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Characterisation of Alternaria species-groups associated with core rot of apples in South Africa

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Alternaria core rot of red apple cultivars is a serious post-harvest disease in South Africa. Thirty isolates of Alternaria spp. previously isolated from apple, together with reference isolates of A. alternata and A. infectoria, were characterised and grouped according to their sporulation patterns and conidial morphology. Isolates were identified as belonging to A. arborescens, A. infectoria and A. tenuissima species-groups. The isolates were also analysed for production of mycotoxins and other secondary metabolites and their cultural characteristics on DRYES medium were recorded. Apple fruit were artificially inoculated with the thirty-two Alternaria isolates and the resulting lesion types were recorded. A data matrix was constructed using all these characters and subjected to cluster analysis to show the similarity between different isolates. Isolates classified as A. infectoria species-group based on sporulation patterns, cultural and biochemical data could be easily differentiated from isolates classified as A. arborescens and A. tenuissima species-groups, which clustered close together. Isolates were further subjected to DNA sequence analysis of the internal transcribed spacers (ITS) 1 and 2 of the nuclear rRNA gene. A phylogeny estimated from the ITS data set delineated two clades, one being typified by the A. infectoria species-group, and the other representing both A. tenuissima and A. arborescens species-groups. The ITS data set clearly separated isolates of A. infectoria species-group from the other species-groups, as all isolates had a distinction of 35 base pair insertions and 6 base pair deletions in the ITS regions. The results obtained in the present study showed that the major pathogens associated with core rot disease of Top Red apples in South Africa belong to the A. tenuissima species-group.

INTRODUCTION

Several different Alternaria spp. are associated with mouldy core of apples (Heald 1921, Ellis & Barrat 1983), while dry core rot (DCR) has been mostly linked to a single species, A. alternata (Carpenter 1942, Miller 1959, Combrink et al. 1985b). DCR chiefly affects the Red Delicious varieties, and although severe to total crop losses have been reported (Mouat 1953, Miller 1959), losses of between 6–8% are generally attributed to this disease (Combrink, Visagie & Grobbelaar 1984). Mouldy core is considered to be of minor economic importance in fruit for immediate consumption, as the flavour of these fruit is unimpaired (Carpenter 1942). However, as this disease is often a precursor to DCR during storage (Ellis & Barrat 1983, Combrink, Kotze & Visagie 1985a), it should also be regarded as serious. Symptoms of both diseases are generally not externally visible, which is why DCR is such a great nuisance to producers and consumers alike. It is only once the apple is cut open through the core that grey mycelium (mouldy core) or even dry, spongy, black core rot (DCR) is noticed in and around the carpel chambers. During storage, this lesion may extend further into the fruit flesh until the whole fruit is rotted (Brien 1937). Presently, there are still no cost-effective methods of controlling these two diseases in South Africa (Fugler 1990), as spraying programmes are not effective once the fungi have invaded the core.

Several species of Alternaria have been reported to shift from a saprophytic to pathogenic state when the host is subjected to environmental stress (e.g. cold-storage), or during senescence of the host, as is the case in ripening fruit (Rotem 1994, Yao & Koller 1995). Alternaria spp. are also some of the most cosmopolitan fungi, and can occur on a variety of food produce, where they are able to produce several types of biologically active metabolites or mycotoxins shown to be harmful to both man and animal (Pero et al. 1973, Bottalico & Logrieco 1998).

Currently, about 400–450 Alternaria species epithets are known in the literature. This number excludes the

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approximately 400 species names in the old literature under Macrosporium, as well as around 200 names published in other genera, which have been or soon will be considered as basionyms for Alternaria species names (Simmons 1992). It is therefore not surprising that controversy and confusion very often accompany their classification. This is especially true for the small-spored species, which share some morphological characteristics as well as overlapping conidial size ranges (Simmons 1992). Conidial size and shape have been used as the sole criterion for identification of Alternaria spp. (Rotem 1994), but other studies show a definite shift, also incorporating molecular characters (Jasalavich et al. 1995, Kusaba & Tsuge 1995, 1996). Simmons & Roberts (1993) pointed out the critical value of the three-dimensional sporulation patterns at 50 magnification, and Roberts, Raymond & Andersen (2000) compared these with molecular data. The production of mycotoxins and other secondary metabolites has also been shown to be of major importance, not only for those species producing host-specific toxins (HST), but also for non-HST producers (Andersen & Thrane 1996, Andersen, Krøger & Roberts 2001, 2002). Andersen & Thrane (1996) showed that metabolite production together with the more traditional morphological and physiological characteristics can provide a much better picture of the species and species-groups within this genus.

Based on a previous study of DCR in South Africa, the pathogen was identified as A. alternata (Combrink et al. 1985b), due to the belief that this was the correct name for the common small-spored Alternaria species. However, as new tools are being employed to characterise small-spored species of Alternaria (Simmons 1992, Roberts et al. 2000, Andersen et al. 2001), one can expect more accurate identifications, resulting in the delimitation of additional species within this disease complex. The objective of this study was thus to identify and characterise the different Alternaria spp. associated with DCR in South Africa, and to determine which species or species-group were primarily responsible for this disease.

MATERIALS AND METHODS

Fungal cultures and growth conditions

During a previous study, several hundred Alternaria strains were isolated throughout the season as endophytes (Serdani et al. 1998) at different stages of apple fruit development. Thirty of these isolates from a Top Red orchard in the Grabouw area of the Western Cape Province, as well as reference isolates of A. alternata (EGS 34-016, CR32) and A. infectoria (EGS 27-193, CR31) were included in this study (Table 1).

For morphology and metabolite profiling, each isolate was transferred to the following nine media: Alkaloid formation agar (ALK; Reshetilova et al. 1992), Dichloran Rose Bengal Yeast Extract Sucrose Agar (DRYES; Frisvad 1983), Malt Extract Agar (MEA; Pitt 1980), Nitrite Sucrose Agar (NS; Frisvad 1981), Oat Meal Agar (OAT; Gams et al. 1979), Potato Carrot Agar (PCA; Simmons 1992), Potato Sucrose Agar (PSA; Booth 1971), Sigma Yeast Extract Sucrose agar (SYES; Filtenborg, Frisvad & Thrane 1990) and V8 Juice agar (V8; Simmons 1992). All media were made with double distilled water and Bie & Berntsen Agar (BBB10030). All media but ALK, PCA and V8 were supplemented with 1·0 ml trace metal solution (1·0 g $ZnSO_4 7H_2O$, 0·5 g $CuSO_4 5H_2O$, 100 ml water; Smith 1949). After inoculation, all dishes except the PCA and V8, were placed in an incubator at 25 C in the dark. The PCA and V8 dishes were incubated unsealed in one layer under an alternating light dark cycle consisting of 8 h of cool-white daylight followed by 16 h darkness at 25 to induce the characteristic sporulation patterns (Simmons 1992).

For pathogenicity testing and storage, a single spore from each isolate was transferred to PCA, and finally aseptically transferred to and stored on Potato Dextrose Agar (PDA; 12 g Biolab agar, 200 g potatoes, 20 g sucrose, 1 l H_2O) slants at room temperature as well as in 20 ml bottles containing sterile water for the purpose of long-term storage. All fungal cultures are held at the culture collection at the Department of Plant Pathology, University of Stellenbosch, South Africa.

Micro- and macromorphological characteristics

Cultures derived from single conidia were examined directly on PCA by transmitted light at 50 magnification after 4–7 d under conditions mentioned previously, and allocated to a sporulation group (Table 1) according to Simmons & Roberts (1993). Isolates were subsequently examined at 400 magnification for micromorphological purposes using tape preparations according to Andersen et al. (2001). Colony colour on DRYES was also recorded and all isolates were categorised into three groups: white (W), dark green (D) or various shades of green (V) (Table 1).

Screening isolates for pathogenicity

Pathogenicity was tested on apples just after picking, and on apples stored for 9 months under conditions of controlled atmosphere (CA) (fi 0–5 , 1–5 % O , 3–10 % CO_2). Two cultivars, Top Red and Granny Smith were inoculated with the 32 Alternaria isolates by means of toothpick-inoculations. Halved toothpicks were autoclaved (five times) in distilled water, followed by potato dextrose broth (once), and then placed on 90 mm diam Petri dishes containing PDA. Toothpicks were inoculated with the Alternaria isolates and colonised within two weeks. Each apple was inoculated (to a depth of 8 mm) by eight toothpicks, each colonised by a different isolate, as well as one toothpick free of fungal growth to act as control. Ten repetitions of each

Table 1. Cultural, morphological, pathological and chemical data used for the characterisation of Alternaria species.

Accession number	Species groups	Colour on DRYES	Sporulation group	Lesion type	Toxins metabolites%
CR1	A. arborescens	D	3	2	T,A,M,X,c
CR2	A. arborescens	D	3	2	T,A,M,c
CR3	A. arborescens	D	3	2	T,A,M,X,c
CR4	A. arborescens	D	3	2	T,A,M,c
CR5	A. arborescens	D	3	2	T,A,M,c
CR6	A. arborescens	D	3	2	T,A,M,c
CR7	A. tenuissima	V	5	2	T,A,M,X,c
CR8	A. arborescens	D	3	2	T,A,M,c
CR9	A. infectoria	W	6	1	2,3,6
CR10	A. arborescens	D	3	2	T,A,M,c
CR11	A. arborescens	D	3	2	T,A,M,X,c
CR12	A. infectoria	W	6	1	2,3,6
CR13	A. tenuissima	V	5	2	T,A,M,X
CR14	A. arborescens	D	3	2	T,A,M,X,c
CR15	A. infectoria	W	6	1	2,6
CR16	A. tenuissima	V	5	3	T,A,M,X,c
CR17	A. tenuissima	V	5	3	T,A,M,X
CR18	A. arborescens	D	3	2	T,A,M
CR19	A. tenuissima	V	5	3	T,A,M,X,c
CR20	A. tenuissima	V	5	3	T,A,M
CR21	A. tenuissima	V	5	3	A,M,L,X,a
CR22	A. tenuissima	V	2	3	T,X,3
CR23	A. arborescens	D	3	2	T,A,M,c
CR24	A. tenuissima	V	5	3	A,M,L,X,c
CR25	A. tenuissima	V	5	3	T,A,M,X
CR26	A. tenuissima	V	5	3	T,A,M,X
CR27	A. tenuissima	V	5	3	T,A,M,X,c
CR28	A. infectoria	W	6	1	2,3,6
CR29	A. infectoria	W	6	1	2,3,6
CR30	A. infectoria	W	6	1	2,3,6
CR31	A. infectoria&	W	6	1	2,3,6
CR32	A. alternata	D	4	2	A,M,L,X,a

Colony colour on the medium DRYES: D, dark olive-green; V, various shades of green; W, white.

Sporulation groups according to Simmons & Roberts (1993).

Lesion types: 1, no lesion; 2, small, dry, black lesion; 3, large, cone-shaped, straw-coloured lesion.

%Known metabolites: T, tenuazonic acid; A, alternariol; M, alternariol monomethyl ether; L, altenuene; X, altertoxin I. Unknown metabolites: 2, metabolite 2; 3, metabolite 3; 6, metabolite 6 (Andersen & Thrane 1996); a, metabolite A; c, metabolite C (Andersen et al. 2001).

& Reference isolate of A. infectoria (ES 27-193).

Reference isolate of A. alternata (ES 34-016).

isolate were done on both apple cultivars and the whole experiment repeated once. Inoculated apples were covered in transparent perforated plastic bags, and incubated for two weeks at 25 . After the incubation period, external lesion size and appearance were noted for each isolate (Table 1). Subsequently, apples were split equatorially and internal lesion diameter and appearance determined.

Extraction procedure

The extraction method used was based on a micro-scale extraction by Smedsgaard (1997) modified into a three-step extraction procedure suited for Alternaria metabolites. Two 14 d-old agar plugs, 6 mm diam, were cut from the middle of a colony from each of the nine media and placed in a 10 ml screw-cap vial. The plugs were first extracted in chloroform methanol, then ethyl acetate containing 1% formic acid and finally in 2-propanol as described in Andersen et al. (2001).

Chemical analysis

HPLC analyses were performed on a HP-1100 high performance liquid chromatograph equipped with a diode array detector. The mobile phase consisted of a linear gradient of water and acetonitrile, both with 0·005% trifluoroacetic acid as described in Andersen et al. (2001). A series of alkylphenones was used to calculate a bracketed Retention Index (RI) for each metabolite (Frisvad & Thrane 1993). Standards of altenuene, alternariols, altersolanol A, altertoxin I, bostrycin, curvularin, dehydrocurvularin, macrosporin, radicinin, tentoxin, tenuazonic acid and 3,4,5-trihydroxy-7-methoxy-2-methylanthraquinone were analysed in the same system and characterised by their RI values and uv-spectra.

Data analysis

An overall metabolite profile was constructed based upon both known and unknown metabolites in all 32

HPLC chromatograms. The HPLC chromatogram of each isolate was compared to the profile, where the presence of a metabolite was given the value 1 and the absence the value 0 , giving a data matrix as described in Andersen et al. (2001). Finally, the data from lesion type and colony colour were combined and added to this matrix. The matrix, containing 32 objects (fungal isolates) and 82 variables [77 metabolites, 3 lesion types and 2 colour categories (dark green and green)], was analysed using the programme NTSYS-pc version 2·02h (Rohlf 1993). The distance between objects was calculated using the Dice coefficient [2a (a b c)] and UPGMA.

Gene amplification and sequencing of ITS

All isolates were subjected to DNA sequence analysis except CR 2, the stocks of which became contaminated, and was therefore excluded from further study. Genomic DNA was isolated from fungal mycelia collected from the plates using the isolation protocol of Lee & Taylor (1990). Template DNA (20 ng) was amplified in a 25 µl PCR reaction mixture consisting of 10 mm KCl, 10 mm $(NH_4)_2SO_4$, 20 mm Tris-HCl (pH 8·8), 6 mm $MgCl_2$, and 500 µm each of dATP, dCTP, dGTP, and dTTP, with 60 pmols ITS1 and ITS4 primers (White et al. 1990), and 2·5 units Biotaq (Bioline, London) DNA polymerase. The reaction was set up as follows: initial denaturation at 96 for 2 min, followed by 30 cycles of denaturation at 96 for 15 s, annealing at 55 for 30 s, extension at 75 for 35 s, and final extension at 75 for 2 min in a Rapidcycler (Idaho Technology, ID). A negative control using water instead of template DNA was set up for each experiment. PCR products were analysed by electrophoresis at 75 v for 2 h in a 0·8 % (w v) agarose gel in 0·5 TAE buffer (0·4 m Tris, 0·05 m NaAc, and 0·01 m EDTA, pH 7·85) and visualised under uv light in a transilluminator (TFX-35C, Vilber Lourmat) following ethidium bromide staining.

PCR products were purified by using a QIAquick PCR Purification Kit (Qiagen GmbH). The purified PCR products were sequenced using the ABI Prism 377 DNA Sequencer (Perkin-Elmer, Norwalk, CN). The cycle sequencing reaction with 20 to 40 ng of DNA template and 3·2 pmol primer in a total volume of 10 µl was carried out with a Dye Terminator Cycle Sequencing Ready Reaction Kit containing AmpliTaq DNA Polymerase (Perkin-Elmer). The reaction was set up as 25 cycles of 96 for 30 s, 50 for 15 s, and 60 for 4 min. The resulting fragments were finally purified using Centri-Sep Spin columns (Princeton Separations, Adelphia, New Jersey) and loaded onto the sequencing gel.

Phylogenetic analysis

Nucleotide sequences of the ITS region of isolates used in this study (GenBank accession nos AF397218–AF397248) as well as Ophiosphaerella herpotricha, O. korrae (U04861 and U04862 respectively) and the outgroup Pleospora herbarum (AF229479) were assembled using Tex-Edit Plus (Bender, TomBB aol.com). The alignment of the sequence files (TreeBASE accession nos S708 and M1130) were conducted using the CLUSTAL W software (Thompson et al. 1994). Adjustments for improvement were made by eye where necessary. Alignment gaps were coded as missing data in the analysis. Phylogenetic analyses were performed with PAUP version 4·0b6 (Swofford 2001). The most parsimonious tree(s) were determined from the ITS data set using the heuristic search option with 1000 random sequence input orders with MULPARS on and TBR branch swapping for the exact solution. The unconstrained topologies of the equally parsimonious trees were compared using the Kishino-Hasegawa test in PAUP, and the most parsimonious tree topology selected. Parsimony bootstrap with 1000 replicates in PAUP was applied to the tree to evaluate the stability. Other measures including tree length, consistency, retention, rescaled consistency and homoplasy indexes (TL, CI, RI, RC and HI) were also calculated. The maximum likelihood and neighbour-joining analyses were also performed to test the parsimonious tree topology.

RESULTS

Micro and macromorphological characteristics

Three distinct species-groups were identified based on sporulation pattern and morphology, namely Alternaria arborescens, A. infectoria and A. tenuissima species-groups. None of the 30 Alternaria isolates associated with core rot of apples could be identified as A. alternata. The reference isolate of A. alternata (CR 32) had well-branched chains (6–12 conidia), originating directly from the agar surface and producing beakless, oblong to ellipsoid conidia. In contrast, the branching chains of A. arborescens species-group isolates were formed on long primary conidiophores rising up to 1000 µm from the surface in Simmons & Roberts 1993: 121, fig. 67. Isolates of the A. infectoria species-group, including the reference isolate of A. infectoria (CR31), were characterised by very long secondary conidiophores in Simmons & Roberts 1993: 125, fig. 70. The secondary conidiophores have the same diameter and distinct septation typical of the primary conidiophores. Conidial shape was ovoid, and mostly transversely septate. The sporulation pattern of the A. tenuissima species-group was characterised by unbranched, moderate to long conidial chains formed on short primary conidiophores on the agar surface in Simmons & Roberts 1993: 124 fig. 69. Within this species-group, there was a certain variation in conidial shape and size.

Isolates classified as A. infectoria species-group on PCA according to the criteria of Simmons & Roberts (1993) consistently produced white colonies on DRYES. Isolates of A. arborescens species-group produced dark,

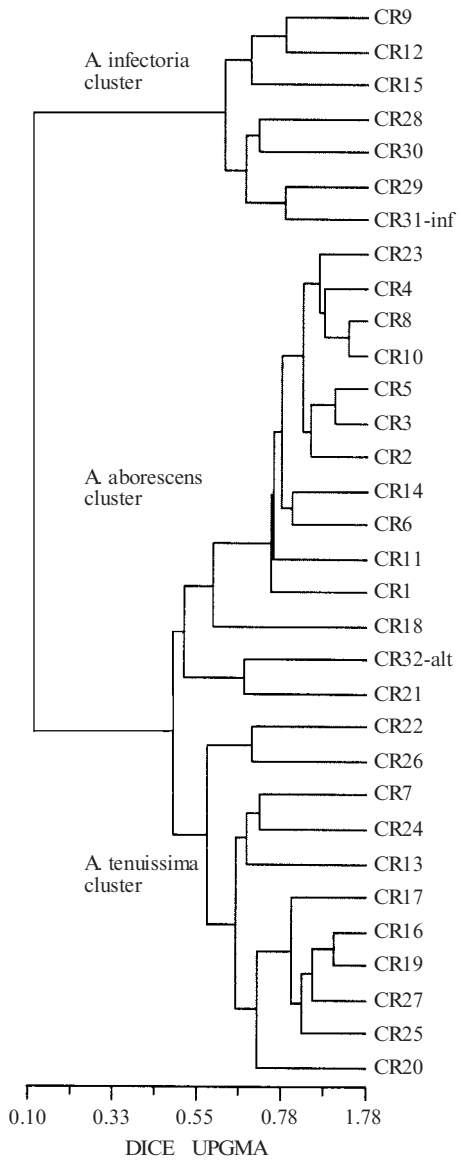

Fig. 1. Dendrogram based on conidial and morphological characteristics, lesion type and secondary metabolites produced by different isolates of Alternaria spp. The cophenetic correlation, r, is 0·96732.

olive-green colonies, while those of A. tenuissima species-group were various shades of green.

Screening isolates for pathogenicity

Three different lesion types were produced on Top Red apples (Table 1). The first resembled the control in that no lesions were formed, while the second showed a region of 1–2 mm around the toothpick to be black, firm, with no external symptoms. The third was straw-coloured, streaked with black, consisting of soft tissue with lesions being visible without splitting the apples. The lesion was cone-shaped, with the widest part at the apple s surface.

Lesion type, rather than lesion size, correlated well with the different Alternaria species-groups (Table 1). Isolates of the A. infectoria species-group were non-pathogenic, did not cause any lesions, and resembled true endophytes. Isolates of the A. arborescens species-group produced the second lesion type, while the third, soft lesion type was caused by A. tenuissima species-group isolates. The only exceptions were two isolates of the A. tenuissima species-group (CR7, 13), which produced the second lesion type. The reference isolates of A. infectoria (CR31) and A. alternata (CR32) produced a type 1 and a type 2 lesion, respectively. There was a tendency for lesions in Granny Smith apples to be more wet and soft than in Top Red apples, thus making the distinction between lesion types less obvious. Furthermore, the three lesion types were not clearly distinguishable in inoculated apples after 9 months storage.

Metabolite production and cluster analysis

Of the 32 isolates tested, 25 produced a variety of both known and unknown metabolites, while the remaining seven isolates only produced unknown secondary metabolites (Table 1). These findings correlate well with the different Alternaria species-groups (Table 1). Alter-solanol A, bostrycin, curvularin, dehydrocurvularin, macrosporin, radicinin, tentoxin or 3,4,5-trihydroxy-7-methoxy-2-methyl-anthraquinone were not detected in any of the 32 Alternaria isolates. None of the seven A. infectoria species-group isolates, including the reference isolate of A. infectoria, produced any known metabolites, which is in accordance with Andersen & Thrane (1996). In general, isolates from A. arborescens and A. tenuissima species-groups produced the same known metabolites, but differed in the production of unknown metabolites. The reference isolates of A. alternata (CR32), together with isolates CR21 and CR24 did not produce tenuazonic acid and isolate CR22 did not produce any of the alternariols (A or M in Table 1). Fig. 1 is derived from the secondary metabolites, sporulation group, and colony colour characteristics, as well as lesion types. It has a cophenetic correlation, r, of 0·96732, meaning that the fit for the cluster analysis is very good (Rohlf 1993) and divides the isolates into one small and one large cluster. The small cluster includes all seven isolates of the A. infectoria species-group, while the large cluster consists of isolates identified as A. arborescens and A. tenuissima species-groups and A. alternata. This cluster can be subdivided into two; one containing all but one of the A. tenuissima species-group isolates, and one with all the A. arborescens species-group isolates. The single A. tenuissima species-group isolate (CR21) that does not cluster with the others, clusters with the single A. alternata isolate.

Phylogenetic analysis

The alignment of the ITS data set spans 604 sites. Parsimony analysis of the alignment with 1000 random sequence input orders produced 74 equally most parsimonious trees (MPT). The best tree topology of

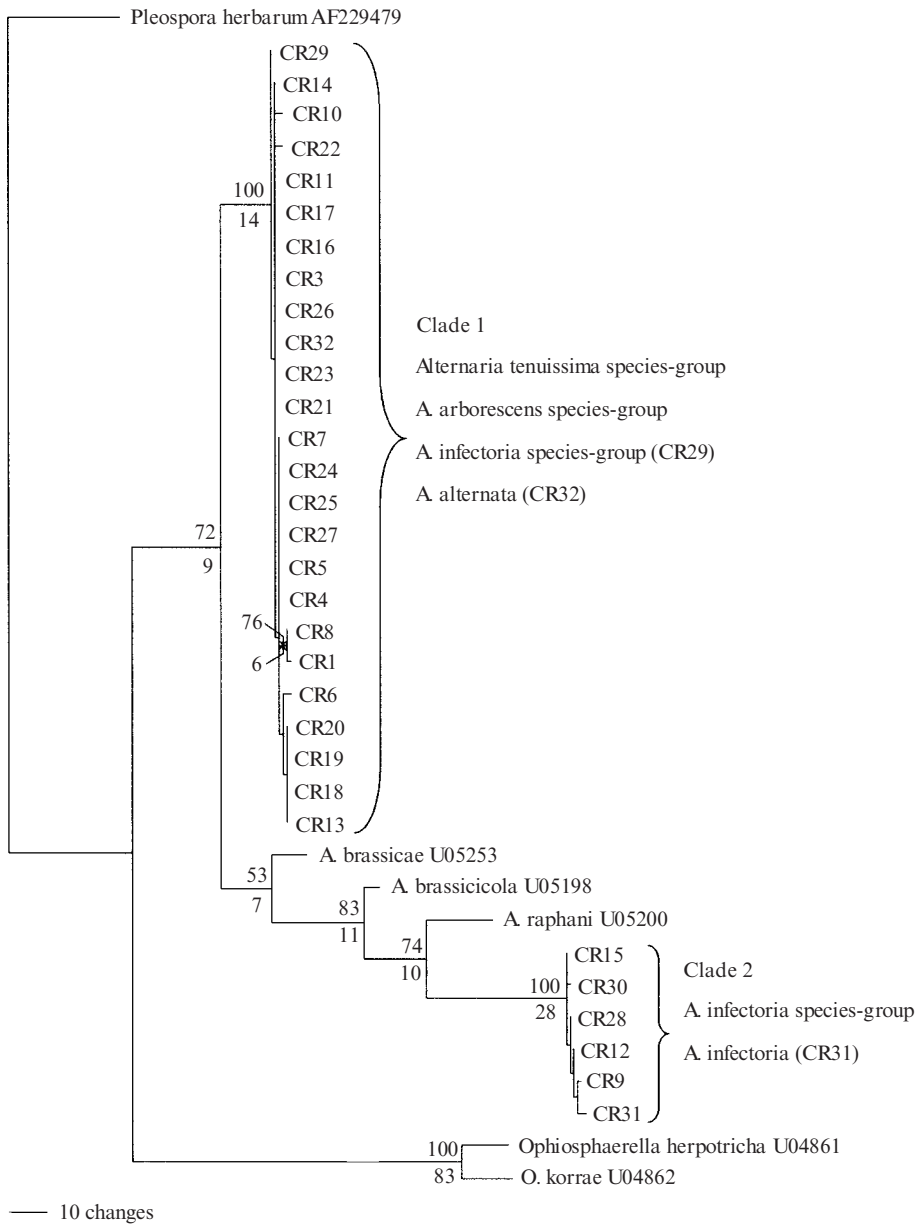

Fig. 2. One of 74 MPTs obtained from parsimony analysis of ITS (including GenBank entries of the same gene from Pleospora herbarum, Ophiosphaerella herpotricha, O. korrae, Alternaria brassicae, A. brassicicola and A. raphani) using a heuristic search in the maximum parsimony analysis with 1000 random sequence input orders. The tree is rooted with the outgroup Pleospora herbarum (TL fl 346 steps, CI fl 0·835, RI fl 0·908, RC fl 0·759, HI fl 0·165). Parsimony bootstrap values of 1000 replicates and decay indices are indicated above and below the branches respectively.

the 74 MPTs was selected as the gene tree (Fig. 2) through the Kishino-Hasegawa likelihood test (data not shown) and evaluated with 1000 bootstrap replications in a heuristic search for clade stability. The neighbour-joining analysis of the data set with 1000 bootstrap replications in PAUP produced a majority-rule consensus tree (data not shown) which was similar to the gene tree (Fig. 2). Maximum-likelihood analysis in heuristic search with 10 random sequence input orders and 100 bootstrap replications were also performed in PAUP for the data set, which produced a tree topology identical to that of the gene tree (Fig. 2).The result in Fig. 2 shows the ITS gene tree dividing the 32 Alternaria isolates into two clades. Clade 1 contains all isolates belonging to the A. tenuissima and the A. arborescens species-groups, the representative A. alternata isolate (CR32), and one A. infectoria species-group isolate (CR29). Clade 2 comprises the remaining six A. infectoria species-group isolates in a clade with strong bootstrap support.

DISCUSSION

Despite the vast amount of variation found among the small-spored Alternaria species, investigation into different aspects of these fungi has proven that these isolates belong to distinct species and species-groups (Roberts et al. 2000, Andersen et al. 2001, 2002). The

results in this study show that Alternaria isolates associated with core rot of apples could be divided into three distinct species-groups on the basis of sporulation patterns. The same divisions were also found when other methods, such as metabolite profiling and pathogenicity testing, were used. As more and different types of data become available, additional taxa will probably be separated and described from these species-groups. Data from the present study already highlights the greater significance of certain characteristics (Table 1). The importance of standard cultural conditions cannot be overstated. It is crucial that none of the Petri dishes is sealed during incubation. This applies to both DRYES and PCA. Furthermore, PCA have to be placed in cool-white daylight and not black-light or nuv-light as is used for Fusarium, because the three-dimensional sporulation patterns cannot develop properly (Andersen et al. 2002).

Sporulation patterns have been grossly overlooked in the past. It is only after Simmons & Roberts (1993) insisted on its importance in Alternaria taxonomy, that it is being acknowledged, though rather hesitantly. Unless a standard method is used, as suggested by Simmons (1992), three-dimensional sporulation patterns will be of no use in comparing data from different researchers. As observed in the present study, Andersen & Thrane (1996) and Andersen et al. (2001, 2002) found colony colour on DRYES to be a stable characteristic, which plays an integral part in the characterisation of Alternaria spp. as long as standard methods are observed. In some cases, however, single conidial colonies may be both white and dark olive-green, but this is the exception rather than the rule. The variation in colony colour of the A. tenuissima species-group isolates suggest that this species-group contains several taxa and further morphological examination will show if these taxa will correspond to Simmons (1995) species 1–5.

Pathogenicity, expressed as host specific toxin production, has been used to integrate various Alternaria species into one (Otani & Kohmoto 1992). Pathogenicity studies on leaves have on the other hand also been used in the past for identification and differentiation of Alternaria spp. (Simmons & Roberts 1993). This is the first study, though, to segregate Alternaria spp. from fruit by looking at lesion types artificially induced on apples. Fulbright (1984) and Elliston (1985) used a similar method to test for relative virulence of Cryphonectria parasitica, though not for identification purposes. It is important to mention that our inoculation methods in no way reflect the reality of DCR infection in the field, as a very large amount of inoculum is inserted into a mechanical wound. However, the purpose of this experiment was to compare isolates with one another and as inoculation methods were standard for all isolates, the results are of great value in separating species.

As tissue from naturally infected fruit is dry, dark brown and corky with air pockets, it is tempting to correlate the second lesion type with dry core rot (DCR). However, when Combrink et al. (1985a) inoculated apple fruit with the Alternaria species isolated from DCR lesions, the lesion type produced was moist, dark brown and streaked with black, very different from lesions occurring naturally. The lesion type formed by the Alternaria sp. of Combrink et al. (1985a) correlates exactly with our lesion type three, formed by A. tenuissima species-group isolates. At the time that Combrink et al. (1985a) identified their Alternaria sp. as A. alternata, the standard method of identification as suggested by Simmons (1992), did not exist. For this reason, one should not exclude the possibility that another species of Alternaria could have been responsible for disease in that particular study. However, during a recent study A. tenuissima was shown to be the predominant Alternaria species isolated from DCR lesions (Serdani et al. 1998). It is thus clear that A. tenuissima species-group is the main Alternaria species-group responsible for DCR with A. arborescens and A. infectoria species-groups merely being secondary invaders. These findings also suggest that it may be worthwhile to rethink current approaches to disease control, as different fungal species can react differently to fungicides.

The larger, spreading lesions of A. tenuissima species-group indicates a higher virulence than that of A. arborescens species-group, specifically just after harvest. Lesion types from these two species are, however, more similar after 9 months of cold storage, which might indicate that the A. arborescens species-group becomes more virulent as storage time increases, or that the host resistance to disease is less after storage. It is therefore essential that studies to determine virulence, when done for the purpose of characterisation, be carried out on freshly harvested apples. The fact that isolates showed similar virulence on Granny Smith apples compared to Top Red apples, suggests that the Alternaria tenuissima species-group responsible for DCR, might also be capable of rotting Granny Smith apples, if these apples had an open calyx tube to allow entry into the core. These findings once again highlight the importance of an open calyx tube as one of the main contributing factors predisposing apples to DCR.

From Fig. 1 it is clear that conidial arrangement, lesion type and colony colour on DRYES, together with secondary metabolite production gives a good segregation of the different species groups, and that they are stable characteristics to be used for future identifications. Throughout this study, isolates of A. infectoria species-group differed from the other Alternaria isolates in all characters examined, and is without doubt a very distinguishable species. Furthermore, no variation was found among isolates from apples. This is in contrast to the study by Andersen & Thrane (1996), where three different taxa within the A. infectoria species-group were identified on malt barley.

In the present study two different approaches were employed based on different data sets of the same

isolates. The dendrogram in Fig. 1 represents phenotypic characters (e.g. morphological and cultural characteristics, virulence and secondary metabolite data) whereas the phylogenetic tree in Fig. 2 represents DNA sequence data from the ITS rRNA genes. Although both data sets were able to distinguish isolates of the A. infectoria species-group, it was not possible to distinguish among A. tenuissima and A. arborescens species-groups using ITS genes. This is in agreement with Gaskell et al. (1997) who also found that a high degree of interspecies similarity within Alternaria in both ITS regions 1 and 2. Our results indicate that ITS genes are not suitable for distinguishing between closely related Alternaria species and should not be used for lumping different pathogenic species as A. alternata as has been done by Kusaba & Tsuge (1995). Phenotypic characters, as used in this study, show that it is possible to distinguish between A. tenuissima species-group and A. arborescens species-group even though they have much in common.

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