Seasonal variation of *Thymus piperella* L. essential oil composition. Relationship among γ-terpinene, *p*-cymene and carvacrol

Juan A. Llorens-Molina[a]*, Sandra Vacas[b], Natalia Escrivá[c], Mercedes Verdeguer[a]

[a]*Mediterranean Agroforestry Institute. Universitat Politècnica de València. Valencia, (Spain)*

[b]*Centre for Agricultural Chemical Ecology. (Mediterranean Agroforestry Institute). Universitat Politècnica de València.*

[c]*School of Agricultural Engineering and Environment. Universitat Politècnica de València.*

** Mediterranean Agroforestry Institute. Universitat Politècnica de València. Camí de Vera. s/n. 46022. Valencia (Spain), e-mail: juallom2@qim.upv.es*

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Abstract

A wild population of *Thymus piperella* L. was monitored for two years in order to correlate phenological stages and meteorological data with yield and essential oil (EO) composition. The plant material was extracted by hydrodistillation and the EO was submitted to GC-MS and GC-FID analysis. To achieve reliable quantification, relative response factors were applied.

The results showed a progressive increase in the proportion of carvacrol according to the sunlight duration and temperature increase, until reaching a maximum at the beginning of flowering stage (52.9 % and 41.0 % in 2018 and 2019, respectively). A simultaneous decrease was found in the amount of *p*-cymene (8.2 % and 12.8 % respectively) in the same stage, whereas γ -terpinene showed a similar evolution to that observed for carvacrol but with lower values (3.3 % - 14.6 %). From statistical analysis, significant changes in the relative proportions of carvacrol and its metabolic precursors: γ-terpinene and *p*-cymene were found. They have been explained based on the proposed metabolic pathways in the literature. On the other hand, when relating the changes in the EO profiles with rainfall data, a strong influence of the water supply on the evolution of the chemical composition was observed.

Keywords: *Thymus piperella*; *p*-cymene; carvacrol, γ-terpinene, rainfall, temperature.

1. Introduction

Thymus piperella L. (Labiatae) is an endemic taxon growing in South Eastern of Iberian Peninsula. (Fig. 1). Although its preferred habitat is the meso-Mediterranean belt, it grows from the thermo-Mediterranean places near the coast up to 1100 m a.s.l in the lower supra-Mediterranean conditions. As typical Mediterranean species, it is adapted to wintry cold temperatures and summer drought, although its optimal development takes place in areas with a certain level of humidity (1). It grows on stony fields with a wide range of lime

content and its capacity to colonize abandoned fields or areas that have suffered forest fires is characteristic. From the ethnobotanical point of view (2), it should be noted its wide and growing use as a culinary herb, particularly, for flavouring green olives. Indeed, investigations on its micropropagation have been conducted in order to promote its cultivation (3).

Its essential oil (EO) is almost entirely made up of monoterpenes, with phenolic compounds such as thymol and carvacrol, to which its antioxidant and antibacterial activity can be attributed. Thus, its applicability as a food preservative has been studied (4) regarding the use of chitosan edible films. Its pharmacological applications related to its traditional use in folk medicine have been also reported (5, 6).

In the literature referred below, as well as in the development of this work, the homogeneity of populations has been assumed, in such a way that the term "chemotype" is used to characterize them. The results of a chemometric investigation based on the study of the infraspecific variability of 31 populations of *T. piperella* (1) led to distinguish three chemotypes with the following average composition: *p*-cymene- carvacrol- γ-terpinene (43.27 %, 15.76 %, 13.98 %); *p*-cymene-thymol (44.82 %, 22.96 %) and carvacrol-*p*-cymene (18.10 %, 52.10 %). Other minor but no negligible compounds accounting for more than 1 % were also reported: α-pinene, camphene, myrcene, limonene, 1,8-cineole, linalool, borneol, β-caryophyllene, and caryophyllene oxide.

The relationship between the distribution of these chemotypes and the environmental factors (bioclimatic and edaphic) affecting wild populations was also investigated (7). The chemotype *p*-cymene-carvacrol-γ-terpinene could be positively related to the aridity index and altitude. The presence of *p*-cymene-thymol chemotype is related to climatic factors affecting water balance both in soils and plants. Less evident is this influence on *p*-cymene-carvacrol chemotype. With respect to *p*-cymene, which is the

common and most abundant compound in the three chemotypes, its positive correlation with the content of soil organic matter is noticeable. However, as far as we know, no data have been reported on the seasonal variations and climatic influences of this EO, which can affect both its EO content and composition (8).

A key issue on the composition of phenolic oils such as *T. piperella* is the relationship between their bioactive compounds, carvacrol or thymol, and their metabolic precursors: γ-terpinene and *p*-cymene (Fig.1). According to studies on biosynthesis of thymol based on $^{14}CO_2$, (9, 10), both thymol and carvacrol come from the aromatization of γ-terpinene to *p*-cymene followed by hydroxylation of this last one. More recently, an alternative pathway has been proposed (11) in which the synthesis of thymol and carvacrol is catalyzed by single P450s from γ-terpinene through two-step oxidation, whereas *p*-cymene could be considered a side product from the premature release of the substrate from the enzyme active site.

A major obstacle to studying seasonal variations in EO profiles is the traditional way of quantification based on the normalization of peak areas from FID chromatograms, since it is not enough accurate to obtain rigorous EO profiles. According to the most recent recommendations (12, 13), quantification based on the use of internal standards and application of response factors does allow true quantification. Furthermore, if the EO content of the plant throughout the vegetative cycle is considered, it is possible to analyze the real changes in the content of secondary metabolites throughout the vegetative cycle, referring to the mass of plant material.

The aim of this work was to study the changes in EO composition affecting the relative rate of p-cymene, γ-terpinene and carvacrol over the vegetative cycle of T. piperella and ~~its relationship with~~ the possible influence of rainfall and temperature. For

this purpose, a representative population in which data from previous research were available was selected.

Fig.1. Geographical distribution of the taxon Thymus piperella L. Datasource: GBIF.org (occurrence search available in [14]:

2. Material and methods

2.1.Plant material

The studied population was situated in Barx (València, Spain). The sampling area (Figure 2) had an approximate extension of 0.4 km^2 and matched one of those locations reported by (1), named "La Drova". A Voucher specimen was kept at the Herbarium of the Universitat Politècnica de València (VALA No 9581).

Figure 2. Location of sampling area

The sampling area was located in a calcareous massif of Cretaceous origin where the abundance of karst forms stands out. From the bioclimatic point of view, it can be assigned at a lower mesomediterranean belt, with humid ombroclimatic conditions (1). The average yearly temperature is 15.9 ºC and the average yearly precipitation 928.6 mm. (15). An important climatological feature of this area is the relative frequency of torrential rain episodes.

Each one of the samples was gathered from 50 individuals at the same phenological stage, randomly selected and collected at 8-9 A.M. They were uniformly distributed along the sampling area in such a way that the plants were > 10 m apart, to avoid individuals from the same parent. The sampling dates and phenological stages over the studied years were the following: 2/1/2018 (beginning vegetative growth), 4/17/2018 (vegetative growth), 6/23/2018 (beginning flowering), 9/1/18 (advanced flowering and

fruiting stage), 4/2/19 (vegetative growth), 6/19/2019 (beginning flowering), 8/1/2019 (full flowering), 8/30/19 (advanced flowering and fruiting stage). It is important to mention that the flowering period is long, beginning to be observed during June, while the fruiting stage begins towards the end of August.

Once the stems and the woody parts were separated, the samples were dried at room temperature and darkness until constant weight. Subsequently, they were stored in sealed polyethylene bags and maintained at -40 ºC until EO extraction.

To ensure the homogeneity in EO profiles of the population, 42 individual plants were submitted to screening by TLC . ( ). No atypical individuals were detected.

2.2.Essential oil extraction

From each sample, three sub-samples weighing approximately 50 g were separated and submitted to hydrodistillation for 3 h with a Clevenger type apparatus. The EO was collected and what remained adhered to the inner wall of the Clevenger was washed with 1-2 ml of dichloromethane (DCM) (≥99.9%, capillary GC grade, Sigma-Aldrich®). Once the water was removed, the DCM extract was dried over anhydrous sodium sulfate (FlukaTM, ≥ 99.0 %) and filtered through a syringe filter OlimPeak (TeknokromaTM). Finally, the DCM was distilled off with a rotary evaporator (Laborota 4001, HeidolphTM) at 25 ° C until constant weight. In this way, the EO content was determined gravimetrically as m (EO) / m (dry material).

2.3.GC and GC/MS Analysis

The analysis of samples was carried out by gas chromatography with flame ionization detector (GC-FID) and mass spectrometry (GC-MS). A Clarus 500 GC (Perkin-Elmer Inc. Wellesley. PA. USA) chromatograph equipped with a FID detector and capillary column ZB-5 (30 m × 0.25 mm i.d. × 0.25 µm film thickness; Phenomenex Inc. Torrance,

CA. USA) was used for quantitative analysis. The injection volume was 1 μL. The GC oven temperature was programmed from 50°C to 250°C at a rate of 3°C min^{-1}. Helium was the carrier gas (1.2 mL min^{-1}). Injector and detector temperatures were set at 250°C. The percentage composition of the EO was calculated from the GC peak areas without correction factors by means of the software Total Chrom 6.2 (Perkin-Elmer Inc., Wellesley. PA. USA).

GC-MS analysis was performed using a Clarus 500 GC-MS (Perkin-Elmer Inc.) apparatus equipped with the same capillary column, carrier and operating conditions described above for GC-FID analysis. Ionization source temperature was set at 200°C and 70 eV electron impact mode was employed. MS spectra were obtained in full scan mode (mass range m/z 45-500 uma). The total ion chromatograms (TIC) and mass spectra were processed with the Turbomass 5.4 software (Perkin-Elmer Inc.). Retention indices were determined by injection of C_8–C_{25} n-alkanes standard (Supelco, Bellefonte, PE, USA) under the same conditions. The EO components were identified by comparison of calculated retention indices and high probability matches according to mass spectra computer library search (NIST MS 2.0) and available data from literature (16). The identification of the following compounds was also confirmed by comparison of their experimental lineal retention index (LRI) with those of authentic reference standards (Merck KGaA, Darmstadt, Germany): α-pinene, β-pinene, camphene, *p*-cymene, myrcene, limonene, (*Z*)-β-ocimene, camphor, terpinolene and terpinen-4-ol.

2.4.Quantification of the EO composition

The quantification of the individual EO constituents was expressed as m/mEO according to the guidelines based on the application of relative response factors to methyl octanoate (99 %, Sigma-Aldrich®) as an internal standard (RRF), (17, 18). For this purpose, the

experimental response factors (ERFs) for the main components were determined (17), whereas predictive RRF were applied for the rest of minor components (18).

The experimental determination of RRF was performed as follows: a weighted amount (10 mg, approx.) of each reference compound and 10 mg of the internal standard (IS) methyl octanoate were made up to 25 mL in a volumetric flask with DCM (≥99.9%, capillary GC grade, Sigma-AldrichTM) (Solution A); afterwards, 5 mL of solution A was made up to 10 mL (Solution B). Two μL of each solution were submitted to GC-FID analysis. From the peaks area values, ERFs were calculated according to:

$$\text{ERF} = \frac{m_i}{A_i} \times \frac{A_{IS}}{m_{IS}}, \qquad (1)$$

Where: m_i and A_i are the mass and peak area for each reference compound, respectively, and m_{IS} and A_{IS}, the mass and peak area of the internal standard. The used pure standards were purchased to Sigma-Aldrich^{TM} with the following purity values: *p*-cymene (99 %), γ-terpinene (97 %), bornyl acetate (95 %), borneol (99 %), carvacrol (97 %), 1,8-cineole (99 %), camphor (96 %).

ERFs calculation was performed twice and their average values were displayed in Table 1 and applied to the determination of % (mi/m_{EO}) of the oil components, according to (13) by the formula below:

$$m_i = \frac{m_{IS} \times ERF \times A_i}{m_{EO} \times A_{IS}} \times 100 \qquad (2)$$

Where m_{EO} is the EO weigh.

To express the percentage of each component per mass of dried leaves, values of EO content expressed as mEO/mm (mm, weight of distilled dried leaves) were calculated according to:

$$\% \left(\frac{m_i}{m_v}\right) = \frac{m_i}{m_{EO}} \times \frac{m_{EO}}{m_m} \times 100 \qquad (3)$$

Results were expressed as mg/100 mg (supplementary materials).

2.5.Statistical data processing

One-way ANOVA analysis followed by multiple range tests were applied to determine the significance of differences in EO content and composition among the sampling times using Statgraphics Centurion XVI® (Statpoint Technologies. Inc.). HSD Tukey test (at $P < 0.05$) was applied to evaluate the significance of the differences. To fulfil the homoscedasticity requirement, original percentage data were subjected to arcsin [square root (%/100)] transformation.

2.6.Meteorological data.

Temperature and precipitation data during 2018 and 2019 were obtained from the meteorological station (Davis Vantage Pro2), located at La Drova-Barx, València (Spain) (coordinates: 39° 0' 18.00" N, 00° 17' 24.36" W) (14). Available historical data for 1994 were obtained from the nearest meteorological weather station located in Oliva (Valencia; coordinates 38° 55' 15", 0° 7' 14" W) (19).

3. Results and discussion

3.1.EO content. Influence of meteorological parameters

EO content values (m EO/ m dried aerial parts) and detailed composition are displayed in Table 1. Significant differences in EO content were observed depending on the phenological stage (Figure 3). Both in 2018 and 2019, the higher values in EO content were verified at the beginning of the flowering stage, decreasing as it progressed. On the other hand, the minimum values were recorded in the initial sampling in winter 2018, in the stage prior to the start of vegetative development. The values observed at the end of August (2.2 % and 1.9 % in 2018 and 2019, respectively) agreed with the general trend by which the synthesis of secondary metabolites advances with the growth of the plant, in parallel with the increase of the photoperiod and consequently of the photosynthetic

activity (20, 21). This pattern of variation was also reported for other *Thymus* species, such as *T. hyemalis* (21) or *T. pulegioides* (22). The same way that for some species belonging to the genus *Origanum,* it increased progressively until reaching a maximum during the full flowering stage (21, 24, 25).

Likewise, these values were also in accordance with those reported coming from the same sampling area during the summer 1994 (reported value: 2.1 % $V_{EO}/m_{dried\ material}$) (1).

With regard to the effect of rainfall, the most notable increase in EO content was observed before flowering during 2019, which could be attributed to the high precipitation recorded in spring of 2019 (Figure 3). Water availability can influence secondary metabolism due to biosynthetic reactions that increase monoterpenes production (26, 27). In contrast, previous studies have reported an increase in some monoterpenes in *T. piperella* EO when the water availability was low (7). However, no general trends can be established on the influence of abiotic stress, such as little water supply, on the biosynthesis of secondary metabolites. As reported in different investigations, the EO content in *Origanum* species was not found related to water deficiency (28). In other studies, an increase (29) was observed. As reported by other authors (30), the relationship between water supply and secondary metabolites content is complex. No general trends can be established as it may favour certain metabolic pathways that increase the content of some compounds, while the opposite may occur with other ones.

If analysed the evolution of EO content (Figure 3), both ways of interpreting the effect of water supply on EO content seem to be complementary. Indeed, the availability of water during the year was high, with rains, occasionally torrential, but regular, favouring the biosynthesis of secondary metabolites and, therefore, the EO content.

During the typical Mediterranean summer drought (months of June, July and August), in which rains were scarce, with high values of evapotranspiration (ETO), a decrease in EO content was noted (historical values of ETO for June, July and August are 100.4, 126.0, 128.6 mm/month, respectively, compared to annual average value of 64.2 mm/month (1)).

Figure 3. Evolution of EO content, average daily temperature and daily precipitation throughout the experimental period (mean ± standard deviations)

3.2.EO composition

3.2.1. Qualitative analysis

The representative chromatogram (TIC) displayed in Fig.4 shows the three main components related with the metabolic route of carvacrol biosynthesis: [6] *p*-cymene, [9] γ-terpinene and [19] carvacrol. It is worth noting the high proportion of camphor [13] (11.3 %) which was not detected again throughout the experimental period, focused on the stages of vegetative development and progress of flowering.

Figure 4. Representative chromatogram of *Thymus piperella* chemotype *p*-cymene-carvacrol (Previous to experimental period: December 2017)

3.2.2. Quantitative analysis. Influence of meteorological parameters.

Full data expressed as % m/m (EO), corresponding to the successive sampling dates, are displayed in Table 1. It is relevant to pay attention to the total % of composition identified according to the phenological stage. The applied quantification method (17, 18) provides the real % of each compound in the EO and, if the EO content is taken into account, the results can be expressed as % with respect to the processed plant material: $m/m_{vegetal\ mat}$ (these data are displayed in supplementary material). Both in 2018 and 2019,

a progressive decrease in the total % of EO composition identified was observed, the lowest values corresponded to advanced flowering stage. This fact could be interpreted by admitting the progressive increase throughout the vegetative cycle of compounds of higher polarity or molecular mass, which were extracted by hydrodistillation but not detected by GC/FID analysis. On the other hand, if compared data depicted in Figure 5 (data expressed as % m/m(EO)) with those displayed in supplementary materials (expressed as % m/m (dried material), (Figure 2, supplementary materials) a similar evolution can be noted, but with stronger differences.

3.2.3. Changes in carvacrol. Influence of meteorological parameters.

Carvacrol was found the major component throughout the experimental period. Analysing the relationship between the yearly evolution of major compounds involved in the metabolic pathways that lead to the synthesis of carvacrol and the meteorological parameters (precipitations and average daily temperatures), some regularities can be emphasized. Firstly, there was a similar pattern between the evolution of the carvacrol %, the EO content and the average daily temperature and sunlight duration, so that the maximum values were reached at the beginning of the warmer periods of 2018 and 2019 (Figures 5, 6). These results agree with those reported for *Origanum vulgare* L. ssp *hirtum* (24). On the other hand, the change in carvacrol % during spring (April-June) in 2018 and 2019 was much more pronounced in the latter. The most marked differential factor in this period was the total rainfall: 55.8 mm and 501.1 mm, in 2018 and 2019, respectively.

The availability of data obtained in the same population during 1994 can contribute to understand these changes. This hypothetical positive influence of rainfall during the vegetative development stage on carvacrol synthesis could be related to the

differences between the proportions *p*-cymene-carvacrol at the advanced flowering stage. These proportions, comparing data of 1994 with those of 2018-2019 were 62.6 %-22.8 % in August-September 1994 [1], against 18.3 %-44.4 %, 26.5-26.8 % in 2018 and 2019, respectively.

To interpret these differences, it is worth comparing the water supply data during the period February-August of 1994 with those of 2018-19. It was warmer and much drier in 1994 than in 2018 and 2019 (Tav = 19.2 ºC, P = 126 mm, in 1994; Tav = 17.7 ºC, P = 309.6 in 2018; Tav = 17.4 ºC, P = 634.4 mm in 2019). The yearly average precipitation during this period, accounting the last ten years was 388.9 mm. Interpreting the relationship between carvacrol content and water supply seems to be difficult. The available data on this matter comes from studies in which water supply is controlled in crop fields (26, 27). Xeric conditions are accepted to be favourable to biosynthesis of phenolic compounds (31) but it can be considered relevant if different habitats are compared (32). However, when studying the same population (and, therefore, the same habitat) in different periods to interpret carvacrol content changes, the influence of their specific meteorological conditions should be considered. The higher biosynthesis of carvacrol in 2018 and 2019 could be explained by the consecutive existence of a period with a high water supply, which favours plant development, followed by the typical Mediterranean summer drought, with high temperatures and intense solar radiation, which are the most favourable conditions for monoterpene synthesis (21)

Figure 5. Changes in γ-terpinene, *p*-cymene and carvacrol content expressed as % (m/m_{EO}), and daily precipitation over the experimental period (error bars represent standard deviations)

Figure 6. Changes in γ-terpinene, *p*-cymene and carvacrol content expressed as % (m/m_{EO}), and average daily temperature over the experimental period (error bars represent standard deviations)

3.2.4. Precursors of carvacrol: p-cymene and γ-terpinene. Other compounds.

If the synchronization of changes affecting the fraction of the major components (γ-terpinene, *p*-cymene and carvacrol) is considered, important and significant variations throughout the vegetative cycle can be noted. The compound *p*-cymene exhibited the lowest values at the beginning of flowering stage (8.2 and 12.8 %), reaching the highest percentage at the initial sampling in winter 2018 (34.2 %), also attaining high values with the progress of flowering stage (32.1 % at the full flowering stage in 2019).

The amounts of γ-terpinene and carvacrol ranged from 3.5 % up to 14.6 % and 26.8 % to 52.9 %, respectively. Both components evolved in a similar way, increasing throughout the vegetative development to reach their highest values at the beginning of flowering stage, and a subsequent progressive decline as flowering stage progressed. Other minor components can be also considered: limonene (t – 3.6 %), linalool (t – 1.5 %), borneol (0.8 – 3.3 %), β-caryophyllene (2.0 - 3.2 %) and caryophyllene oxide (0.7 – 3.1 %). These minor compounds coincide with those cited by (1) when studying this same population in 1994: limonene (0.8 %), linalool (1.1 %), borneol (2.3 %), β-caryophyllene (0.4 %) and caryophyllene oxide (0.1 %). It should be noted that all the proportions reported in previous studies (1) were calculated by applying the normalization method of the peak areas, so the comparisons are merely indicative.

Regarding the classes of compounds, hydrocarbon monoterpenes accounted for 23.5–42.6 %, oxygenated monoterpenes ranged from 34.3–57.5 %, the hydrocarbon

sesquiterpenes were 2.3 – 4.0 % and oxygenated sesquiterpenes 1.0 – 4.3 %. The main difference regarding the previous reported data (1) were referred to hydrocarbon/oxygenated monoterpenes balance, as consequence of *p*-cymene-carvacrol rates.

According to the composition initially reported from this population (1), it was considered belonging to *p*-cymene + carvacrol chemotype, *p*-cymene accounted for 62.6 % of the EO composition, γ-terpinene content was 2.5 % and carvacrol represented 22.8 %. In general, these authors reported for this chemotype the following average composition: 52.10 % of *p*-cymene, 4.29 %, of γ-terpinene and 18.10 % of carvacrol (1). This population could be classified as belonging to this chemotype if the data corresponding to the advanced flowering stage were considered. However, based on the values observed during vegetative growth and the beginning of flowering, the population could fit into the *p*-cymene chemotype and γ-terpinene-carvacrol chemotype, respectively. This fact highlights criticism of the concept of chemotype already raised by various authors (33). Indeed, the distinction between chemotypes based on quantitative differences between metabolically interrelated compounds, may not be adequate when considering ontogenetic or environmental variability factors.

These seasonal changes can be interpreted taking into account the metabolic pathways involving *p*-cymene, γ-terpinene and carvacrol, which are similar to those reported for *Origanum vulgare* L. ssp. *hirtum* [23]. In the same way, if the May-July period is considered, an increase in carvacrol for *Thymus capitatus* L. was also found [24]. During vegetative development, the conversion of γ-terpinene to *p*-cymene slowed down, whereas the conversion of *p*-cymene to carvacrol was intensified. This tendency, related to thymol, the other endpoint of this metabolic pathway, has been also observed

in other phenolic oils, such as *Origanum syriacum* (20). This fact leads to a depletion of *p*-cymene, while γ-terpinene and carvacrol increased later to a greater extent.

During the flowering stage, the conversion of γ-terpinene to *p*-cymene predominated over the synthesis of carvacrol from *p*-cymene, in such a way that an accumulation of *p*-cymene took place. This interpretation, based on the relative predominance of γ-terpinene synthesis, conversion of γ-terpinene to *p*-cymene and synthesis of carvacrol from *p*-cymene, is easier to interpret from the well-known metabolic pathway, in which *p*-cymene is an intermediate stage in the synthesis of carvacrol (9). More difficult is to understand these changes from the alternative metabolic route (11), since the changes in *p*-cymene and carvacrol, coming from γ-terpinene as only source, should go in the same way, conversely to the γ-terpinene change observed.

4. Conclusions

Seasonal changes in EO content and composition of a *T. piperella* population were studied for two years, relating them to meteorological data: daily mean temperature and precipitation. The results were compared with those from previous studies (1, 7) carried out on the same population. This species is an endemism located in a relatively small area in the Eastern of the Iberian Peninsula and its seasonal evolution has not been studied to date, to the best of our knowledge.

Carvacrol and its metabolic precursors, *p*-cymene and γ-terpinene, resulted the main components. Taking into account the statistical significance of the differences observed between the successive samplings, it can be affirmed that their seasonal changes have followed the same pattern throughout the two studied years: the proportion of carvacrol increased, as it did the temperature and the sunlight period duration, until reaching a maximum at the beginning of flowering, at first of the warmest period, to later decrease as the flowering progressed. In fact, the temperature variation was practically

parallel to the evolution in the carvacrol content. The compound γ-terpinene showed similar pattern of variation, but with smaller values and less pronounced changes, while *p*-cymene showed an inverse evolution regarding carvacrol and γ-terpinene, reaching for some samples a value close or similar to that of carvacrol. This evolution could be interpreted based on the widely accepted metabolic pathway for carvacrol biosynthesis (9).

The occasional occurrence of intense rains did not affect the evolution of the EO chemical profile, since considering they even took place at dates relatively close to the samplings, they did not seem to cause a remarkable effect. More interesting is the comparison with the data from 1994. The typical xeric conditions of the Mediterranean summer, favourable to the synthesis of phenolic compounds, occurred in both 1994 and 2018 – 2019. However, the striking higher water supply during vegetative growth in 2018 and 2019, compared to 1994, could be related to the increased carvacrol biosynthesis. The enhancement of carvacrol biosynthesis, as a result of combining high water supply during vegetative development and later water deficiency, could be the subject of further investigations because of its possible usefulness from the agronomical point of view.

The studied population, at some points during the flowering period, was considered as belonging to the *p*-cymene-carvacrol chemotype, according to previous studies (1). If this very long flowering period is considered as a whole, it could also be determined as chemotype carvacrol- *p*-cymene- γ-terpinene, since the latter compound reached at some times during the flowering period values higher than those of *p*-cymene. This fact supports the convenience of defining chemotypes according to the involved

metabolic pathways instead of the presence of some major components at a certain point of the vegetative cycle.

In this work, a methodology for quantification based on the consideration of relative response factors was applied. Throughout the two years, the same pattern of variation of the total percentage of EO composition identified was repeated, decreasing as the vegetative cycle progresses. This could be explained by admitting the progressive presence in the EO of metabolites not recorded by the chromatograph in the applied working conditions.

In this manner, the method used allowed a much more rigorous determination of the EO composition. Despite the fact that the composition changed throughout the vegetative cycle, the values obtained through this methodology are basically identical to those got through the normalization of the peak areas (Fig.1, supplementary materials, when compared with Fig. 5). However, more significant differences were observed when taking into account the EO content, in order to express the composition based on the mass of processed plant material instead of the mass of EO (Fig. 2, supplementary materials).

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Figure captions

Fig.1. Geographical distribution of the taxon Thymus piperella L. Datasource: GBIF.org (occurrence search available in: . https://www.gbif.org/occurrence/search?has_coordinate=true&has_geospatial_issue=false&taxon_key=8354272

Figure 2. Location of sampling area

Figure 3. Evolution of EO content, average daily temperature and daily precipitation throughout the experimental period (error bars mean +/- standard deviations)

Figure 4. Representative chromatogram of *Thymus piperella* chemotype *p*-cymene-carvacrol (Previous to experimental period: December 2017)

Footnote:

Compounds accounting > 0.2 % (total ion chromatogram). 1. α-Thujene (0.2 %), 2. α-Pinene (0.7 %), 3. Camphene (1.2 %), 4. Myrcene (0.5 %), 5. α-Terpinene (0.4 %), 6. p-Cymene (16.8 %), 7. Limonene (0.8 %), 8. 1,8-cineole (0.5 %), 9. γ-terpinene (7.9 %), 10. *cis*-Sabinen hydrate (0.3 %), 11. *trans*-Linalool oxide (0.5 %), 12. Linalool (3.8 %), 13. Camphor (11.3 %), 14. Borneol (3.3 %), 15. Terpinen-4-ol (1.2 %), 16. p-Cymen-8-ol (0.2 %), 17. Thymol (1.2 %), 18. Carvacrol (40.2 %), 19. β-Caryophyllene (2.8 %), 20. Germacrene-D (0.3 %), 21. Bicyclogermacrene (0.4 %), 22. Spathulenol (0.5 %), 23. Caryophyllene oxide (2.2 %)

Figure 5. Changes in γ-terpinene, *p*-cymene and carvacrol content expressed as % (m/m_{EO}), and daily precipitation over the experimental period (error bars represent standard deviations)

Figure 6. Changes in γ-terpinene, *p*-cymene and carvacrol content expressed as % (m/m_{EO}), and average daily temperature over the experimental period (error bars represent standard deviations)

Figure 1 (Supplementary materials). Changes in γ-terpinene, *p*-cymene and carvacrol content expressed as % ($m/m_{dried\ material}$), and daily precipitation over the experimental period (error bars represent standard deviations)