Colletotrichum fioriniae and *Colletotrichum godetiae* Causing Postharvest Bitter Rot of Apple in South Tyrol (Northern Italy)

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Abstract

South Tyrol (northern Italy) harbors one of the largest interconnected apple farming areas in Europe, contributing approximately 10% to the apple production of the European Union. Despite the availability of sophisticated storage facilities, postharvest diseases occur, one of which is bitter rot of apple. In Europe, this postharvest disease is mainly caused by the *Colletotrichum acutatum* species complex. This study aimed to characterize the *Colletotrichum* spp. isolated from decayed apple fruit collected in 2018 and 2019 in South Tyrol. The characterization of *Colletotrichum* spp. was accomplished based on multilocus DNA sequences of four different genomic regions—actin (ACT), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), histone H3 (HIS3), and the internal transcribed spacer (ITS) region—as well as morphological and pathogenicity assessment. A phylogenetic analysis based on multilocus DNA sequences showed that the isolates obtained from apples with symptoms of bitter rot belonged to the species *Colletotrichum godetiae* and *Colletotrichum fioriniae*, which are part of the *Colletotrichum acutatum* species complex. A third species isolated from apples

belonging to the same species complex, *Colletotrichum salicis*, was described in this area. Moreover, the *Colletotrichum* isolates found in this study proved to be virulent on Cripps Pink, Golden Delicious, and Roho 3615/Evelina. To the best of our knowledge, *C. godetiae* and *C. fioriniae* have so far never been mentioned as postharvest pathogens of apple in Italy, although the reanalysis of samples collected in the past indicates that these pathogens have been occurring in Italy for at least a decade. So far, bitter rot seems to play a minor role as a postharvest disease in South Tyrol, but it was disproportionately represented on a few scab-resistant apple cultivars, which are increasingly planted in organically managed orchards. Considering that the expansion of organic apple production and the conversion to new potentially *Colletotrichum*-susceptible cultivars will continue, the present study represents an important contribution toward a better understanding of bitter rot in this geographic area.

Keywords: fungi, fruit, tree fruits, etiology, pathogen diversity

Colletotrichum acutatum and *Colletotrichum gloeosporioides* species complexes (SCs) have been associated with bitter rot of apple (*Malus domestica* Borkh.) all over the world. However, the *C. acutatum* SC is considered to be more prevalent in Europe (Grammen et al. 2019) and comprises species of pathogenic fungi, which cause the most significant losses during the vegetative stage in the orchard. In warmer climates, *C. acutatum* SC infects apple fruits during the summer growing period, in which the first symptoms can become visible as small, black spots that later become depressed and expand to form tan to dark-brown or black, usually circular lesions. Finally, salmon-orange spores are formed in acervuli that arise concentrically from the center of the lesion while the fruits are still on the trees. However, in cooler growing areas, probably because of climatic effects, this SC is more often noticed as a latent pathogen; it does not induce any symptoms at harvest, but the development of rot occurs during storage (Sutton et al. 2014; Wencker and Thomma 2020; Wharton and Diéguez-Urbeondo 2004).

Multilocus sequence typing (MLST), which is based on the analysis of several protein-coding genes, in addition to the internal transcribed spacer (ITS) region, has become widely accepted as a tool for DNA

barcoding and species identification (Taylor and Fisher 2003). In 2012, Damm et al. (2012) performed a taxonomic revision based principally on multilocus phylogenetic data, in which the *C. acutatum* SC was subdivided into >30 species, confirming the existence of at least nine distinct molecular genetic groups (A1 to A9) proposed by Sreenivasaprasad and Talhinhas (2005). To name some species of these genetic groups associated with bitter rot of apple, A3 group was described as *Colletotrichum fioriniae*, A4 as *Colletotrichum godetiae* (syn. *Colletotrichum clavatum*), and A7 as *Colletotrichum salicis* (Amaral Carneiro et al. 2021; Damm et al. 2012; Faedda et al. 2011; Grammen et al. 2019; Shivas and Tan 2009). To distinguish the species within the *C. acutatum* SC, different gene regions can be analyzed. Generally, actin (ACT), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), histone H3 (HIS3), chitin synthase 1 (CHS-1), and β -tubulin (TUB2) gene regions are considered to represent suitable markers to characterize the *C. acutatum* SC (Damm et al. 2012; Weir et al. 2012). In recent years, two species of the *C. acutatum* SC, *C. fioriniae* and *C. godetiae*, were recognized as the most important pathogens causing bitter rot of apple in Europe (Baroncelli et al. 2014; Børve and Stensvand 2015; Grammen et al. 2019; Munda 2014; Nodet et al. 2016). These two species show distinct morphological features, such as differences in the shape and size of conidia, in the color of colonies, and in the growth rates of the mycelium. In addition, *C. godetiae* proved to be more aggressive in the infection of apple fruit (Grammen et al. 2019). *C. salicis* is another species of the *C. acutatum* SC associated with bitter rot of apple in Europe, although so far it has been reported in few studies (Amaral Carneiro et al. 2021; Grammen et al. 2019).

South Tyrol (northern Italy) harbors the largest interconnected apple-producing area in Europe. Approximately 974,000 metric tons of apple were produced on 18,333 hectares (ha) of land in 2019 (Autonomous Province of Bozen/Bolzano 2020). Over the past years, researchers in European countries such as Belgium, England, France, The Netherlands, Norway, and Slovenia have published several reports describing the emergence of species of the *C. acutatum* SC that have not been associated with bitter rot of apple in the past (Baroncelli et al. 2014; Børve and Stensvand 2015; Grammen et al. 2019; Munda 2014;

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Nodet et al. 2016; Wenneker et al. 2016). However, thus far no scientific information is available about the *Colletotrichum* spp. causing bitter rot of apple in South Tyrol, which harvests approximately 10% of the total apple production in the European Union. Therefore, the main objective of this study was to collect apples with postharvest symptoms of bitter rot from several packinghouses in South Tyrol and perform phenotypic and molecular genetic characterization of the isolates identified as *Colletotrichum* spp. Furthermore, pathogenicity assays were performed with selected isolates. These results will lead to an increased understanding of the causative agents of bitter rot of apple in this region and northern Italy and will provide a foundation for developing specific diagnostic methods and implementing disease management strategies.

Materials and Methods

Sampling and isolation of postharvest pathogens of apple. In 2018 and 2019, collection of apples with decay symptoms was conducted after 6 to 8 months of storage under controlled conditions in six packinghouses representing different cultivation areas in the Autonomous Province of Bozen/Bolzano, such as Eisacktal/Valle Isarco, the area around Bozen/Bolzano, Unterland/Bassa Atesina, the area around Meran/Merano, and Vinschgau/Val Venosta. Four packinghouses stored apples grown by following integrated production standards, one packinghouse specialized in organic apples, and one packinghouse had separate lines for the storage of apples derived from organic and integrated production. Whereas first-year sampling involved apples that displayed any kind of postharvest disease, second-year sampling focused on so-called *Gloeosporium* SC (Giraud 2012), which traditionally comprises several species of the genera *Neofabraea* spp. and *Colletotrichum* spp.

More than 1,000 apple fruits that were inspected for the presence of symptoms of postharvest diseases were taken to the laboratory for microbiological isolation to determine the pathogens involved in the disease. A photo setup was established to document external and internal symptoms during the process of pathogen isolation. Rotten apples were washed with curd soap, rinsed well with tap water, and cleaned with ethanol. From each apple, four transplants from the border between healthy and diseased pulp tissue were dissected and transferred to a Petri dish containing potato dextrose agar (PDA; VWR BDH Chemicals, Poole, UK) nutrient medium. The cultures were incubated in the laboratory at room temperature (approximately 24°C), and fungal growth was observed after 14 days. Of the 740 fungal cultures that were isolated in 2018 and 331 that were obtained in 2019, 19 isolates were identified as *Colletotrichum* spp. based on morphological features on PDA and DNA sequencing of the ITS region. Once the cultures were identified as belonging to the genus *Colletotrichum*, monospore cultures were prepared by adapting the procedure described by Choi et al. (1999) (Amaral Carneiro and Baric 2021). Three additional *Colletotrichum* isolates obtained from apple fruit and leaf spots in previous years in South Tyrol were included in this study. To store the isolates, small blocks of medium that contained mycelium were excised and conserved in slant culture tubes on malt agar (Biolab, Budapest, Hungary) at 4°C. All isolates characterized in the present study are reported in Table 1.

DNA-based analysis. The 22 pure cultures identified as *Colletotrichum* spp. were transferred on fresh PDA medium and incubated at 24°C for 2 weeks to obtain sufficient amounts of mycelium for DNA isolation. The DNA extraction protocol of Cassago et al. (2002) was followed, with some modifications. The mycelium was harvested from the growth medium with a sterile blade and was placed in a 2-ml microcentrifuge tube containing 500 µl of lysis buffer (50 mM Tris-HCl, pH 7.5; 10 mM EDTA; 1% sodium dodecyl sulfate). Subsequently, two 3-mm tungsten carbide beads (Qiagen, Hilden, Germany) were added to the 2-ml tube, which was then mixed vigorously at a frequency of 30 Hz in the TissueLyser II (Qiagen, Hilden, Germany) for 2 min and left to rest for 1 min on ice. This step was followed by protein precipitation with potassium acetate (pH 4.8), avoiding the use of phenol-chloroform. DNA was precipitated with isopropanol and washed with 70% ethanol, and the pellet was dried under laminar flow. Finally, the DNA was resuspended in 30 µl of Tris-EDTA-Buffer (pH 8), and DNA quantity and quality were assessed with a DeNovix DS-11 Spectrophotometer (DeNovix Inc., Wilmington, DE).

To identify the *Colletotrichum* isolates at the species level, PCR amplifications with specific primers were performed for the ACT (Carbone and Kohn 1999), GAPDH (Guerber et al. 2003), and HIS3 (Crous et al. 2004) gene regions in addition to the ITS region (Lévesque and de Cock 2004). Each PCR reaction contained 7.5 µl of the 2× PCRBIO Taq Mix (PCR Biosystems Ltd., Oxford, United Kingdom), which contains a premix of Taq DNA polymerase, dNTPs and the appropriate buffer system, 0.75 µl of each forward and reverse primer (5 µM), and 5 µl of sterile, nuclease-free water. The final volume of the PCR reaction of 15 µl was reached by the addition of 1 µl of template DNA (initial concentration of 10 ng µl⁻¹). The PCR conditions were as follows: an initial denaturation step of 2 min at 95°C, followed by 35 cycles of 95°C for 30 s, the primer-specific annealing temperature for 30 s (Table 2) and 72°C for 45 s, and a final extension step of 7 min at 72°C. In addition to the annealing temperatures, Table 2 indicates the expected amplicon lengths produced by each pair of primers. PCR products were separated and visualized on agarose gels stained with MIDORI Green (NIPPON Genetics, Saitama, Japan). All PCR products displaying sharp and distinct bands of the expected size were purified with the A'SAP PCR clean-up reagents (ArticZymes, Tromsø, Norway) and sent for Sanger sequencing with the forward primer to Microsynth SeqLab GmbH (Göttingen, Germany). To confirm each distinct DNA sequence, selected PCR products were also sequenced with the reverse primers. Raw sequence data were controlled and aligned by using the Clustal Omega alignment tool implemented in the software program Geneious Prime, version 2019.2 (Biomatters Ltd., Auckland, New Zealand).

To identify the *Colletotrichum* isolates, all sequences were subjected to nucleotide basic local alignment search tool (BLASTn) analysis (National Center for Biotechnology Information, Bethesda, MD) (Boratyn et al. 2013). All the sequences generated in this study were deposited in GenBank; accession numbers can be found in Table 1. Sequences of the same set of genomic regions as analyzed in the present study of 35 reference isolates representing different species of the genus were selected from the works of Da Lio et al. (2018) and Damm et al. (2012) to perform phylogenetic analyses (Supplementary Table S1). The phylogenetic analyses were accomplished for each gene separately (data not shown because each genomic region provided the same topology) and for a multilocus concatenated alignment combining four genes (ACT, GAPDH, HIS3, and ITS) using the maximum likelihood algorithm as implemented in the software Molecular Evolutionary Genetic Analysis (MEGA X) (Kumar et al. 2018). The alignment comprised all the sequences obtained in the present study and the sequences of the reference isolates, whereas *C. lindemuthianum* was used as an outgroup. Ambiguously aligned regions were excluded from analyses. Branch-support values for each tree obtained by maximum likelihood analysis were assessed by bootstrapping with 1,000 replications.

Morphological characterization. Morphological assessment based on in vitro growth rates and colony morphology was conducted with 19 *Colletotrichum* spp. isolates. A 5-mm mycelial plug of an actively growing colony was excised and placed in the center of a fresh Petri dish with PDA medium, sealed with Parafilm (Beemis, Neenah, WI), and incubated at 20°C for 2 weeks in the dark. Four technical replicates were prepared for each isolate. Colony diameter (two perpendicular diameters) was measured after 3, 5, 7, 10, and 14 days and the mycelial area ($A = \pi r^2$) was calculated. The experiment was performed twice, with 4 weeks' time separation. Colony color was evaluated visually after 14 days. To compare conidia shape and size, two isolates of *C. fioriniae* (18-DSS-BS-CF-1-002 and 18-DSS-BS-NT0-1-010) and two isolates of *C. godetiae* (18-DSS-BS-EL-1-016 and 18-DSS-BS-EL-1-028) were selected. A conidial spore suspension was made by flooding the Petri dishes with 3 ml of distilled water, scraping over the mycelium, and filtering the solution through a sterile gauze. The conidial spore suspension was diluted with distilled water until a spore concentration of 10⁴ conidia ml⁻¹ was obtained. For each isolate, the length and width of 80 randomly selected conidia were measured using an optical microscope coupled to a digital camera (Leica DMLS, Leica Microsystems, Wetzlar, Germany) with DeltaPix Insight software version 3.2.X (DeltaPix, Smorum, Denmark). The software was calibrated with a micrometer-measuring scale. Conidia were

classified according to Damm et al. (2012) and Velho et al. (2015) as cylindrical or obclavate.

Statistical analyses were performed on the quantitative morphological features (which excluded colony color and shape of conidia) to test for differences between the isolates. Before 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 R Statistical Software (R Development Core Team 2019).

An enzymatic characterization with the APIZYM assay (Bio-Merieux SA, Paris, France) was performed for selected isolates to assess the

extracellular activity of 19 enzymes: alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, a-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α -galactosidase, β -galactosidase, β -glucuronidase, α -glucosidase, β -glucosidase, N-acetyl- β -glucosaminidase, α -mannosidase, and α -fucosidase. Two representative isolates of *C. fioriniae* (18-DSS-BS-CF-1-002 and 18-DSS-BS-NTO-1-010) and two isolates of *C. godetiae* (18-DSS-BS-EL-1-016 and 18-DSS-BS-EL-1-028) were selected for this analysis. Small pieces of fungal PDA cultures were transferred to 30 ml of potato dextrose broth (Biolab, Budapest, Hungary), and the cultures were

Table 1. Isolates of *Colletotrichum* spp. obtained from apples with symptoms of bitter rot that were collected from packinghouses in South Tyrol in 2018 and 2019 and obtained in previous years at the Laimburg Research Centre

No.	Sample code	Apple cultivar	Fungal species	Year	Apple growing area	Farming practice	GenBank accession numbers ^a			
							ACT	GAPDH	HIS3	ITS
1	18-DSS-BS-CF-1-002	Coop 38/Gold Rush	<i>Colletotrichum fioriniae</i>	2018	Meran/Merano	Organic	MT980849	MT980873	MT980892	MT812218
2	18-DSS-BS-EL-1-012	Roho 3615/Evelina	<i>Colletotrichum salicis</i>	2018	Unterland/Bassa Atesina	Organic	MT347599*	MT347600*	MT347598*	MT33738*
3	18-DSS-BS-EL-1-016	Roho 3615/Evelina	<i>Colletotrichum godetiae</i>	2018	Unterland/Bassa Atesina	Organic	MT980859	MT980875	MT980899	MT812203
4	18-DSS-BS-EL-1-022	Roho 3615/Evelina	<i>Colletotrichum godetiae</i>	2018	Unterland/Bassa Atesina	Organic	MT980866	MT980888	MT980908	MT812204
5	18-DSS-BS-EL-1-023	Roho 3615/Evelina	<i>Colletotrichum godetiae</i>	2018	Unterland/Bassa Atesina	Organic	MT980860	MT980876	MT980906	MT812205
6	18-DSS-BS-EL-1-028	Roho 3615/Evelina	<i>Colletotrichum godetiae</i>	2018	Unterland/Bassa Atesina	Organic	MT980858	MT980885	MT980909	MT812206
7	18-DSS-BS-EL-1-031	Roho 3615/Evelina	<i>Colletotrichum godetiae</i>	2018	Unterland/Bassa Atesina	Organic	MT980861	MT980877	MT980895	MT812207
8	18-DSS-BS-EL-1-032	Roho 3615/Evelina	<i>Colletotrichum godetiae</i>	2018	Unterland/Bassa Atesina	Organic	MT980862	MT980878	MT980900	MT812208
9	18-DSS-BS-EL-1-033	Roho 3615/Evelina	<i>Colletotrichum godetiae</i>	2018	Unterland/Bassa Atesina	Organic	MT980863	MT980886	MT980907	MT812209
10	18-DSS-BS-LF-1-001	Coop 38/Gold Rush	<i>Colletotrichum fioriniae</i>	2018	Meran/Merano	Organic	MT980850	MT980869	MT980891	MT812215
11	18-DSS-BS-LF-1-005	Coop 38/Gold Rush	<i>Colletotrichum fioriniae</i>	2018	Meran/Merano	Organic	MT980851	MT980872	MT980893	MT812216
12	18-DSS-BS-NTO-1-010	SQ 159/Natyra	<i>Colletotrichum fioriniae</i>	2018	Meran/Merano	Organic	MT980852	MT980870	MT980894	MT812217
13	19-DSS-BS-EL-2-001	Roho 3615/Evelina	<i>Colletotrichum godetiae</i>	2019	Eisacktal/Valle Isarco	Organic	MT980864	MT980879	MT980896	MT812210
14	19-DSS-BS-EL-2-010	Roho 3615/Evelina	<i>Colletotrichum godetiae</i>	2019	Eisacktal/Valle Isarco	Organic	MT980865	MT980880	MT980904	MT812211
15	19-DSS-BS-EL-2-019	Roho 3615/Evelina	<i>Colletotrichum godetiae</i>	2019	Meran/Merano	Organic	MT980867	MT980887	MT980901	MT812212
16	19-DSS-BS-3-001	Roho 3615/Evelina	<i>Colletotrichum godetiae</i>	2019	Meran/Merano	Organic	MT980855	MT980883	MT980903	MT812199
17	19-DSS-BS-3-002	Roho 3615/Evelina	<i>Colletotrichum godetiae</i>	2019	Meran/Merano	Organic	MT980868	MT980889	MT980910	MT812200
18	18-DSS-CAFA-DEC	Golden Delicious	<i>Colletotrichum godetiae</i>	2018	Meran/Merano	Integrated	MT980857	MT980874	MT980897	MT812202
19	18-DSS-BS-DEC	Roho 3615/Evelina	<i>Colletotrichum godetiae</i>	2018	Meran/Merano	Organic	MT980856	MT980884	MT980898	MT812198
20	Lb 215/08	Granny Smith	<i>Colletotrichum godetiae</i>	2008	n/a ^b	n/a	MT980853	MT980881	MT980905	MT812213
21	CA/2 (Apple fruit)	n/a	<i>Colletotrichum godetiae</i>	2013	n/a	n/a	MT980854	MT980882	MT980902	MT812201
22	1A_01 (Apple leaf spots)	n/a	<i>Colletotrichum fioriniae</i>	2018	n/a	n/a	MT980848	MT980871	MT980890	MT812214

^a The accession numbers marked with an asterisk (*) were taken from Amaral Carneiro et al. (2021). ACT, actin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HIS3, histone 3; ITS, internal transcriber spacer.

^b n/a, not available.

Table 2. Genomic regions analyzed in this study with information on the applied primer pairs, their annealing temperatures, and the expected amplicon length

Target genomic region	Primer names	Annealing temperature (°C)	Amplicon length (bp)	Reference
Actin (ACT)	ACT-512F/ACT-783R	58	~200	Carbone and Kohn (1999)
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	GDF1/GDR1	60	~200	Guerber et al. (2003)
Histone H3 (HIS3)	CYLH3F/CYLH3R	58	~400	Crous et al. (2004)
Internal transcribed spacer (ITS)	UP18S42/LO28S22	60	~600	Levesque and de Cock (2004)

incubated on a reciprocating shaker at 100 rpm at 20°C for 10 days. After that, the mycelium was removed by filtration through three layers of sterile gauze and the filtrates were tested (Żukiewicz-Sobczak et al. 2016). API ZYM tests were carried out by placing 65 µl of filtrate at the appropriate positions of the test strip and incubating the strips for 3 days at 20°C. Apart from the initial step, the assay was performed according to the manufacturer's instructions.

Pathogenicity test. Based on molecular genetic data, three of the four *Colletotrichum* isolates investigated in the enzymatic assay (18-DSS-BS-NT0-1-010, 18-DSS-BS-EL-1-016, and 18-DSS-BS-EL-1-028) were selected to perform a pathogenicity test and prove their capacity to induce postharvest decay of apples. Pathogenicity assessment was accomplished on the cultivars Cripps Pink, Golden Delicious, and Roho 3615/Evelina. Twelve apples were used for each apple cultivar and fungal isolate. The apple fruits were washed with curd soap and incubated in a 0.5% sodium hypochlorite solution for 5 min, rinsed with distilled water, and dried with sterile paper soaked with 70% ethanol. Conidial suspensions from 15-day-old cultures on PDA were prepared as previously described, and the conidial concentration was determined with a hemocytometer and adjusted to 10^6 conidia ml⁻¹. Three wounds were made on the equatorial plane of the fruit with a sterile piercing tool (depth 6 mm × width 1 mm) at 0°, 90°, and 180°. Inoculation was performed with 20 µl of spore suspension, whereas control fruits were inoculated with 20 µl of sterile distilled water. Inoculated fruits were incubated for 21 days in moist chambers at 20°C in the dark. External symptoms were assessed, and the size of the lesions was measured at 3, 5, 7, 10, 14, and 21 days post inoculation (dpi). At the end of the experiment, three infected apples of each cultivar were selected and fungal pathogens were reisolated from the internal lesions on PDA medium. The cultures were grown for 10 days under the conditions described previously to obtain sufficient mycelium for DNA extraction. The ITS region was amplified by PCR, and Sanger sequencing was performed to confirm the identity of the isolates as described previously.

Results

Distribution and disease symptoms. In 2018 and 2019, a total of 1,071 fungal cultures was obtained from apples with symptoms of post-harvest diseases that were collected in six packinghouses in South Tyrol. Only 19 of these isolates were determined to be *Colletotrichum* spp. Apple rot symptoms caused by this pathogen ranged from small, circular, slightly sunken, brown lesions to larger lesions with copious quantities of spores, produced in acervuli arising in concentric circles from the infection point. Most isolates were obtained from the cultivar Roho 3615/Evelina, and almost all the apples from which this genus was isolated derived from organic cultivation (Table 1).

Phylogenetic analysis. A total of 22 *Colletotrichum* isolates (19 obtained in the present study and three isolated in previous years at the Laimburg Research Centre) were further identified to the species level based on DNA sequence analysis of four genomic regions, ACT, GAPDH, HIS3, and ITS. Each DNA sequence acquired in the present study was individually trimmed at the same position at either end, and the final sequence lengths ranged from 184 to 186 bp, 155 to 156 bp, 313 to 317 bp, and 505 to 507 bp for ACT, GAPDH, HIS3, and ITS, respectively. The DNA sequences obtained in the present study were aligned with those of 35 reference isolates belonging to different species of the *C. acutatum* SC available in GenBank (Table 1 and Supplementary Table S1), resulting in a concatenated multilocus alignment with 1,194 positions. The phylogenetic tree based on maximum likelihood analysis (Fig. 1) showed that most *Colletotrichum* isolates found in South Tyrol (16/22) clustered in the monophyletic *C. godetiae* group. This group, among others, comprised type specimens 117418/2015 and CBS 131331 of this species, which were described in the studies by Da Lio et al. (2018) and Damm et al. (2012). A smaller number of isolates from South Tyrol (5/22) clustered in the monophyletic *C. fioriniae* group. This group contained the type specimens 117288/2015 and CBS 125396 of *C. fioriniae*, which were also described by Lio et al. (2018) and Damm et al. (2012). Only 1 of the 22 isolates from South Tyrol was grouped with type specimens CBS 113.14 and CBS 115.14 of *C. salicis* described by Damm et al.

(2012) (Amaral Carneiro et al. 2021). All the clades comprising distinct isolates of the three species *C. godetiae*, *C. fioriniae*, and *C. salicis* that have been identified thus far in South Tyrol were supported by bootstrap values of 100%.

Pairwise distance matrices obtained separately for each genomic region and comprising DNA sequences of each isolate from South Tyrol revealed the presence of two different ITS haplotypes for *C. fioriniae* (ITS-a: 4 isolates and ITS-b: 1 isolate), whereas only one ITS haplotype (ITS-c) was observed for all 16 isolates of *C. godetiae*. The haplotypes ITS-a and ITS-b of *C. fioriniae* differed in a single point mutation (Supplementary Table S2). The 186-bp segment of ACT displayed one haplotype for all the isolates of *C. fioriniae* (ACT-a) and two haplotypes for *C. godetiae* (ACT-b: 15 isolates and ACT-c: 1 isolate). ACT-b and ACT-c differed in two point mutations (Supplementary Table S3).

The DNA sequences of the GAPDH and HIS3 genes (157 bp and 317 bp, respectively) showed the highest degree of variability among the isolates obtained in the present study. While the GAPDH gene segment revealed one haplotype for all the isolates of *C. fioriniae* (GAPDH-a), three haplotypes were found for *C. godetiae* (GAPDH-b: seven isolates, GAPDH-c: eight isolates, and GAPDH-d: one isolate). The GAPDH-b, GAPDH-c and GAPDH-d sequences differed in two point mutations (Supplementary Table S4). Regarding the HIS3 gene segment, also in this case, one haplotype was found in all the isolates of *C. fioriniae* (HIS3-a) and three haplotypes were found for *C. godetiae* (HIS3-b: 12 isolates, HIS3-c: 3 isolates, and HIS3-d: 1 isolate). For the latter, variable sites were found on five positions (Supplementary Table S5). With the combination of the individual genotypes of the different genomic regions analyzed, six different multilocus genotypes (labeled from I to VI) were observed (Supplementary Tables S6). As observed in Figure 1, two multilocus genotypes of *C. fioriniae* (I and II) and four multilocus genotypes of *C. godetiae* (III to VI) were found in the present work, whereas the seventh multilocus genotype, identified as *C. salicis*, was described by Amaral Carneiro et al. (2021).

Morphological characterization. Morphological characterization focused on isolates of *C. godetiae* and *C. fioriniae*, whereas *C. salicis* was described in a previous study (Amaral Carneiro et al. 2021). The appearance of colonies of *C. godetiae* on the upper side of PDA was mostly flat with a distinct margin, the surface was covered with short floccose aerial mycelium, and the color ranged from light gray to dark gray, whereas the reverse side appeared gray to dark gray with black streaking and black spots inside the growth medium. Isolate 18-DSS-BS-EL-1-016 differed considerably from other *C. godetiae* isolates because it was flat with an undulate margin, it lacked aerial mycelium, the color was dark green olivaceous on both the upper and reverse sides, and sporulation was observed already on the third day of growth. The other *C. godetiae* isolates showed orange-salmon conidial masses and dark melanized structures similar to acervuli on the seventh day of growth. Two isolates identified as *C. fioriniae*, 18-DSS-BS-CF-1-002 and 18-DSS-BS-NT0-1-010, appeared light gray on the upper side with pink nuances and light pink on the reverse side with green-olivaceous spots. Salmon-orange conidial masses were observed to be randomly distributed on the growing mycelium after 7 days' growth. The two remaining *C. fioriniae* isolates, 18-DSS-BS-LF-1-001 and 18-DSS-BS-LF-1-005, did not show pinkish mycelium typical of *C. fioriniae* (Grammen et al. 2019), but they were light gray to bluish gray with whitish margins on the upper side and whitish gray to dark green on the reverse side (Supplementary Fig. S1). No sporulation was observed in these isolates after 2 weeks of growth.

Morphological characteristics such as shape and size of conidia and colony color of two representative isolates of *C. fioriniae* and *C. godetiae* are described in Table 3, and mycelium growth rates are shown in Supplementary Figure S2. *C. fioriniae* samples showed a larger in vitro mycelium growth area (25.1 ± 0.9 and 24.6 ± 1.4 cm²) than *C. godetiae* samples (13.9 ± 1.2 and 5.7 ± 0.1 cm²) after 7 days' growth. On the 14th day, the mycelium of the two isolates of *C. fioriniae* and the *C. godetiae* isolate 18-DSS-BS-EL-1-028 covered the whole area of the Petri dish with PDA medium, whereas the mycelium area of the *C. godetiae* isolate 18-DSS-BS-EL-1-016 increased to 17.7 ± 3.0 cm². Spore characteristics (length and width) were significantly different between the two species. The spores of the *C. godetiae* isolates,

18-DSS-BS-EL-1-016 and 18-DSS-BS-EL-1-028 (15.8 ± 1.5 and $14.8 \pm 1.2 \mu\text{m}$, respectively), were larger than those of the *C. fioriniae* isolates, 18-DSS-BS-CF-1-002 and 18-DSS-BS-NTO-1-010 (13.9 ± 1.2 and $13.2 \pm 0.8 \mu\text{m}$, respectively; $P < 0.05$) (Table 3). The shape

of conidia of the two *C. godetiae* isolates were typically cylindrical, whereas the conidia of the two *C. fioriniae* isolates had an obclavate shape with acute ends. Colony color and conidia shape and size of *C. salicis* are described by Amaral Carneiro et al. (2021).

Fig. 1. Multigene phylogenetic maximum-likelihood tree constructed using MEGA X software (Kumar et al. 2018) with concatenated sequences of actin (ACT), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), histone 3 (HIS3), and internal transcriber spacer (ITS) of *Colletotrichum* isolates causing bitter rot of apple. The phylogenetic analysis was based on an alignment with 1,194 positions. *Colletotrichum lindemuthianum* was used as an outgroup. Sequences of the same set of genomic regions as analyzed in the present study of 35 reference isolates representing different species of the genus were selected from the works of Da Lio et al. (2018) and Damm et al. (2012) (Supplementary Table S1). The numbers at the branches of the tree (>95%) represent bootstrap values obtained after 1,000 replications. The 22 *Colletotrichum* isolates characterized in this study are highlighted in bold (19 obtained in the present study and three isolated in previous years at the Laimburg Research Centre). Roman numerals I to VI, marked in red, indicate the different multilocus genotypes found in this study. The isolate of *Colletotrichum salicis* described in the work of Amaral Carneiro et al. (2021) is underlined and marked with an asterisk (*).

The API ZYM test indicated enzyme activity of alkaline phosphatase, esterase lipase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, β -galactosidase, and β -glucosidase for the two isolates of *C. fioriniae* (18-DSS-BS-CF-1-002 and 18-DSS-BS-NT0-1-010) and two isolates of *C. godetiae* (18-DSS-BS-EL-1-016 and 18-DSS-BS-EL-1-028). For none of the investigated fungal isolates was activity of leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α -chymotrypsin, β -glucuronidase, α -glucosidase, or α -fucosidase observed. Positive reactions for esterase (C4) were revealed for both *C. godetiae* isolates but not for the *C. fioriniae* isolates. The isolates 18-DSS-BS-CF-1-002 (*C. fioriniae*) and 18-DSS-BS-EL-1-028 (*C. godetiae*) were found to produce N-acetyl- β -glucosaminidase and α -mannosidase, whereas isolate 18-DSS-BS-NT0-1-010 was the only one with a positive reaction for lipase enzyme activity (see Supplementary Table S7).

Pathogenicity test. *C. fioriniae* and *C. godetiae* were pathogenic on all three apple cultivars tested, Cripps Pink, Golden Delicious, and Roho 3615/Evelina, because 93% of the inoculated wounds were successfully infected and showed typical symptoms of bitter rot. The lesions became evident at 5 dpi on all the three apple cultivars tested, and the funnel-shaped decay of the pulp, which is typical of bitter rot, was noticed at 10 dpi. Isolate 18-DSS-BS-NT0-1-010 of *C. fioriniae* induced brown circular lesions with salmon-orange spore masses on the lesion surface, whereas the two *C. godetiae* isolates (18-DSS-BS-EL-1-016 and 18-DSS-BS-EL-1-028) formed dark brown to black lesions with concentric rings of black conidial masses on the lesion surface. Isolate 18-DSS-BS-EL-1-016 was the only one to induce black decay of the pulp tissue on the cultivars Cripps Pink and Golden Delicious (Supplementary Figs. S3 to S5).

C. godetiae isolates expanded faster on Golden Delicious and Cripps Pink over the entire incubation period of 21 days. At the end of the experiment, the lesion diameters induced by isolates 18-DSS-BS-EL-1-016 and 18-DSS-BS-EL-1-028 were 2.4 ± 0.6 and 2.6 ± 0.3 cm (mean $\pm$ SD), respectively, compared with 2.0 ± 0.9 cm of isolate 18-DSS-BS-NT0-1-010. Conversely, on Roho 3615/Evelina, *C. godetiae* isolates showed higher growth rates compared with *C. fioriniae* until 14 days of incubation; however, after 21 days of incubation, the lesion diameter of *C. fioriniae* 18-DSS-BS-NT0-1-010 (3.3 ± 1.0 cm) was larger than that induced by the two *C. godetiae* isolates, 18-DSS-BS-EL-1-016 and 18-DSS-BS-EL-1-028 (3.2 ± 1.0 cm and 2.8 ± 1.2 cm, respectively) (Fig. 2). Based on statistical analyses of lesion diameters, Golden Delicious appeared to be more tolerant ($P < 0.01$) to the *Colletotrichum* isolates tested, whereas Roho 3615/Evelina was the most susceptible apple cultivar in this study ($P < 0.01$).

Discussion

The present study is the first to address the pathogen species associated with bitter rot of apple in South Tyrol. Although this region is one of the largest apple-producing areas in Europe, contributing approximately 10% to apple production of the European Union, most of which are stored in sophisticated storage facilities (Autonomous Province of Bozen-Bolzano 2020), scientific data about postharvest pathogens in South Tyrol are not readily available. Several years ago, the genetic diversity of the genus *Tilletiopsis* spp., which can induce a postharvest disorder known as white haze as well as affect apple fruit before harvest, was assessed in South Tyrol (Baric et al. 2010). Marshall and

Rizzolli (2015) characterized the occurrence of different postharvest diseases and concluded that bitter rot of apple so far did not represent a major postharvest disease in South Tyrol. Nevertheless, knowledge about the species associated with this disease can be of practical

Fig. 2. Increase of the average lesion diameters ($\pm$ standard error) induced by three *Colletotrichum* isolates over a period of 21 days and measured on 6 different days post inoculation (dpi). Three apple cultivars **A**, Cripps Pink, **B**, Golden Delicious, and **C**, Roho 3615/Evelina were inoculated. The white column refers to *Colletotrichum fioriniae* isolate 18-DSS-BS-NT0-1-010, whereas the gray and black columns refer to *Colletotrichum godetiae* isolates 18-DSS-BS-EL-1-016 and 18-DSS-BS-EL-1-028, respectively.

Table 3. Morphological features, such as conidia shape and size and colony color, of four representative isolates of *Colletotrichum fioriniae* and *Colletotrichum godetiae*

Species	Isolate	Conidia length (μ m)	Conidia width (μ m)	Ratio (L/W) ^a	Conidia shape	Colony color	
						Upper side	Reverse side
<i>Colletotrichum fioriniae</i>	18-DSS-BS-CF-1-002	13.8 ± 1.1	5.1 ± 0.6	2.7	Obclavate	Light gray with pink nuances	Light pink with green-olivaceous stains
	18-DSS-BS-NT0-1-010	13.8 ± 1.2	4.9 ± 0.5	2.8	Obclavate	Light gray with pink nuances	Light pink
<i>Colletotrichum godetiae</i>	18-DSS-BS-EL-1-028	14.5 ± 1.3	4.9 ± 0.6	2.9	Cylindrical	Gray	Light gray
	18-DSS-BS-EL-1-016	15.7 ± 1.2	5.8 ± 0.5	2.7	Cylindrical	Dark green olivaceous	Dark green olivaceous

^a L, length; W, width.

importance. The characterization of *Colletotrichum* spp. in the present study was performed by combining molecular genetic analysis with a morphological and pathogenicity assessment. The results of the phylogenetic analysis of multilocus DNA sequences showed that the isolates obtained from decayed apples with symptoms of bitter rot belonged to the species *C. godetiae*, *C. fioriniae*, and *C. salicis*, all being part of the *C. acutatum* SC (Damm et al. 2012). *C. godetiae* was the most common species (16/22 isolates) associated with bitter rot of apple in our study, especially on the cultivar Roho 3615/Evelina derived from organic production. In Europe, most of the identified causal agents of bitter rot of apple belong to the *C. acutatum* SC (Baroncelli et al. 2014; Børve and Stensvand 2015; Grammen et al. 2019; Ivic et al. 2013; Munda 2014; Nodet et al. 2016; Wenneker et al. 2016), whereas species belonging to the *C. gloeosporioides* SC have been reported from the United States (Munir et al. 2016), South America (Velho et al. 2015), Korea (Park et al. 2018), and France and Belgium (Grammen et al. 2019; Nodet et al. 2019).

Although an extensive sampling of apples with postharvest diseases was performed in 2018 and 2019 in South Tyrol, the number of isolates identified as the genus *Colletotrichum* was rather low and was observed in 1.8% of the decayed apples investigated. Among these isolates, no species belonging to the *C. gloeosporioides* complex was found. Bitter rot is considered one of the most important fungal diseases of apple responsible for major economic losses throughout the world (Khodadadi et al. 2020). However, data about the incidence of the disease principally refer to the period before harvest (Grammen et al. 2019; Khodadadi et al. 2020; Munda 2014; Munir et al. 2016; Nodet et al. 2016, 2019; Park et al. 2018; Velho et al. 2015), whereas data about the extent of postharvest losses of apple induced by *Colletotrichum* spp. are rarely available. It may well be the case that a higher number of isolates of *Colletotrichum* spp. would be obtained if the sampling were conducted in the orchards during the vegetative season than on apples that were collected after a period of prolonged storage in a controlled atmosphere.

Previous studies on the occurrence of *Colletotrichum* spp. on rotten apples after several months of storage in Italy are few, and they reported the presence of *C. acutatum* in the regions of Emilia-Romagna and South Tyrol (Mari et al. 2011; Marschall and Rizzolli 2015). The identification of *C. acutatum* isolates in the work by Mari et al. (2011) was based on the description of morphological characteristics, such as colony color and conidia shape and size, and was further supported by 100% identification of the DNA sequence of the ITS region with a GenBank accession (AB626881), which was identified as *C. acutatum* (syn. *Glomerella acutata*). The ITS sequence with the accession number AB626881 was submitted to GenBank in 2011, and identification of the fungal isolate from which the DNA sequence was derived as *C. acutatum* was accomplished before the taxonomic revision of the *C. acutatum* SC performed by Damm et al. (2012). Therefore, it is probable that the fungal isolate was not identified as *C. acutatum* sensu stricto but as belonging to the *C. acutatum* SC.

Considering the revised species nomenclature within the *C. acutatum* SC, DNA sequences of the ITS region representative of several species of the complex described by Da Lio et al. (2018) and Damm et al. (2012) were reanalyzed together with GenBank accession AB626881 and all the ITS sequences obtained in the present study (Supplementary Fig. S6). The phylogenetic tree revealed that GenBank accession AB626881 did not cluster in the *C. acutatum* clade sensu stricto but in the monophyletic *C. fioriniae* group. Moreover, the DNA sequence showed 100% identity with the sequence of *C. fioriniae* type specimen CBS 125396 and with one of the isolates obtained in the present study (18-DSS-BS-CF-1-002; Supplementary Fig. S6). This finding would imply that *C. fioriniae* is not a new species in Italy but has already been present as a causal agent of bitter rot of apple since at least 2011. Similarly, we can speculate that *C. acutatum* reported by Marschall and Rizzolli (2015) refers to the SC, but the main causal agent of bitter rot of apple in this area has in fact been the species *C. godetiae*. This assumption is supported by the fact that two *Colletotrichum* isolates obtained from Laimburg Research Centre that were collected in 2008 and 2013 were identified as *C. godetiae* by MLST in the present study. Furthermore, the finding of four different

multilocus genotypes among 16 isolates of *C. godetiae* investigated in the present study may be an indication that this species has been present in this geographic area for a longer time. To the best of our knowledge, *C. godetiae* and *C. fioriniae* have so far never been mentioned as postharvest pathogens of apple in Italy, although there are several indications that these species have been isolated from apples with bitter rot for at least a decade. Consequently, all the isolates obtained from apple in this geographic area and previously identified as *C. acutatum* would need to undergo a revision.

The recognition of ITS as one of the official DNA barcode markers for identification of fungal species has been of great benefit to the scientific community (Schoch et al. 2012). To identify an unknown fungus, generally BLAST (Boratyn et al. 2013), which is associated with the National Center for Biotechnology Information (NCBI) database, is used to compare the ITS sequence fragment of a query to all other DNA sequences contained in the database to identify identical or most similar matches. The BLAST search typically relies on the nucleotide collection, in which a query sequence is compared with all the sequences present in the database. However, it has been recommended that BLAST search identifications against GenBank should be made with caution, because approximately 27% of fungal ITS sequences contained in GenBank were submitted with insufficient taxonomic identification (Raja et al. 2017). To avoid misidentification of fungi, it is possible to perform a more stringent BLAST search by checking the “sequences from type material” box on the general BLAST page and, whenever possible, by selecting DNA sequences of type material or other authenticated isolates for phylogenetic analyses. In addition to this, several curated databases have been established containing DNA sequences of the ITS region and other ribosomal and protein-coding genes of different groups of fungi (Federhen 2012; Raja et al. 2017). In the specific case of *Colletotrichum*, the database Q-bank has a dedicated section for the identification of fungi, which is curated by Dr. Ulrike Damm, and in which the taxonomic description and MLST data of various type specimens are provided (<https://qbank.eppo.int/fungi/>) (EPPO-Q-bank 2021).

In the present study, four molecular markers were analyzed by Sanger sequencing, ACT, GAPDH, HIS3, and ITS to be considered in the multigene phylogenetic analysis. Nevertheless, each of the four gene segments alone proved to be a good marker to distinguish *C. fioriniae*, *C. godetiae*, and *C. salicis* from each other but also from other species of the *C. acutatum* SC. The highest degree of genetic distance at the interspecific level was found for partial DNA sequences of the GAPDH and HIS3 genes, which also represent recommended markers to be used for species identification within the *C. acutatum* SC (Da Lio et al. 2018; Damm et al. 2012). Damm et al. (2012) analyzed six gene regions to obtain a phylogeny (ITS, TUB2, HIS3, GAPDH, CHS-1, and ACT) and showed that not all gene fragments were informative to be used in phylogenetic studies. For example, CHS-1 is not suitable for separating *C. godetiae* from other species in the *C. acutatum* SC, and TUB2 was insufficient to make a reliable distinction between *Colletotrichum* spp. in the study by Grammen et al. (2019). In the present work, it was not possible to obtain a specific PCR product for CHS-1. Despite intensive efforts to optimize the PCR reaction, multiple bands were observed on the agarose gel. The same results were obtained for the TUB2 region when using the primers described by Damm et al. (2012) and Grammen et al. (2019).

Colletotrichum species were traditionally classified using morphological features based on colony color and the shape and size of conidia. However, the investigation of morphological characters performed in the present study, in particular the colony features, showed more variability in phenotypic traits than Grammen et al. (2019). For example, the pinkish colony color and salmon- to orange-colored conidial masses of *C. fioriniae* were not observed in the isolates 18-DSS-BS-LF-1-001 and 18-DSS-BS-LF-1-005. Furthermore, the colony color of *C. godetiae* isolates in the present study ranged from light gray to dark gray, whereas the reverse side appeared gray to dark gray with black streaking and black spots inside the growth medium; Grammen et al. (2019) reported the color of the colonies as light brown on the upper side and a mix of orange, gray, and yellow on the reverse side. In fact, several studies have demonstrated that the morphological traits may not be stable and may differ in dependence of the

experimental conditions. Thus, the morphological approach alone is not reliable for species discrimination within the genus (Cannon et al. 2012; Damm et al. 2012; Velho et al. 2015; Weir et al. 2012).

In this work, extracellular enzymatic profiles of two representative isolates of each species, *C. fioriniae* and *C. godetiae*, were investigated after 10 days of growth in liquid nutrient medium, revealing a high degree of similarity, such as the common activity of alkaline phosphatase, acid phosphatase, and naphthol-AS-BI-phosphohydrolase. These enzymes are necessary in the degradation of organic phosphorus compounds, which are components of the phospholipid bilayer of cell membranes (De Oliveira et al. 2013). Likewise, esterase lipases, important enzymes in the breakdown of ester bonds, which link the monomers of fatty acids such as cutin, were produced by all the isolates. The extracellular enzymes released by plant pathogenic fungi are connected to their ability to degrade the plant cuticle and cell wall components, which causes the onset of the disease. Few studies have focused on extracellular enzymatic profiles of the genus *Colletotrichum*. Fernando et al. (2001) showed that isolates of *C. acutatum* are capable of secreting cellulolytic enzymes, such as β -glucosidase, both in vitro and in vivo. Other works demonstrated the ability of *Colletotrichum fructicola* and *C. gloeosporioides* to produce hydrolytic enzymes such as amylases, pectinases, and lipase, which are important in pathogenesis (Armesto et al. 2019; Velho et al. 2018).

In the present work, we observed that all the investigated *Colletotrichum* isolates manifested activity of β -glucosidase, which plays a key role in the degradation of cellulose and β -galactosidase and represents one of the cell wall-modifying enzymes that plays a role in fruit ripening and softening (Tiwari et al. 2013; Yang et al. 2018). In fact, in the pathogenicity test, both *C. fioriniae* (18-DSS-BS-NT0-1-010) and *C. godetiae* (18-DSS-BS-EL-1-016 and 18-DSS-BS-EL-1-028) were able to colonize and cause putrefaction of the fleshy pulp tissue independent of the apple cultivar. However, some differences were noticed: esterase C4 was observed only in *C. godetiae* samples and lipase (C14) in isolate 18-DSS-BS-NT0-1-010 (*C. fioriniae*). Moreover, isolates 18-DSS-BS-CF-1-002 (*C. fioriniae*) and 18-DSS-BS-EL-1-0128 (*C. godetiae*) differed from the others because they produced N-acetyl- β -glucosaminidase and α -mannosidase, enzymes involved in the degradation of chitin and responsible for the hydrolysis of mannan and other hemicellulose components, respectively (Eades and Hintz 2000).

The API ZYM system is a semiquantitative method designed to detect the activity of 19 enzymes in a single test, and it has been applied as a simple and fast tool for differentiation of bacterial and fungal species, especially for microorganisms that cause human diseases and are used in food processing (Colombo et al. 2018; Humble et al. 1977; Tharagonnet et al. 1977; Tofalo et al. 2011; Żukiewicz-Sobczak et al. 2016). A few studies concerning phytopathogens exist; however, to the best of our knowledge, this method has never been applied for the enzymatic characterization of fungal species belonging to the genus *Colletotrichum*. De Oliveira et al. (2013) recommended the API ZYM test for the characterization of the fungus *Corynespora cassicola*, which displays a wide host plant range. Although differences in enzymatic profiles have been observed, the small number of samples involving only five isolates did not permit obtaining more conclusive results (De Oliveira et al. 2013). Likewise, some enzymatic profile differences were observed in the present study; however, more samples of *C. fioriniae* and *C. godetiae* should be tested to confirm and reproduce these results.

Breeding new apple cultivars and rootstocks is a crucial factor for the implementation of more sustainable fruit-growing systems such as organic agriculture. Some of the new apple cultivars that have increasingly been grown in organic production systems are Coop 38/Gold Rush, Roho 3615/Evelina, and SQ 159/Natyra because they carry resistance genes to apple scab (Kienzle and Kelderer 2017). In 2001, Biggs and Miller (2001) demonstrated that some scab-resistant apple cultivars are particularly susceptible to *C. acutatum*, however. For example, Pristine was described as the most susceptible cultivar, whereas Enterprise showed a high degree of susceptibility, and Coop 38/Gold Rush presented moderate levels of susceptibility.

The total organic farming area in the countries of the European Union was 13.4 million ha in 2018 and is expected to grow in the coming years (Eurostat 2020). In Italy, organic apples were grown on 8,235

ha in 2019 (Sinab 2020). As for apple production in South Tyrol, approximately 10% of the cultivation area is organically managed (Südtiroler Bauernbund 2017) and approximately 60,500 metric tons of organic apples were harvested in 2019 (Autonomous Province of Bozen-Bolzano 2020). A remarkable observation in the present work was that 18 of 19 *Colletotrichum* isolates were obtained from organically grown apples. Moreover, 13 of 16 *C. godetiae* isolates were obtained from Roho 3615/Evelina, whereas three of the four *C. fioriniae* isolates found in our 2-year sampling survey were obtained from the cultivar Coop 38/Gold Rush, and one was isolated from SQ 159/Natyra. Considering that one of the goals of South Tyrolean agriculture is to double the area of organically managed land in the next years (Südtiroler Bauernbund 2017), one would expect a further increase in the cultivation of scab-resistant but potentially *Colletotrichum*-susceptible apple cultivars.

For this reason, research should focus on assessing the susceptibility of the most important apple cultivars grown organically in South Tyrol to *Colletotrichum* species but also to other preharvest and postharvest pathogens. Furthermore, it is crucial to conduct a large-scale survey on the incidence of *Colletotrichum* in organically and conventionally managed orchards and compare the incidence and species composition to those occurring after a period of prolonged storage in a controlled atmosphere. This survey would provide insight into whether geographic distinctiveness or cultivation practices in this region influence the frequency of various species of the genus *Colletotrichum* and their ability to remain latent in apple fruit. An additional aspect to emphasize is that species of the genus *Colletotrichum* cause devastating diseases on a wide range of fruit crops and that *C. acutatum* SC isolated from other host species were shown to be pathogenic on apple fruit as well (Dowling et al. 2020; Grammen et al. 2019). Zapparata et al. (2017) reported *C. godetiae* as a causal agent of grape berry rot (*Vitis vinifera*) in the Tuscany region (Italy). Considering that grapevine is the second most important permanent culture in South Tyrol and vineyards cover an area of 5,553 ha (Autonomous Province of Bozen-Bolzano 2020), it is important to understand whether and to which degree cross-infections with *Colletotrichum* among grapevine and apple can occur.

The present study demonstrated that different species of the *C. acutatum* SC are present as postharvest pathogens of apple in South Tyrol but so far play a minor role compared with *Penicillium expansum*, *Phlyctema vagabunda* (syn. *Neofabraea alba* and *Neofabraea vagabunda*), or *Botrytis cinerea* (unpublished data). Nevertheless, the expansion of organic apple production and the conversion to new potentially susceptible cultivars could be factors leading to an increase of the incidence of bitter rot. Therefore, further knowledge about the biology and epidemiology of *Colletotrichum* spp. is of utmost importance to predict whether and to which degree bitter rot could become a problem in South Tyrol, as was observed in other apple-growing areas.

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