***Penicillium* Species Associated with Preharvest Wet Core Rot in South Africa and Their Pathogenicity on Apple**

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ABSTRACT

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Symptoms associated with the core region of apple fruits (*Malus domestica*) can be classified as moldy core (MC), wet core rot (WCR), and dry core rot (DCR). Infections leading to WCR are thought to occur primarily postharvest, although in South Africa preharvest symptoms also have been reported. The first aim of this study was to investigate the causative agent(s) of preharvest WCR by isolating fungi from eight internal positions in asymptomatic, MC, WCR, and DCR fruits. Secondly, the pathogenicity and virulence of all *Penicillium* isolates were investigated using three apple fruit inoculation methods: surface wounding, deep wounding, and nonwounding. Isolation of fungi from WCR fruits showed that *Penicillium* was the predominant fungal genus from most isolation positions including the lesion area. *Penicillium ramulosum* was the predominant species isolated from all fruits. However, in WCR fruits, the incidence (58%) of *P. ramulosum* was much higher than in MC (6%), DCR (7%), or asymptomatic (7%) fruits. Less frequently isolated *Penicillium* species included *P. expansum* and a few other species. Pathogenicity testing using the nonwounding method was best at discriminating highly virulent isolates. *P. expansum* was the most virulent species, followed by a putative new *Penicillium* species with closest sequence similarity to *P. dendriticum*. *P. ramulosum* isolates, although showing varying degrees of virulence, all had low virulence, causing only small lesions in wounded apple fruits.

In South Africa, core rots are among the most important postharvest diseases of apple (*Malus domestica*) and are mainly associated with susceptible Delicious strains that have open calyx tubes (3,4,25). Postharvest losses can range between 5 and 8% (25), with additional losses occurring prior to harvest as a result of premature fruit drop in orchards (3,28). Core rots are categorized as dry core rot (DCR) and wet core rot (WCR), depending on the appearance of the rot in the fleshy tissue surrounding the core. WCR is economically important, since symptoms expand rapidly during storage causing complete fruit rot (4). Another symptom associated with apple core regions is moldy core (MC). MC is not economically important

in most countries, since visible symptoms are restricted to the core (1,2,4,28,30). Although many fungi have been associated with DCR and WCR, *Alternaria* and *Penicillium* are considered to be the predominant genera involved (4,5,28).

WCR is mainly known as a postharvest disease but has been reported as a preharvest disease in South Africa. Postharvest infections occur when pathogen spores enter the apple core when fruit are dipped in contaminated water in packinghouses (3,30). In South Africa, postharvest WCR was first reported in 1973 as a serious disease, affecting 4 to 20% of Starking apples (3). These losses were mostly associated with apples that were dipped in diphenylamine (DPA) emulsions, which increased infection by *Penicillium* (3). For preharvest WCR, incidences in the range of 0.3 to 1.7% have recently been reported by van der Walt (34). The mode and time of infection for preharvest WCR are unknown.

The specific *Penicillium* species causing WCR have not been studied extensively. In South Africa and the United States, *P. expansum* has been reported as the cause of extensive WCR during postharvest handling of fruit (3,30). *P. roquefortii* has also been isolated from apple fruit with post-

harvest WCR, but it did not cause WCR when inoculated preharvest (30). For preharvest WCR, *P. funiculosum* and *P. expansum* have been reported as causative agents in South Africa (4,5,25).

Identifying *Penicillium* species through the sole use of morphological characteristics is difficult. Therefore, sequencing of the internal transcribed spacer (ITS) region, the cytochrome c oxidase 1 gene (*COI*), and the β -tubulin gene is helpful for identification (12,13,24,27). The β -tubulin gene is the most divergent of these regions, but unfortunately databases for species outside of *Penicillium* subgenus *Penicillium* are limited (24).

Several methods have been published for evaluating the pathogenicity and virulence of *Penicillium* isolates toward apple fruits. These include the application of spore suspensions to surface wounds (29), inoculation of conidia and/or hyphae into 3-mm-deep surface wounds (4,30), and injection of spores into the core region using a syringe (3,15,30). An alternative method for evaluating the pathogenicity of dry core rot pathogens, such as *Alternaria* spp., is by wounding fruit surfaces with a colonized toothpick (25).

WCR is becoming increasingly problematic in South Africa. Therefore, the first aim of the study was to determine which fungi were associated with apple fruit showing symptoms of WCR, DCR, and MC, as well as asymptomatic fruit. The second aim was to identify the *Penicillium* species associated with apples showing symptoms of WCR, DCR, and MC, as well as asymptomatic fruit, using morphological and molecular methods. Lastly, the pathogenicity of the isolates was determined using three inoculation methods.

MATERIALS AND METHODS

Sampling strategy. Apples were collected during 2005–06 (one orchard) and 2006–07 (11 orchards) in three production regions in South Africa: Ceres (six orchards), Grabouw (four orchards), and Ermelo (one orchard). In each orchard, 238 to 300 fruits were sampled by randomly harvesting two to three mature fruits per tree throughout the orchard.

Isolation of fungi from apples. Isolations were made from eight internal posi-

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*The e-Xtra logo stands for “electronic extra” and indicates that Figures 4, 8, and 9 appear in color online.

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tions in asymptomatic, WCR, DCR, and MC fruits (Fig. 1). Fungal isolation was attempted from all the WCR apples, whereas from asymptomatic, DCR, and MC apples, only randomly selected subsets of fruit representative of the different orchards were selected for isolations. During the 2005–06 and 2006–07 seasons, fungi were isolated from three and 23 WCR apples, respectively. Fungi also were isolated from 25 asymptomatic, nine MC, and one DCR apple in the 2005–06 season, and from 48 asymptomatic, 27 MC, and 43 DCR apples in the 2006–07 season. Apples were first cut in half longitudinally, and halves used for isolations were surface-sterilized for 30 s in 70% ethanol, 2 min in 1% NaOCl, and 15 s in 70% ethanol. Small pieces (1 to 3 mm) were plated onto potato dextrose agar (Difco Laboratories, Detroit, MI) supplemented with 0.04 g streptomycin/liter (PDA⁺). Plates were incubated at 25 ± 4°C on a laboratory bench and inspected regularly for fungal growth. Fungi were subcultured by transferring to fresh PDA⁺ for identification to the genus level. Single-spore cultures of all *Penicillium* isolates were prepared and stored at 4°C on PDA slants as well as in 20-ml bottles containing sterile water.

Morphological characterization of *Penicillium* isolates. Single-spored *Penicillium* isolates ($n = 84$) were first characterized using several morphological traits as previously described (7,16,18,21). Spore suspensions of each isolate, prepared in semi-solid agar (0.2% agar, 0.05% Tween 80) were inoculated at three points on Czapek yeast autolysate agar (CYA), malt extract agar (MEA), and 25% glycerol nitrate agar (G25N) (18,21). The inoculated unwrapped 90-mm petri dishes, containing 20 ml of media, were incubated at 25°C (CYA, MEA, G25N), 5°C (CYA), and 37°C (CYA) in the dark for 7 days (16). Codes and color names for the isolates were assigned according to Kornerup and Wanscher (10).

Molecular characterization of *Penicillium* isolates. *DNA extraction.* Isolates were grown for 14 days on PDA⁺ plates. Mycelia and spores were scraped from plates and genomic DNA was extracted using a slightly modified method of Lee and Taylor (11).

β-tubulin polymerase chain reaction (PCR)–restriction fragment length polymorphism (RFLP) analyses. PCR amplification of the partial β -tubulin gene was conducted using primers Bt2a (5' GGT AACCAATCGGTGCTGCTTTC 3') (8) and PentubR (5' GACGGACGACAT CGAGAACCTG 3'). The PCR reaction mixture consisted of 0.2 μ M of each primer (Bt2a and PentubR), 0.2 mM of each dNTP, 1× PCR buffer (Bioline USA Inc., Taunton, MA), 0.7 U BIOTAQ DNA polymerase (Bioline), 0.2 mg bovine serum albumin (BSA) Fraction V (Roche Diagnostics, South Africa, Randburg), 1.3

μ l DNA, and 3 mM MgCl₂ in a final volume of 50 μ l. Amplifications were conducted in a 2700 Applied Biosystems (Foster City, CA) PCR machine, starting with an initial denaturation cycle of 5 min at 94°C, followed by 40 cycles of 45 s at 94°C, 45 s at 55°C, and 60 s at 72°C and a final extension cycle of 7 min at 72°C. Successful amplification was checked on a 1% agarose gel containing ethidium bromide.

The amplified PCR products were digested using the restriction enzymes *Hae*III and *Rsa*I. The *Hae*III digest reaction consisted of 1× enzyme buffer (Fermentas Inc., Glen Burnie, MD), 1.5 μ l *Hae*III (Fermentas), and 8 μ l PCR product in a total volume of 25 μ l. The *Rsa*I digestion reaction was similar, except that 0.5 μ l *Rsa*I (Fermentas) was used. Mixtures were incubated overnight at 37°C, and 15 μ l of the restriction digestions were run in a lane adjacent to a 50-bp DNA ladder (Fermentas) on a 3% agarose gel (MS-8 agarose, Molecular screen, Hispanagar, Burgos, Spain) containing ethidium bromide. Isolates that exhibited the same restriction pattern for both enzymes were classified into the same β -tubulin PCR-RFLP group.

β-tubulin gene and ITS region sequencing. A subset ($n = 25$) of the isolates (Table 1) that represented the different β -tubulin PCR-RFLP groups were selected for sequencing of the ITS region and β -tubulin gene. These isolates also included representatives of all the morphologically identified species. ITS amplification was conducted using the same PCR reaction conditions as for the β -tubulin gene, except that the ITS1 and ITS4 primers (36) and an annealing temperature of 52°C were used. ITS and β -tubulin PCR products were purified using the MSB Spin PCRapace kit (Invitex, Haus, Berlin) according to manufacturer's instructions. Sequence analyses were conducted by the Central Analytical

Sequencing Facility at Stellenbosch University using the BigDye system (version 3.1 dye terminators, Applied Biosystems) and an ABI 3130XL Genetic Analyzer. Geneious Pro (Biomatters Ltd., Auckland, New Zealand) was used to view ABI trace files, and to obtain consensus double-strand sequences for each isolate. Sequence data were submitted to GenBank (<http://www.ncbi.nlm.nih.gov/Genbank/>), and all the sequenced isolates were deposited in the Stellenbosch University culture collection (STEU) at the Department of Plant Pathology (Table 1).

Species identification and phylogenetic analyses. The consensus double-strand sequences of the isolates ($n = 25$) were subjected to BLAST analyses in GenBank. The BLAST analyses of both gene regions were able to confirm the identity of isolates within *Penicillium* subgenus *Penicillium* (*P. expansum*) and *Penicillium* subgenus *Aspergilloides* (*P. chermisinum* and *P. glabrum*), since 100% sequence similarity was obtained. However, for isolates with similarity to species within *Penicillium* subgenus *Biverticillium*, phylogenetic analyses were required since some isolates only had low sequence similarity to known species in GenBank. Furthermore, isolates that were morphologically identified as *P. ramulosum* showed some sequence variation, which also required further investigation.

Phylogenetic analysis of the ITS region was conducted using a dataset containing sequences of *Penicillium* subgenus *Biverticillium* and associated *Talaromyces* species. Reference sequences were from the studies of Lobuglio et al. (12), Peterson (17), Heredia et al. (9), Seifert et al. (23), and Visagie et al. (35). The dataset was aligned in ClustalX (33), with manual adjustments to alignments made in Se-Al (19). Seven ambiguously aligned regions were identified and replaced with codes,

Fig. 1. Eight positions (lesion area [LS1 and LS2], calyx tube area [CX1 to CX3], core region [CR], blossom end [BL], and stem end [ST]) within an apple fruit from which fungi were isolated.

with step matrices calculated to assign different weights to these codes using INAASE 2.3b (14).

Phylogenetic analysis was also conducted for the β -tubulin gene of *Penicillium* subgenus *Biverticillium* and associated *Talaromyces* species, using only a small dataset with reference sequences from the study of Visagie et al. (35). Introns were aligned manually in Se-Al (19), whereas exons were aligned using ClustalX (33) and then adjusted manually in Se-Al (19).

The aligned ITS and β -tubulin datasets were analyzed in PAUP*v4.0b10 (31) using the neighbor-joining option. *Talaromyces thermophilus* was chosen as an outgroup for the ITS phylogeny based on the study by Heredia et al. (9). For the β -tubulin phylogeny, *Talaromyces trachyspermus* was chosen as the outgroup. Confidence levels in the nodes were determined by performing a bootstrap analysis (1,000 replicates using the neighbor-joining option).

Pathogenicity and virulence of *Penicillium* isolates. The pathogenicity (ability to cause a lesion) and virulence (size of lesion caused) of *Penicillium* isolates were investigated using three inoculation methods that entailed (i) surface wounding, (ii) deep wounding, or (iii) nonwounding of apple fruits with toothpicks colonized by *Penicillium* isolates. Toothpicks were sterilized by autoclaving five times in deionized water, with the water being replaced

after each autoclave session in order to remove bleaching reagents. The toothpicks were then transferred to potato dextrose broth (PDB; Difco Laboratories) and autoclaved again (26). Sterile toothpicks infused with PDB were placed aseptically onto 90-mm PDA dishes, and each dish was inoculated with one agar plug (5 mm diameter) of a 7-day-old *Penicillium* colony growing on PDA⁺. For all inoculation methods, Top Red (strain of cultivar Delicious) apples were first surface-sterilized by immersion for 30 s in 70% ethanol, followed by 2 min in 1% NaOCl and 15 s in 70% ethanol. The apples were air-dried in a laminar flow cabinet.

Surface wounding inoculation method. The pathogenicity and virulence of 84 *Penicillium* isolates, which were identified to species level, were evaluated. Toothpicks colonized with the *Penicillium* isolates, as well as controls consisting of toothpicks that were placed on noninoculated PDA⁺ plates, were first cut in half and then inserted 8 mm into fruits. Each fruit contained six toothpicks: five colonized with a different isolate and a noncolonized control. Four replicates of each isolate were inoculated once into four different fruits. Two of these independent trials were conducted. Inoculated fruits were incubated in moisture chambers at high relative humidity (85 to 90%) for 10 days at 25 $\pm$ 4°C. At the end of the incubation period, toothpicks were removed from apples, and the apples were split longitudinally

across the inoculation points with a sterile knife. Lesions were characterized according to diameter (mm), color (medium brown or light brown), and texture (dry, very wet, or spongy). The diameter of the lesions formed on the surface (skin) of fruits was also measured. Isolations were made from a subset of the apples onto PDA⁺ to fulfill Koch's postulates.

Data of the lesion sizes produced by each *Penicillium* isolate, as well as lesion sizes of different lesion types (T1 to T3), were analyzed using Statistica version 8.0 (Statsoft Inc. 2008, STATISTICA [data analysis software system], version 8.0. www.statsoft.com). Factorial analyses of variance (ANOVA) were done on external lesion diameters with factors being (i) trial repeats (two repeats) and *Penicillium* isolates and also (ii) trial repeats and lesion types. Since the interaction between isolates and trial repeats were significant ($P < 0.001$), as well as the interaction between lesion type and trial repeats ($P < 0.001$), the lesion diameters were compared to lesion types and isolates per trial repeat with ANOVA. Appropriate Bonferroni multiple comparisons were done to investigate where significant differences (interactions) occurred. Where the residuals were not normally distributed, nonparametric tests such as the Kruskal-Wallis analysis of variance test or a bootstrap test was done to confirm the results of the ANOVA. All analyses were conducted at the 5% significance level.

Deep wounding inoculation method. The pathogenicity and virulence of 10 *Penicillium* isolates representing the main *Penicillium* species identified in the study (Table 1) were evaluated. This included one *P. expansum* isolate, seven *P. ramulosum* isolates, and one isolate each of *Penicillium* sp. (aff. *dendriticum*) and *P. glabrum*. Isolates were inoculated by inserting one *Penicillium*-colonized toothpick off-center from the calyx tube at an angle into the fruit until it reached the core cavity. For each isolate, five fruits were inoculated per trial. Controls consisted of five fruits inoculated with an uncolonized toothpick. Two independent trials were conducted. Inoculated fruits were incubated under the same conditions as for the surface wounding experiment, except for the incubation period being longer (2 to 4 weeks), depending on the virulence of isolates. The lesions were characterized for color and texture as described for the surface wounding method.

Nonwounding inoculation method. The same isolates that were used in the deep wounding experiment were also evaluated in the nonwounding method. The nonwounding method was conducted in the same manner as the deep wounding method, except that a colonized toothpick was inserted directly into the calyx tube. This did not result in wounding of fruit tissue since calyx tubes were large enough to allow free passage of the toothpick.

Table 1. *Penicillium* isolates used in the study and identification and cataloging information

STEU culture no. ^a	PCR-RFLP group ^b	Species ID ^c	ITS accession number ^d	β -tubulin accession no. ^d
6561	1	<i>P. expansum</i>	FJ491783	FJ491757
6562	1	<i>P. expansum</i>	FJ491784	FJ491758
6563	1	<i>P. expansum</i>	FJ491785	FJ491759
6564	1	<i>P. expansum</i>	FJ491786	FJ491760
6565	2	<i>P. ramulosum</i>	FJ491796	FJ491771
6566	2	<i>P. ramulosum</i>	FJ491792	FJ491763
6567	2	<i>P. ramulosum</i>	FJ491790	FJ491762
6568	2	<i>P. ramulosum</i>	FJ491787	FJ491761
6569	2	<i>P. ramulosum</i>	FJ491789	FJ491772
6570	2	<i>P. ramulosum</i>	FJ491791	FJ491773
6571	2	<i>P. ramulosum</i>	FJ491794	FJ491774
6572	2	<i>P. ramulosum</i>	FJ491797	FJ491766
6573	2	<i>P. ramulosum</i>	FJ491798	FJ491765
6574	2	<i>P. ramulosum</i>	FJ491793	FJ491767
6575	3	<i>P. sp. (aff. dendriticum)</i>	FJ491806	FJ491781
6576	3	<i>P. sp. (aff. dendriticum)</i>	FJ491807	FJ491780
6577	4	<i>P. glabrum</i>	FJ491804	FJ491776
6578	4	<i>P. glabrum</i>	FJ491805	FJ491777
6579	5	<i>P. ramulosum</i>	FJ491800	FJ491768
6580	5	<i>P. ramulosum</i>	FJ491799	FJ491770
6581	5	<i>P. ramulosum</i>	FJ491795	FJ491764
6582	6	<i>P. sp. (aff. cecidicola)</i>	FJ491803	FJ491779
6583	7	<i>P. chermisinum</i>	FJ491802	FJ491778
6584	8	<i>P. ramulosum</i>	FJ491788	FJ491769
6588	2	<i>P. ramulosum</i>	FJ491801	FJ491775

^a All *Penicillium* cultures were submitted to the Stellenbosch University culture collection (STEU).

^b The polymerase chain reaction (PCR) restriction fragment length polymorphism (RFLP) groups were identified by conducting restriction digestion on β -tubulin PCR products using the enzymes *RsaI* (R) and *HaeIII* (H). Isolates that had the same restriction digest pattern for both enzymes were classified into the same PCR-RFLP group.

^c Based on sequence and morphological identification.

^d ITS = internal transcribed spacer. Accession numbers in GenBank.

RESULTS

Isolation of fungi from apples. *Penicillium* was the dominant fungal genus isolated from WCR fruits. Members of the genus were isolated from 92% (24 out of 26) of WCR fruits, with *Penicillium* recovered from all but one of the orchards in Ermelo. The incidence of *Penicillium* was highest (>51%) in five of the eight isolation positions including lesion area 1 (LS1), lesion area 2 (LS2), calyx tube area 1 (CX1), calyx tube area 2 (CX2), and the core region (CR). *Alternaria* was only prevalent at the CX1 position (56%) and the blossom end (BL) (Figs. 1 and 2).

Penicillium was also isolated in healthy, DCR, and MC fruits, although the inci-

dences were much lower than in WCR fruits for all isolation positions (Fig. 3). MC fruits had the second highest *Penicillium* incidence (<14%). *Penicillium* was isolated at a low frequency (<10%) from DCR fruits (Fig. 3), considering that isolations were made from 44 DCR fruits, as opposed to 26 WCR fruits. Similarly, in asymptomatic apples, the incidence of *Penicillium* was low (<10%) in the 73 analyzed fruits (Fig. 3).

Morphological characterization of *Penicillium* isolates. *Penicillium* isolates were obtained from 40 fruits that included 24 WCR fruits, seven DCR fruits, three MC fruits, and six asymptomatic fruits. Since *Penicillium* isolates were obtained

from more than one of the eight isolation positions within an apple, a total of 84 isolates were obtained, of which 60 isolates were from WCR, 13 from DCR, five from MC, and six from asymptomatic fruits (Table 2).

Characterization based on macro- and micromorphology of the *Penicillium* isolates (7,16,18,21) led to the identification of six distinct groups, which represented *P. expansum*, *P. glabrum*, *P. chermisinum*, *P. ramulosum*, as well as two putative new species (Fig. 4). Although isolate STEU 6583 produced brown exudates and a light brown soluble pigment (Fig. 4) that is not characteristic of *P. chermisinum*, the isolate conformed to all other characters of

Fig. 2. Percentage of wet core rot apples containing different fungal genera within eight internal isolation positions (LS = lesion area, CX = calyx tube region, CR = core region, ST = stem region, and BL = blossom parts). The LS1 and LS2 positions and CX1 to CX3 positions were located in different regions of the apple, as shown in Figure 1. Fungi that were only represented by three or four isolates were grouped under “Other fungi”.

Fig. 3. Percentage of apples with wet core rot (WCR), dry core rot (DCR), moldy core (MC), and asymptomatic apples (Asymp) containing *Penicillium* within eight different isolation positions (LS = lesion area, CX = calyx tube region, CR = core region, ST = stem region, and BL = blossom parts). The L1 and L2 positions and CX1 to CX3 positions were located in different regions of the apple, as shown in Figure 1.

this species and was identified as such. Isolates identified as *P. ramulosum* typically produced strongly funiculose colonies as well as a large amount of glutinous exudates. Isolates also produced biverticillate conidiophores, with the thin acerose phialides giving rise to ellipsoidal to subspheroidal conidia. All of these characters are typical of this recently described new species (35).

Three isolates (STEU 6575, 6576, and 6582) may represent two putative new *Penicillium* species. The isolates showed characters closely resembling species from a synnema-producing clade (35) consisting of *P. cecidicola*, *P. dendriticum*, and *P. ramulosum*. These species typically produce strongly funiculose colonies, with similar conidiophore dimensions (Metulae, 9–17 × 2–3.5 µm; Phialides, 8–15 × 2–3 µm; Conidia, 2–4.5 × 1.5–2.5 µm). Isolates STEU 6575 and 6576, however, were distinct from the synnema-producing clade (*P. cecidicola*, *P. dendriticum*, and *P. ramulosum*) since they characteristically produced colonies with light green conidia *en masse*, a pink soluble pigment on CYA (Fig. 4), and no synnemata. Similarly, isolate STEU 6582 was distinct from the synnema-producing clade since the isolate grew fast on both CYA (5 to 5.5 mm/day) and MEA (6 to 7 mm/day) (25°C), and produced olive brown conidia *en masse* which were light green at the colony margins (Fig. 4).

Molecular characterization of *Penicillium* isolates. *β-tubulin* PCR-RFLP analyses. Eight *β-tubulin* PCR-RFLP groups were identified among the 84 *Penicillium* isolates (Fig. 5). The eight PCR-RFLP groups included representatives of all six morphologically identified species (Table 1).

Species identification and phylogenetic analyses. Analyses of the ITS (±600 bp) and *β-tubulin* (±500 bp) gene sequences confirmed the identity of isolates that were morphologically identified as *P. expansum* (PCR-RFLP group 1), *P. chermisinum* (PCR-RFLP group 7), and *P. glabrum* (PCR-RFLP group 4) (Table 1).

Phylogenetic analyses of *Penicillium* subgenus *Biverticillium* isolates (PCR-RFLP groups 2, 3, 5, 6, and 8) showed that isolates morphologically identified as *P. ramulosum* (PCR-RFLP groups 2, 5, and 8) grouped in a well-supported clade in the ITS (98% bootstrap) and *β-tubulin* (89% bootstrap) phylogenies, along with reference isolates of *P. ramulosum* (35) (Figs. 6 and 7). Although the *P. ramulosum* isolates contained sequence variation in both the ITS and *β-tubulin* regions, no consistent morphological differences could be observed between these isolates. Therefore, the isolates were all considered to belong to this species, according to the species concept of Pitt (18) that was adopted in our study.

The ITS and *β-tubulin* phylogenies also provided support for isolates STEU 6575, 6576, and 6582 (PCR-RFLP groups 3 and 6) representing two putative new species, as also suggested by the morphological analyses. Isolates in PCR-RFLP group 3 (STEU 6575 and 6576) were resolved in a well-supported clade, separate from all known species in the ITS and *β-tubulin* phylogenies, with *P. dendriticum* being its closest relative (Figs. 6 and 7). These isolates will hereafter be referred to as *P. sp.* (aff. *dendriticum*). Isolate STEU 6582 in PCR-RFLP group 6 was unresolved in the ITS phylogeny and grouped separately from all species for which *β-tubulin* sequences were available (Figs. 6 and 7). Since this isolate had *P. cecidicola* as its closest relative (Fig. 6), the isolate will hereafter be referred to as *P. sp.* (aff. *cecidicola*).

Association of different *Penicillium* species with symptomatic and asymptomatic apples. Various *Penicillium* species were found in asymptomatic, DCR, and MC apples. The two predominant *Penicillium* species obtained from DCR apples were *P. expansum* (6% of all isolates) and *P. ramulosum* (7%) (Table 2). In asymptomatic apples, the predominant species was *P. ramulosum* (6%), isolated from within the calyx tube regions (CX1 to CX3) or the core (CR). In the MC apples, only *P. ramulosum* (6%) was obtained,

Table 2. Pathogenicity of 84 *Penicillium* isolates, grouped according to their species identity and the surface lesion type (T1 to T3) that each isolate caused, in a surface inoculation experiment

<i>Penicillium</i> species	Isolation position from apple (number of isolates identified from each position) ^a	Lesion type ^b	Average lesion diam. (mm) ± SE ^c
<i>Penicillium</i> isolates obtained from apples with dry core rot symptoms			
<i>P. expansum</i>	CX3(2), LS1(2), LS2(1)	T1	58.1 ± 4.7
<i>P. ramulosum</i>	LS2(1), CX3(2), CR(1)	T2	11.3 ± 1.5
<i>P. ramulosum</i>	LS2(1)	T3	3.8 ± 3.8
<i>P. ramulosum</i>	CX2(1)	T2 or T3	4.9 ± 2.6
<i>P. glabrum</i>	CX3(1)	T3	2.5 ± 0.4
<i>P. sp.</i> (aff. <i>cecidicola</i>)	LS1(1)	T3	2.0 ± 0.3
<i>Penicillium</i> isolates obtained from asymptomatic apples			
<i>P. ramulosum</i>	CX3(1), CX1(1)	T3	1.1 ± 0.7
<i>P. ramulosum</i>	CR(1)	T1 or T3	3.3 ± 0.5
<i>P. ramulosum</i>	CX1(1), CX2(1)	T2	7.2 ± 2.0
<i>P. glabrum</i>	CX1(1)	T3	0
<i>Penicillium</i> isolates obtained from apples with moldy core			
<i>P. ramulosum</i>	CX2(1)	T2 or T3	6.6 ± 0.9
<i>P. ramulosum</i>	LS2(1), CX2(1), CX3(2)	T3	1.1 ± 0.4
<i>Penicillium</i> isolates obtained from apples with wet core rot symptoms			
<i>P. expansum</i>	LS1(1), LS2(4), CX3(1), CR1(1)	T1	59.6 ± 4.8
<i>P. ramulosum</i>	LS1(8), LS2(1), CX3(6), CX2(5), CR(7), CX1(6), BL(2)	T2	9.2 ± 0.4
<i>P. ramulosum</i>	LS1(5), LS2(1), CR(1), CX2(2), ST(1)	T2 or T3	4.6 ± 0.9
<i>P. ramulosum</i>	CX2(1), CR(3)	T3	1.8 ± 0.9
<i>P. sp.</i> (aff. <i>dendriticum</i>)	LS1(1), CX3(1)	T2	17.2 ± 1.5
<i>P. glabrum</i>	BL(1)	T3	0.0
<i>P. chermisinum</i>	CX3(1)	NL	0.0

^a Isolates were obtained from different isolation positions within apples, as shown in Figure 1, including the two lesion areas (LS1 and LS2), three calyx tube areas (CX1 to CX3), blossom end (BL), stem end (ST), and core region (CR). The number in brackets following the isolation position indicates the number of isolates obtained from a specific isolation position, and that were also tested for pathogenicity.

^b Lesion type produced by *Penicillium* isolates was determined by characterizing color and texture (type) of lesions. The three lesion types that were distinguished included T1 = light brown and very wet, T2 = medium brown and spongy, T3 = medium and dry, very small lesion, and NL = no lesion. The lesion types that are indicated are those that were produced in two independent trials, with each trial containing four inoculated fruits for each isolate. For isolates that produced two different lesion types in the four inoculated apples within a trial or between trials, both lesion types are shown and indicated by “or”.

^c Lesion size is the average of two independent repeat trials, with four apples inoculated within each trial. Lesion size averages and standard errors (SE) are those for the isolates that are grouped within each row.

mainly from tissue surrounding the calyx tube (CX2 and CX3), but it was also found in tissue external to the core region (LS2) (Table 2, Fig. 1).

In WCR fruits, *P. ramulosum* was the dominant species (58% of all isolates) that was isolated from several internal isolation positions, with *P. expansum* (8%) only being isolated occasionally. *P. ramulosum* was also the dominant species isolated from the WCR lesion area (positions LS1 and LS2). *Penicillium* species (aff. *dendriticum*) was also isolated from the lesion area (LS1). *P. expansum* was mainly iso-

lated from the lesion areas (LS1 and LS2) (Table 2).

Some of the *Penicillium* species were present in most of the investigated orchards, whereas the distribution of others was limited. *P. ramulosum* was isolated from almost all of the orchards, except for one orchard in Grabouw and the single orchard that was sampled in Ermelo. *P. expansum* and *P. glabrum* were isolated from four and three orchards, respectively. *P. sp.* (aff. *cecidicola*) and *P. chermisinum* were each only isolated in one Grabouw orchard, whereas *P. sp.* (aff.

dendriticum) was only isolated from one Ceres orchard.

Pathogenicity and virulence of *Penicillium* isolates. Surface wounding inoculation method. The surface wounding inoculation showed that the isolates varied in the size of lesions that they caused, as well as the appearance (color and texture) of the lesions (Table 2, Fig. 8). The appearance of lesions was reproducible for most, but not all isolates between experiments. Three distinct lesion types were identified: T1 (light brown and very wet), T2 (medium brown and spongy), and T3 (medium

Fig. 4. Growth of *Penicillium* spp. on Czapek yeast autolysate agar (left) and malt extract agar (right) after 7 days. Species that were identified from apples included **A**, *P. expansum* isolate STEU 6562, **B**, *P. ramulosum* isolate STEU 6568, **C**, *P. chermisinum* isolate STEU 6583, **D**, *P. sp.* (aff. *dendriticum*) isolate STEU 6575, **E**, *P. glabrum* isolate STEU 6577, and **F**, *P. sp.* (aff. *cecidicola*) isolate STEU 6582.

Fig. 5. Eight polymerase chain reaction (PCR) restriction fragment length polymorphism (RFLP) groups that were identified among *Penicillium* isolates obtained from apples. The PCR-RFLP groups were identified by conducting *Rsa*I (R) and *Hae*III (H) restriction digests on PCR amplified products of the partial β -tubulin gene. A 50-bp DNA ladder (L) was run along with the digest products.

brown color, dry, and very small) (Fig. 8). For 13 of the *P. ramulosum* isolates, the lesion types were inconsistent, ranging between T2 and T3 lesions within the same experiment (Table 2). The T2 lesion most closely resembled the WCR lesions that were found at the time of harvest. The two *P. sp.* (aff. *dendriticum*) isolates both caused a T2 lesion. Lesions caused by *P. expansum* were all of the T1 lesion type (light brown and very wet appearance).

Only one isolate of *P. chermisinum* was found, and this isolate was nonpathogenic (Table 2).

In *Penicillium*-inoculated fruits, this genus was reisolated from lesions, fulfilling Koch's postulates. The control, inoculated with uncolonized toothpicks, never displayed lesions (Fig. 8D), and *Penicillium* was not isolated from the inoculation site.

There were significant differences between the lesion sizes associated with

some lesion types (T1 to T3). The T1 lesion was significantly larger than the T2 and T3 lesions, but no significant differences were present between lesion sizes of the T2 and T3 lesions (data not shown). The lesion size of the T1 lesions was distinct, and the average lesion sizes of isolates in the two experiments ranged from 54 to 65 mm. However, lesion size averages of the T2 lesions (6 to 18 mm) and T3 lesions (0.0 to 6 mm) were not distinct and overlapped.

P. ramulosum was the only species for which there was significant differences among isolates for lesion sizes. For all the other species, isolates within the same species did not differ significantly in the lesion sizes they caused (data not shown). The lesion size averages of the two experiments for *P. expansum* ranged from 54 to 65 mm, *P. ramulosum* lesions ranged from 1 to 15 mm, *P. sp.* (aff. *dendriticum*) lesions ranged from 16 to 18 mm, and *P. glabrum* lesions ranged from 0 to 3 mm.

Deep wounding inoculation method. In the deep wounding experiment, most of the isolates caused lesions after a 4-week incubation period. The exception was the *P. expansum* isolate that caused extensive rotting 2 weeks after inoculation, causing a T1 lesion. Most of the *P. ramulosum* isolates caused the same lesion type (T2), as seen for the surface toothpick inoculation trial. The exception was one isolate that caused a T3 lesion in the surface wounding inoculation, but in the deep wounding inoculation caused either a T1, T2, or T3 lesion. Similarly, the *P. glabrum* isolate caused a T3 lesion in surface wounding, but a T1 or T2 lesion in the deep wounding experiment. The *P. sp.* (aff. *dendriticum*) isolate caused a T2 lesion in surface and deep wounding experiments. The control of the wounding experiment did not cause a lesion, only a slight browning where wound healing occurred.

Nonwounding inoculation method. In the nonwounding method, only two of the isolates, representing *P. expansum* and *P. sp.* (aff. *dendriticum*), caused distinct lesions. The *P. expansum* isolate caused a T1 lesion and complete fruit rotting after 2 weeks (Fig. 9A). The *P. sp.* (aff. *dendriticum*) isolate caused a T2 lesion after 4 weeks of incubation (Fig. 9B). No lesions were observed in the *P. ramulosum* and *P. glabrum* inoculated or control apples (Fig. 9C).

DISCUSSION

Our study showed that *Penicillium* was the predominant genus present within the lesion area (LS1 and LS2) of preharvest WCR fruits. *Penicillium* was also isolated at a high incidence (>51%) in the calyx tube (CX1 and CX2) and core region (CR) of WCR fruits, which may suggest that this is the infection route of these pathogens. Alternatively, colonization of these regions may have occurred as a result of secondary

Fig. 6. Neighbor-joining tree of *Penicillium* species in subgenus *Biverticillium*, based on the internal transcribes spacer (ITS) regions and 5.8S region of nuclear rDNA. The aligned ITS dataset was 514 base pairs long, with seven regions replaced with weighted codes (14). *Penicillium* isolates from apples are those with Stellenbosch University culture collection codes (STEU).

spread from the lesion. Although *Penicillium* was also isolated from several isolation positions in asymptomatic, DCR, and MC apples, the incidence was much lower (<17%) than in WCR fruits. Several other studies have also reported that *Penicillium* is not generally associated with the internal regions of asymptomatic apple fruits (6,25,32).

Six *Penicillium* species were identified from fruits, of which *P. ramulosum* was the dominant species isolated from all fruits (symptomatic and asymptomatic) and all but two orchards. However, the incidence was much higher in WCR fruits (58%) than in other symptomatic fruits (<7%). *P. ramulosum* has recently been described as a new species from fynbos soil and *Protea* infructescences from South Africa, as well as moth damaged Riesling grapes from Canada (35). Most previous studies in South Africa morphologically identified *P. funiculosum* as the causative agent of pre-harvest WCR (4,5,25). This discrepancy with our current study may have been due to Pitt's (18) somewhat broad morphological concept of *P. funiculosum* that could have in the past resulted in the misidentification of *P. ramulosum*, *P. sp.* (aff. *dendriticum*) and *P. sp.* (aff. *cecidicola*) isolates as *P. funiculosum*. The second most common *Penicillium* species in fruits was *P. expansum* that was isolated in four orchards from DCR and WCR fruits, followed by *P. glabrum* that was only isolated in three orchards from asymptomatic, DCR, and WCR fruits. The distribution of *P. chermisinum* and *P. sp.* (aff. *dendriticum*) was each limited to WCR fruits obtained from one orchard each. *P. sp.* (aff. *cecidicola*) was also only obtained in one orchard from asymptomatic fruit.

The pathogenicity and virulence of *Penicillium* isolates were evaluated using three different toothpick inoculation methods (surface, nonwounding, and deep wounding). The toothpick inoculations provided a rapid method for screening a large number of isolates without the labor-intensive task of quantifying spores that is required for inoculations with spore suspensions. Furthermore, toothpick inoculations are especially suited for inoculations into the core region, since we previously were unable to obtain consistent results with inoculations into the core using spore suspensions (unpublished data). The surface inoculation method was useful for pathogenicity investigations and revealed three lesion types based on color and texture (T1 to T3). These lesion types were relatively reproducible, but did vary for some isolates within and between experiments. The T2 lesion type most closely resembled the initial WCR symptoms seen in harvested orchard fruits. The lesion sizes of the T1 lesions were significantly larger than T2 and T3 lesions. Results from the deep wounding experiments were not consistent, which may be due to the fact

that wounding occurs in the core region where endogenous fungi can then also cause infections along with the inoculated isolate. This method, therefore, is not recommended for pathogenicity testing. The lesion sizes measured in the surface inoculation method were somewhat predictive of the virulence of isolates as seen in the nonwounding inoculation method. The nonwounding method showed that some isolates and species were able to cause disease without artificial wounding, suggesting that these isolates are more virulent

and that this method can be used to differentiate the virulence of isolates.

The *Penicillium* species varied in pathogenicity and virulence. *P. expansum* was the most virulent species, since it caused the largest lesions (T1 type) in the surface inoculation method, as well as complete fruit rot within 2 weeks in the nonwounding experiments. This species is also well known for causing blue mold of apples, which is an external rot of great economic importance (20,30). The second most virulent species was *P. sp.* (aff. *dendriticum*),

Fig. 7. Neighbor-joining tree of *Penicillium* species in subgenus *Biverticillium*, based on the β -tubulin region. The aligned β -tubulin dataset was 425 base pairs long. *Penicillium* isolates from apples are those with Stellenbosch University culture collection codes (STEU).

Fig. 8. Three main lesion types caused by *Penicillium* isolates in a surface toothpick inoculation trial: **A**, lesion type 1, caused by *P. expansum*, light brown and very wet; **B**, lesion type 2, caused by *P. ramulosum* and *P. sp.* (aff. *dendriticum*), medium brown and spongy; and **C**, lesion type 3, caused by *P. ramulosum*, *P. glabrum*, and *P. sp.* (aff. *cecicicola*), medium brown, dry, and small. **D**, Control toothpicks consisting of toothpicks that were placed on uninoculated potato dextrose agar plates were also included in the trial.

Fig. 9. Two main lesion types caused by *Penicillium* isolates that were inoculated with a *Penicillium*-colonized toothpick inserted through the calyx tube into the core region, not causing wounding: **A**, lesion type 1, light brown and very wet and only caused by *P. expansum*, and **B**, lesion type 2, medium brown and spongy and caused by *P. sp.* (aff. *dendriticum*). **C**, Control toothpicks, *P. ramulosum* and *P. glabrum* inoculations resulted in no lesions.

which caused relatively large lesions (T2 type) in the surface inoculation method (16 to 18 mm), as well as fruit rot in the non-wounding experiment. The *P. ramulosum* isolates all appeared to be weak pathogens with low virulence, since they caused only small-sized type T2 and T3 lesions in the wounding experiments. Furthermore, in the nonwounding experiments, none of the seven evaluated *P. ramulosum* isolates caused fruit rot. However, since the *P. ramulosum* isolates differed significantly in the lesion sizes that they caused in the surface inoculation method, more isolates need to be evaluated with the nonwounding method to confirm this result. The three *P. glabrum* isolates only caused T3 lesions that were very small. The *P. chermisimum* isolate was the only isolate that was considered to be nonpathogenic.

Results from our study suggest that pre-harvest WCR may be caused by at least three *Penicillium* species, including *P. expansum*, *P. ramulosum*, and *P. sp.* (aff. *dendriticum*). Although the most virulent species, *P. expansum* and *P. sp.* (aff. *dendriticum*), were rarely isolated from a limited number of orchards, the incidence of these species may be obscured in isolation studies since lesions have usually pro-

gressed substantially when isolations are made. At this stage of disease development, successive colonization by saprophytic or facultative and minor pathogens, such as *P. ramulosum*, may have occurred. The highly virulent WCR *Penicillium* species may be of importance in contributing to premature fruit drop in orchards, which can be as high as 2.3% (unpublished data), due to the rapidly expanding lesion that they cause. Although *P. ramulosum* isolates seem to have low virulence, they may have the potential to initiate WCR infections if (i) wounds are present such as growth cracks in the core and calyx tube that are formed during abnormally fast growth, (ii) mite damage occurs in the calyx tube and core, or (iii) fruits experience stress conditions. Furthermore, it is also possible that *P. ramulosum* may predispose apples to infection by more virulent species. Such an additional role of weak pathogens in fruit decay was suggested by Sanderson and Spotts (22) for *P. solitum*, which predisposed otherwise resistant wounds to infection by *P. expansum*.

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