Differentiation between *Eutypa lata* and *Cryptovalsa* cf *ampelina* by means of Cellular Fatty Acid Analysis

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***Eutypa lata* is generally accepted to cause dieback in grapevines. *Cryptovalsa* cf *ampelina* may possibly contribute to this phenomenon but cannot be distinguished from the former species, due to the identical morphology of the anamorph stages. Cellular fatty acids were recovered from both fungi and analysed by capillary gas chromatography using a polar column. Fatty acids were identified by GC-MS analyses of picolinyl esters. The mean relative percentages of C16:0, C16:1(9) and C18:0 differed significantly ($p \leq 0,01$) between the species and allowed rapid differentiation. The 16:0/C16:1(9) and (C16:1(9)/C18:0 ratios were of similar diagnostic value.**

The Ascomycete *Eutypa lata* (Pers.: Fr.) Tul., syn. *E. armeniacae* (Hansf. & Carter) has long been recognised as a pathogen on apricot (Carter, 1955). This fungus also proved to be the cause of dieback in grapevine (Moller & Kasimatis, 1978). The teleomorph of this fungus forms only on wood which has been dead for four to five years (Carter, 1957). Perithecia form in stromata but do not develop unless the annual rainfall exceeds 300 mm (Bolay & Moller, 1977), and do not form on artificial media (Glawe, Dilley & Moller, 1983). Isolates from ascospores of the fungus and from wood of dieback infected vines give rise to the anamorph, *Libertella blepharis* A. L. Smith.

A search for *E. lata* stroma conducted in several vineyards in the Western Cape revealed the presence of another, relatively abundant Ascomycete, that appeared on one-year-old prunings as well as older dead wood (Ferreira, 1988). Perithecia and ascospores of this fungus resembled those of *E. lata* but multispored asci (32 spores) were formed in perithecia. This is in contrast to the eight-spored asci formed by the later organism. In addition, perithecia of *E. lata* are always found in a definite stroma (Carter, 1957) whereas perithecia of the second fungus occur solitarily. Isolates from ascospores of this fungus [*Cryptovalsa* cf *ampelina* (Nits.) Fuckel according to F. Rappaz, Microbiologische Institut, Swiss Federal Institute of Technology, Zurich, Switzerland] also gave rise to an anamorph morphologically indistinguishable from that of *E. lata.*

This indistinguishable morphology of the anamorph stages made it impossible to study the involvement of *C.* cf *ampelina* in the dieback phenomenon.

Cellular fatty acid analysis (CFAA) has found wide application as a rapid technique for differentiating between species of micro-organisms (Komagata & Suzuki, 1987). In spite of initial reservations (Shaw, 1966), CFAA has also been used to differentiate between yeast species (Kock, Cottrell & Lategan, 1986; Tredoux, Kock & Lategan, 1987). Wassef (1977) noted that, with a few exceptions, there were no apparent significant differences in cellular fatty acids found in the Ascomycete taxa. While this statement is true for fungi in general (Shaw, 1966), Augustyn (1989) was able to differentiate between 46 strains of *Saccharomyces cerevisiae* by utilising the strain specific differences in fatty acid relative percentages.

This paper, therefore, reports on an attempt to differentiate between the anamorph stages of *E. lata* and *C.* cf *ampelina* by means of CFAA.

MATERIALS AND METHODS

Isolation of fungi

Dead wood of vines containing perithecia of *E. lata* and *C.* cf *ampelina* respectively was collected from two sites for each species on the VORI Experimental Farm at Stellenbosch. After washing the wood under running tap water for 15 min., the tops of the softened perithecia were cut through with a sterile scalpel. Ten perithecia per isolate were removed and placed in separate McCartney bottles each containing 10 ml sterile distilled water. After 30 min. the bottles were shaken and the respective ascospore suspensions poured onto water agar in a petri dish. After swirling, the water was decanted.

Germination of spores and cultivation of anamorphs

The four petri dishes were subsequently incubated at 25°C for 24 hours. Four single germinated spores for each isolate were subsequently transferred to individual petri dishes containing potato dextrose agar and chloromycetin (500 mg/l) and incubated at 25°C for 7 days. Three replicates of ten discs (6 mm diameter) containing mycelium and agar were then cut from each single spore isolate and placed separately in petri dishes containing 10 ml Czapek Dox medium and chloromycetin (500 mg/l). The petri dishes were then incubated at 25°C for 10 days and the mycelium harvested by combining the contents of the 10 petri dishes for each replicate. The mycelium was washed by centrifugation with sterile distilled water (x 3) and freeze dried. The freeze-dried mycelium was subsequently ground in a Wiley mill to pass through a 0,25 mm sieve. A total of 12 samples for each of the two isolates per species was prepared in this way.

Fatty acid analysis

Fatty acids were recovered and esterified using the method of Augustyn & Kock (1989). Fatty acid methyl esters (FAME) were analysed on a J & W DB wax capillary column (30 m x 0,32 mm with a 0,15 micron layer) as described by Augustyn & Kock (1989). Fatty acids were identified after preparation of picolinyl es-

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ters (Harvey, 1984) and analysed on a Finnigan 4600 quadrupole gas chromatograph-mass spectrometer (GC-MS) system (Augustyn & Kock, 1989).

In this paper fatty acids are named according to the delta system in which the carboxyl carbon atom is designated number one. Abbreviated notation, e.g. C17:2(9, 12) designates: number of carbon atoms, number of double bonds (position of double bonds).

Data manipulation

The FAME are expressed as relative percentages. Total area count from C14:0 to C18:3 inclusive was considered as 100%.

Mean relative percentages (MRP's) were calculated for the individual fatty acids per ascospore, isolate and species and these data subjected to analysis of variance. Fatty acid MRP's differing at the 1% level were used to distinguish between the species.

The Index of Relationship (R) between the isolates of a species and between the species was calculated using Holman's formula (Holman, 1978).

RESULTS AND DISCUSSION

The fatty acids of *Eutypa lata* and *Cryptovalsa* cf *ampelina*

All FAME found in *E. lata* were also present in *C.* cf *ampelina*. A typical gas chromatogram is depicted in Fig. 1 (*C* cf *ampelina)*. Sixteen FAME, viz. C12:0, C13:0, C14:0, C14:1(9), C15:0, C16:0, C16:1(9), C17:0, C17:1(9), C17:1(11), C17:2(9, 12), C18:0, C18:1(9), C18:1(11), C18:2(9, 12) and C18:3(9, 12, 15) were identified.

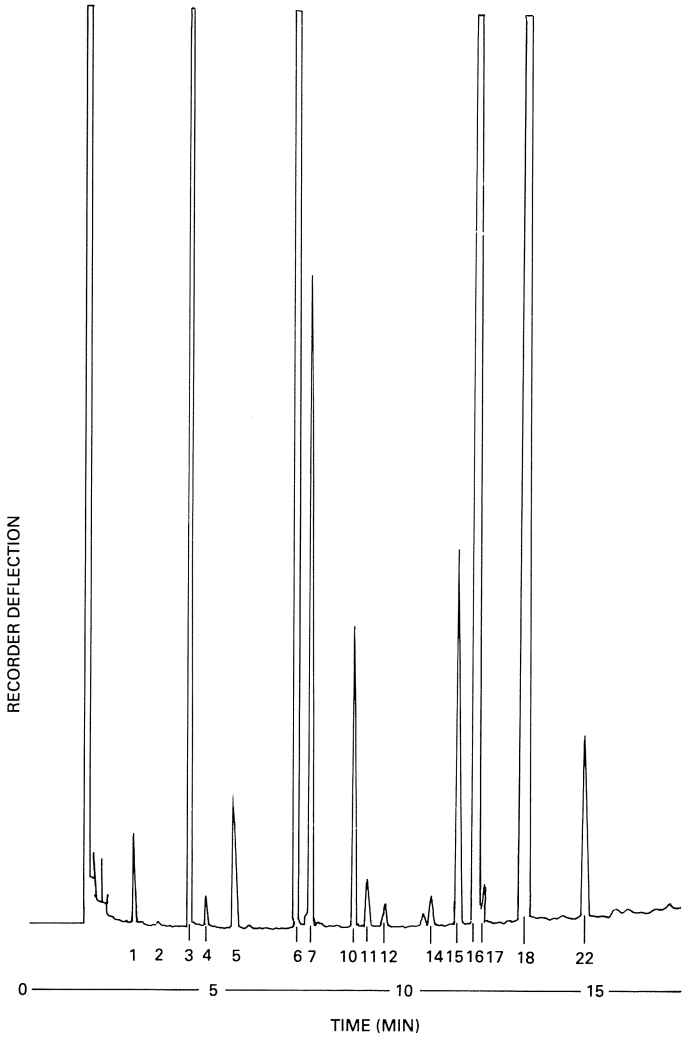

FIG. 1.

Profile of fatty acid methyl esters from *C.* cf *ampelina* recorded on a DB wax capillary column. **Peak** 1. C12:0; 2: C13:0; 3: C14:0; 4: C14:1(9); 5: C15:0; 6: C16:0; 7: C16:1(9); 10: C17:0; 11: C17:1(9); 12: C17:1(11); 14: C17:2(9, 12); 15: C18:0; 16; C18:1(9); 17: C18:1(11); 18: C18:2(9, 12); 22: C18:3(9, 12, 15)

Although reference standards for all fatty acids were not available, interpretation of picolinyl ester mass spectra allowed unambiguous identification of the various fatty acids (Harvey, 1982; Christie, Brechany & Holman, 1987). The picolinyl ester mass spectrum interpreted as belonging to C17:2(9, 12) is depicted in Fig. 2. The 40 amu gap between $^{m}/_{z}$ 220 and $^{m}/_{z}$ 260, the 26 amu gaps between $^{m}/_{z}$ 234 and $^{m}/_{z}$ 260 and $^{m}/_{z}$ 274 and $^{m}/_{z}$ 300 respectively, as well as the molecular ion at $^{m}/_{z}$ 357 clearly indicate C17:2(9, 12) (Christie *et al.*, 1987). The intensity of some of the diagnostic ions was less than that found by Christie *et al.* (1987), a problem also experienced by Augustyn & Kock (1989).

Unsaturated C17 fatty acids have not been identified in many fungi (Wassef, 1977; Lösel, 1988), possibly due to the fact that many authors tend to ignore fatty acids present at or below the 1% level. Whereas the lower fungi tend to accumulate a larger variety of polyunsaturated fatty acids (PUFA) only two PUFA [C18:2(9, 12) and C18:3(9, 12, 15) have been found in the Ascomycete taxa (Wassef, 1977; Nes & Nes, 1979). The identification of C17:2(9, 12), therefore, indicates that more PUFA may be present amongst the minor fatty acids present in Ascomycete taxa.

Small amounts of iso- and anteiso-branched fatty acids were present in the samples analysed at the onset of this study. Branched-chain fatty acids are not common in fungi and when present they are associated with the sphingolipid fraction (Lösel, 1988). Substantial amounts of iso-and anteiso-branched fatty acids have, however, been found in *Conidiobolus* sp. (Zygomycetes) (Tyrell, 1968). Both iso- and anteiso-branched fatty acids disappeared from the cellular fatty acid profiles (CFAP's) of *E. lata* and *C.* cf *ampelina* upon addition of chloromycetin to the culture medium, indicating bacterial contamination of the original cultures.

Fatty acid relative percentages as basis for differentiation between *E. lata* and *C.* cf *ampelina*

In fungi the relative percentages of the fatty acids produced by different ascospores from the same ascus may vary considerably (Mantle, Morris & Hall, 1969). Such variation was also recorded in this study. The respective MRP's for 12 fatty acids per isolate (derived from three replicates per spore, four spores per isolate) are presented in Table 1.

The data presented in Table 2 show highly significant interspecies differences between the MRP's of C16:0, C16:1(9) and C18:0. This indicated that differentiation between species was possible.

Perusal of the fatty acid relative percentages revealed the utility of the C16:0/C16:1(9) and C16:1(9)/C18:0 ratios as diagnostic tools for rapid species differentiation. The data presented in Table 3 confirm that these ratios allow differentiation between *C.* cf *ampelina* and

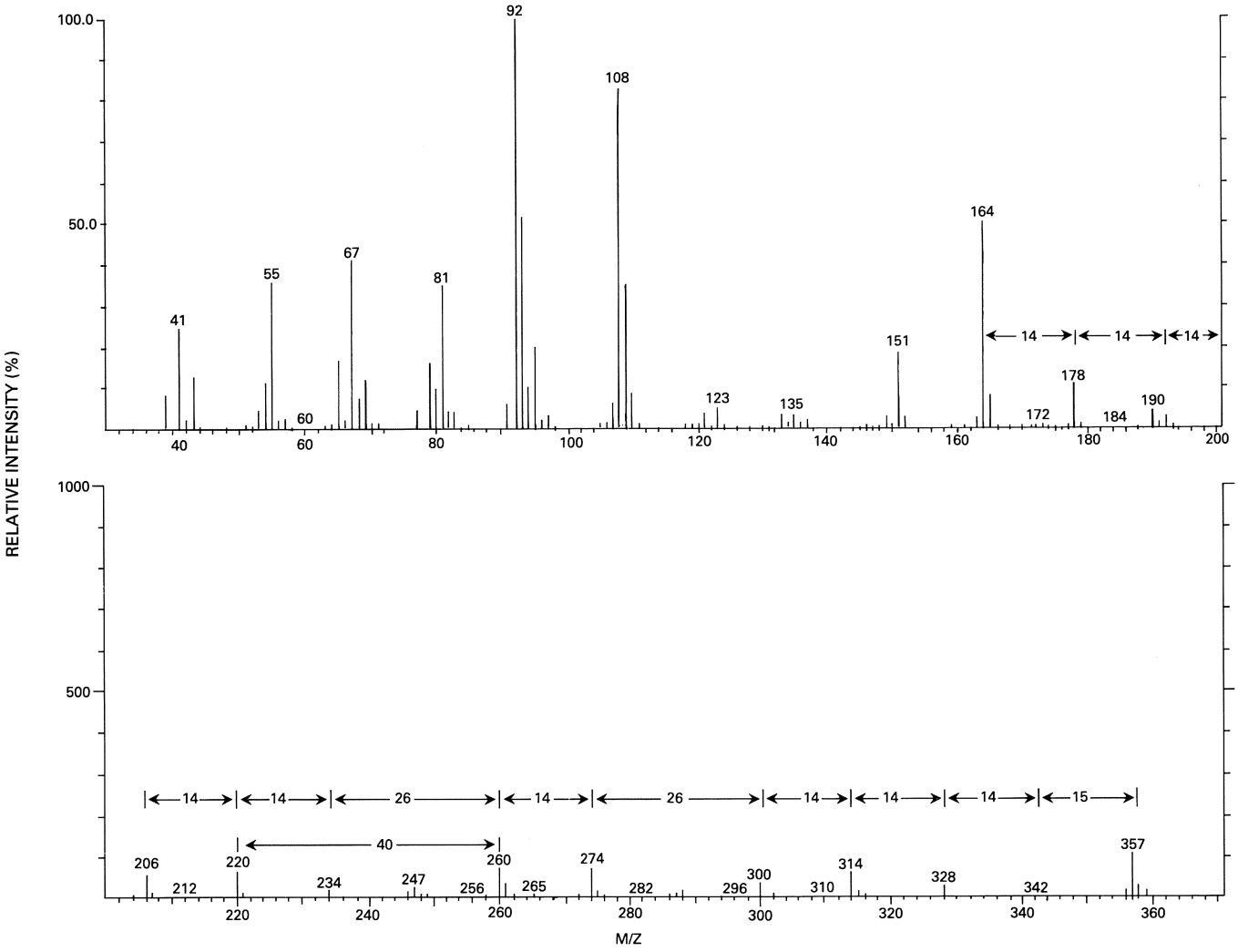

FIG. 2
Picolinyl ester mass spectrum of C17:2(9, 12)

TABLE 1

Mean relative percentages (MRP's) for 12 fatty acids in two isolates each of two Ascomycete species

SPECIES	FATTY ACID (MRP)											
	14:0	15:0	16:0	16:1(9)	17:0	17:1(9)	17:1(11)	17:2(9,12)	18:0	18:1(9)	18:2(9,12)	18:3(9,12,15)
C. cf *ampelina*												
Isolate 1												
Mean 4 spores:	14,01	0,48	31,01	1,36	0,46	0,27	0,19	0,21	1,27	18,06	30,20	1,28
SD	2,58	0,08	1,26	0,24	0,11	0,03	0,01	0,05	0,21	1,57	1,34	0,38
Isolate 2												
Mean: 4 spores	16,48	0,31	31,84	1,64	0,75	0,17	0,13	0,14	1,26	15,43	30,21	0,67
SD	1,80	0,07	0,92	0,13	0,10	0,05	0,03	0,04	0,12	0,96	1,90	0,05
Mean SP	15,25	0,39	31,43	1,50	0,61	0,22	0,16	0,18	1,27	16,75	30,21	0,97
SD SP	2,52	0,11	1,16	0,24	0,18	0,06	0,04	0,05	0,17	1,85	1,63	0,41
E. lata												
Isolate 1												
Mean: 4 spores	15,37	1,48	19,85	6,19	0,81	0,14	0,81	0,53	0,61	15,63	35,74	0,78
SD	2,56	0,45	1,46	0,64	0,16	0,04	0,21	0,12	0,10	3,34	3,31	0,27
Isolate 2												
Mean: 4 spores	18,59	2,21	20,06	6,45	0,84	0,21	1,01	0,73	0,45	16,73	30,63	0,66
SD	2,89	0,52	3,37	0,92	0,16	0,06	0,21	0,20	0,14	2,99	3,06	0,17
Mean SP	16,98	1,85	19,96	6,32	0,82	0,18	0,91	0,63	0,53	16,18	33,18	0,72
SD SP	3,14	0,60	2,54	0,79	0,16	0,06	0,23	0,19	0,14	3,15	4,06	0,23

SD: standard deviation
SP: species

TABLE 2

Mean relative percentages (MRP's) for three fatty acids that allow differentiation between *C.* cf *ampelina* and *E. lata*.

Species	(Isolate)	FATTY ACIDS (MRP) 16:0	16:1(9)	18:0
C. cf *ampelina*	1	31,01 a	1,36 a	1,27 a
	2	31,84 a	1,64 a	1,26 a
E. lata	1	19,85 b	6,19 b	0,61 b
	2	20,06 b	6,45 b	0,45 b

Values for the same fatty acid followed by different letters differ significantly ($p \leq 0,01$)

E. lata, viz. > 15 and < 6 for C16:0/C16:1(9), and < 3 and > 6 for C16:1(9)/C18:0 respectively.

The R value calculated for the isolates per species was 0,921 for *Cryptovalsa* and 0,877 for *Eutypa*. These R values clearly reflected the similarity between the MRP's of the major fatty acids present in the CFAP's of the various isolates as was also found by Augustyn (1989) for *Saccharomyces cerevisiae* strains. This also indicated a greater variation between fatty acid MRP's of the *Eutypa* isolates (Table 1). The much lower R (0,810) calculated from the fatty acid MRP's between species (Table 1) confirmed that differentiation between species based on consideration of all fatty acid MRP's was also possible.

Various authors have expressed doubts concerning the utility of fatty acid data in fungal taxonomy. These conclusions were based on the relative lack of variation in fungal fatty acids (Shaw, 1966; Wassef, 1977). Results of this study, however, show that *C.* cf *ampelina* and *E. lata* can be distinguished by studying the composition of their CFAP's. Further evaluation of this technique would thus seem feasible.

CONCLUSIONS

It was possible to differentiate between the strains of *E. lata* and *C.* cf *ampelina* occurring on the VORI experimental farm in Stellenbosch. The general utility of this technique has to be confirmed by analysing a large number of isolates of both species from widely divergent localities.

TABLE 3

Mean relative percentages (MRP's) and significant ratios for three diagnostically important fatty acids in two Ascomycete species

		C. cf *ampelina*					*E. lata*				
					16:0	16:1(0)				16:0	16:1(9)
		16:0	16:1(9)	18:0	16:1(9)	18:0	16:0	16:1(9)	18:0	16:1(9)	18:0
Isolate 1											
Spore	1	31,35	1,30	1,36	24,12	0,96	21,26	6,38	0,53	3,33	12,04
	2	31,44	1,16	1,43	27,10	0,81	18,76	6,28	0,65	2,99	9,66
	3	30,38	1,41	1,22	21,55	1,16	20,10	6,43	0,65	3,13	9,89
	4	30,48	1,58	1,09	19,29	1,45	19,29	5,67	0,60	3,40	9,45
Mean		31,01	1,36	1,27	22,80	1,07	19,85	6,19	0,61	3,21	10,15
Isolate 2											
Spore	1	31,72	1,65	1,27	19,22	1,30	22,23	6,33	0,40	3,51	15,83
	2	31,92	1,66	1,23	19,23	1,35	19,27	6,67	0,47	2,89	14,19
	3	31,34	1,58	1,29	19,84	1,22	22,09	5,69	0,57	3,85	9,98
	4	32,38	1,68	1,27	19,27	1,32	16,66	7,11	0,36	2,34	19,75
Mean		31,84	1,61	1,26	19,41	1,30	20,06	6,45	0,45	3,11	14,33

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