual species of the *Colletotrichum acutatum* complex.

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