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ORIGINAL ARTICLE

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# Monitoring the emission of volatile organic compounds from the leaves of *Calocedrus macrolepis* var. *formosana* using solid-phase micro-extraction

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**Abstract** In this study, solid-phase micro-extraction (SPME) fibers coated with polydimethylsiloxane/divinylbenzene (PDMS/DVB), coupled with gas chromatography/mass spectrometry, were used to monitor the emission patterns of biogenic volatile organic compounds (BVOCs) from leaves of *Calocedrus macrolepis* var. *formosana* Florin. in situ. In both sunny and rainy weather, the circadian profile for BVOCs from *C. macrolepis* var. *formosana* leaves has three maximum emission cycles each day. This kind of emission pattern might result from the plant's circadian clock, which determines the rhythm of terpenoid emission. Furthermore, emission results from the leaves demonstrated that the circadian profile of $\alpha$-pinene observed was opposite to the profiles of limonene and myrcene, a difference that may be attributable to two different subpathways for terpenoid biosynthesis.

**Key words** Biogenic volatile organic compounds (BVOCs) · *Calocedrus macrolepis* var. *formosana* · Chemotype · Gas chromatography-mass spectrometry · Solid-phase micro-extraction (SPME)

## Introduction

Plants produce a great many organic compounds, such as carbohydrates, amino acids, fatty acids, cytochromes, and chlorophylls, through primary metabolism. At the same time, they also produce numerous organic compounds through secondary metabolism in the process of growth and development. The terpenes derived from isoprenoids constitute the largest class of secondary products, and they are also the most important precursors for phytoncides in forest materials. Phytoncides are volatile organic compounds released by plants, and they resist and break up hazardous substances in the air. Scientists have confirmed that phytoncides can reduce dust and bacteria in the air, and exposure to essential oils from trees has also been reported to lessen anxiety and depression, resulting in improved blood circulation and blood pressure reduction in humans and animals.[1] However, the chemical compositions of phytoncides emitted from various trees are very different and not yet clearly identified. Therefore, further investigations into the compositions are worth pursuing.

*Calocedrus macrolepis* var. *formosana*, one of the five most valuable conifers in Taiwan, is an endemic tree that grows at elevations of 300–1900 m in Taiwan's central mountains.[2] Many previous studies have demonstrated that the leaf essential oil of *C. macrolepis* var. *formosana* has excellent inhibitory effects against termites, mildew, and fungi, as well as functioning as an antioxidant, antiinflammatory agent, and anti-mosquito larvicide.[3–5] However, information about the phytoncides released by *C. macrolepis* var. *formosana* is limited, and the composition and the dynamic variations of concentration have not been completely understood. It is, therefore, imperative to find an effective method to monitor the phytoncides emitted from this plant, allowing its diurnal variations to be understood. A fast, simple, and effective preconcentration and extraction technique, such as solid-phase micro-extraction (SPME), has been used as an alternative tool in biochemical research and applied to the analyses of volatile compounds.[6,7]

The objectives of this study are to characterize and quantify the biogenic volatile organic compounds (BVOCs) from leaves of *C. macrolepis* var. *formosana* using SPME and to monitor the dynamic variations of volatile compounds emitted by living leaves of *C. macrolepis* var. *formosana* using a sampling chamber specially designed for this task.

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## Materials and methods

### Plant materials

The seedlings of 2-year-old *C. macrolepis* var. *formosana* (Lien Hua-Chin No. 18) were obtained from the Lien Hua-Chin Research Center located in Nantou County in central Taiwan. The species was confirmed by Dr. Yen-Ray Hsu of the Taiwan Forestry Research Institute, and voucher specimens (CM0001) were deposited at the Laboratory of Wood Chemistry (School of Forestry and Resource Conservation, National Taiwan University). BVOCs from juvenile leaves of *C. macrolepis* var. *formosana* were sampled on March 26 and 30, 2006.

### Essential oil distillation

Fresh leaves (200 g) of *C. macrolepis* var. *formosana*, in triplicate, were subjected to hydrodistillation using a modified Clevenger-type apparatus for 12 h.[8] Essential oils were stored in airtight containers before analysis.

### Circadian profiles for BVOCs emitted by *C. macrolepis* var. *formosana*

To obtain and analyze the BVOCs emitted by live *C. macrolepis* var. *formosana*, a glass chamber (120 mm wide, $\psi$ = 60 mm) was designed and assembled as described by Zini et al.,[9] with slight modifications (Fig. 1). Before sampling, the SPME fiber was conditioned for 10 min at 250°C. The leaves of *C. macrolepis* var. *formosana* were put into the glass sampling chamber, and then a SPME coupled with polydimethylsiloxane/divinylbenzene (PDMS/DVB) fiber was introduced into the chamber and exposed to the headspace of the chamber for 15 min, where a thermometer monitored the temperature variation. The conditions and fiber selected in this study were based on a previous experiment in which a complete optimization of the extraction conditions was carried out.[10] This procedure was performed every 1 h for continuous periods over 24 h on a sunny day (average temperature, 21.8°C; relative humidity, 61%) and a rainy day (average temperature, 17.8°C; relative humidity, 91%).

### Gas chromatography-flame ionization detector (GC-FID) and GC-mass spectroscopy (MS) analyses

GC analysis was carried out using a Finnigan Trace GC with a flame ionization detector (FID) equipped with a 30 m × 0.25 mm WCOT column coated with 0.25 μm 5% diphenyl dimethyl silicone supplied by J&W (DB-5MS). Diluted essential oil samples (1.0 μl, 1/100, v/v, in ethyl acetate) were injected automatically in the split mode, and a 10:1 split ratio was used for all samples. The column temperature program was from 60° to 80°C at 3°C/min, and then heated at 5°C/min up to 120°C, subsequently heated at 1°C/min up to 14°C, and finally heated at 30°C/min up to 200°C. The carrier gas used was helium at a flow rate of 1.0 ml/min. The oils were analyzed using a Finnigan Trace GC–Polaris Q mass instrument (Finnigan-Spectronex) fitted with the same column and temperature program as above. MS parameters were ionization voltage (EI), 70 eV; peak width, 20 s; mass range, 50–650 amu; and ion source temperature, 230°C. The Kovats retention indices[11] were calculated for all volatile constituents using a homologous series of *n*-alkanes, $C_9$–$C_{22}$, on the DB-5MS column. Quantification was performed using percentage peak area calculations using the GC-FID, and the identification of individual components was done using the Wiley/NBS Registry of Mass Spectral Database and NIST MS Search, published literature, and several authentic reference compounds. The quantity of compounds was obtained by integrating the peak area of the spectrograms.

## Results and discussion

### Chemical constituents of essential oils

Table 1 shows the constituents identified, the percentage composition, and their Kovats index (KI) values listed in order of elution from the DB-5MS capillary column. A total of 69 compounds in the *C. macrolepis* var. *formosana* leaf

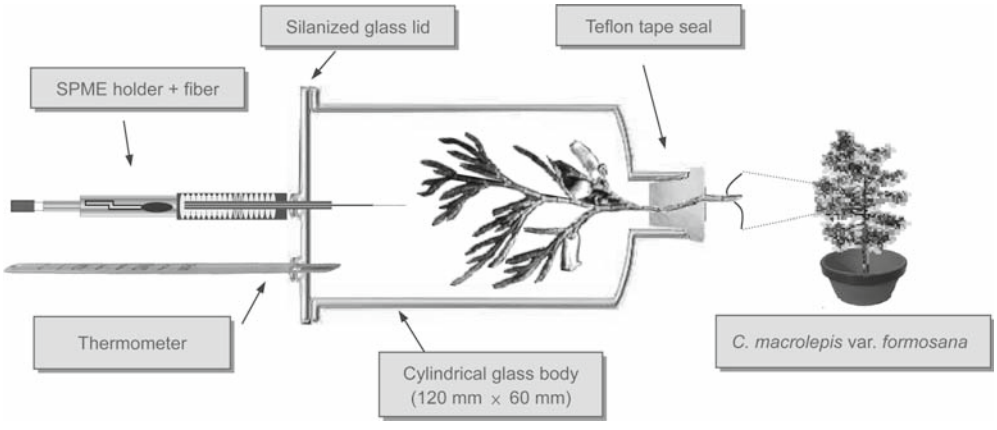


**Fig. 1.** Glass chamber for solid-phase micro-extraction (*SPME*) sampling of volatiles emitted from *C. macrolepis* var. *formosana* live leaves

**Table 1.** Constituents of essential oils from the leaves of *Calocedrus macrolepis* var. *formosana* using hydrodistillation

| KI[a] | Composition[b] | Relative concentration (%) | | Identification[c] |
|---|---|---|---|---|
| | | Mean | SD | |
| **Monoterpene hydrocarbons (%)** | | **35.85** | | |
| 938 | α-Pinene | 11.19 | 1.04 | MS, KI, ST |
| 953 | Camphene | 0.09 | 0.00 | MS, KI, ST |
| 992 | Myrcene | 2.69 | 0.18 | MS, KI, ST |
| 1006 | α-Phellandrene | 0.02 | 0.00 | MS, KI, ST |
| 1013 | 3-Carene | 0.03 | 0.00 | MS, KI, ST |
| 1033 | Limonene | 16.38 | 1.53 | MS, KI, ST |
| 1063 | γ-Terpinene | 0.59 | 0.03 | MS, KI, ST |
| 1090 | Terpinolene | 3.17 | 0.07 | MS, KI |
| 1099 | α-Terpinolene | 0.55 | 0.03 | MS, KI |
| 1118 | 1,3,8-*p*-Menthatriene | 0.15 | 0.01 | MS, KI |
| 1136 | (*E,Z*)-Alloocimene | 0.02 | 0.01 | MS, KI |
| 1142 | (*E,E*)-Alloocimene | 0.06 | 0.01 | MS, KI |
| 1152 | (–)-3-Neoiso thujanol | 0.06 | 0.00 | MS, KI |
| 1180 | Terpinen-4-ol | 0.85 | 0.01 | MS, KI, ST |
| **Oxygenated monoterpenes (%)** | | **3.09** | | |
| 1020 | Isocineole | 0.24 | 0.02 | MS, KI |
| 1124 | Dehydro-sabina ketone | 0.04 | 0.00 | MS, KI |
| 1192 | Dihydro carveol | 1.66 | 0.02 | MS, KI |
| 1221 | α-Fenchyl acetate | 0.34 | 0.01 | MS, KI |
| 1229 | α-Aenchyl acetate | 0.13 | 0.00 | MS, KI |
| 1241 | Nojigiku acetate | 0.01 | 0.00 | MS, KI |
| 1285 | Bornyl acetate | 0.58 | 0.01 | MS, KI, ST |
| 1297 | 3-Methoxyacetophenone | 0.09 | 0.01 | MS, KI |
| **Sesquiterpene hydrocarbons (%)** | | **50.71** | | |
| 1338 | Presilphiperfol-7-ene | 0.04 | 0.01 | MS, KI |
| 1350 | (–)-α-Cubebene | 0.66 | 0.08 | MS, KI |
| 1377 | Isoledene | 0.31 | 0.03 | MS, KI |
| 1393 | β-Elemene | 1.65 | 0.15 | MS, KI |
| 1401 | β-Longipinene | 0.2 | 0.04 | MS, KI |
| 1420 | β-Caryophyllene | 17.64 | 0.75 | MS, KI, ST |
| 1430 | β-Copaene | 0.27 | 0.04 | MS, KI |
| 1445 | *cis*-Muurola-3,5-dinene | 0.15 | 0.02 | MS, KI |
| 1449 | *trans*-Muurola-3,5-dinene | 0.23 | 0.03 | MS, KI |
| 1453 | α-Humulene | 1.96 | 0.11 | MS, KI, ST |
| 1462 | allo-Aromadendrene | 0.56 | 0.11 | MS, KI |
| 1467 | *trans*-9-*epi*-Caryophyllene | 0.59 | 0.05 | MS, KI |
| 1472 | β-Acoradiene | 0.44 | 0.06 | MS, KI |
| 1475 | β-Cadinene | 1.34 | 0.16 | MS, KI |
| 1479 | Germacrene D | 5.78 | 0.51 | MS, KI |
| 1484 | β-Selinene | 0.34 | 0.04 | MS, KI |
| 1490 | *trans*-Muurola-4(14),5-dinene | 0.24 | 0.04 | MS, KI |
| 1493 | Valencene | 0.78 | 0.08 | MS, KI |
| 1499 | α-Muurolene | 1.27 | 0.14 | MS, KI |
| 1512 | γ-Cadinene | 0.98 | 0.15 | MS, KI |
| 1521 | δ-Cadinene | 5.84 | 0.72 | MS, KI |
| 1529 | 1,4-Cadinadiene | 0.23 | 0.03 | MS, KI |
| 1535 | α-Cadinene | 0.17 | 0.03 | MS, KI |
| 1546 | α-Calacorene | 0.71 | 0.13 | MS, KI |
| 1568 | β-Calacorene | 0.04 | 0.01 | |
| 1674 | Cadalene | 8.29 | 0.67 | MS, KI |
| **Oxygenated sesquiterpenes (%)** | | **0.16** | | |
| 1588 | Caryophyllene oxide | 0.16 | 0.03 | MS, KI |
| **Other (%)** | | **10.07** | | |
| 911 | Sorbaldehyde | 0.04 | 0.02 | MS, KI |
| 926 | Artemesia triene | 0.04 | 0.00 | MS, KI |
| 980 | 1-Octen-3-ol | 0.56 | 0.05 | MS, KI |
| 1052 | β-Ocimene | 0.06 | 0.00 | MS, KI |
| 1149 | Not identified | 0.03 | 0.01 | MS, KI, ST |
| 1170 | (*E,Z*)-1,3,5-Undecatriene | 0.08 | 0.01 | MS, KI |
| 1267 | *trans*-Cinnamaldehyde | 0.02 | 0.00 | MS, KI, ST |
| 1280 | *trans*-Ethyl chrysanthemumate | 0.74 | 0.07 | MS, KI |
| 1307 | Not identified | 0.11 | 0.02 | MS, KI |

**Table 1.** *Continued*

| KI[a] | Composition[b] | Relative concentration (%) | | Identification[c] |
|---|---|---|---|---|
| | | Mean | SD | |
| 1322 | Not identified | 0.05 | 0.01 | MS, KI |
| 1329 | *trans*-Patchenol | 0.02 | 0.01 | MS, KI |
| 1372 | Orcinol | 0.03 | 0.00 | MS, KI |
| 1436 | Not identified | 0.08 | 0.01 | MS, KI |
| 1506 | Not identified | 0.31 | 0.04 | MS, KI |
| 1576 | Spathulenol | 1.06 | 0.20 | MS, KI |
| 1635 | Not identified | 0.85 | 0.70 | MS, KI |
| 1640 | Not identified | 1.03 | 0.11 | MS, KI |
| 1645 | Not identified | 0.26 | 0.43 | MS, KI |
| 1663 | Not identified | 3.1 | 1.80 | MS, KI |
| 1669 | Not identified | 1.6 | 0.89 | MS, KI |

[a] KI, Kovats index relative to $C_9$–$C_{22}$ *n*-alkanes on the DB-5MS column
[b] Identification based on comparison of the mass spectrum, Kovats index on a DB-5MS column in Adams,[11] and coinjection with authentic compounds
[c] KI, Kovats index; MS, mass spectroscopy; ST, co-injection with authentic standard compounds

essential oil were identified (Table 1). Among these compounds, 50.71% were sesquiterpene hydrocarbons and 35.85% were monoterpene hydrocarbons; they also included 3.09% oxygenated monoterpenes and 0.16% oxygenated sesquiterpenes. The major constituents in the *C. macrolepis* var. *formosana* leaf essential oil were $\beta$-caryophyllene (17.64%), limonene (16.38%), and $\alpha$-pinene (11.19%), followed by cadalene (8.29%), $\delta$-cadinene (5.84%), germacrene D (5.78%), terpinolene (3.17%), and myrcene (2.69%).

Because of the difference in volatility, low molecular weight compounds with higher volatility were readily released from the leaves and detected in this study. On a sunny day, the major volatile compounds emitted from *C. macrolepis* var. *formosana* at midday were $\alpha$-pinene (19.88%), myrcene (16.10%), (–)-limonene (54.29%), terpinolene (1.26%), and $\beta$-caryophyllene (8.46%). On a rainy day, the readings were $\alpha$-pinene (52.14%), myrcene (9.84%), (–)-limonene (31.21%), terpinolene (0.51%), and $\beta$-caryophyllene (6.29%).

## Circadian profiles for BVOCs emitted by *C. macrolepis* var. *formosana* leaves

To identify and monitor the emission patterns of BVOCs from leaves of *C. macrolepis* var. *formosana* on a sunny day and a rainy day, short extractions (15 min) were performed every 1 h over 24 h. The results of BVOC changes in the chromatographic profiles obtained for a 24-h period are shown in Fig. 2; changes in the relative content of each major compound at different times are shown in Fig. 3.

Three of the major constituents of the fragrance, $\alpha$-pinene, limonene, and myrcene, were emitted in a rhythmic manner over a 24-h period, which showed three maximum emission patterns on both a sunny day and rainy day (see Fig. 2), whereas the other two minor compounds, $\beta$-caryophyllene and terpinolene, were not significantly varied. On a sunny day, the maximum emission times were 3 a.m.–4 a.m., 11 a.m.–1 p.m., and 11 p.m., and the relative abundance in the chromatographic profile of both $\alpha$-pinene and limonene was higher than that of myrcene. Interestingly, the relative level of limonene suddenly increased at 10 a.m., and then dropped back to the same level at 2 p.m.; $\beta$-caryophyllene showed a similar tendency. On a rainy day, the maximum emission times were 2 a.m.–6 a.m., 3 p.m.–5 p.m., and 8 p.m.–11 p.m., and the relative abundance in the chromatographic profile of $\alpha$-pinene and limonene was significantly higher than that of myrcene. A previous study of circadian and seasonal variation in the essential oil from *Virola suriamensis* leaves[12] used four samplings obtained from leaves collected at 6 a.m., 12 a.m., 6 p.m., and 9 p.m. during February, June, and October. The results showed that the relative levels of both limonene and myrcene at 6 a.m. dropped to approximately half at 6 p.m. and finally increased to nearly the same content at 9 p.m. Both $\alpha$-pinene and $\beta$-pinene were only detected at 6 a.m. and 9 p.m. However, the circadian variation of the essential oil from *V. suriamensis* leaves was not discussed in depth. However, in a previous study of the monoterpene and sesquiterpene formation pathway in snapdragon flowers (*Antirrhinum majus*), the results indicated that the methyl-erythritol phosphate (MEP) pathway operates in a rhythmic manner controlled by the circadian clock, which determines the rhythmicity of terpenoid emission.[13] Accordingly, we have concluded that this circadian variation of volatile compounds was caused by the circadian clock, and a complete explanation requires further investigation.

Furthermore, the volatile amount of limonene increased with temperature from 11 a.m. to 1 p.m. on a sunny day, and the amount was far higher than $\alpha$-pinene and myrcene (Fig. 2). There have been many publications demonstrating that secondary metabolic composition and volatile amount were influenced by temperature,[14,15] photoperiod, intensity of light, and season.[16,17] For instance, a previous study on the influence of light and temperature on monoterpene emission rates from slash pine[18] found that the monoterpene emission rate was strongly influenced by temperature,

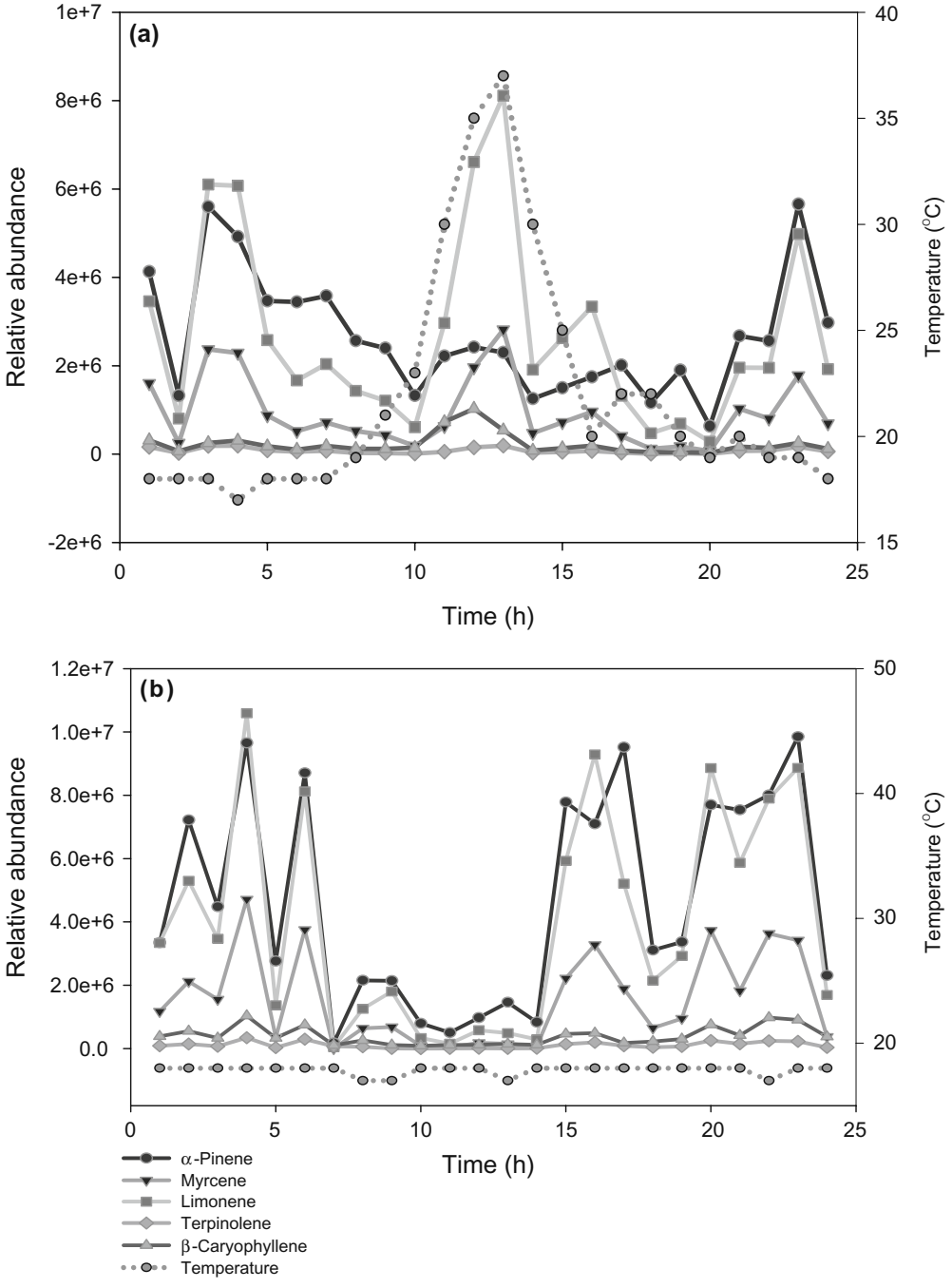


**Fig. 2.** Circadian profile for major volatile compounds emitted from *C. macrolepis* var. *formosana* leaves on a sunny day (**a**) and a rainy day (**b**)

and other studies showed that the relationship between monoterpene emissions and temperature showed plant-to-plant variation.[18,19] In this current study, the sudden increase in emission of limonene at noon might have been caused by the high temperature, whereas the high emission at early morning and midnight might be regulated by the circadian clock. Thus, the emission of BVOC is a complex process, and the variations in its behavior require future investigations.

The results of relative contents (area percent) of volatile compounds emitted from *C. macrolepis* var. *formosana* leaves varied with the time of the day, showing that limonene and myrcene had a similar emission tendency (Fig. 3), and the quantity of limonene was significantly higher than myrcene. Conversely, the circadian profile of $\alpha$-pinene observed was opposite to those of limonene and myrcene. This kind of emission behavior was also noted in the publication of Angioni et al.,[20] who indicated that the average values in leaves of fenchone and camphor showed an opposite behavior, which can be tentatively explained by the different biochemical pathways of formation. Among the major compounds of *Lavandula stoechas* L. ssp. *stoechas*, camphor, camphene, borneol, and bornyl acetate derive from the bornyl cation intermediate, whereas fenchone, $\alpha$-pinene, myrtenal, and myrtenyl acetate derive from the pinyl cation intermediate. When fenchone increased,

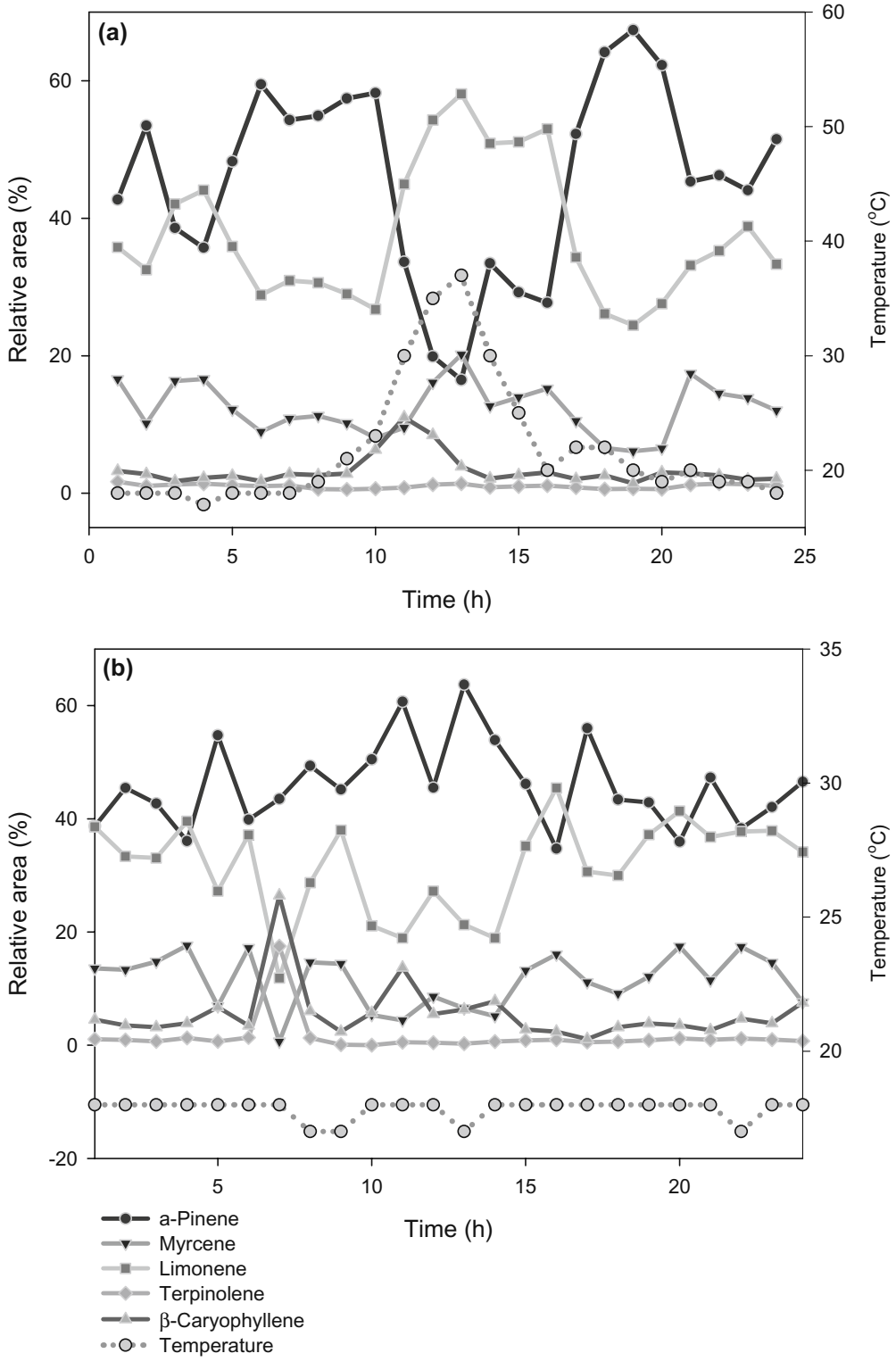


**Fig. 3.** Relative contents (area percent) of the major volatile compounds emitted from *C. macrolepis* var. *formosana* leaves varied with the time of the day on a sunny day (**a**) and a rainy day (**b**)

camphor decreased. The compounds, which belong to the same subpathway of fenchone and camphor, showed a similar behavior. Limonene, which derives directly from the $\alpha$-terpinyl cation, showed a trend similar to that of camphor.[20]

The biosynthetic pathway of major volatile compounds from *C. macrolepis* var. *formosana* leaves is illustrated in Fig. 4. The catalytic mechanism of monoterpene syntheses proceeds through the ionization of geranyl diphosphate (GPP) and subsequent isomerization to form linalyl diphosphate (LPP). After ionization of LPP, the resulting linalyl carbocation can undergo deprotonation at the C-3 methyl group to form myrcene (1A pathway) or to form the $\alpha$-terpinyl cation intermediate by cyclization (1B pathway).

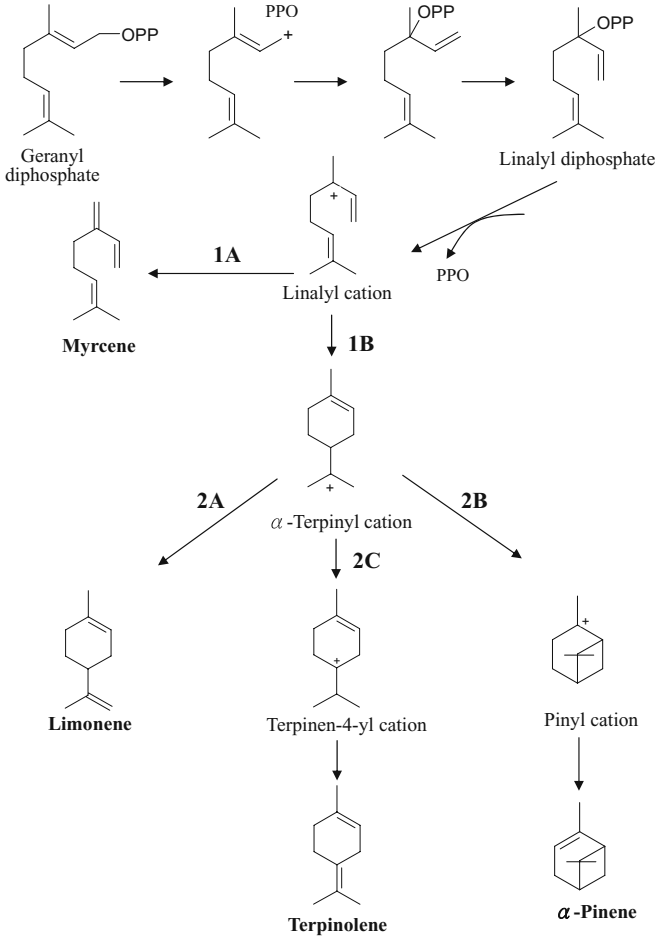


**Fig. 4.** Biosynthetic pathway of the major volatile compounds from *C. macrolepis* var. *formosana* leaves

Formation of monoterpenoids is through the $\alpha$-terpinyl cation intermediate, yielding different products by three subpathways. First, the elimination of a proton from the $\alpha$-terpinyl cation allows the formation of limonene (2A subpathway). Second, the $\alpha$-terpinyl cation undergoes 2,8-closure (2B subpathway), the pinyl cation is formed, and subsequent deprotonation of this pinyl cation produces $\alpha$-pinene. Third, the $\alpha$-terpinyl cation undergoes a 1,2-hydride shift (from C-4 to C-8) to form the terpinen-4-yl cation and the subsequent loss of a proton from terpinen-4-yl cation to form the terpinolene. From the foregoing description, it appears that $\alpha$-pinene and limonene were derived from two different subpathways, 2A and 2B. This could be the reason why the amount of $\alpha$-pinene and limonene emitted from leaves of *C. macrolepis* var. *formosana* showed an opposite trends, because the secondary metabolites may be under enzyme control, resulting in qualitative variability. Furthermore, the relative content of $\beta$-caryophyllene on a rainy day was greater than on a sunny day, and we assumed that the rainy day was beneficial to the emission of $\beta$-caryophyllene. The $\beta$-caryophyllene as an infochemical has been suggested by numerous studies, such as elicitor of a neuron response in the Egyptian cotton leaf worm (*Spodoptera littoralis* B.),[21] and of antennal responses in the female codling moth *Cydia pomonella* L. Moreover, it also showed toxicity toward the generalist herbivore *Spodoptera exigua* Hübn[22] when released from freshly cut or damaged tissue.[10]

Phytoncides are a variety of compounds released by plants to resist and decompose hazardous substances in the air; thus, a phytoncide can be called a "natural defense system." Phytoncides include a large number of organic substances, such as isoprene and terpenoid compounds, alkanes, alkenes, carbonyl compounds, alcohols, and esters. Isoprene and terpenoid compounds are the principal components. It has been shown that phytoncide has numerous functions, as described above, and the essential oil from *C. macrolepis* var. *formosana* is rich in limonene, $\alpha$-pinene, myrcene, and so forth, which are used as fragrant components in aromatic products. The components of these oils are believed to aid health, beauty, and hygiene in a variety of ways.

This is the first time that variation of volatile compounds emitted from *C. macrolepis* var. *formosana* leaves in situ under different weather conditions has been reported. The report provides important guidelines for the state government, county agencies, and other organizations.

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