Gas Chromatography-Mass Spectroscopy Analysis of Aromatic Compounds of Leaves and Peel from Healthy and Viroid-infected Citron Plants

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ABSTRACT. The aroma profile and the influence of viroid infection on the oil composition of leaves and peel of citron plants were studied. Oil components were separated, identified and quantified by Gas Chromatography-Mass Spectroscopy. Limonene was one of the main components in the essential oil of leaves and peel. Viroid infection reduced oil content of leaves and caused pronounced alteration in the oil composition of the peel when compared with leaves. Significant changes were observed in the concentration of volatile terpene constituents. Aldehydes and esters were found in significantly higher concentration in the oil of healthy fruit peel compared with peel from viroid plants.

Index words. Citron leaves, citrus peel, Essential oil, Viroid infection, GC-MS analysis.

Citron was the first citrus cultivated in the Mediterranean region and the island of Crete, and it is considered a basic species and a parent plant in citrus evolution (13). Improved genetic material has been created by mutation and introduction of foreign isolates into the island.

Viroids are the smallest agents of infectious diseases. Citrus may be infected by several citrus viroids which include the exocortis and cachexia agents (5, 21). Viroid diseases usually originate by accidental introduction of viroids from wild plants into susceptible cultivated plants (1). These diseases appear in nearly all areas where citrus is grown, and are transmitted by propagation of infected budwood and by mechanical means, such as pruning with contaminated tools (10, 16, 19). According to Roistacher (16), there are several varieties of citron sensitive to *Citrus exocortis viroid* (CEVd) and other citrus viroids. The reaction of different citron to citrus viroids was initially reported by Frolich et al. (6), who studied the symptoms of the disease by inoculating various cultivars and clones with different viroid sources. Symptoms of viroid infection include chlorosis, epinasty of the newly formed and developing leaves, curling of the leaves and cracking of the lower side of midvein (2).

Etrog citron is one of the oldest cultivated citrus and is a host of all the citrus viroids (CVds). It was initially used as an indicator plant for CEVd, but today it is used as a general indicator for citrus viroids. Lota et al., (12) used the Gas Chromatography-Mass Spectroscopy (GC-MS) technique to analyze the essential oil composition of peel and leaves of different citron varieties cultivated in Corsica. Limonene and citral were the main substances identified in both peel and leaves.

The aim of this work was to study the effects of viroid infection on the profile and composition of the essential oil components extracted from citron leaves and peel and analyzed by the GC-MS technique.

MATERIALS AND METHODS

Sampling. Leaves and fruits were collected from 16-yr-old citron plants (healthy and viroid infected) in the Chania region of Crete island. Tissue was collected around the top of the tree at a height of 1.7 m. Seedlings trees of cultivar Diamante,

certified genetically by isoenzymatic analysis, were used to obtain material free of viruses and viroids (14, 15). Four isoenzymatic systems, malate deshydrogenase, tetrazolium oxidase, glutamate oxaloacetate transaminase and esterases, in correlation with biometric characteristics, were applied for the taxonomy of the citron variety. All the trees (seedlings) possessed a unique banding for the four isoenzymatic systems. The viroid-infected trees were produced by inoculating some trees with tissue from an experimental tree of Etrog citron, carrying a virulent strain of CEVd. The presence of CEVd was confirmed by the severe reaction obtained in citron from 469 field sources and the infection of *Gynura*, a selective host of CEVd (20). The symptoms of CEVd on citron trees were yellow blotch and slight cracking in stems. In general the trees of Diamante presented bark mottling without appreciable dwarfing. The trees had already been infected for 12 yr before the assays.

Care was taken to collect leaves of approximately 5-7 mo old, representative of the variety. Samples of 10 leaves from each of 10 healthy and 10 diseased trees were collected at different time intervals over a period of greater than 1 yr. The fruits were collected 3 mo after the anthesis from the four compass points of the trees to minimize position variables.

Essential oil extraction and analysis. The hydrodistillation method with the Clevenger apparatus was used to extract the essential oil from leaves and peel. Samples of 150 g were washed, cut and extracted for 3 h (9). Oil samples were collected and stored in vials at -18°C until used.

For gas chromatography analysis (17, 18) a 30 m × 0.32 mm i.d., SE-52 capillary column and 0.25 µm film thickness was used. The column was installed in a Perkin Elmer Autosystem GC equipped with a glass injection splitter with a split ratio 1/20 and flame ionization detector (FID). The oven temperature was programmed from 50°C (5 min isothermal), to 230°C at 4°C/min. The temperature of the injector and detector were 200 and 240°C, respectively. Helium (0.6 ml/min) was used as a carrier gas. Injection volume was 0.2 µl. Chromatographic analysis was run in six replicates. Standards previously identified as constituents of citron leaves were added to the sample for peak assignment based on peak enrichment. The results were calculated on the basis of an internal standard method (octyl acetate as internal standard).

A Hewlett-Packard P5890 GC with a split/splitless injection port and split ratio 1/100, coupled with a HP5970 Mass Spectrometer system (electron impact mode) was used. A 25 m × 0.2 mm i.d., HP-1 fused silica capillary column, 0.2 µm film thickness was used. The temperature program was isothermal for 3 min at 60°C and then raised to 230°C at 5°C/min. The injector temperature was 200°C and the transfer line temperature was 250°C. The column outlet was inserted directly into the ion source block. Ultrapure helium was used as a carrier gas with flow rate 1 ml/min. Injection volume was 0.2 µl. Identification of components was confirmed by comparison of the experimental retention index and Mass Spectrum with that of authentic reference standards.

The data were subjected to the analysis of variance (ANOVA) and the test of Duncan was performed in order to identify significant differences at $P < 005$ between healthy and diseased citrons.

RESULTS AND DISCUSSION

Yield of the essential oil. The yield of the essential oil was expressed as ml/kg of the fresh material (Table 1). Healthy leaves yielded higher quantity of essential

TABLE 1
AVERAGE YIELD OF ESSENTIAL OIL, FROM LEAVES AND PEEL OF HEALTHY CITRON PLANTS AND THOSE INFECTED BY EXOCORTIS VIROID

	Oil Content	
	Viroid-infected	Healthy
Leaves	3.8 ± 0.2[z]	8.4 ± 0.7 a[y]
Peels	5.8 ± 0.3	5.9 ± 0.4 a

[z]Values are mean of six samplings expressed as ml/kg fresh weight.
[y]Values horizontally followed by different letters are statistically significant at $P < 0.05$.

oil compared with diseased ones, while no significant differences were observed in the quantity of the oil extracted from fruit peel. It seems that these differences are the result of cytological effect on the mesophyll tissues as a result of viroid infection.

Essential oil composition. In this study the main monoterpene hydrocarbon was limonene. Other hydrocarbons which were identified were the monoterpenes α, β-pinene, myrcene, δ-3-carene, α-terpinene, and terpinolene. Among the sesquiterpenes caryophyllene, farnasene, humulene were the most important. Esters on a quantitative basis, account for only a small fraction of the citrus oil, while ethyl acetate, linalyl acetate, geranyl acetate are the components most frequently appeared (Tables 2 and 3). Large amounts of alcoholic components were also found among the volatile flavor substances of citrus. However, their contribution to the overall flavor and aroma is probably less than that of the carbonyl compounds and terpenes.

The mean value and other data for the components of the essential oil from leaves from six samplings and six replicates which come from healthy and infected plants are presented in Table 2. Limonene, geranial, geraniol, neral, nerol and citronellol were the major components of both healthy and infected leave oils respectively (4). The amount of limonene was statistically different between healthy and infected leaves. Significant differences were also observed in the minor components of the essential oil from leaves such as: the monoterpene hydrocarbon δ-3-carene, the aldehyde decanal, the ester geranyl propionate, and the sesquiterpene hydrocarbons, β-caryophyllene, bergamotene and humulene.

CEVd accumulates in mesophyll tissues and causes cytological resolution (3). Although no biological function of monoterpenes is yet known, their penetrating odor probably acts positively in attracting or repelling insects (7).

Peel oil from citron fruits infected with CEVd had relatively high concentrations of terpenes while aldehydes and esters were found in lower quantities (Table 3). Significant differences in the α and β-pinene, (Z) β- and (E) β-ocimene, citral, citronellal, neryl and geranyl acetate and neryl propionate components of the essential oil of peel among healthy and viroid infected citron plants were found. Citral is considered as a key aroma compound of citrus flavor and its content is indicative of the quality of oils. It is of great scientific and technological significance in aromatic and medicinal chemistry (8).

The essential oil data (Table 3) from the viroid-infected plants also showed significant reductions in the content of alcohols: nerol + citronellol and geraniol. The only exception was noticed in the amount of α-terpineol which was significantly higher in the infected peel.

TABLE 2
ESSENTIAL OIL COMPONENTS OF HEALTHY AND VIROID INFECTED CITRON LEAVES

Components	Viroid infected leaves	Healthy leaves
Monoterpene hydrocarbons		
α-pinene	2.1[z] ± 0.21	1.3 ± 0.32 a[y]
β-pinene	2.7 ± 0.79	2.8 ± 0.96 a
myrcene	17.8 ± 2.04	15.7 ± 5.68 a
δ,3-carene	5.0 ± 1.76	1.7 ± 0.59 b
limonene	288.1 ± 44.07	234.2 ± 46.41 b
(Z) β-ocimene	3.6 ± 0.82	2.7 ± 1.02 a
(E) β-ocimene	6.4 ± 1.96	4.0 ± 0.59 a
γ-terpinene	1.1 ± 0.01	1.9 ± 0.87 a
Sesquiterpene Hydrocarbons		
β-caryophyllene	2.3 ± 0.79	4.1 ± 0.91 b
bergamotene	0.08 ± 0.01	1.8 ± 0.51 b
humulene	1.4 ± 0.52	2.4 ± 0.85
farnasene	2.1 ± 0.11	3.7 ± 0.49 a
Esters		
citronellyl acetate	1.3 ± 0.69	1.7 ± 0.63 a
neryl acetate	8.7 ± 1.75	11.2 ± 2.84 a
geranyl acetate	27.2 ± 3.78	32.1 ± 4.44
neryl propionate	1.3 ± 0.39	2.0 ± 0.6 9a
geranyl propionate	0.5 ± 0.21	4.4 ± 0.48 b
Alcohols		
linalool	9.7 ± 1.41	11.2 ± 1.38 a
α-terpineol	3.1 ± 0.89	3.6 ± 0.38 a
nerol+citronellol	12.4 ± 2.60	10.1 ± 1.5 a
geraniol	15.4 ± 3.20	12.5 ± 2.5 a
C12-ol	2.0 ± 0.80	4.2 ± 1.1 b
Aldehydes		
C9-al	2.2 ± 0.77	4.2 ± 1.51 a
citronellal	13.2 ± 1.06	16.1 ± 1.88 a
iso-citral cis	5.0 ± 1.88	4.8 ± 1.07 a
iso-citral trans	6.7 ± 1.39	6.0 ± 1.03 a
C10-al	10.8 ± 1.13	1.1 ± 0.57 b
neral	165.4 ± 15.76	163.5 ± 20.73 a
geranial	178.3 ± 18.23	171.4 ± 22.86 a
C11-al	2.3 ± 0.44	3.6 ± 0.04 a
C12-al	2.4 ± 1.13	2.1 ± 1.01 a
Oxygenated sesquiterpenes		
caryophyllenoxide	1.9 ± 0.18	4.1 ± 0.72 a

[z]Values expressed as mg/kg fresh weight are mean of six samplings ± standard deviation.
[y]Values horizontally followed by different letters differ significantly at $P < 0.05$.

Generally the content of the essential oil extracted by viroid infected leaves is higher in citral and total aldehydes while that from peels is rich in hydrocarbons (terpene and sesquiterpene).

There is a probability that the detected differences are due to the fact that the viroid affects the flavedo of the oleiferous glands, but the mechanism of oil biosynthesis in the diseased tissues is still unknown (11).

TABLE 3
ESSENTIAL OIL COMPONENTS, IN CITRON PEEL FROM HEALTHY AND VIROID INFECTED TREES

Components	Viroid infected peels	Healthy peels
Monoterpene hydrocarbons		
α-pinene	15.1[z] ± 1,30	2.2 ± 0.12 b[y]
β-pinene	132.3 ± 4,20	12.5 ± 1.30 b
Myrcene	13.6 ± 1,80	12.6 ± 1.50 a
δ,3-carene	2.1 ± 0,80	0.7 ± 0.04 b
limonene	255.3 ± 10.60	249.3 ± 18.30 a
(Z) β-ocimene	8.1 ± 1.20	3.5 ± 0.86 b
(E) β-ocimene	13.4 ± 2.10	6.8 ± 1.12 b
γ-terpinene	3.2 ± 0.60	3.3 ± 0.95 a
Sesquiterpene hydrocarbons		
β-caryophyllene	4.8 ± 1.10	4.1 ± 1.10
bergamotene	4.2 ± 1.00	3.3 ± 1.20 a
humulene	7.0 ± 1.50	5.3 ± 1.86 a
farnasene	2.9 ± 0.55	1.4 ± 0.55 a
terpinolene	1.2 ± 0.08	0.4 ± 0.03 a
Esters		
citronellyl acetate	1.1 ± 0.20	0.6 ± 0.08 a
neryl acetate	2.1 ± 0.85	10.5 ± 1.30 b
geranyl acetate	1.7 ± 0.50	6.3 ± 1.16 b
neryl propionate	2.2 ± 0.90	0.1 ± 0.02 b
geranyl propionate	0.9 ± 0.70	1.1 ± 0.43 a
Alcohols		
linalool	4.4 ± 0.90	5.3 ± 1.10 a
terpinen-4-ol	4.2 ± 1.20	2.6 ± 0.93 a
α-terpineol	7.1 ± 2.30	2.3 ± 0.78 b
nerol+citronellol	3.5 ± 1.10	20.5 ± 4.15 b
geraniol	6.8 ± 1.60	22.3 ± 3.80 b
Aldehydes		
C9-al	1.7 ± 0.50	1.6 ± 0.50 a
citronellal	0.9 ± 0.20	2.5 ± 0.92 b
iso-citral cis	0.5 ± 0.10	1.7 ± 0.23 b
iso-citral trans	0.6 ± 0.15	0.6 ± 0.06 a
C10-al	2.8 ± 0.70	1.3 ± 0.32 a
neral	14.5 ± 3.40	80.8 ± 11.80 b
geranial	31.2 ± 5.40	133.7 ± 15.20 b
C11-al	0.9 ± 0.09	1.5 ± 0.21 a
C12-al	1.2 ± 0.30	0.7 ± 0.05 a

[z]Values expressed as mg/kg fresh weight are mean of six samplings ± standard deviation.
[y]Values horizontally followed by different letters differ significantly at $P < 0.05$.

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