RESULTS

CHAPTER THREE

RESULTS

3.1 Essential oils extracted from leaves of selected *Citrus* species

Essential oils were extracted from fresh leaves of *Citrus halimii, C. hystrix, C. madurensis, C. micrantha* var. *microcarpa* and citrumello (*C. paradisi* X *P. trifoliata*) by steam distillation. The oils obtained were light yellowish to yellowish orange in colour (Table 3.1).

Table 3.1: Characteristics of the essential oils obtained from leaves of selected *Citrus* species

Species	Characteristics of the essential oils
Citrus halimii	Yellowish oil
C. hystrix	Light yellowish
C. madurensis	Deep yellowish
C. micrantha var. *microcarpa*	Yellowish
Citrumello (*C. paradisi* X *P. trifoliata*)	Yellowish orange

3.2 Analysis of essential oil components by GCMS

Citrus essential oils were subjected to GCMS. Total ion chromatograms of essential oils of *C. halimii, C. hystrix, C. madurensis, C. micrantha* var. *microcarpa* and citrumello are shown in Fig. 3.1, 3.2, 3.3, 3.4 and 3.5 respectively and the components are listed in Table 3.2, 3.3, 3.4, 3.5 and 3.6 according to increasing retention time. A list of compounds identified to relate the species is tabulated in Table 3.7 and divided on the basis of hydrocarbon, hydroxy, carbonyl and ester. Identification of essential oil components was done by comparing the mass spectra with those in the literature (Masada, 1976). A total of 25 compounds were able to identified from all the species studied. Percentages of the compounds are shown in Table 3.8.

Table 3.2: continued

Peak No.	Scan	R.T.	M.W.	Mass Spectrum Fragmentation	M.F. / Comments
6	165	10.20	136	M^+ 136 (45%), 121 (98%), 105 (22%), 93 (100%), 79 (38%), 77 (46%), 43 (42%), 41 (51%), 39 (45%).	$C_{10}H_{16}$ α-terpinene
7	176	10.75	136	M^+ 136 (10%), 121 (12%), 107 (14%), 93 (51%), 79 (30%), 68 (100%), 53 (33%), 41 (47%), 39 (52%).	$C_{10}H_{16}$ Limonene
8	185	11.20	136	M^+ 136 (4%), 121 (12%), 105 (17%), 93 (100%), 79 (50%), 63 (17%), 53 (34%), 41 (89%).	$C_{10}H_{16}$ 3,7-dimethyl-1,3,7-octatriene
9	192	11.55	136	M^+ 136 (27%), 121 (24%), 105 (10%), 93 (100%), 77 (44%), 65 (11%), 53 (12%), 43 (46%), 39 (35%).	$C_{10}H_{16}$ 3-carene
10	210	12.45	136	M^+ 136 (62%), 121 (75%), 105 (2%), 93 (100%), 79 (47%), 67 (16%), 53 (26%), 41 (67%), 39 (61%).	$C_{10}H_{16}$ Ocimene

Table 3.2: continued

Peak No.	Scan	R.T.	M.W.	Mass Spectrum Fragmentation	M.F. / Comments
11	215	12.70	154	M^+ 154, 139 (1%), 121 (9%), 93 (38%), 80 (20%), 71 (75%), 69 (32%), 55 (99%), 43 (95%), 41 (100%), 39 (31%).	$C_{10}H_{18}O$ Linalool
12	265	15.20	154	M^+ 154 (8%), 136 (6%) 121 (1%), 111 (33%), 93 (36%), 86 (18%), 71 (100%), 55 (32%), 43 (85%).	$C_{10}H_{18}O$ β-terpineol
13	288	16.35	154	M^+ 154, 139 (3%), 136 (15%), 121 (20%), 107 (3%), 93 (32%), 81 (26%), 67 (17%). 59 (100%), 43 (67%), 31 (27%).	$C_{10}H_{18}O$ α-terpineol
14	377	20.80	196	M^+ 196, 181, 136 (12%), 121 (27%), 107 (4%), 93 (28%), 79 (8%), 67 (9%), 59 (12%), 43 (100%), 31 (4%).	$C_{12}H_{20}O_2$ Terpinyl acetate

Table 3.2: continued.

Peak No.	Scan	R.T.	M.W.	Mass Spectrum Fragmentation	M.F. / Comments
15	415	22.70		M^+ 204 (3%), 189 (5%), 161 (25%), 147 (7%), 133 (22%), 120 (26%), 105 (28%), 93 (38%), 79 (33%), 69 (34%), 55 (29%), 41 (100%).	Unknown compound
16	426	23.25		M^+ 204 (3%), 189 (1%), 161 (14%), 147 (2%), 133 (11%), 120 (10%), 105 (11%), 93 (30%), 79 (18%), 69 (69%), 55 (29%), 41 (100%).	Unknown compound
17	460	24.95	204	M^+ 204 (3%), 189, 161 (29%), 147 (2%), 133 (9%), 119 (18%), 165 (34%), 91 (42%), 79 (30%), 67 (19%), 55 (30%), 41 (100%)	$C_{15}H_{24}$ Caryophyllene

Table 3.2: continued

Peak No.	Scan	R.T.	M.W.	Mass Spectrum Fragmentation	M.F. / Comments
18	487	26.30		M^+ 204, 189 (1%), 161 (6%), 148 (1%), 136 (8%), 121 (6%), 107 (17%), 93 (33%), 81 (17%), 69 (67%), 55 (32%), 43 (71%), 41 (100%).	Unknown compound
19	508	27.35		M^+ 218 (3%), 176 (44%), 161 (6%), 147 (9%), 134 (31%), 119 (48%), 105 (34%), 91 (55%), 81 (15%), 67 (25%), 55 (29%), 41 (100%).	Unknown compound
20	530	28.45	222	M^+ 222 (1%), 204 (14%), 189 (3%), 161 (14%), 148 (3%), 136 (2%), 121 (32%), 105 (15%), 93 (16%), 81 (20%), 71 (18%), 69 (13%), 55 (19%), 43 (100%).	$C_{15}H_{26}O$ Cedrol

Table 3.3: Essential oil composition of *Citrus hystrix*

Peak No.	Scan	R.T.	M.W.	Mass Spectrum Fragmentation	M.F. / Comments
1	123	8.10	136	M^+ 136 (12%), 121 (5%), 93 (100%), 91 (40%), 79 (29%), 77 (43%), 69 (15%), 43 (19%), 41 (63%), 39 (36%).	$C_{10}H_{16}$ β-pinene
2	129	8.40	136	M^+ 136 (4%), 121 (3%), 93 (60%), 91 (18%), 77 (18%), 69 (40%), 53 (11%), 41 (100%), 39 (33%).	$C_{10}H_{16}$ β-myrcene
3	186	11.25		M^+ 170, 155 (2%), 111 (16%), 94 (28%), 68 (24%), 59 (100%), 55 (40%), 43 (76%), 41 (36%), 39 (19%). 31 (26%).	Unknown compound
4	204	12.15	154	M^+ 154 (1%), 139 (3%), 121 (10%), 93 (44%), 80 (21%), 71 (86%), 55 (67%), 41 (100%).	$C_{10}H_{18}O$ Linalool
5	242	14.05	154	M^+ 154 (1%), 139 (2%), 95 (19%), 69 (45%), 55 (33%), 43 (21%), 41 (100%), 39 (30%).	$C_{10}H_{18}O$ Nerol

Table 3.3: continued

Peak No.	Scan	R.T.	M.W.	Mass Spectrum Fragmentation	M.F. / Comments
6	251	14.50	154	M^+ 154 (9%), 136 (7%), 121 (7%), 107 (3%), 93 (36%), 86 (18%), 71 (100%), 55 (42%), 43 (93%).	$C_{10}H_{18}O$ β-terpineol
7	289	16.40	156	M^+ 156 (1%), 138 (3%), 123 (6%), 109 (5%), 95 (16%), 82 (22%), 69 (49%), 55 (40%), 41 (100%), 39 (21%).	$C_{10}H_{20}O$ Citronellol
8	297	16.80	154	M^+ 154, 139 (1%), 123 (4%), 111 (3%), 93 (5%), 82 (4%), 69 (65%), 55(13%), 41 (100%).	$C_{10}H_{18}O$ Geraniol
9	347	19.30		M^+ 154 (2%), 139 (5%), 96 (35%), 81 (88%), 68 (22%), 59 (97%), 43 (100%), 31 (25%),	Unknown compound

Table 3.3: continued

Peak No.	Scan	R.T.	M.W.	Mass Spectrum Fragmentation	M.F. / Comments
10	365	20.20	222	M^+ 222, 204 (2%), 189 (1%), 161 (3%), 154 (1%), 137 (1%), 121 (8%), 109 (1%), 93 (17%), 81 (6%), 69 (89%), 53 (11%), 41 (100%), 31 (1%).	$C_{15}H_{20}O$ Farnesol
11	371	20.50	204	M^+ 204 (14%), 189 (4%), 161 (82%), 147 (4%), 133 (12%), 119 (98%), 105 (100%), 93 (56%), 81 (35%), 77 (26%), 55 (39%), 41 (89%).	$C_{15}H_{24}$ Copaene
12	397	21.80	204	M^+ 204 (2%), 189 (6%), 161 (11%), 147 (10%), 133 (31%), 119 (15%), 105 (23%), 93 (45%), 79 (36%), 69 (51%), 55 (29%), 41 (100%).	$C_{15}H_{24}$ α-caryophyllene
13	411	22.50	204	M^+ 204 (4%), 189 (2%), 161 (3%), 147 (14%), 136 (3%), 121 (24%), 93 (100%), 80 (39%), 67 (18%), 53 (20%), 41 (54%).	$C_{15}H_{24}$ α-caryophyllene

Table 3.3: continued

Peak No.	Scan	R.T.	M.W.	Mass Spectrum Fragmentation	M.F. / Comments
14	434	23.65	204	M^+ 204 (15%), 189 (10%), 161 (32%), 121 (64%), 105 (63%), 93 (73%), 79 (43%), 67 (28%), 55 (35%), 41 (100%).	Unknown compound
15	447	24.30	204	M^+ 204 (48%), 189 (17%), 161 (100%), 134 (61%), 119 (76%), 105 (71%), 91 (46%), 81 (44%), 77 (22%), 55 (31%), 41 (87%).	Unknown compound
16	465	25.20	222	M^+ 222, 204 (1%), 189 (2%), 161 (7%), 148 (1%), 136 (9%), 121 (7%), 107 (18%), 93 (36%), 81 (20%), 69 (75%), 55 (34%), 41 (100%), 31 (2%).	Unknown compound
17	664	35.15		M^+ 290 (2%), 275 (1%), 95 (12%), 81 (13%), 69 (47%), 55 (36%), 41 (100%).	Unknown compound

Table 3.4: Essential oil composition of *Citrus madurensis*

Peak No.	Scan	R.T.	M.W.	Mass Spectrum Fragmentation	M.F. / Comments
1	107	7.35	136	M^+ 136 (7%), 121 (12%), 105 (11%), 93 (100%), 77 (33%), 67 (11%), 53 (12%), 41 (38%).	$C_{10}H_{16}$ α-pinene
2	129	8.45	136	M^+ 136 (7%), 121 (10%), 107 (4%), 93 (100%), 79 (28%), 69 (43%), 53 (15%), 41 (94%).	$C_{10}H_{16}$ β-pinene
3	157	9.85	154	M^+ 154 (8%), 139 (7%), 121 (10%), 111 (12%), 108 (16%), 93 (61%), 81 (28%), 79 (25%), 68 (64%), 55 (26%), 43 (100%).	$C_{10}H_{18}O$ Cineole
4	168	10.40	136	M^+ 136 (5%), 121 (13%), 105 (15%), 93 (100%), 79 (49%), 67 (17%), 53 (28%), 43 (32%), 39 (55%).	$C_{10}H_{16}$ 3,7-dimethyl-1,3,7-octatriene
5	201	12.05	154	M^+ 154, 136 (3%), 121 (11%), 107 (4%), 93 (45%), 80 (23%), 71 (87%), 55 (64%), 43 (95%), 31 (4%).	$C_{10}H_{18}O$ Linalool

Table 3.4: continued.

Peak No.	Scan	R.T.	M.W.	Mass Spectrum Fragmentation	M.F. / Comments
6	243	14.15	154	M^+ 154 (7%), 136 (7%), 111 (33%), 93 (36%), 71 (100%), 55 (31%), 43 (86%), 39 (22%).	$C_{10}H_{18}O$ β-terpineol
7	250	14.50	154	M^+ 154, 136 (18%), 121 (24%), 93 (37%), 81 (27%), 67 (16%), 59 (100%), 43 (55%), 39 (17%).	$C_{10}H_{18}O$ α-terpineol
8	346	19.30	204	M^+ 204 (2%), 189 (4%), 161 (28%), 148 (2%), 136 (59%), 121 (100%), 105 (20%), 93 (94%), 77 (29%), 67 (12%), 53 (20%), 41 (67%).	Unknown compound
9	375	20.75	204	M^+ 204, 189 (8%), 161 (12%), 147 (17%), 133 (13%), 121 (21%), 107 (37%), 93 (63%), 81 (70%), 67 (57%), 55 (47%), 41 (100%).	$C_{15}H_{24}$ Elemene

Table 3.4: continued

Peak No.	Scan	R.T.	M.W.	Mass Spectrum Fragmentation	M.F. / Comments
10	393	21.65	204	M^+ 204, 189 (5%), 161 (9%), 147 (8%), 133 (26%), 121 (10%), 105 (21%), 93 (43%), 79 (36%), 69 (51%), 55 (27%), 41 (100%), 39 (33%).	$C_{15}H_{24}$ Caryophyllene
11	408	22.40	204	M^+ 204 (3%), 189 (2%), 161 (4%), 147 (14%), 136 (4%), 121 (23%), 107 (14%), 93 (100%), 80 (35%), 67 (19%), 55 (19%), 41 (59%).	$C_{15}H_{24}$ α-caryophyllene
12	429	23.45		M^+ 204 (9%), 189 (2%), 161 (76%), 147 (7%), 133 (19%), 119 (36%), 105 (66%), 91 (59%), 81 (48%), 67 (27%), 55 (41%), 41 (100%).	Unknown compound
13	435	23.75		M^+ 204 (15%), 189 (18%), 161 (31%), 147 (12%), 133 (18%), 121 (33%), 105 (76%), 93 (68%), 81 (42%), 67 (26%), 55 (42%), 41 (100%).	Unknown compound

Table 3.4: continued

Peak No.	Scan	R.T.	M.W.	Mass Spectrum Fragmentation	M.F. / Comments
14	460	25.00		M^+ 204 (2%), 189 (8%), 175 (2%), 161 (20%), 149 (5%), 135 (11%), 121 (17%), 107 (23%), 93 (46%), 81 (26%), 67 (27%), 59 (100%), 43 (61%).	Unknown compound
15	478	25.90		M^+ 204 (5%), 189 (6%), 107 (25%), 93 (31%), 81 (34%), 67 (29%), 59 (69%), 43 (100%).	Unknown compound
16	492	26.60		M^+ 222 (4%), 204 (24%), 189 (49%), 161 (51%), 93 (34%), 81 (39%), 59 (100%), 43 (94%).	Unknown compound
17	511	27.55		M^+ 222 (1%), 204 (5%), 189 (6%), 149 (22%), 109 (13%), 93 (13%), 81 (14%), 59 (100%), 43 (32%).	Unknown compound

Table 3.5: Essential oil composition of *Citrus micrantha* var. *microcarpa*

Peak No.	Scan	R.T.	M.W.	Mass Spectrum Fragmentation	M.F / Comments
1	219	12.90	154	M^+ 154, 139 (1%), 121 (8%), 107 (3%), 93 (35%), 71 (69%), 55 (58%), 43 (100%).	$C_{10}H_{18}O$ Linalool
2	255	14.70	154	M^+ 154 (1%), 139 (2%), 95 (17%), 69 (39%), 67 (12%), 55 (29%), 41 (100%).	$C_{10}H_{18}O$ Nerol
3	306	17.25	156	M^+ 156 (1%), 138 (2%), 123 (5%), 109 (4%), 95 (12%), 81 (20%), 69 (44%), 55 (35%), 41 (100%).	$C_{10}H_{20}O$ Citronellol
4	320	17.95	152	M^+ 152, 139 (1%), 123 (4%), 111 (3%), 93 (5%), 84 (4%), 69 (66%), 53 (8%), 41 (100%).	$C_{10}H_{16}O$ Neral

Table 3.5: continued

Peak No.	Scan	R.T.	M.W.	Mass Spectrum Fragmentation	M.F. / Comments
5	369	20.40		M^+ 204 (1%), 189 (2%), 136 (25%), 121 (46%), 93 (45%), 81 (29%), 69 (34%), 55 (33%), 41 (100%).	Unknown
6	389	21.40	154	M^+ 154 (1%), 136 (5%), 121 (7%), 93 (14%), 80 (8%), 69 (87%), 41 (100%).	$C_{10}H_{18}O$ Geraniol
7	399	21.90	204	M^+ 204 (1%), 189 (9%), 161 (12%), 147 (17%), 133 (12%), 121 (20%), 107 (31%), 93 (55%), 81 (61%), 67 (54%), 55 (43%), 41 (100%).	$C_{15}H_{24}$ Elemene
8	419	22.90		M^+ 204 (4%), 189 (5%), 161 (30%), 147 (6%), 133 (10%), 121 (40%), 105 (29%), 93 (39%), 79 (28%), 67 (32%), 55 (39%), 41 (100%).	Unknown compound

Table 3.5: continued

Peak No.	Scan	R.T.	M.W.	Mass Spectrum Fragmentation	M.F. / Comments
9	425	23.20		M+ 204 (17%), 189 (12%), 161 (39%), 147 (20%), 133 (20%), 119 (44%), 105 (92%), 91 (47%), 77 (26%), 69 (14%), 55 (34%), 41 (100%).	Unknown compound
10	432	23.55	204	M^+ 204 (4%), 189 (2%), 161 (4%), 147 (12%), 136 (4%), 121 (25%), 107 (13%), 93 (100%), 80 (38%), 67 (20%), 53 (22%), 41 (68%).	$C_{15}H_{24}$ α-caryophyllene
11	451	24.50	204	M^+ 204 (8%), 161 (61%), 105 (53%), 91 (56%), 81 (44%), 79 (42%), 41 (100%), 39 (43%).	$C_{15}H_{24}$ Caryophyllene
12	460	24.95		M^+ 204 (50%), 161 (75%), 119 (52%), 105 (64%), 93 (39%), 91 (51%), 55 (41%), 41 (100%).	Unknown compound

Table 3.5: continued

Peak No.	Scan	R.T.	M.W.	Mass Spectrum Fragmentation	M.F. / Comments
13	463	25.10		M^+ 204 (14%), 161 (61%), 105 (42%), 93 (48%), 91 (46%), 79 (41%), 43 (49%), 41 (100%).	Unknown compound
14	478	25.85		M^+ (222), 204 (4%), 189 (10%), 43 (49%), 67 (36%), 55 (37%), 43 (87%), 39 (30%).	Unknown compound
15	487	26.30		M^+ 204 (11%), 189 (13%), 121 (50%), 93 (50%), 67 (52%), 53 (43%), 41 (100%), 39 (49%).	Unknown compound
16	497	26.80		M^+ 222 (1%), 204 (7%), 161 (28%), 105 (21%), 93 (15%), 81 (18%), 55 (25%), 43 (100%).	Unknown compound

Table 3.5: continued

Peak No.	Scan	R.T.	M.W.	Mass Spectrum Fragmentation	M.F. / Comments
17	520	27.95		M^+ 204 (27%), 189 (34%), 161 (59%), 105 (100%), 91 (36%), 59 (87%), 43 (78%).	Unknown compound
18	531	28.50		M^+ 222 (1%), 204 (8%), 189 (7%), 149 (22%), 95 (24%), 79 (19%), 59 (100%), 43 (58%).	Unknown compound

Table 3.6: Essential oil composition of citrumello

Peak No.	Scan	R.T.	M.W.	Mass Spectrum Fragmentation	M.F. / Comments
1	98	6.90	136	M$^+$ 136 (5%), 121 (9%), 105 (10%), 93 (100%), 91 (42%), 79 (24%), 77 (26%), 67 (11%), 53 (13%), 41 (44%), 39 (40%).	$C_{10}H_{16}$ α-pinene
2	120	8.00	136	M$^+$ 136 (10%), 121 (4%), 107 (2%), 93 (100%), 91 (41%), 79 (30%), 77 (43%), 69 (13%), 53 (13%), 41 (61%), 39 (40%).	$C_{10}H_{16}$ β-pinene
3	131	8.55	136	M$^+$ 136 (1%), 121 (2%), 107 (1%), 93 (40%), 77 (8%), 69 (38%), 53 (9%), 41 (100%), 39 (27%).	$C_{10}H_{16}$ β-myrcene
4	144	9.20	136	M$^+$ 136 (45%), 121 (90%), 105 (20%), 93 (100%), 91 (55%), 79 (35%), 77 (40%), 65 (13%), 53 (14%), 41 (41%), 39 (39%).	$C_{10}H_{16}$ α-terpinene

Table 3.6: continued

Peak No.	Scan	R.T.	M.W.	Mass Spectrum Fragmentation	M.F./ Comments
5	151	9.55	136	M^+ 136 (18%), 121 (17%), 107 (16%), 93 (93%), 91 (36%), 79 (38%), 77 (35%), 68 (100%), 53 (34%), 41 (53%), 39 (56%).	$C_{10}H_{16}$ Limonene
6	167	10.35	136	M^+ 136 (4%), 121 (13%), 105 (17%), 93 (100%), 91 (48%), 79 (51%), 77 (42%), 67 (18%), 53 (31%), 43 (34%), 41 (85%), 39 (69%).	$C_{10}H_{16}$ 3,7-dimethyl-1,3,7-octatriene
7	172	10.60	136	M^+ 136 (27%), 121 (23%), 105 (10%), 93 (100%), 91 (51%), 79 (26%), 77 (41%), 65 (11%), 51 (12%), 43 (44%), 41 (36%), 39 (32%).	$C_{10}H_{16}$ 3-carene

Table 3.6: continued

Peak No.	Scan	R.T.	M.W.	Mass Spectrum Fragmentation	M.F. / Comments
8	187	11.35	136	M^+ 136 (65%), 121 (80%), 105 (24%), 93 (100%), 91 (56%), 79 (51%), 77 (42%), 67 (17%), 53 (26%), 43 (44%), 41 (68%), 39 (62%).	$C_{10}H_{16}$ Ocimene
9	240	14.00	154	M^+ 154 (9%), 136 (6%), 121 (1%), 111 (36%), 93 (36%), 86 (19%), 71 (100%), 69 (21%), 55 (32%), 43 (86%), 41 (49%), 39 (24%), 31 (5%).	$C_{10}H_{18}O$ β-terpineol
10	268	15.40	156	M^+ 156 (1%), 138 (3%), 123 (6%), 109 (5%), 93 (13%), 81 (20%), 69 (47%), 67 (27%), 55 (35%), 43 (25%), 41 (100%), 31 (25%).	$C_{10}H_{18}O$ Unknown compound

Table 3.6: continued.

Peak No.	Scan	R.T.	M.W.	Mass Spectrum Fragmentation	M.F. / Comments
11	343	19.15	204	M^+ 204 (1%), 189 (4%), 161 (23%), 136 (42%), 121 (100%), 107 (27%), 93 (88%), 91 (39%), 79 (36%), 67 (22%), 55 (27%), 43 (47%), 41 (91%), 39 (44%).	Unknown compound
12	379	20.95	204	M^+ 204 (1%), 189 (3%), 161 (6%), 148 (5%), 133 (19%), 120 (10%), 105 (17%), 93 (31%), 79 (31%), 69 (41%), 55 (27%), 41 (100%), 39 (36%).	$C_{15}H_{24}$ Elemene
13	400	22.00	204	M^+ 204 (1%), 189 (3%), 175 (2%), 161 (6%), 148 (5%), 133 (19%), 121 (7%), 105 (17%), 93 (31%), 79 (31%), 69 (41%), 55 (27%), 41 (100%).	$C_{15}H_{24}$ Caryophyllene

Table 3.6: continued

Peak No.	Scan	R.T.	M.W.	Mass Spectrum Fragmentation	(M.F.) / Comments
14	419	22.95	204	M^+ 204 (3%), 189 (2%), 161 (3%), 147 (13%), 136 (3%), 121 (23%), 107 (14%), 93 (100%), 80 (38%), 67 (19%), 55 (20%), 43 (22%), 41 (58%), 39 (29%),	$C_{15}H_{24}$ α-caryophyllene
15	430	23.50		M^+ 204 (8%), 189 (1%), 161 (74%), 147 (5%), 133 (18%), 121 (7%), 105 (59%), 91 (59%), 81 (46%), 67 (25%), 55 (39%), 43 (31%), 41 (100%), 39 (44%)	Unknown compound
16	439	23.95		M^+ 204 (4%), 189 (4%), 161 (9%), 147 (3%), 133 (5%), 121 (26%), 107 (29%), 93 (53%), 91 (29%), 79 (32%), 69 (24%), 55 (40%), 41 (100%), 39 (36%)	Unknown compound

Table 3.6: continued

Peak No.	Scan	R.T.	M.W.	Mass Spectrum Fragmentation	M.F. / Comments
17	446	24.30	204	M^+ 204 (50%), 189 (19%), 161 (100%), 147 (9%), 134 (57%), 119 (75%), 105 (73%), 91 (49%), 81 (42%), 61 (19%), 55 (34%), 41 (93%).	Unknown compound
18	478	25.90	222	M^+ 220 (1%), 205 (7%), 187 (3%), 162 (6%), 147 (7%), 133 (5%), 119 (14%), 107 (13%), 93 (24%), 79 (27%), 69 (22%), 55 (24%), 43 (100%).	Unknown compound
19	483	26.15	222	M^+ 222 (1%), 204 (6%), 189 (8%), 161 (15%), 149 (7%), 133 (6%), 121 (13%), 109 (25%), 93 (21%), 81 (23%), 69 (31%), 55 (28%), 43 (100%), 41 (58%).	Unknown compound

Table 3.7: Compounds identified in essential oils of five selected *Citrus* species

Compound	Species				
	C. halimii	*C. hystrix*	*C. madurensis*	*C. micrantha* var. *microcarpa*	Citrumello (*C. paradisi* X *P. trifoliata*)
1) β-myrcene	+	+	-	-	+
2) α-terpinene	+	-	-	-	+
3) β-phellandrene	+	-	-	-	-
4) Limonene	+	-	-	-	+
5) 3-carene	+	-	-	-	+
6) α-pinene	+	-	+	-	+
7) β-pinene	+	+	+	-	+
8) Camphene	+	-	-	-	-
9) α-caryophyllene	-	+	+	+	+
10) Caryophyllene	+	+	+	+	+
11) Copaene	-	+	-	-	-
12) Ocimene	+	-	-	-	+
13) Elemene	-	-	+	+	+
14) 3,7-dimethyl-1,3,7-octatriene	+	-	+	-	+
15) Geraniol	-	+	-	+	-
16) Nerol	-	+	-	+	-
17) Linalool	+	+	+	+	-
18) Citronellol	-	+	-	+	+
19) α-terpineol	+	-	+	-	-
20) β-terpineol	+	+	+	-	+
21) Cedrol	+	-	-	-	-
22) Farnesol	-	-	-	-	-
23) Cineol	-	-	+	-	-
24) Neral	-	-	-	+	-
25) Terpinyl acetate	+	-	-	-	-

Table 3.8: Comparison of the essential oils composition of five selected *Citrus* species

Compound	Relative amount (%)				
	Species				
	C. halimii	*C. hystrix*	*C. madurensis*	*C. micrantha* var. *microcarpa*	Citrumello (*C. paradisi* X *P. trifoliata*)
a) Hydrocarbon					
i) Monoterpenes					
1) β-myrcene	3.6	1.2	-	-	6.0
2) α-terpinene	0.1	-	-	-	1.5
3) β-phellandrene	1.6	-	-	-	-
4) Limonene	1.0	-	-	-	1.4
5) 3-carene	4.1	-	-	-	2.3
6) α-pinene	4.9	-	0.7	-	0.2
7) β-pinene	13.1	0.4	11.7	-	3.8
8) Camphene	tr	-	-	-	-
9) Ocimene	1.5	-	-	-	1.7
ii) Sesquiterpenes					
1) α-caryophyllene	-	0.8	1.3	1.2	0.5
2) Caryophyllene	2.5	4.8	7.3	8.5	40.8
3) Copaene	-	1.1	-	-	-
4) Elemene	-	-	2.4	1.2	0.3

tr = trace < 0.1 %

Table 3.8: continued

Compound	Relative amount (%)				
	Species				
	C. halimii	*C. hystrix*	*C. madurensis*	*C. micrantha* var. *microcarpa*	Citrumello (*C. paradisi* X *P. trifoliata*)
b) Hydroxy					
i) Terpenoid					
1) Geraniol	-	0.8	-	7.7	-
2) Nerol	-	44.1	-	20.9	-
3) Linalool	1.4	3.4	12.1	0.7	-
4) Citronellol	-	22.3	-	11.1	1.1
5) α-terpineol	35.4	-	1.7	-	-
6) β-terpineol	1.5	2.6	1.2	-	2.6
7) Cedrol	0.2	-	-	-	-
8) Farnesol	-	-	-	-	-
9) Cineole	-	-	3.5	-	-
c) Carbonyl					
i) Terpenoid					
1) Neral	-	-	-	6.3	-
d) Ester					
i) Terpenoid					
1) Terpinyl acetate	22.3	-	-	-	5.3
e) Miscellaneous					
1) 3,7-dimethyl-1,3,7-octatriene	0.9	-	3.0	-	

Fig. 3.1: Total ion chromatogram of *Citrus halimii* leaf oil

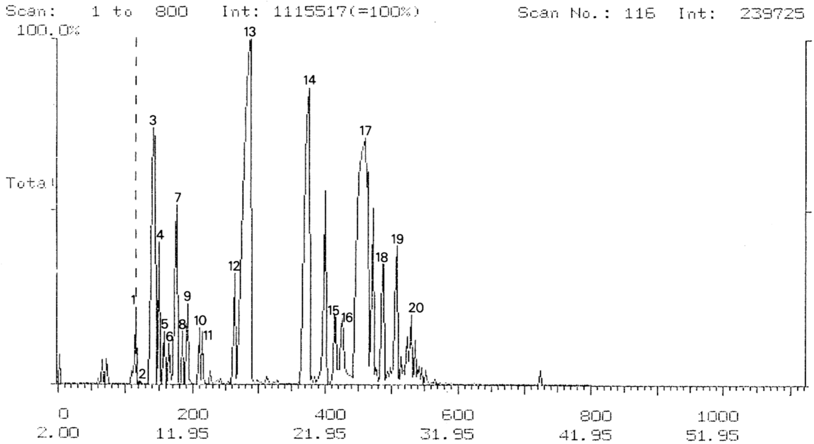


Fig. 3.2: Total ion chromatogram of *Citrus hystrix* leaf oil

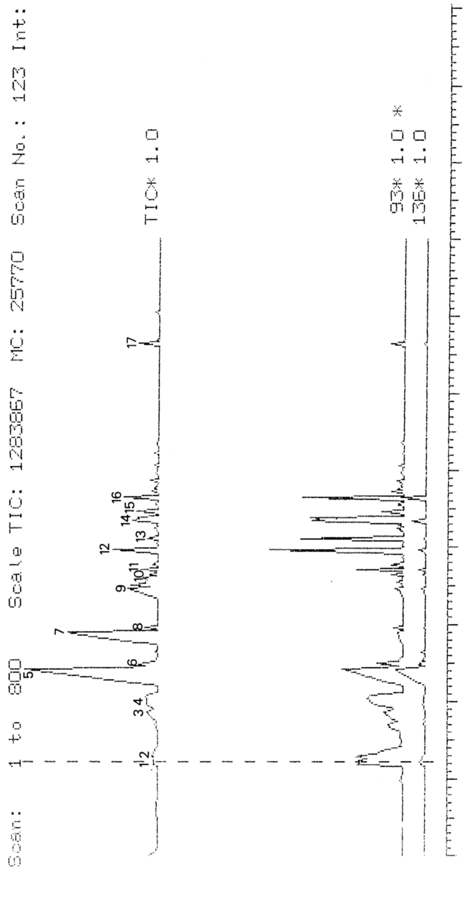


Fig. 3.3: Total ion chromatogram of *Citrus madurensis* leaf oil

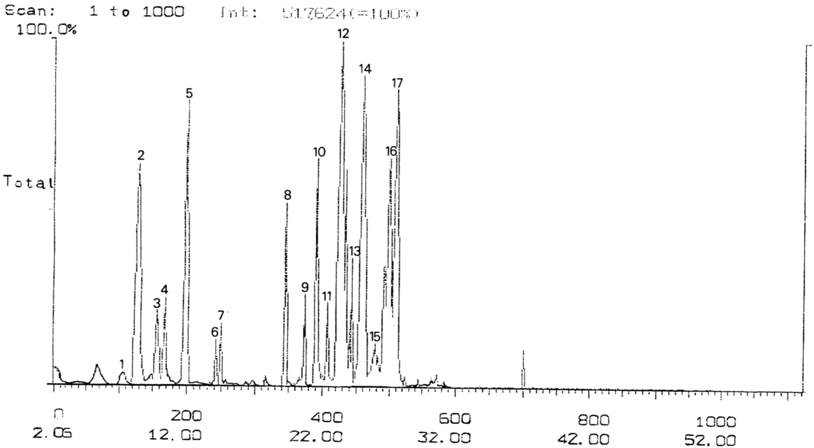


Fig. 3.4: Total ion chromatogram of *Citrus micrantha* var. *microcarpa* leaf oil

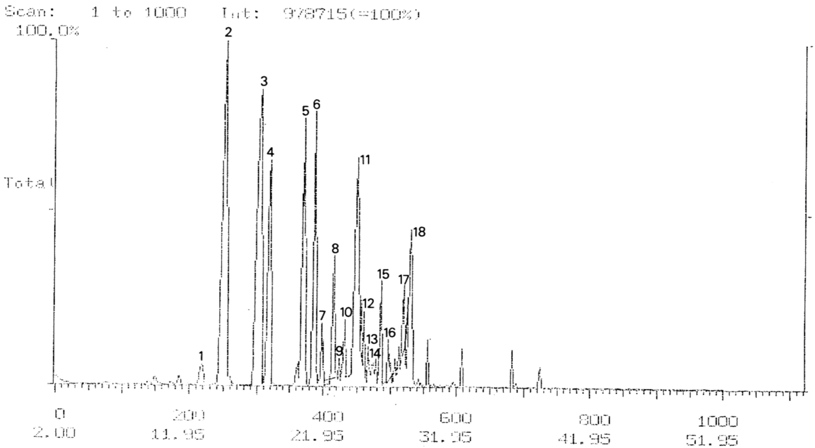


Fig. 3.5: Total ion chromatogram of citrumello leaf oil

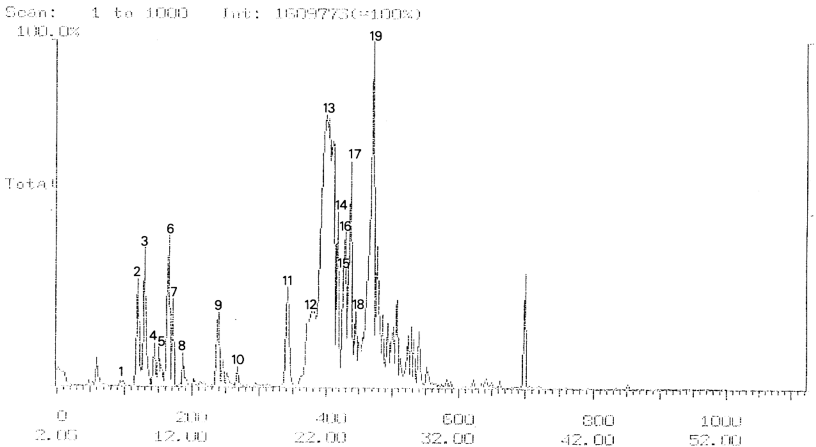


Fig. 3.6: Infrared spectrum of *C. halimii*

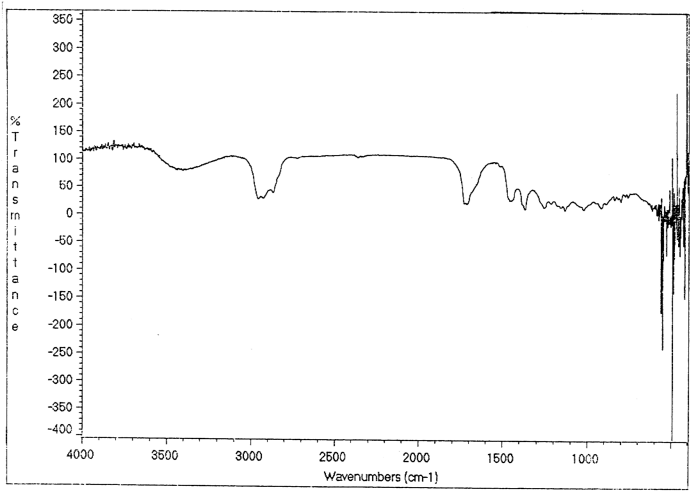


Fig. 3.8 : Infrared spectrum of *C. madurensis*

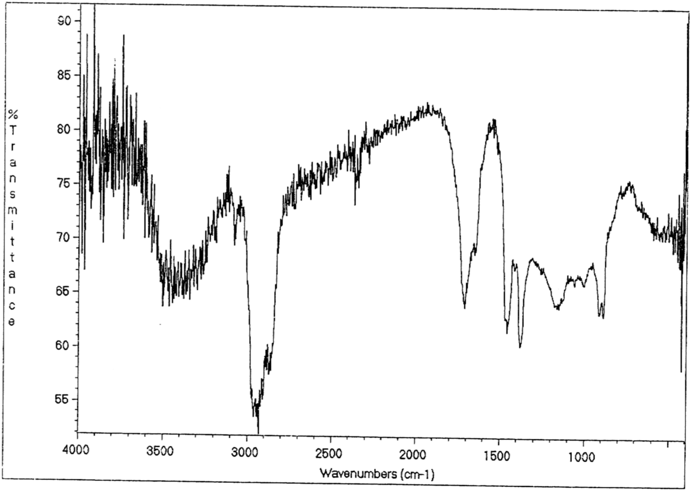


Fig. 3.9 : Infrared spectrum of *C. micrantha* var. *microcarpa*

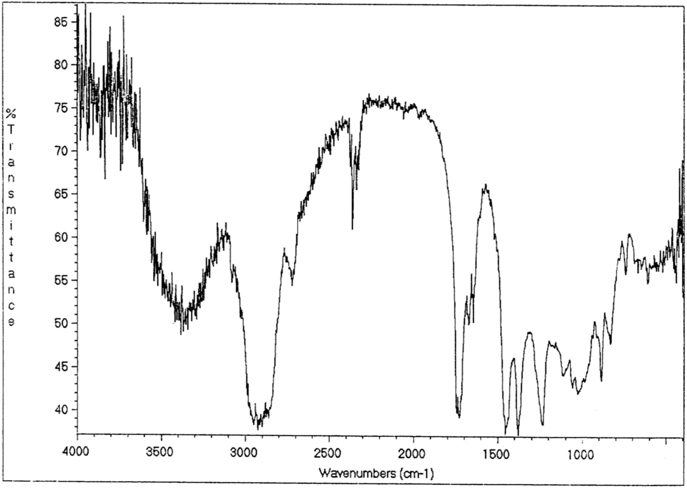
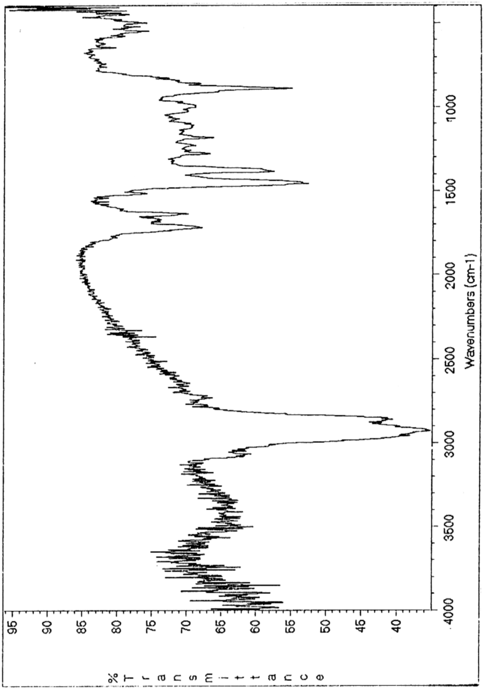


Fig. 3.10: Infrared spectrum of citrumello



3.3 Isoenzymes analysis in leaves of selected *Citrus* species

Five isoenzymes (esterase, glutamate oxaloacetate transaminase, peroxidase, malate dehydrogenase and polyphenoloxidase) were investigated for *Citrus halimii, C. hystrix, C. madurensis, C. micrantha* var. *microcarpa* and citrumello. However banding for peroxidase and esterase were not observed in citrumello. No band was observed for the isoenzyme studies on malate dehydrogenase and polyphenol oxidase for all the species studied. In certain cases, clear bands were difficult to obtain. The reason could be the presence of phenolic compounds which interfered with enzymatic assays (Torres *et al.*, 1978; Coombs and Hind, 1985) or the level of activity was too low to be detected. Rf values for each bands were calculated and zymogram for each isoenzyme was drawn .

3.3.1 Glutamate oxaloacetate transaminase

A total of three bands were observed (Figure 3.6, Plate 6). Band 3 was typical to all species. Band 2 was present in *Citrus halimii* and *C. micrantha* var. *microcarpa*, while band 1 was shown in *Citrus hystrix* and citrumello. Most of the species had a total of two bands except *C. madurensis* (only one band was detected). The colour of band 3 was more intense compared to bands 1 and 2.

3.3.2 Peroxidase

In this enzyme system, a total of 4 bands were detected in all species (Figure 3.7, Plate 7). Bands 1, 2 and 4 were specific for *Citrus madurensis*. Band 3 was shown in all species except in *C. madurensis*. Most of the species had only one band except *C. madurensis*. The four bands exhibited in this systems showed the same colour intensity.

3.3.3 Esterase

A total of 5 bands were exhibited (Figure 3.8, Plate 8). There were two specific bands observed in this system. Band 4 was specific for *C. micrantha* var. *microcarpa,* while band 5 for *C. halimii*. Band 3 was found common in *C. madurensis* and *C. micrantha* var. *microcarpa*. Band 2 which was shown in *C. halimii*, *C. hystrix* and *C. micrantha* var. *microcarpa* was missing in *C. madurensis*. Band 1 was observed in *C. halimii* and *C. hystrix*. *C. halimii* and *C. micrantha* var. *microcarpa* had the most number of bands (a total of three), while *C. hystrix* had two bands and *C. madurensis* had only one band. Colours of band 2 (in *C. hystrix*) and band 3 (*C. madurensis*) were very intense compared to the other bands. Band 1 and 4 had the same colour intensity whereas band 5 was very faint .

Table 3.9: Isoenzyme systems studied in selected *Citrus* species

	Species				
Enzyme	*C. halimii*	*C. hystrix*	*C. madurensis*	*C. micrantha* var. *microcarpa*	Citrumello (*C. paradisi* X *P. trifoliata*)
Acid phosphatase	-	-	-	-	-
Esterase	+	+	+	+	-
Glutamate oxaloacetate transaminase	+	+	+	+	+
Malate dehydrogenase	-	-	-	-	-
Peroxidase	+	+	+	+	-
Laccase	-	-	-	-	-

Experiments were repeated 3 times for each isoenzyme

Key: + bands detected
- no bands detected

Plate 6: Zymogram of glutamate oxaloacetate transaminase of *Citrus* species studied

Plate 7: Zymogram of peroxidase of *Citrus* species studied

Plate 8: Zymogram of esterase of *Citrus* species studied

Key to species:

a: Citrumello

b: *C. micrantha* var. *microcarpa*

c: *C. madurensis*

d: *C. hystrix*

e: *C. halimii*

Plate 6

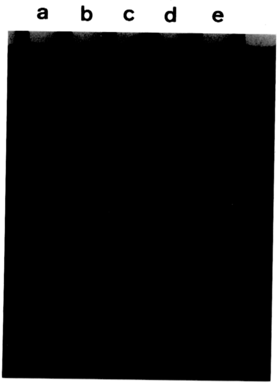


Plate 7

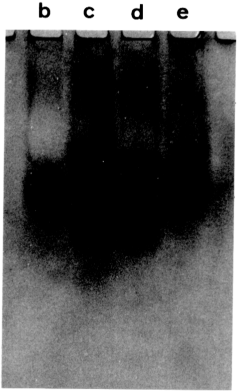


Plate 8

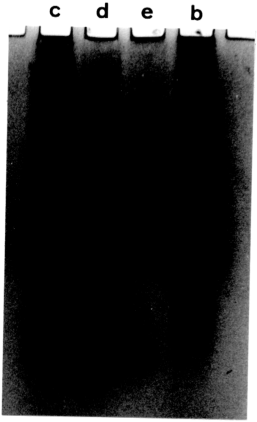
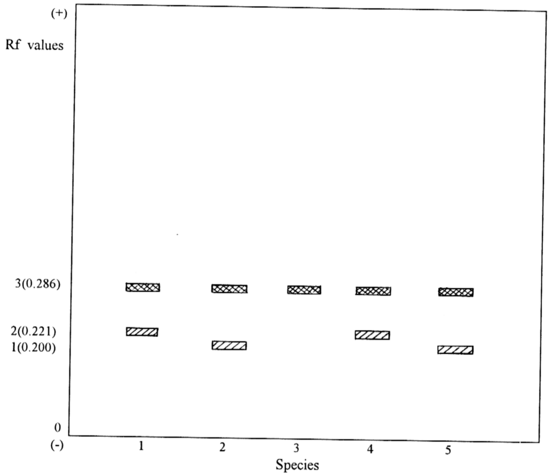


Figure 3.11: Schematic zymogram of glutamate oxaloacetate transaminase in *Citrus* studied



Key:

1 = *Citrus halimii*

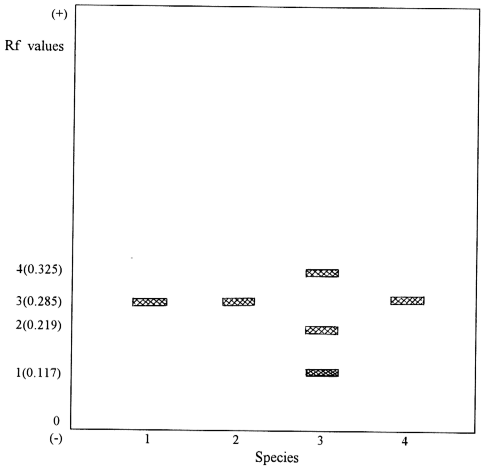
2 = *C. hystrix*

3 = *C. madurensis*

4 = *C. micrantha* var. *microcarpa*

5 = Citrumello (*C. paradisi* X *P. trifoliata*)

Figure 3.12: Schematic zymogram of peroxidase in *Citrus* studied



Key:

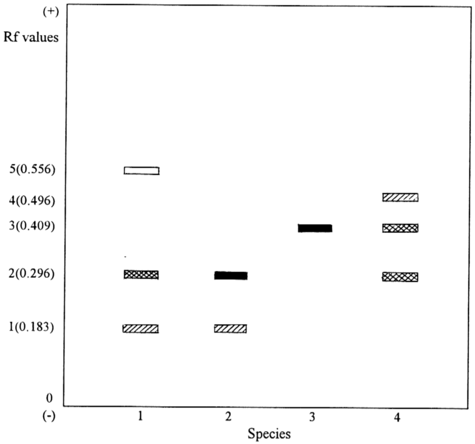
1 = *Citrus halimii*

2 = *C. hystrix*

3 = *C. madurensis*

4 = *C. micrantha* var. *microcarpa*

Figure 3.13: Schematic zymogram of esterase in *Citrus* studied



Key:

1 = *Citrus halimii*

2 = *C. hystrix*

3 = *C. madurensis*

4 = *C. micrantha* var. *microcarpa*

3.4 Analysis of browning of young shoot extracts of selected *Citrus* species

Analysis of browning of young shoot extracts was performed on *C. halimii*, *C. hystrix*, *C. madurensis*, *C. micrantha* var. *microcarpa* and citrumello (*C. paradisi* X *P. trifoliata*).

Observations on the colours of shoot extracts were made prior to classifications. Colours upon extraction varied from light yellow (citrumello) to greenish yellow (*C. halimii*, *C. micrantha* var. *microcarpa* and *C. madurensis*). However in *C. hystrix* the colour was dark green with slight purplish tinge. This was a result of anthocyanin pigmentation (Esen and Soost, 1974b).

Species studied are then classified into two phenotypes based on the development or nondevelopment of browning of shoot extracts when left standing for 24 hours:

1) Browning: The colour of extracts turned brown .

2) Nonbrowning: Extracts failed to develop brown colour.

Observations were also made after 1 week to confirm the results. Results obtained in this study is tabulated in Table 3.10. The browning process was comparatively more rapid in shoot extracts from *C. madurensis* where browning was observed on the surface of supernatant after 20 minutes of centrifugation and then gradually turned brownish yellow within 24 hours. Shoot extracts of *C. hystrix* developed brown colour after 24 hours left standing and the intensity was least comparatively. Subsequently, the colour of the latter extracts turned brownish green. Extracts from the other species did not turn brown after 24 hours.

From these observations, species can be classified into two phenotypes as below:

Browning species - *C. madurensis*

- *C. hystrix*

Nonbrowning species - *C. halimii*

- *C. micrantha* var. *microcarpa*

- citrumello (*C. paradisi* X *P. trifoliata*)

Experiments on effect of inhibitors were employed to confirm that the enzyme extracted was polyphenol oxidase (PPO). Diethyldithiocarbamate (DIECA) and potassium metabisulphite which are known as PPO inhibitors were used in this experiment. Potassium metabisulphite, DIECA and cystein which were included in extraction buffer totally inhibited browning (Table 3.11, 3.12, 3.13, 3.14 and 3.15). Insoluble polyvinylpyrrolidone (PVP) did not prevent browning. Observations after 1 week were done to confirm the results.

Table 3.10: Distribution of enzymatic browning of young shoot homogenates in selected *Citrus* species

Species	Colour of extract	
	0 hour	24 hours
Citrus halimii	greenish yellow	greenish yellow
C. hystrix	dark green with slight purplish tinge	brownish green
C. madurensis	greenish yellow	brownish yellow
C. micrantha var. *microcarpa*	greenish yellow	greenish yellow
Citrumello (*Citrus paradisi* X *Poncirus trifoliata)*	light yellow	light yellow

Shoots were extracted in 0.05 M Phosphate buffer (pH 7.2) and observations were made at 0 and 24 hours. Experiments were repeated 3 times.

Table 3.11: Extract colour of shoot homogenates of *Citrus halimii* in various inhibitors after centrifugation

Extraction solutions	Extract colour after centrifugation		
	0 hour	24 hours	1 week
1) 0.05 M phosphate buffer pH 7.2 (control)	greenish yellow	greenish yellow	greenish yellow
2) 10 mM potassium metabisulfite ($K_2S_2O_5$)	greenish yellow	greenish yellow	greenish yellow
3) 20 mM DIECA	greenish yellow	greenish yellow	greenish yellow
4) 2% insoluble PVP	greenish yellow	greenish yellow	greenish yellow
5) 5 mM $K_2S_2O_5$	greenish yellow	greenish yellow	greenish yellow
6) 20 mM L-cystein HCl	greenish yellow	greenish yellow	greenish yellow

Table 3.12: Extract colour of shoot homogenates of *Citrus hystrix* in various inhibitors after centrifugation

Extraction solutions	Extract colour after centrifugation		
	0 hour	24 hours	1 week
1) 0.05 M phosphate buffer pH 7.2 (control)	dark green with slight purplish tinge	brownish green	brownish green
2) 10 mM potassium metabisulfite ($K_2S_2O_5$)	light greenish yellow	yellow	yellow
3) 20 mM DIECA	dark green	dark green	dark green
4) 2% insoluble PVP	dark green	dark brownish green	dark brownish green
5) 5 mM $K_2S_2O_5$	yellowish green	yellowish green	light orange
6) 20 mM L-cystein HCl	greenish yellow	greenish yellow	greenish yellow

Table 3.13: Extract colour of shoot homogenates of *Citrus madurensis* in various inhibitors after centrifugation

Extraction solutions	Extract colour after centrifugation		
	0 hour	24 hours	1 week
1) 0.05 M phosphate buffer pH 7.2 (control)	greenish yellow browning on the surface of the supernatant	brownish yellow	light brown
2) 10 mM potassium metabisulfite ($K_2S_2O_5$)	greenish yellow	greenish yellow	greenish yellow
3) 20 mM DIECA	greenish yellow	yellow	yellow
4) 2% insoluble PVP	deep yellow	light orange	deep orange
5) 5 mM $K_2S_2O_5$	greenish yellow	greenish yellow	greenish yellow
6) 20 mM L-cystein HCl	greenish yellow	greenish yellow	greenish yellow

Table 3.14: Extract colour of shoot homogenates of *Citrus micrantha* var. *microcarpa* in various inhibitors after centrifugation

Extraction solutions	Extract colour after centrifugation		
	0 hour	24 hours	1 week
1) 0.05 M phosphate buffer pH 7.2 (control)	greenish yellow	greenish yellow	greenish yellow
2) 10 mM potassium metabisulfite ($K_2S_2O_5$)	greenish yellow	greenish yellow	greenish yellow
3) 20 mM DIECA	greenish yellow	greenish yellow	greenish yellow
4) 2% insoluble PVP	greyish yellow	greyish yellow	greyish yellow
5) 5 mM $K_2S_2O_5$	greenish yellow	greenish yellow	greenish yellow
6) 20 mM L-cystein HCl	light yellow	light yellow	light yellow

Table 3.15 : Extract colour of shoot homogenates of citrumello (*Citrus paradisi* X *Poncirus trifoliata*) in various inhibitors after centrifugation

Extraction solutions	Extract colour after centrifugation		
	0 hour	24 hours	1 week
1) 0.05 M phosphate buffer pH 7.2 (control)	light yellow	light yellow	light yellow
2) 10 mM potassium metabisulfite ($K_2S_2O_5$)	light yellow	light yellow	light yellow
3) 20 mM DIECA	light greenish yellow	light greenish yellow	light greenish yellow
4) 2% insoluble PVP	light greenish yellow	light greenish yellow	light greenish yellow
5) 5 mM $K_2S_2O_5$	light yellow	light yellow	light yellow
6) 20 mM L-cystein HCl	light yellow	light yellow	light yellow

3.5 Morphogenetic responses of explants of *C. hystrix*

3.5.1 Leaf explants

Callus was produced in a medium which contained 1 mg/l 2,4-D alone. Similar results were observed when 0.5 mg/l of 2,4-D combined with 1 mg/l NAA or with an addition of 0.5 mg/l of kinetin (Table 3.16). Callus was initiated in all these media after 3 weeks in culture. The friable callus produced was brownish yellow in colour. Upon subculture, callus displayed slow growth rate and remained non-morphogenic. Roots formation were obtained in media which supplemented with 1 mg/l NAA singly and combination of NAA and kinetin at 1 mg/l each. Shoots regeneration were observed in MS based media incorporated with combinations of 5 mg/l 6-BAP and 1 mg/l NAA, 2 mg/l IAA and 4 mg/l 6-BAP and combination of IAA, 6-BAP and GA_3 at 2, 4 and 1 mg/l respectively. Shoots were produced from the cut edge of midrib via direct organogenesis. Shoots regeneration started only after 2 weeks in culture, in a medium which contained 4 mg/l 6-BAP and 2 mg/l IAA. 1-2 shoots were obtained per explant. Leaf explants did not respond to MS based medium supplemented with 5 mg/l 6-BAP alone.

3.5.2 Stem explants

Roots formation were observed from the cut end of explant in MS based medium supplemented with 1 mg/l NAA within 3 to 4 weeks in culture (Table 3.16). Multiple shoots initiation through direct organogenesis were observed after 3 weeks in culture, in media supplemented with 6-BAP singly or in combination with NAA, IAA and GA_3. From one explant about 6-7 shoots were typically produced. However no stem elongation was observed upon subculture. Even in a medium supplemented with GA_3 (1 mg/l) shoots regenerated failed to elongate. No callus formation was observed from stem explants. The explants did not respond to MS based media which contained 2,4-D singly or in combination with NAA or combination of NAA and kinetin at all concentrations.

3.5.3 Cotyledon explants

Cotyledon explants developed callus in a medium which contained 1 mg/l 2,4-D (Table 3.16). The callus produced was brownish yellow in colour. Callus growth was slow even after subculturing and remain non-morphogenic. Roots formation were observed in media supplemented with 1 mg/l NAA and combination of NAA and 2,4-D (0.5 and 1 mg/l respectively). Roots initiation was faster (1 week) in the former media compared to the latter. Shoots regeneration were shown in a medium with 2 mg/l IAA and 4 mg/l 6-BAP or with 1 mg/l GA_3 added. Shoots were obtained only after 8 weeks in culture in both media. Stem elongation occured after one passage of subculture. Regeneration pathway was by direct organogenesis (Plate 9c).

Table 3.16: Responses of different explants of *C. hystrix* to MS based media with various plant growth regulator combinations. Observations were made after 12 weeks in culture.

Key:

N	(NAA)
K	(Kinetin)
B	(6-BAP)
A	(IAA)
D	(2,4-D)
G	(GA_3)
0	No response
1	Callus initiations after 1 week
2	Callus initiations after 2 weeks
R	Roots growing directly from explants
S	Shoots growing directly from explants
	1 shoot per explant
	2-3 shoots per explant
	> 4 shoots per explant

Explants	MS salt + plant growth regulator combinations (mg/l)								
	N 1	N + K 1, 1	D 1	B + N 5, 1	B 5	I + B 2, 4	I + B + G 2, 4.1	D + N 0.5, 1	N + D + K 1, 0.5, 0.5
Leaf	R	R	2	S	0	S	S	2	2
Stem	R	0	0	S	S	S	S	0	0
Cotyledon	R	-	1	-	-	S	S	R	0

3.6 Morphogenetic responses of explants of *C. madurensis*

3.6.1 Leaf explants

The leaf explants were responsive to certain media forming either callus (Plate 9b), roots or shoots (Table 3.17). The responses were observed after 1-2 weeks but recorded only after 12 weeks upon organogenesis if there was any. Callus was formed in media supplemented with 2,4-D alone and when combined with another auxin NAA. Comparatively the formation of callus was the fastest (within 1 week) in a medium with 1 mg/l 2,4-D whereas the time taken in a medium supplemented with 0.5 mg/l 2,4-D and 1 mg/l NAA was the slowest (4 weeks). Rapid callus growth was observed in the former medium but growth declined after the second subculture. The callus was creamy in colour, in a medium with 2,4-D alone but yellowish green in the other medium. However, all the calli were non-morphogenic. Shoots regenerations were observed in media supplemented with 5 mg/l 6-BAP singly or combination of 2 mg/l IAA with 4 mg/l 6-BAP. Multiple shoots initiation (3 per explant) were shown in the latter medium. The shoots were regenerated directly from the cut edge of midrib and were only observed after 1 month in culture. The regeneration pathway was by direct organogenesis without callus intermediary stage (Plate 9d).

3.6.2 Stem explants

The stem explants were responsive in terms of callus production (Table 3.17) but no plant regeneration was observed. Callus was obtained in all the media tried except in media which were added with combination of 2 mg/l IAA and 4 mg/l 6-BAP or with the addition of 1 mg/l GA_3. Rapid callus initiation was displayed in media which were supplemented either with NAA (1 mg/l) alone or in combination with 1 mg/l kinetin and also those with 2,4-D singly (1 mg/l) and combination of 0.5 mg/l 2,4-D with 1 mg/l NAA. Callus formation was first observed after 1 week in culture. The callus produced was greenish yellow in colour, however in the media which were supplemented with 2,4-D the colour was creamy. All the callli were non-morphogenic and friable in texture. The callus initiated in a medium which contained 1 mg/l 2,4-D failed to grow and became necrotic after 2 weeks in culture.

3.6.3 Cotyledon explants

Results are tabulated in 3.17. Cotyledon explants produced only roots and callus. Callus was obtained only in MS based medium supplemented with 1 mg/l NAA. The callus was greenish in colour, soft in texture and non-morphogenic. Callus initiation was observed after 1 week in culture. However, callus growth was slow. Simultaneously roots were also produced directly from explants cultured on the same medium. Roots production were also observed in a medium supplemented with combination of NAA, 2,4-D and kinetin (1, 0.5 and 0.5 mg/l respectively). No shoot regeneration was observed from cotyledon explants in all the media tried.

Table 3.17: Responses of different explants of *C. madurensis* to MS based media with various plant growth regulator combinations. Observations were made after 12 weeks in culture.

Key:

N	(NAA)
K	(Kinetin)
B	(6-BAP)
A	(IAA)
D	(2,4-D)
G	(GA_3)
0	No response
1	Callus initiations after 1 week
2	Callus initiations after 3 weeks
R	Roots growing directly from explants
S	Shoots growing directly from explants
(dotted box)	1 shoot per explant
(hatched box)	2-3 shoots per explant

Explants	MS salt + plant growth regulator combinations (mg/l)								
	N 1	N + K 1, 1	D 1	B + N 5, 1	B 5	I + B 2, 4	I + B + G 2, 4.1	D + N 0.5, 1	N + D + K 1, 0.5, 0.5
Leaf	R	-	1	-	S	S	0	2	0
Stem	1	1	1	1	1	0	0	1	2
Cotyledon	1 + R	-	0	-	0	-	-	-	R

3.7 Morphogenetic responses of explants of *C. micrantha* var. *microcarpa*

3.7.1 Leaf explants

Caulogenesis and rhizogenesis were observed from leaf explants (Table 3.18). Observations were recorded after 12 weeks in culture whereby all explants had given full response to the media tried. Explants were responsive when cultured in media supplemented with 2,4-D singly or in combination with NAA, producing callus. The calli were yellowish green to greenish in colour and friable in texture. However the callus failed to grow and became necrotic. Media incorporated with NAA (1 mg/l) or when combined with kinetin (1 mg/l) seemed to be the best root inducing media. Roots formation were shown after 1 week in culture. Callusing was observed after 1 week in culture followed by rhizogenesis after 4 weeks, in media which contained NAA, 2,4-D and kinetin. The leaf explants did not however regenerate.

3.7.2 Stem explants

Results are summarised in Table 3.18. Callus was produced in most of the media tried except in MS based media added with 6-BAP (5 mg/l) alone, or in combination with IAA (2 mg/l) or IAA and GA_3 (2 and 1 mg/l respectively). Upon subculture, callus and explants did not show any organogenesis. Calli were soft and the colours range from yellowish green to greenish. Initiation of callus was observed within 1 week whereas in media supplemented with NAA, 6-BAP and kinetin, the response took about 4 weeks. Subsequently the growth of callus was very slow.

3.7.3 Cotyledon explants

Shoots regeneration, rhizogenesis and callus production were observed from cotyledon explants (Table 3.18). Explants responded to all the media used in this experiment except in MS based medium incorporated with 5 mg/l of 6-BAP. Roots were developed only in a medium which contained 1 mg/l NAA. Callus was observed in media supplemented with 2,4-D alone or in combination with NAA and kinetin. Similar observations were recorded in a medium incorporated with 1 mg/l NAA and 1 mg/l kinetin. The initiation of callus was initially observed after 1 week in culture in most of the media tried. Callus texture was soft and displayed rapid growth rate. Shoots were regenerated in culture media incorporated with 6-BAP in combination with NAA or IAA or with the addition of 1 mg/l GA_3. Shoots were present after 2 months in culture. 1 shoot was produced per explant. However in a medium supplemented with combination of 5 mg/l 6-BAP and 1 mg/l NAA 2 shoots per explant were observed. Shoots were regenerated by direct organogenesis (Plate 9e).

Table 3.18: Responses of different explants of *C. micrantha* var. *microcarpa* to MS based media with various plant growth regulator combinations. Observations were made after 12 weeks in culture.

Key:

N	(NAA)
K	(Kinetin)
B	(6-BAP)
A	(IAA)
D	(2,4-D)
G	(GA_3)
0	No response
1	Callus initiations after 1 week
2	Callus initiations after 3 weeks
R	Roots growing directly from explants
S	Shoots growing directly from explants
	1 shoot per explant
	2-3 shoots per explant

Explants	MS salt + plant growth regulator combinations (mg/l)								
	N 1	N + K 1, 1	D 1	B + N 5, 1	B 5	I + B 2, 4	I + B + G 2, 4.1	D + N 0.5, 1	N + D + K 1, 0.5, 0.5
Leaf	R	R	1	0	0	0	0	1	R
Stem	1	2	1	2	0	0	0	1	1
Cotyledon	R	1	1	S	0	S	S	1	2

3.8 Morphogenetic responses of explants of citrumello (*Citrus paradisi* X *Poncirus trifoliata*)

3.8.1 Leaf explants

The leaf explants were responsive in terms of shoots production (Table 3.19). Shoots were produced from explants cultured in MS based medium incorporated with 2 mg/l IAA, 4 mg/l 6-BAP and 1 mg/l GA_3. Shoot primordia was formed. One shoot was produced per explant. Shoots were produced from the cut edge of the midrib without prior to callus phase. Explants did not respond to MS based media which supplemented with combinations of 1 mg/l NAA and 1 mg/l kinetin, 5 mg/l 6-BAP and 1 mg/l NAA, and 5 mg/l 6-BAP alone.

3.8.2 Stem explants.

Results are summarised in Table 3.19. Shoots initiation were observed from explants in MS based medium which contained 5 mg/l 6-BAP and combination of IAA, 6-BAP and GA_3 (2, 4 and 1 mg/l respectively). Shoots were produced after one month in culture. On the average, 5-6 shoots were produced per explant. However the shoots failed to elongate. The explants did not respond to the media supplemented with NAA singly or in combination with kinetin and 6-BAP as employed in this study.

3.8.3 Cotyledon explants

Direct shoots regeneration (Plate 9a) and rhizogenesis were observed from cotyledon explants (Table 3.19). Shoots were produced from explants cultured in media supplemented with 6-BAP alone or in combination with IAA and NAA and in addition with GA_3 at all concentrations. Shoots initiation were observed after 4 weeks in culture. 1 shoot was produced per explant except in a medium which contained 2 mg/l of IAA and 4 mg/l of 6-BAP. 2 shoots per explant were obtained. Roots formation were shown in media supplemented with 1 mg/l NAA singly or in combination with 0.5 mg/l 2,4-D and 0.5 mg/l kinetin.

Table 3.19: Responses of different explants of citrumello (*C. paradisi* X *P. trifoliata*) to MS based media with various plant growth regulator combinations. Observations were made after 12 weeks in culture.

Key:

N	(NAA)
K	(Kinetin)
B	(6-BAP)
A	(IAA)
D	(2,4-D)
G	(GA_3)
0	No response
R	Roots growing directly from explants
S	Shoots growing directly from explants
	1 shoot per explant
	> 4 shoots per explant

Explants	MS salt + plant growth regulator combinations (mg/l)								
	N 1	N + K 1, 1	D 1	B + N 5, 1	B 5	I + B 2, 4	I + B + G 2, 4.1	D + N 0.5, 1	N + D + K 1, 0.5, 0.5
Leaf	-	0	-	0	0	-	S	-	-
Stem	-	0	-	0	S	-	S	-	-
Cotyledon	R	0	0	S	S	S	S	-	R

Plate 9:

a Plant regeneration from cotyledon explant of citrumello

b Callus from leaf explant of *C. madurensis*

c Regenerants from cotyledon explant of *C. hystrix*

d Regenerants from leaf explant of *C. madurensis*

e Regenerants from cotyledon explant of *C. micrantha* var. *microcarpa*

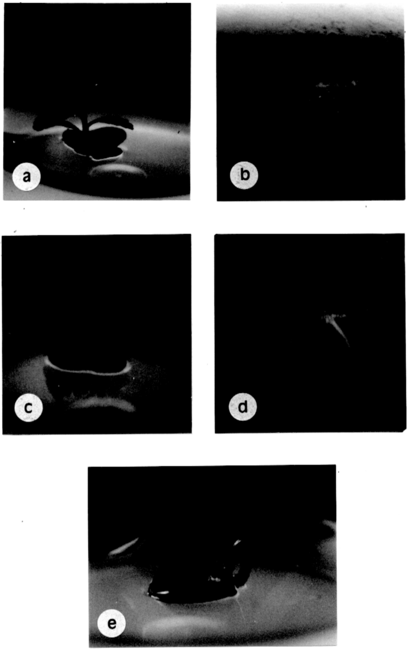

a
b
c
d
e

Table 3.20: Percentage of regeneration from different explants of *C. hystrix*, *C. madurensis*, *C. micrantha* var. *microcarpa* and citrumello

Species	Plant growth regulator (mg/l)	Percentage of shoot formation — Explant: leaf	stem	cotyledon
C. *hystrix*	6-BAP (5)	0	6.7 ± 5.7	-
	6-BAP (5) NAA (1)	26.7 ± 5.8	13.3 ± 5.7	-
	6-BAP (4) IAA (2)	16.7 ± 5.8	23.3 ± 5.7	36.7 ± 5.7
	6-BAP (4) IAA (2) GA_3 (1)	33.3 ± 5.8	13.3 ± 5.7	33.3 ± 5.8
C. *madurensis*	6-BAP (5)	10 ± 10	0	-
	6-BAP (5) NAA (1)	-	0	0
	6-BAP (4) IAA (2)	6.7 ± 5.7	0	-
	6-BAP (4) IAA (2) GA_3 (1)	0	0	-

Table 3.20: continued

Species	Plant growth regulator (mg/l)	Percentage of shoot formation		
		Explant		
		leaf	stem	cotyledon
C. micrantha var. *microcarpa*	6-BAP (5)	0	0	0
	6-BAP (5) NAA (1)	0	0	13.3 ± 11.5
	6-BAP (4) IAA (2)	0	0	10 ± 10
	6-BAP (4) IAA (2) GA_3 (1)	0	0	3.3 ± 5.7
Citrumello	6-BAP (5)	0	47 ± 5.7	23.3 ± 5.7
	6-BAP (5) NAA (1)	0	0	13.3 ± 5.7
	6-BAP (4) IAA (2)	-	-	16.7 ± 5.8
	6-BAP (4) IAA (2) GA_3 (1)	37 ± 5.7	53 ± 11.5	16.7 ± 5.8