Identification and pathogenicity assessment of *Colletotrichum* isolates causing bitter rot of apple fruit in Belgium

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ABSTRACT

Worldwide *Colletotrichum* spp. have been identified as a problem in the apple production. This is the first study executed and confirming the presence of *Colletotrichum* spp. causing the postharvest disease bitter rot on apple fruits in Belgium. The identification, genetic diversity of *Colletotrichum* isolates (present in Belgian apple orchards) their morphological traits and pathogenicity on two apple cultivars (cvs. Pinova and Nicoter) with a different level of susceptibility were studied. Based on sequence analysis of six different gene regions beta-tubuline (TUB2), histone H3 (HIS3), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), chitin synthase 1 gene (CHS-1), actin (ACT) and the Internal Transcriber Spacer (ITS) gene region, six different *Colletotrichum* spp., belonging to either the *C. acutatum* or *C. gloeosporioides* complexes, were isolated from twenty-one apple cultivars in three Belgian orchards: *C. fiorinae*, probably *C. kahawae*, *C. salicis*, *C. rhombiforme*, *C. acutatum* and *C. godetiae*. *Colletotrichum godetiae* was found to be the most present and pathogenic species in Belgian orchards. The species *C. rhombiforme* was found and identified on apple fruit for the first time. Reliable morphological discrimination between species, based on features such as in vitro growth rate, colony colour and spore measurements, is not possible. As such, molecular identification appears to outperform morphological analysis and was in this study the most ideal tool for identifying unknown isolates of *Colletotrichum* species. Inoculation assays on two apple cultivars revealed a significant difference in pathogenicity among isolates and among *Colletotrichum* species. The pathogenicity tests also showed that isolates coming from another host species, e.g. strawberry, are also pathogenic on apple fruits. Cultivar Pinova appeared to be more susceptible to bitter rot than cv. Nicoter. Given the difficulties with managing *Colletotrichum* infections, additional knowledge on the pathogen and the plant-pathogen interaction is essential for effective disease control.

KEYWORDS

Postharvest fungal disease; apple bitter rot; molecular multilocus phylogeny; inoculation assays

Introduction

Colletotrichum spp. are stated to be one of the most important polyphagous fungal pathogens worldwide to cause anthracnose on vegetables, wheat, fruit and ornamental plants (Dean et al. 2012; Alaniz et al. 2015; Cannon et al. 2012; Freeman et al. 1998). Bitter rot of apple fruit caused by *Colletotrichum* spp. can become an increasing postharvest problem in Belgium. The exact loss in the apple production industry is still not clear today, due to a lack of research and knowledge. Neighbouring European countries such as Slovenia, England, The Netherlands, Norway and France are sending out increasing numbers of first reports over the past years describing shortly the upcoming occurrence of *Colletotrichum* spp. on apple fruits (Munda 2014; Munir et al. 2016; Wenneker et al. 2016; Børve and Stensvand 2015; Nodet et al. 2016). Studies executed in Sweden and Germany showed a postharvest loss of apple fruits of respectively 25 and 10% by *Colletotrichum acutatum* (Weber and Palm 2010; Børve and Stensvand 2015). Worldwide Velho et al. (2015) describes that up to 50% of the apple production can be lost due to bitter rot. This pathogen *Colletotrichum* is able to cause pre- and postharvest diseases on apple. In northern regions, due to temperature causes, only postharvest problems are noticed until now. Disease symptoms of bitter rot on apple fruits start with the development of small dark brown spots expanding to light brown sunken lesions during or after storage. Afterwards, on the host, conidia are formed in acervuli concentrically in the centre of the lesion (Agrios 2005a; Damm et al. 2012).

In Europe *C. godetiae* and *C. fioriniae*, belonging to the *C. acutatum* species complex, are the main species contributing to this bitter rot problem and symptoms on apple fruit (Ivic et al. 2013; Baroncelli et al. 2014; Munda 2014; Velho et al. 2015; Wenneker et al. 2016; Nodet et al. 2016). In more southern regions, such as Brazil and the USA, both species of the *C. acutatum* and *C. gloeosporioides* complexes are stated to be the causal agents (Crusius et al. 2002; Gonzales et al. 2006; Munir et al. 2016). So in general both the *Colletotrichum acutatum* species complex and *C. gloeosporioides* complex, have been associated with bitter rot on apple (Alaniz et al. 2015; Baroncelli et al. 2015; Kou et al. 2013; Velho et al. 2015). Which specific *Colletotrichum* spp. are associated with the problem of bitter rot in Belgium nowadays, is still unknown.

Taxonomical delineation and naming of the different species belonging to the *Colletotrichum* genus has always been difficult and complex (Cannon et al. 2012). Until 1957, the speciation was only based on the host from which it was isolated (Corda 1831). From then on, von Arx proposed to base the species name on morphological characteristics, such as size and shape of conidia and appressoria, colony colour and growth rate, making a reduction from 750 to 11 species (von Arx 1957). Those characteristics were not reliable for species identification. In 1980 *Colletotrichum* species were revised, combining morphological with pathological characteristics (Sutton 1980), which resulted in a distinction between *C. gloeosporioides* and *C. acutatum*. Afterwards these species were actually considered to be species complexes (*C. gloeosporioides* sensu lato and *C. acutatum* sensu lato), based on molecular and morphological analysis, and containing different ‘new’ species such as *C. fioriniae* and *C. godetiae* (Damm et al. 2012).

At present, pathogens are identified using molecular methods alongside morphological characterization (Cai et al. 2009). Molecularly, different gene regions have been used and show potential to make a clear distinction between several *Colletotrichum* spp., including *C. acutatum* and *C. gloeosporioides* complexes, with DNA sequence analysis: Velho et al. (2015) used three gene regions (ITS, GAPDH and TUB2), Ivic et al. (2013) only used ITS1 and ITS2 regions, Ismail et al. (2015) used TUB2 and HIS3, Baroncelli et al. (2015) ITS, TUB2, MAT1–2 and GAPDH, and Munir et al. (2016) used ITS, GAPDH and TUB2. All these authors found a clear identification of the isolates in their studies.

The suggested increase of this *Colletotrichum* problem in Europe can be explained by different hypothetical aspects such as the use of more specific fungicides before harvest, differences in sensitivity for fungicides, cross-infections of different host plants and/or the effect of climate change (Munir et al. 2016; Velho et al. 2015; Runion 2003; Phoulivong et al. 2012). A first important step is to identify the true species present in orchards. *Colletotrichum* species differ in pathogenicity and in their response to fungicides, making it necessary to study fungicide sensitivity (Adaskaveg and Hartin 1997; Bernstein et al. 1995; Bragança et al. 2016; Freeman et al. 1998; Peres et al. 2002, 2004; Valero et al. 2010; Velho et al. 2014; Munir et al. 2016). By doing this, management in orchards can be optimized.

Bitter rot of apple fruit is an increasing problem in Belgium, where no information is available about the causal agent of this disease. Therefore, the main goal of this contribution is to gain first insights in the different *Colletotrichum* spp. present in Belgian apple orchards. The specific objectives are: 1) identify the species of *Colletotrichum* that are associated with bitter rot on apple based on molecular and morphological characteristics, and 2) determine the pathogenicity of different isolates on a more (cv. Pinova) and less (cv. Nicoter) susceptible apple cultivar. Outcomes of this study will lead to an increased understanding of this disease, which could aid the development of improved management strategies.

Materials and methods

Sampling and preparation of the fungal isolates

In September and October 2014, fruits from 21 different apple cultivars (Alkmene, Braeburn, Cox Orange Pippin (Cox O.P.), Elstar, Eysden, Gala, Gloster, Golden Delicious, Granny Smith, Henri Van Dievoet, Idared, Jonathan, Keuleman, Merlijn, Marie Joseph d'Othée, Nicoter, Nicogreen, Pinova, Red Prince and Topaz) were collected at the optimal harvest window for cold storage (based on the Streif index for long term storage of apple fruit (DeLong et al. 1999)) from three different professional managed dwarf tree orchards in Belgium (50° 59' N, 4° 50' E; 50° 46' N, 5° 11' E; 50° 44' N, 5° 45' E). Forty fruits of each cultivar were randomly picked and stored at room temperature with a 15/9-h day-night light regime and relative humidity of 60%. Twice a week apples were examined for the presence of brown concentric necrotrophic lesions of at least 15 mm, which morphologically resembled symptoms of *Colletotrichum*. These lesions appeared approximately after one month. Apples with other typical postharvest infections of fruit pathogens, such as *Monilinia* spp. and *Penicillium* spp., were excluded from this study. Fungi from the necrotrophic lesions were isolated by disinfecting the infected area with 70% ethanol followed by cutting out a small fruit part at the edge of the lesion (approximately 5 mm × 5 mm × 8 mm). The apple tissues were positioned with the inner part on fructic agar medium (166.67 ml apple juice, 1.67 g CaCO₃, 6.25 g agar (Fisher Scientific), 6.25 g Potato Dextrose Agar (PDA; Sigma Aldrich)) supplemented with streptomycin and tetracycline (working solution of 150 µg/ml) to suppress the growth of bacteria. Isolations were incubated at room temperature for at least 14

days, with a 15/9-h day-night light regime and relative humidity of 60%. Pure cultures were prepared based on the ‘hyphal tips isolation’, because not all the isolates sporulated easily. This method is described to have an equal efficiency to produce pure isolates and demands less time (Leyronas et al. 2012). To store the isolates, small cubical blocks of medium, containing mycelium, were excised and conserved in 25% glycerol at -80°C .

A total of 99 isolates were used in this study, 85 isolates were collected from apple fruits as described above, and 14 isolates from different hosts (Table 1). One isolate from cherry and 13 isolates from strawberry in Belgium were obtained from the Research Station of Fruit Cultivation, Sint-Truiden, for genetic comparison between isolates from different hosts.

Table 1: The 99 fungal isolates reported in this study: host fruit, the location of the orchard, year of sampling and the identified species. Fourteen *Colletotrichum* isolates were selected for further analysis (marked with *).

Isolate	Host (cultivar)	Location orchard	Year	Species
Cherry-1*	Cherry	Sint-Truiden	2008	<i>C. godetiae</i>
Apple-1	Pinova	Sint-Truiden	2013	<i>C. godetiae</i>
Apple-2	Golden	Sint-Truiden	2014	<i>C. godetiae</i>
Apple-3	Golden	Sint-Truiden	2013	<i>C. godetiae</i>
Apple-4	Golden	Sint-Truiden	2013	<i>C. fioriniae</i>
Apple-5	Golden	Sint-Truiden	2013	<i>C. fioriniae</i>
Strawberry-1	Strawberry	Sint-Truiden	2014	<i>C. nymphaeae</i>
Strawberry-2*	Strawberry	Sint-Truiden	2014	<i>C. nymphaeae</i>
Strawberry-3	Strawberry	Sint-Truiden	2014	<i>C. nymphaeae</i>
Strawberry-4	Strawberry	Sint-Truiden	2014	<i>C. nymphaeae</i>
Strawberry-5	Strawberry	Sint-Truiden	2014	<i>C. nymphaeae</i>
Strawberry-6	Strawberry	Sint-Truiden	2014	<i>C. nymphaeae</i>
Apple-6	Alkmene	Rillaar	2014	<i>C. kahawae</i>
Apple-7	Alkmene	Rillaar	2014	<i>C. kahawae</i>
Apple-8	Braeburn	Rillaar	2014	<i>C. godetiae</i>
Apple-9	Braeburn	Rillaar	2014	<i>C. godetiae</i>
Apple-10	Braeburn	Rillaar	2014	<i>C. salicis</i>
Apple-11	Braeburn	Rillaar	2014	<i>C. kahawae</i>
Apple-12	Braeburn	Rillaar	2014	<i>C. salicis</i>
Apple-13	Cox O.P.	Rillaar	2014	<i>C. salicis</i>
Apple-14*	Cox O. P.	Rillaar	2014	<i>C. fioriniae</i>
Apple-15	Cox O. P.	Bolbeek	2014	<i>C. godetiae</i>
Apple-16	Cox O. P.	Bolbeek	2014	<i>C. godetiae</i>
Apple-17	Cox O. P.	Bolbeek	2014	<i>C. godetiae</i>
Apple-18	Elstar	Rillaar	2014	<i>C. godetiae</i>
Apple-19*	Elstar	Bolbeek	2014	<i>C. godetiae</i>
Apple-20	Eysden	Rillaar	2014	<i>C. kahawae</i>
Apple-21	Eysden	Rillaar	2014	<i>C. godetiae</i>
Apple-22*	Gala	Rillaar	2014	<i>C. godetiae</i>
Apple-23	Gloster	Rillaar	2014	<i>C. salicis</i>
Apple-24	Golden	Rillaar	2014	<i>C. salicis</i>
Apple-25	Golden	Rillaar	2014	<i>C. salicis</i>
Apple-26	Granny Smith	Bolbeek	2014	<i>C. rhombiforme</i>
Apple-27	Granny Smith	Bolbeek	2014	<i>C. godetiae</i>
Apple-28	Granny Smith	Bolbeek	2014	<i>C. rhombiforme</i>
Apple-29	Granny Smith	Bolbeek	2014	<i>C. godetiae</i>
Apple-30	Granny Smith	Bolbeek	2014	<i>C. godetiae</i>
Apple-31*	Henri Van Dievoet	Rillaar	2014	<i>C. kahawae</i>
Apple-32	Henri Van Dievoet	Rillaar	2014	<i>C. kahawae</i>
Apple-33	Henri Van Dievoet	Rillaar	2014	<i>C. kahawae</i>
Apple-34	Idared	Rillaar	2014	<i>C. salicis</i>
Apple-35	Idared	Rillaar	2014	<i>C. salicis</i>
Apple-36	Idared	Rillaar	2014	<i>C. godetiae</i>
Apple-37	Idared	Rillaar	2014	<i>C. godetiae</i>
Apple-38	Idared	Rillaar	2014	<i>C. salicis</i>
Apple-39	Idared	Rillaar	2014	<i>C. salicis</i>
Apple-40	Idared	Rillaar	2014	<i>C. godetiae</i>
Apple-41	Idared	Rillaar	2014	<i>C. godetiae</i>
Apple-42	Idared	Rillaar	2014	<i>C. godetiae</i>
Apple-43	Idared	Rillaar	2014	<i>C. salicis</i>
Apple-44	Jonathan	Rillaar	2014	<i>C. salicis</i>
Apple-45	Jonathan	Rillaar	2014	<i>C. salicis</i>
Apple-46	Jonathan	Rillaar	2014	<i>C. salicis</i>
Apple-47	Jonathan	Rillaar	2014	<i>C. salicis</i>
Apple-48*	Merlijn	Rillaar	2014	<i>C. fioriniae</i>
Apple-49*	Marie Josée Othée	Rillaar	2014	<i>C. kahawae</i>
Apple-50*	Marie Josée Othée	Rillaar	2014	<i>C. salicis</i>
Apple-51*	Marie Josée Othée	Rillaar	2014	<i>C. rhombiforme</i>
Strawberry-7	Strawberry	Rummen	2008	<i>C. fioriniae</i>
Strawberry-8	Strawberry	Geetbets	2008	<i>C. godetiae</i>
Strawberry-9	Strawberry	Diepenbeek	2008	<i>C. godetiae</i>
Strawberry-10	Strawberry	Grazen	2008	<i>C. godetiae</i>
Strawberry-11	Strawberry	Widoote	2008	<i>C. nymphaeae</i>
Strawberry-12*	Strawberry	Borgloon	2008	<i>C. nymphaeae</i>
Strawberry-13*	Strawberry	Sint-truiden	2008	<i>C. salicis</i>
Apple-59*	Pinova	Sint-Truiden	2008	<i>C. acutatum</i>
Apple-60	Pinova	Rillaar	2014	<i>C. godetiae</i>
Apple-61	Pinova	Rillaar	2014	<i>C. godetiae</i>
Apple-62	Pinova	Rillaar	2014	<i>C. salicis</i>
Apple-63*	Pinova	Sint-Truiden	2014	<i>C. rhombiforme</i>

Apple-64	Topaz	Rillaar	2014	<i>C. godetiae</i>
Apple-65	Topaz	Rillaar	2014	<i>C. godetiae</i>
Apple-66	Jonathan	Rillaar	2014	<i>Fusarium</i> spp.
Apple-67	Marie Josée Othée	Rillaar	2014	<i>Fusarium</i> spp.
Apple-68	Alkmene	Rillaar	2014	<i>Fusarium</i> spp.
Apple-69	Jonathan	Rillaar	2014	<i>Fusarium</i> spp.
Apple-70	Jonathan	Rillaar	2014	<i>Fusarium</i> spp.
Apple-71	Gloster	Rillaar	2014	<i>Fusarium</i> spp.
Apple-72	Pinova	Bolbeek	2014	<i>Fusarium</i> spp.
Apple-73	Pinova	Bolbeek	2014	<i>Fusarium</i> spp.
Apple-74	Pinova	Bolbeek	2014	<i>Fusarium</i> spp.
Apple-75	Nicoter	Rillaar	2014	<i>Fusarium</i> spp.
Apple-76	Gloster	Rillaar	2014	<i>Fusarium</i> spp.
Apple-77	Pinova	Rillaar	2014	<i>Fusarium</i> spp.
Apple-78	Pinova	Sint-Truiden	2014	<i>Neofabraea</i> spp.
Apple-79	Henri Van Dievoet	Rillaar	2014	<i>Neofabraea</i> spp.
Apple-80	Pinova	Merdorp	2014	<i>Neofabraea</i> spp.
Apple-81	Pinova	Merdorp	2014	<i>Neofabraea</i> spp.
Apple-82	Pinova	Merdorp	2014	<i>Neofabraea</i> spp.
Apple-83	Pinova	Merdorp	2014	<i>Neofabraea</i> spp.
Apple-84	Topaz	Merdorp	2014	<i>Neofabraea</i> spp.
Apple-85	Pinova	Rillaar	2014	<i>Neofabraea</i> spp.
Apple-86	Pinova	Merdorp	2014	<i>Neofabraea</i> spp.
Apple-87	Cox	Rillaar	2014	<i>Alternaria</i> spp.
Apple-88	Pinova	Merdorp	2014	<i>Alternaria</i> spp.
Apple-89	Pinova	Merdorp	2014	<i>Alternaria</i> spp.
Apple-90	Alkmene	Rillaar	2014	<i>Alternaria</i> spp.
Apple-91	Nicoter	Rillaar	2014	<i>Alternaria</i> spp.
Apple-92	Nicoter	Rillaar	2014	<i>Penicillium</i> spp.

133 Molecular identification of *Colletotrichum* isolates

134 The 99 isolates were transferred onto liquid fructic medium (166.67 ml apple juice, 1.67 g
135 CaCO₃, 6.25 g Potato Dextrose Broth (PDB; Sigma Aldrich)) in order to obtain sufficient mycelium for
136 DNA extraction. After 14 days at room temperature, the mycelium was harvested from the medium, put
137 into 2 ml tubes, centrifuged at 8000 rpm (Hettich, MIKRO 220R), followed by removing the
138 supernatant. The mycelium was washed twice with sterile distilled water, centrifuged at 8000 rpm; the
139 supernatant was removed at each washing step. The mycelium was finally frozen in liquid nitrogen and
140 freeze dried during 72 h before storage at −20 °C.

141 DNA extraction was performed on 13 mg freeze dried material in a 2 ml tube. Four beads were
142 added to each sample and put in the bead beater (Fastprep Precellys 24® (Bertyn)) for 30 s at a speed
143 of 6.5 m s^{−1}. The protocol of the DNeasy Plant Mini Kit (QIAGEN) was followed according to the
144 manufacturer's instructions, except for an additional washing step: the extract derived from the
145 QIAshredder® (purple) column was washed with chloroform and centrifuged for 3 min at 10200 rpm.
146 The upper phase was used to continue with the QIAGEN® protocol. Finally, DNA was quantified by

measuring the absorbance at 260 nm using a NanoDrop® spectrophotometer (2000 UV-Vis, Thermo Fisher Scientific, Inc.).

In order to allow selection of pure *Colletotrichum* isolates, the Internal Transcriber Spacer (ITS) region consisting of the 5.8S rRNA gene with two flanking internal transcribed spacers (UN-UP18S-42 and UN-LO28S-22), was amplified per isolate using the primers of Gariepy et al. (2003) (Bakkeren et al. 2000; Cannon et al. 2012). To identify the *Colletotrichum* isolates at species level, PCR amplifications with *Colletotrichum* specific primers were performed for the beta-tubuline (TUB2) (O'Donnell and Cigelnik 1997; Glass and Donaldson 1995), histone H3 (HIS3) (Crous et al. 2004), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Guerber et al. 2003), chitin synthase 1 gene (CHS-1) (Carbone and Kohn 1999) and actin (ACT) (Carbone and Kohn 1999) genes. Each PCR reaction contained: 0.2 mM dNTP, 3 mM MgCl₂, 1X PCR buffer, 0.6 µM of each primer, 1 U Taq DNA polymerase (Promega) and 8.33 µl template DNA (concentration of 1 ng µl⁻¹). The final volume of the PCR master mix was adjusted to 50 µl with distilled water. PCR thermocycling conditions were the same as in the reference from which primers were selected. Quality and quantity of the PCR product was checked on a 1% agarose gel (1.5 g agarose in 150 ml 1X TAE buffer with 7.5 µl ethidium bromide) which ran for 30 min at 120 V and 500 mA.

For each gene region, PCR products were purified and sequenced with the forward primer at MacroGen Inc. (the Netherlands). Raw sequence data were edited using Seqman (Lasergene® DNASTAR) and aligned using the ClustalW alignment tool (MEGA 6.0) with default alignment parameters (gap opening/gap extension costs of 15/6.66) (Tamura et al. 2013). In order to identify *Colletotrichum* isolates, all isolate sequences were subjected to BLASTn analysis (GenBank, National Center for Biotechnology Information) (Boratyn et al. 2013), taking into account the limitations as describe in Raja et al. (2017) and using a curated molecular database, Q-bank (Crous et al. 2015). The phylogenetic analyses of the data were performed by using the software MEGA 6.0. *Colletotrichum lindemuthianum* was added as outgroup. Reference isolates for each group of species is shown in Suppl. Table 1 (Damm et al. 2012; Martin-Felix et al. 2017). Phylogenetic trees were generated from each gene separately and by combining the six genes in a multilocus alignment together with the reference isolates

(Suppl. Table 1). Trees were constructed using the maximum likelihood bootstrap (100 bootstrap replications) statistical method in MEGA 6.0. Pairwise uncorrected p-distances between *Colletotrichum* species were computed using MEGA 6.0. Insertions and deletions were pairwise deleted. All obtained sequences were deposited in GenBank (Suppl. Table 2) and the alignments and phylogenetic trees in TreeBASE. The interspecific and intraspecific minimum-maximum of uncorrected p-distances was determined for each gene region, giving information about the variation that exists between species and between isolates of each species.

Morphological comparison of *Colletotrichum* isolates

One or two pure isolates of each of the different *Colletotrichum* spp. were selected for morphological assessment. This resulted in a total of fourteen *Colletotrichum* isolates (Table 1). The isolates were grown on PDA at 22 °C in darkness for hyphal tips isolation. An agar plug with actively growing mycelium (colony diameter at least 5 mm diameter) was excised from the growing edge and put in the centre of a new PDA plate. Five new Petri dishes for each isolate were made from a single plate, constituting technical replicates. Colony diameter (two perpendicular diameters) was measured after five and ten days. Colony colour was evaluated visually after ten days.

Conidia of the selected isolates from 14-day-old colonies were studied microscopically. A conidial spore suspension was made by flooding the Petri dish with 5 ml distilled water, scraping over the mycelium and filtering the solution through a medical compress and sieve. The conidial spore suspension was diluted with distilled water until a spore concentration of 5.0×10^5 conidia ml⁻¹ and a minimal volume of 10 ml was reached. Conidial spores were counted using a haemocytometer under the light microscope. Isolates were given an additional near UV treatment (360 nm wavelength) (Philipps, type TL 8 W BLB) of 16 h in order to increase the sporulation until at least 4.0×10^5 conidia ml⁻¹ were recovered. For each isolate, the length and width of 60 randomly selected conidial spores were measured using a microscope coupled to a digital camera (ColorView Soft Imaging System digital camera) and measuring software Cell B (Olympus Europe). Conidia were classified according to Velho et al. (2015): cylindrical, fusiform or obclavate.

Statistical analysis was performed on each of the morphological features, except for colony colour, to test whether different isolates showed significant differences. Prior to a one-way analysis of variance (ANOVA), homogeneity of variances was checked for each dataset. Pairwise comparisons were performed using a post-hoc Tukey HSD test. All statistical analyses were performed using the statistical software JMP Pro® (SAS Institute Inc. 2013).

Pathogenicity of *Colletotrichum* isolates on apple

Preliminary inoculation experiments on ten apple cultivars (Pinova, Nicoter, Nicogreen, Braeburn, Golden Delicious, Henri Van Dievoet, Marie Joseph d'Othée, Gloster, Idared and Topaz) revealed that cv. Pinova apples were most susceptible (based on lesion diameter) to *Colletotrichum* infections and cv. Nicoter fruits were least susceptible. Both cultivars were hence collected at their optimal harvest window (based on Streif-index): 29th of September 2015. Last fungicide treatments before harvest on trees of both cultivars were with Frugico® (active ingredient (a.i.) diethofencarb (9/07/2015), Bellis® (a.i. pyraclostrobin and boscalid) (16/07/2015) and Switch® (a.i. fludioxonil and cyprodinil) (5/08/2015) (standard practices against storage rot), with Delan® (a.i. dithianon) (30/06/2015) against scab or mildew. Prior to the experiment, all fruits were stored four months in a completely dark storage room at 1 °C. After storage, fruits were surface-sterilized in 5% sodium hypochlorite (bleach) for two minutes, rinsed in distilled water for two minutes and then air-dried at room temperature. Before infection, fruits were wounded (depth: 4 mm) with a needle on three locations around the equator, separated by 120°. Six fruits were used for each isolate-cultivar combination. The 14 selected *Colletotrichum* isolates, representing the different *Colletotrichum* spp., were grown on PDA for 14 days followed by a near UV light treatment for 16 h prior to inoculation. Spore solutions were made for each isolate as described above. Artificial infection was performed by injecting a 5 µl drop of spore solution (5.0×10^5 conidia ml⁻¹) in the wound, while control fruits were injected with 5 µl sterile distilled water. Fruits were placed in a dark incubation room at 22 °C and a constant high relative humidity of 95%. The development of the necrotic lesions was examined by measuring two perpendicular diameters at 4, 8, 12 and 15 days post infection (dpi). The experiment was performed twice with two weeks of time separation (repetitions).

Fungi were re-isolated from the lesions on fructic agar medium. To confirm the true identity of the isolations, both the ACT and GAPDH gene region were sequenced and compared with the sequences of the isolate with which fruits were infected, thus completing Koch's postulates (Agrios 2005b).

The lesion diameter of each isolate per fruit was obtained by taking the mean diameter of the three wounds. Per experiment, each isolate-cultivar combination was performed on six apples (replicates). Both experiments were analysed by means of a multiple analysis of variance (MANOVA), after checking for normality and homogeneity of variances. MANOVA allowed us to analyse three different factors: experiment, apple cultivar and isolate and revealed that 'experiment' did not contributed significantly. Therefore, data of both experiments were combined into a single dataset and analysed using a two-way ANOVA (factor apple cultivar and isolate and interaction) to gain better insight in differences between the apple cultivars and the pathogenicity of the different isolates. Pairwise comparison was performed using the Tukey HSD test.

Results

Molecular identification of *Colletotrichum* isolates

Out of the 99 isolates 72 were identified as *Colletotrichum* spp., 12 isolates were identified as *Fusarium* spp., nine as *Neofabraea* spp., five as *Alternaria* spp. and one isolate was identified as *Penicillium* spp. based on the ITS sequence. The 72 *Colletotrichum* isolates were identified further at species level based on TUB2, HIS3, GAPDH, CHS-1 and ACT sequence fragments. The multigene analysis was generated by combining the DNA fragments of the six gene regions mentioned above, respectively approximately 600, 700, 400, 300, 300 and 300 bp. The alignment used for the phylogenetic analysis had a length of 2011 bp. In total, seven *Colletotrichum* species were found by this multigene comparison: *C. fioriniae*, probably *C. kahawae*, *C. nymphaeae*, *C. salicis*, *C. rhombiforme*, *C. acutatum* and *C. godetiae*. On apple, 58 isolates were identified: *C. godetiae* (20 isolates), *C. salicis* (19), probably *C. kahawae* (9), *C. fioriniae* (5), *C. rhombiforme* (4) and *C. acutatum* (1). The isolate from cherry fruit was identified as *C. godetiae* (1). Thirteen isolates from strawberry were characterised as follows: *C. nymphaeae* (8), *C. godetiae* (3), *C. fioriniae* (1) and *C. salicis* (1). Out of these available *Colletotrichum* samples, 14 isolates were selected (Table 1) for further experiments: two isolates of *C. salicis*, *C. godetiae*, *C. fiorinia*, *C. rhombiforme*, probably *C. kahawae* and *C. nymphaeae*, one isolate of *C. acutatum* and one cherry isolate of *C. nymphaea*.

Of these seven species, only *C. kahawae* belonged to the *C. gloeosporioides* species complex (Weir et al. 2012), while the six other species belonged to the *C. acutatum* species complex (Damm et al. 2012), which was confirmed in the phylogenetic tree (Fig. 1). Both species complexes were separated in distinct clades with a high support (bootstrap value of 98–99). Bootstrap values for *C. fioriniae* were rather low (90). Meanwhile, both *C. salicis*, *C. godetiae* and *C. nymphaeae* isolates had a bootstrap value of 99. It must be noted that the topology of the species in the *C. acutatum* species complex remains ambiguous (Fig. 1). When looking at the single-gene results and phylogenetic trees (Supplementary Figs. 2, 3, 4, 5, 6 and 7), discrimination between the *C. gloeosporioides* species complex and *C. acutatum* species complex is possible with high support in the single sequences from ITS, ACT and

GAPDH. Onto species level, the ACT and HIS3 gene regions showed a good separation for all the individual *Colletotrichum* spp. and was highly supported (bootstrap 98–100) (Supplementary Fig. 2). This was in contrast with single-gene region phylogenetic trees based on ITS, GAPDH and CHS-1 (Supplementary Figs. 3, 4 and 5), which showed low bootstrap values and no good support for clustering the *Colletotrichum* spp. The single-gene region phylogenetic tree of TUB2 did not give reliable results at all, since the different *Colletotrichum* spp. did not cluster but were spread throughout the whole tree. The phylogenetic tree combining ACT, HIS3 and GAPDH (Supplementary Fig. 1) showed that a good separation for almost all the seven *Colletotrichum* species is possible, except for *C. salicis* and *C. godetiae*.

Overall, the lowest and highest interspecific minimum-maximum of uncorrected P-distances were, respectively, 0.0–4.9% for TUB2 and 5.2–50.0% for GAPDH. This means that the difference between species for TUB2 is low. For GAPDH this is much higher, pointing out that GAPDH as a gene region can be more informative in separating *Colletotrichum* species. Interspecific uncorrected P-distances for ITS, CHS-1, HIS3, ACT ranged between 0.5–9.0%, 0.5–14.2%, 3.2–16.4%, 5.3–26.7%, and, respectively. Intraspecific uncorrected p-distances never exceeded 1.6%, except for TUB2 (e.g. *C. fiorinae* 4.9%, *C. godetiae* 4.7%, *C. kahawae* 3.2%, *C. nymphaea* 3.0%, *C. rhombiforme* 4.7% and *C. salicis* 4.7%), meaning that the different isolates of one species differs for TUB2.

Fig. 1: multigene phylogenetic maximum-likelihood tree was constructed with concatenated sequences of ITS, TUB2, HIS3, GAPDH, CHS-1 and ACT of *Colletotrichum* isolates causing bitter rot on apple fruits. *C. lindemuthianum* was used as an outgroup. Reference isolates and corresponding accession numbers can be found in Suppl. Table 1. Information about the names representing the isolates in this figure can be found in Table 1. The numbers at the branching of the tree represent the bootstrap values.

Morphological comparison of *Colletotrichum* isolates

Seven different *Colletotrichum* spp., i.e. *C. fiorinae*, *C. kahawae*, *C. nymphaeae*, *C. salicis*, *C. rhombiforme*, *C. acutatum* and *C. godetiae*, identified by sequence analysis, were used for morphological analysis. The morphological characteristics (colony colour, growth rate in vitro and spore characteristics) of 14 selected isolates are shown in Table 2.

Table 2: Morphological characteristics of 14 selected representative isolates of seven *Colletotrichum* species: conidia length (μm), conidia width (μm), mycelium growth rate (mm day^{-1}), conidia shape and colony colour (upper and reverse side of the Petri dishes)

Species	Isolate	Conidia Length (μm)*	Conidia width (μm)*	Mycelium growth rate (mm day^{-1})*	Conidia shape	Colour colony UPPER side	REVERSE side
<i>C. salicis</i>	Apple-50	17.5 (± 1.38)	7.0 (± 0.72)	8.0 (± 0.91)	Cylindrical	Grey/brown	Yellow
	Strawberry-13	14.0 (± 1.52)	5.1 (± 0.61)	7.4 (± 0.64)			White and pink
<i>C. fioriniae</i>	Apple-48	14.3 (± 0.78)	4.7 (± 0.41)	5.8 (± 0.99)	Obclavate	Pink	Pink
	Apple-14	13.0 (± 1.09)	4.8 (± 0.42)	7.0 (± 1.12)			
<i>C. godetiae</i>	Apple-19	15.6 (± 0.98)	5.3 (± 0.65)	7.3 (± 1)	Fusiform	Light brown	Mix orange, grey and yellow
	Apple-22	15.2 (± 0.93)	5.0 (± 0.46)	8.0 (± 1.23)			
	Cherry-1	14.7 (± 0.98)	5.3 (± 0.54)	6.9 (0.58)			
<i>C. kahawae</i>	Apple-31	13.9 (± 0.99)	5.5 (± 0.51)	6.3 (± 1.26)	Cylindrical	Mix of white, pink and yellow	Yellow
	Apple-49	18.0 (± 1.68)	6.9 (± 0.79)	5.8 (± 1.78)		Mix of white and grey	White and grey
<i>C. nymphaeae</i>	Strawberry-2	14.6 (± 0.98)	4.7 (± 0.49)	6.8 (± 0.79)	Obclavate	White	White/yellow
	Strawberry-12	15.3 (± 1.09)	5.2 (± 0.44)	3.8 (± 1.71)		White and grey	White/yellow
<i>C. rhombiforme</i>	Apple-51	17.0 (± 1.12)	5.3 (± 0.57)	7.0 (± 0.46)	Fusiform	Grey/brown	Grey/black
	Apple-63	17.7 (± 1.28)	6.1 (± 0.52)	6.7 (± 0.53)			
<i>C. acutatum</i>	Apple-59	12.3 (± 1.33)	4.3 (± 0.50)	7.3 (± 1)	Cylindrical	Pink and white	Pink

*The data shown in this table are the mean $\pm$ standard deviation.

The characteristic colony colour of the fungal pathogen on the upper side of the Petri dish showed that differences between species are present, but also between isolates of one species. Both *C. salicis* and *C. rhombiforme* mycelia are brown to grey and the difference is nearly impossible to detect (Fig. 2, upper side, A1–2 and F1–2). *Colletotrichum fioriniae* isolates have a rather pinkish mycelium and sporulation with orange conidial masses (Fig. 2, upper side, B1–2). *Colletotrichum godetiae* isolates have light brown mycelium in mass with orange conidial masses developing in concentric circles (Fig. 2, upper side, C1–2-3). Isolates from *C. acutatum* have a mix of pink and white mycelium and orange sporulation masses (Fig. 2, upper side, D1). Apart from this, there was sometimes a clear difference between the colony colour of two isolates from a single species, such as *C. nymphaeae* (Fig. 2, upper side, E1–2) and *C. kahawae* (Fig. 2, G1–2). The characteristics of the reverse side of an isolate on a Petri dish show that *C. fioriniae* (Fig. 2, reverse side, B1–2) and *C. acutatum* (Fig. 2, reverse side, D1) are typically pink and *C. nymphaea* (Fig. 2, reverse side, E1–2) white. *Colletotrichum rhombiforme* isolates (Fig. 2, reverse side, F1–2) are typically black/grey on the bottom of the Petri dish, a clear difference with all the other available species. *Colletotrichum godetiae* (Fig. 2, reverse side, C1–2-3) is also on the reverse side characterized with the typical concentric rings in a mix of orange/grey/yellow colour. Both isolates of *C. kahawae* (Fig. 2, reverse side, G1–2) and *C. salicis* (Fig. 2, reverse side, A1–2) differ on the reverse side; respectively yellow vs. white and yellow vs. pink-white.

Fig. 2: Colony characteristics of the 14 selected *Colletotrichum* isolates on PDA medium, upper side and reverse side: *C. salicis* (A1/Apple-50 and A2/Strawberry-13), *C. fioriniae* (B1/Apple-48 and B2/Apple-14), *C. godetiae* (C1/Apple-19, C2/Apple-22 and C3/Cherry-1), *C. acutatum* (D1/Apple-59), *C. nymphaeae* (E1/Strawberry-2 and E2/Strawberry-12), *C. rhombiforme* (F1/Apple-51 and F2/Apple-63) and *C. kahawae* (G1/Apple-49 and G2/Apple-31). More detailed isolate information is described in Table 1.

The in vitro growth rate (PDA Petri dishes) of the 14 selected isolates from 5.8 to 8.7 mm day⁻¹. Of all the isolates tested, strawberry-12 isolate (*C. nymphaeae*) had the lowest growth rate per day with 3.8 mm day⁻¹ and isolate apple-50 (*C. salicis*) the highest growth rate with 8.0 mm day⁻¹ (Table 4). *C. nymphaeae* (5.8 mm day⁻¹) was significantly lower than *C. salicis* (7.7 mm day⁻¹; $P = 0.0013$) and *C. godetiae* (7.4 mm day⁻¹; $P = 0.0020$). Significant differences ($P < 0.01$) were observed between the two isolates of the species *C. kahawae*, *C. nymphaeae* and *C. godetiae*.

The apple-40 (*C. salicis*), strawberry-13 (*C. salicis*), apple-49 (*C. kahawae*), apple-31 (*C. kahawae*), apple-51 (*C. rhombiforme*) and apple-63 (*C. rhombiforme*) isolates showed limited or no sporulation. However, a 16 h near UV treatment resulted in acceptable sporulation levels of at least 4.0×10^5 conidia ml⁻¹. Differences in capability to sporulate in vitro were observed in the 14 selected isolates (data not shown). Spore characteristics (conidia length, width and shape) were significantly different between all possible pairwise combinations of *Colletotrichum* ($P < 0.05$), except for species combinations *C. nymphaeae* - *C. fioriniae* ($P > 0.5$), *C. nymphaeae* - *C. godetiae* ($P > 0.5$) and *C. salicis*

- *C. kahawae* ($P > 0.5$). *Colletotrichum fioriniae* showed no significant difference ($P > 0.05$) in spore characteristics of both isolates, whereas for all other *Colletotrichum* spp., spore characteristics, length and/or width differed significantly ($P < 0.05$) between the two or three isolates.

Pathogenicity of *Colletotrichum* isolates on apple

All 14 isolates of the seven *Colletotrichum* spp. were pathogenic on both cvs. Nicoter and Pinova apples (Fig. 3). Typical symptoms of *Colletotrichum* infections were round lesions, expanding towards the core of the apple like a reversed cone, and in some cases the typical orange sporulation patterns became visible. The first lesions appeared three to four days post inoculation. Re-isolation from these lesions, followed by sequencing, confirmed the isolates that were originally inoculated on the apple.

Each error bar is constructed using 1 standard error from the mean.

Fig. 3: Mean expansion rate of the lesion diameter (mm day^{-1}) and standard error for the *Colletotrichum* species, isolates and apple cultivars (Nicoter and Pinova). *C. salicis* (A1/Apple-50 and A2/Strawberry-13), *C. fiorinae* (B1/Apple-48 and B2/Apple-14), *C. godetiae* (C1/Apple-19, C2/Apple-22 and C3/Cherry-1), *C. acutatum* (D1/Apple-59), *C. nymphaeae* (E1/Strawberry-2 and E2/Strawberry-12), *C. rhombiforme* (F1/Apple-51 and F2/Apple-63) and *C. kahawae* (G1/Apple-49 and G2/Apple-31). More detailed isolate information is described in Table 1.

Colletotrichum species expanded faster on cv. Pinova (higher expansion rate of the pathogen based on the lesion diameter) than cv. Nicoter, for example *C. acutatum* (Fig. 3, D1/Apple-59). Differences between species and isolates are present. *C. kahawae* (isolate G1/apple-49) and *C. salicis* (isolate A1/Apple-50) are the least pathogenic isolates, with respectively an average of 1.03 mm day^{-1} and 1.14 mm day^{-1} of lesion expansion for cv. Nicoter and resp. 2.14 mm day^{-1} and 2.35 mm day^{-1} of lesion expansion for cv. Pinova. Isolate C2/Apple-22, belonging to *C. godetiae*, is the most pathogenic on cvs. Nicoter and Pinova with approximately 4 mm day^{-1} . Both isolates of one *Colletotrichum* species can show a high variability in pathogenicity for the same cultivar. *Colletotrichum kahawae* isolates (G1

and G2, cultivar Pinova), for example, differed significantly ($P = 0.0001$) in pathogenicity from average 1.26 till 3.15 mm day⁻¹. There was a significant interaction ($P = 0.0001$) between the factors ‘isolate’ and ‘apple cultivar’.

Discussion

Postharvest diseases have become a more and more important problem in the production of apple fruits. The pathogens involved are not always well known, although this is a prerequisite to control the problem. We reported about the first study executed on the presence of *Colletotrichum* spp. causing the postharvest disease bitter rot on apple fruits in Belgian orchards. Seven *Colletotrichum* spp., belonging to either the *C. acutatum* or *C. gloeosporioides* complexes, were found to cause bitter rot of apple (and strawberry) in Belgium. Molecular and morphological tools showed an identification of different *Colletotrichum* species, but a reliable discrimination between species was only possible by molecular approaches. Pathogenicity of all species was confirmed using inoculation assays on two apple cultivars, Pinova and Nicoter. Overall, *C. godetiae* was found to be the most aggressive species. Variation between isolates and between cultivars was observed. The pathogenicity tests also showed that isolates coming from another host species, e.g. strawberry, are pathogenic on apple fruits as well.

This study revealed seven different *Colletotrichum* spp., isolated from three different apple orchards, causing bitter rot in Belgium on apple and strawberry: *C. godetiae*, *C. fioriniae*, *C. salicis*, probably *C. kahawae*, *C. nymphaeae*, *C. rhombiforme* and *C. acutatum*, belonging to the *C. acutatum* and *C. gloeosporioides* species complex. No new or unknown *Colletotrichum* spp. were identified. *C. godetiae* is the most common species causing bitter rot on apple in our study. In other European countries *C. godetiae* and *C. fioriniae* were found to be the most common *Colletotrichum* spp. (Ivic et al. 2013; Baroncelli et al. 2014; Nodet et al. 2016; Munda 2014; Wenneker et al. 2016). However in contrast, *C. salicis* was more often isolated than *C. fioriniae* in this study. Interestingly, *C. rhombiforme* and *C. kahawae* were not yet found on apple in Europe, which highlights the polyphagous characteristics of *Colletotrichum* species. *Colletotrichum rhombiforme* is mainly known to cause anthracnose on olives and cranberries (Damm et al. 2012). Meanwhile, *C. kahawae* is known to have several hosts in countries such as Australia, Italy and the USA (Weir et al. 2012; Liu et al. 2013; Ismail et al. 2015). Research on

this topic is in European regions mainly limited to first reports on apple fruits with limited research depth. Today it is necessary to encounter such a possible upcoming problem and doing specific research by following standardized protocols.

Over the last decades, molecular tools for species identification and delineation have been used extensively, often resulting in taxonomic changes. The multigene approach enabled identification of the seven *Colletotrichum* species. Identification based on ITS could only distinguish fungal pathogens on genus level and give a clear discrimination (bootstrap value >95) between the *Colletotrichum acutatum* and *C. gloeosporioides* species complex. The interspecific variability of the ITS gene region is insufficient for identification at species level because of the low nucleotide diversity. Therefore multilocus sequencing is currently more executed (Yokosawa et al. 2017). By combining different gene regions, with large interspecific distances, species delineation can be done more accurately. In studies from Munir et al. (2016), Prihastuti et al. (2009) and Weir et al. (2012) three to eight gene regions were used for identification and phylogenetic analyses. This multigene approach with six gene regions (ITS, TUB2, HIS3, GAPDH, CHS-1 and ACT) used in this paper in most cases lead to identification at species level. It resulted in a clear distinction between both *Colletotrichum* spp. complexes, but the resolution at species level, was not always highly supported. Some species could not be separated in an unambiguous way. The use of gene regions, which support the differences and similarities between *Colletotrichum* spp., is an important feature to reliably identify new species. For this purpose it is important to review single gene regions. CHS-1, TUB2 and ITS as single gene regions cannot separate the species identified in this study. The low interspecific distances between isolates for for example TUB2 is insufficient to make a reliable distinction between all seven species (Suppl. Fig. 3). Damm et al. (2012) used the same six gene regions in their phylogenetic analysis as well and confirmed that not all gene fragments were informative to use in phylogenetic studies. For example CHS-1 is not suitable for separating *C. godetiae* from other species in the *Colletotrichum acutatum* species complex (Damm et al. 2012). A multi-gene approach, including genes with larger interspecific distances such as HIS3, ACT and GAPDH, is recommended and better suited for species identification, as has been proven in this study (Suppl. Fig. 7). A multigene approach, based on ITS, GAPDH and TUB2, has been previously

proposed as a suitable way of differentiating *Colletotrichum* spp. (Velho et al. (2015), Alaniz et al. (2015) and Munir et al. (2016)). However, our results can indicate that less variable gene regions such as TUB2 and ITS will in fact decrease the resolution of phylogenetic trees. We recommend to restrict species identification to a few carefully selected highly polymorphic gene regions rather than adding less valuable information of ITS and TUB2 sequences. To further characterize correctly specific *Colletotrichum* species, it can be necessary to sequence some other loci. Example given in this study is the finding of probably *C. kahawae*. Sequencing the loci of CAL, ApMat and GS is necessary to be able to confirm certainly the identity of these isolates, as shown in Liu et al. (2015). By standardizing genes used in this type of studies, a similar degree of discrimination and delineation can be achieved in each species complex, a principle proposed by Everett (2014).

The morphological characteristics (colony colour, in vitro growth rate, spore sizes and spore shape) studied, do not make it possible to distinguish between different *Colletotrichum* species. The characteristics of in vitro fungal growth and the spore size were not sufficiently consistent to discriminate isolates at the species level in this study, which is in agreement with Velho et al. (2015) and Munir et al. (2016). Our study showed that only conidia width gave a significant difference between the two species complexes, as in accordance with Phoulivong et al. (2010). The characteristic colony colour appears to be an inefficient feature to reliably distinguish *Colletotrichum* spp. and even within one *Colletotrichum* species the variability was high, as was also described for example for *C. fructicola* in Brazil and Uruguay which shows five different colour groups (Velho et al. 2015; Du et al. 2005; Weir et al. 2012; Munir et al. 2016). Some *Colletotrichum* species show however less variability, such as *C. nymphaeae* and *C. melonis* (Velho et al. 2015). The presence of fewer haplotypes or less diversity for both species might result in a lower variability in colony colour in our study. Conclusions have to be taken with care, taking into account the number of isolates to provide conclusive evidence.

The morphological characteristics, of the isolates studied here, were not all stable within one single species and next to this there is a clear variation in species delineation according to the gene region studied in the phylogenetic analysis. There is no variation when assigning the isolates to a species complex. This could be an evidence that it is difficult to exactly assign a correct specific species name

to different isolates, compared to assigning it only to a species complex. The question can rise whether it isn't just the population variability that is described in a species complex (and not single species), making it possible to refute the super speciation of the *Colletotrichum* complexes that exists today (Everett 2014; Børve and Stensvand 2017).

The *Colletotrichum* spp. found on strawberry and cherry in Belgium, *C. godetiae*, *C. fioriniae* and *C. salicis*, were also present and pathogenic on apple fruits. The ability of all different species, even isolates which originated from cherry and strawberry fruits, found in this study to artificially infect wounds on apple cvs. Pinova and Nicoter clearly supports the polyphagous character of the pathogen. It is known in literature that cross-infections of several *Colletotrichum* spp. can occur and might lead to serious problems (Phoulivong et al. 2012). Future studies should pay more attention to *Colletotrichum* spp. originating from other host plants to provide even more insight into this polyphagous pathogen. The pathogenicity tests in this study were performed by wounding fruits. The removal of the physical barrier might result in a higher virulence of the fungal isolates, might affect fungal growth rates and can result in differences in susceptibility compared to unwounded fruits. Whether there is infection on unwounded fruits and which entries are used by the *Colletotrichum* spp. was not studied and remains unclear.

The two apple cultivars used in this study, Nicoter and Pinova, showed variation in susceptibility to the *Colletotrichum* isolates. Biggs and Miller (2001) studied the relative susceptibility of 18 different apple cultivars to *Colletotrichum acutatum* and showed that a clear difference in susceptibility between cultivars is present, but the cultivars used in this study were not included. A possible explanation for the differences in susceptibility between apple cultivars in general can be based on biochemical or physical differences during ripening, which effects the fungal growth inside the fruit (Nour et al. 2010; Munir et al. 2016). It has been implied that time and ripening dependent changes of cultivars including ethylene biosynthesis, apple firmness, sugar content, antifungal components (e.g. chlorogenic acid) leads to an increase in susceptibility to postharvest fungi (Lattanzio et al. 2001; Prusky et al. 2013; Angay et al. 2014).

This study proves that *Colletotrichum* spp. are commonly present as a postharvest pathogen in Belgium, while this wasn't expected. Seven different *Colletotrichum* spp. were found, of which six on apple, showing a different level of pathogenicity (taking into account the limited amount of tested isolates). *Colletotrichum godetiae* was the most common and aggressive species, confirming the studies of surrounding European countries. Our study revealed also a clear cultivar-isolate interaction concerning the pathogenicity. Increasing the amount of orchards, including several geographical locations, might lead to changes in frequency of specific *Colletotrichum* spp. and may lead to the discovery of new species. It is also important that the species identification procedure is standardised, taken into account bottlenecks such as sample size, molecular techniques, choice and number of gene regions (Everett 2014). Our findings confirm a possible upcoming problem of bitter rot, making clear that research and follow-up is necessary for future disease management in case of occurrence, spreading and pathogen-host relations. Next to this, the study of the mechanism behind the differences in susceptibility of apple cultivars could be a key factor to learn more about the development of non-chemical control measures for *Colletotrichum* e.g. by breeding for less susceptible cultivars.

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1. Supplementary tables

Supplementary table 1: Sequence information of accepted *Colletotrichum* spp. and GenBank accession numbers used as a reference in this manuscript.

GenBank accession numbers

Species	Isolates	ITS	ACT	GAPDH	HIS3	CHS-1	TUB2
<i>C. godetiae</i>	CBS 129951	JQ948430	JQ949751	JQ948762	JQ949421	JQ949091	JQ950081
<i>C. fioriniae</i>	CBS 129916	JQ948317	JQ949638	JQ948647	JQ949308	JQ948978	JQ949968
<i>C. nymphaea</i>	CBS 129929	JQ948229	JQ949550	JQ948559	JQ949220	JQ948890	JQ949880
<i>C. salicis</i>	CBS 607.49	JQ948460	JQ949781	JQ948791	JQ949451	JQ949121	JQ950111
<i>C. rhombiforme</i>	CBS 129953	JQ948457	JQ949778	JQ948788	JQ949448	JQ949118	JQ950108
<i>C. kahawae</i>	CBS 12988	JX010236	JX009475	JX009965	/	JX009832	JX010428
<i>C. acutatum</i>	CBS 129924	JQ948375	JQ949696	JQ948706	JQ949366	JQ949036	JQ950026
<i>C. lindemuthianum</i>	CBS 144.31	JX546806	JQ005842	JX546708	JQ005821	JQ005800	JQ005863

651 **Supplementary table 2:** GenBank accession numbers of the 72 identified *Colletotrichum* isolates. Sequences
652 missing were marked with a forward slash. Fourteen isolates were selected for further analysis (indicated with *).

Isolate	GAPDH	ITS	HIS3	ACT	TUB2	CHS-1
Cherry-1*	/	/	KY576272	KY576339	KY576408	KY576473
Apple-1	KY576144	KY576210	KY576273	KY576340	KY576409	KY576474
Apple-2	KY576145	KY576211	KY576274	KY576341	KY576410	KY576475
Apple-3	KY576146	KY576212	KY576275	KY576342	KY576411	KY576476
Apple-4	KY576147	KY576213	KY576276	KY576343	KY576412	KY576477
Apple-5	KY576148	KY576214	KY576277	KY576344	KY576413	KY576478
Strawberry-1	KY576149	/	/	/	KY576414	/
Strawberry-2*	KY576150	KY576215	KY576278	KY576345	KY576415	KY576479
Strawberry-3	KY576151	/	KY576279	/	/	KY576480
Strawberry-4	KY576152	/	KY576280	/	KY576416	KY576481
Strawberry-5	KY576153	KY576216	KY576281	KY576346	KY576417	KY576482
Strawberry-6	KY576154	KY576217	KY576282	KY576347	KY576418	KY576483
Apple-6	KY576155	KY576218	KY576283	KY576348	KY576419	KY576484
Apple-7	KY576156	KY576219	KY576284	KY576349	/	KY576485
Apple-8	KY576157	/	KY576285	KY576350	KY576420	KY576486
Apple-9	KY576158	/	KY576286	KY576351	KY576421	KY576487
Apple-10	KY576159	KY576220	KY576287	KY576352	KY576422	KY576488
Apple-11	KY576160	KY576221	KY576288	KY576353	/	KY576489
Apple-12	KY576161	KY576222	KY576289	KY576354	KY576423	KY576490
Apple-13	KY576162	KY576223	KY576290	KY576355	KY576424	KY576491
Apple-14*	KY576163	KY576224	KY576291	KY576356	KY576425	KY576492
Apple-15	KY576164	KY576225	KY576292	KY576357	KY576426	KY576493
Apple-16	KY576165	KY576226	KY576293	KY576358	KY576427	KY576494
Apple-17	KY576166	KY576227	KY576294	KY576359	KY576428	KY576495
Apple-18	KY576167	KY576228	KY576295	KY576360	KY576429	KY576496
Apple-19*	KY576168	KY576229	KY576296	KY576361	KY576430	KY576497
Apple-20	KY576169	KY576230	KY576297	KY576362	KY576431	KY576498
Apple-21	KY576170	KY576231	KY576298	KY576363	KY576432	KY576499
Apple-22*	KY576171	KY576232	KY576299	KY576364	KY576433	KY576500
Apple-23	KY576172	KY576233	KY576300	KY576365	KY576434	KY576501
Apple-24	/	KY576234	KY576301	KY576366	KY576435	KY576502
Apple-25	KY576173	KY576235	KY576302	KY576367	KY576436	KY576503
Apple-26	/	KY576236	KY576303	KY576368	KY576437	KY576504
Apple-27	KY576174	KY576237	KY576304	KY576369	KY576438	KY576505
Apple-28	KY576175	KY576238	KY576305	KY576370	KY576439	KY576506
Apple-29	/	KY576239	KY576306	KY576371	KY576440	KY576507
Apple-30	KY576176	KY576240	KY576307	KY576372	KY576441	KY576508
Apple-31*	KY576177	KY576241	KY576308	KY576373	/	KY576509
Apple-32	KY576178	KY576242	KY576309	KY576374	KY576442	KY576510
Apple-33	KY576179	KY576243	KY576310	KY576375	/	/
Apple-34	KY576180	KY576244	KY576311	KY576376	KY576443	KY576511
Apple-35	KY576181	/	KY576312	KY576377	KY576444	KY576512
Apple-36	KY576182	KY576245	/	KY576378	KY576445	KY576513
Apple-37	/	KY576246	KY576313	KY576379	KY576446	KY576514
Apple-38	KY576183	KY576247	KY576314	KY576380	KY576447	KY576515
Apple-39	KY576184	KY576248	KY576315	KY576381	KY576448	KY576516
Apple-40	KY576185	KY576249	KY576316	KY576382	KY576449	KY576517
Apple-41	KY576186	KY576250	KY576317	KY576383	KY576450	KY576518
Apple-42	KY576187	KY576251	KY576318	KY576384	KY576451	KY576519
Apple-43	KY576188	KY576252	KY576319	KY576385	KY576452	KY576520
Apple-44	KY576189	KY576253	KY576320	KY576386	KY576453	KY576521
Apple-45	KY576190	KY576254	KY576321	KY576387	KY576454	KY576522
Apple-46	/	/	/	KY576388	KY576455	KY576523
Apple-47	KY576191	KY576255	KY576322	KY576389	KY576456	KY576524
Apple-48*	KY576192	/	KY576323	KY576390	KY576457	KY576525
Apple-49*	KY576193	KY576256	KY576324	KY576391	/	KY576526
Apple-50*	KY576194	/	KY576325	KY576392	KY576458	KY576527
Apple-51*	KY576195	KY576257	KY576326	KY576393	KY576459	KY576528
Strawberry-7	KY576196	KY576258	KY576327	KY576394	KY576460	KY576529
Strawberry-8	KY576197	KY576259	/	KY576395	KY576461	KY576530
Strawberry-9	KY576198	KY576260	KY576328	KY576396	KY576462	KY576531
Strawberry-10	KY576199	KY576261	KY576329	KY576397	KY576463	KY576532
Strawberry-11	KY576200	KY576262	KY576330	KY576398	KY576464	KY576533
Strawberry-12*	KY576201	KY576263	KY576331	KY576399	KY576465	KY576534
Strawberry-13*	KY576202	KY576264	/	KY576400	KY576466	KY576535
Apple-59*	KY576203	KY576265	KY576332	KY576401	/	KY576536
Apple-60	KY576204	KY576266	KY576333	KY576402	KY576467	KY576537
Apple-61	KY576205	KY576267	KY576334	KY576403	KY576468	KY576538
Apple-62	KY576206	KY576268	KY576335	KY576404	KY576469	KY576539

Apple-63*	KY576207	KY576269	KY576336	KY576405	KY576470	KY576540
Apple-64	KY576208	KY576270	KY576337	KY576406	KY576471	KY576541
Apple-65	KY576209	KY576271	KY576338	KY576407	KY576472	KY576542

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2. SUPPLEMENTARY FIGURES

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Supplementary Fig. 1: Multi-gene maximum-likelihood tree was constructed with concatenated sequences of ACT, GAPDH and HIS3 of *Colletotrichum* isolates causing bitter rot on apple fruits. *Colletotrichum lindemuthianum* data are used as an outgroup. Information about the names representing the isolates in this figure can be found in Table 1. The numbers at the branching of the tree represent the bootstrap values.

- *C. salicis*
- *C. fioriniae*
- ▽ *C. acutatum*
- ◆ *C. nymphaea*
- ▼ *C. godetiae*
- *C. rhombiforme*
- *C. kahawae*

Supplementary Fig. 2: Single gene maximum-likelihood tree was constructed with concatenated sequences of ACT of *Colletotrichum* isolates causing bitter rot on apple fruits. *Colletotrichum lindemuthianum* data was used as an outgroup. Information about the names representing the isolates in this figure can be found in Table 1. The numbers at the branching of the tree represent the bootstrap values.

Supplementary Fig. 3: Single gene maximum-likelihood tree was constructed with concatenated sequences of ITS of *Colletotrichum* isolates causing bitter rot on apple fruits. *Colletotrichum lindemuthianum* data was used as an outgroup. Information about the names representing the isolates in this figure can be found in Table 1. The numbers at the branching of the tree represent the bootstrap values.

Supplementary Fig. 4: Single gene maximum-likelihood tree was constructed with concatenated sequences of TUB2 of *Colletotrichum* isolates causing bitter rot on apple fruits. *Colletotrichum lindemuthianum* data was used as an outgroup. Information about the names representing the isolates in this figure can be found in Table 1. The numbers at the branching of the tree represent the bootstrap values.

Supplementary Fig. 5: Single gene maximum-likelihood tree was constructed with concatenated sequences of CHS-1 of *Colletotrichum* isolates causing bitter rot on apple fruits. *Colletotrichum lindemuthianum* data was used as an outgroup. Information about the names representing the isolates in this figure can be found in Table 1. The numbers at the branching of the tree represent the bootstrap values.

Supplementary Fig. 6: Single gene maximum-likelihood tree was constructed with concatenated sequences of GAPDH of *Colletotrichum* isolates causing bitter rot on apple fruits. *Colletotrichum lindemuthianum* data was used as an outgroup. Information about the names representing the isolates in this figure can be found in Table 1. The numbers at the branching of the tree represent the bootstrap values.

Supplementary Fig. 7: Single gene maximum-likelihood tree was constructed with concatenated sequences of HIS3 of *Colletotrichum* isolates causing bitter rot on apple fruits. *Colletotrichum lindemuthianum* data was used as an outgroup. Information about the names representing the isolates in this figure can be found in Table 1. The numbers at the branching of the tree represent the bootstrap values.