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Seasonal variability in essential oil of aerial parts and roots of an Artemisia absinthium L. population from a Spanish area with supramediterranean climate (Teruel, Spain)

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In this study, the essential oil composition of aerial parts and roots of *Artemisia absinthium* L. was characterized by gas chromatography with flame ionization detector (GC/FID) and confirmed by gas chromatography coupled to mass spectrometry (GC/MS). Seasonal variations have been studied throughout the whole vegetative cycle according to phenological stage of plants in a supramediterranean climate. The two most characteristic chemotypes in north-eastern of Iberian Peninsula co-occur in this population with *cis*-epoxyocimene and *cis*-epoxyocimene + *cis*-chrysanthemyl acetate as major compounds, respectively. The results show that, despite of high intrapopulational variability, some significant variations can be appreciated in major components. The composition of the root essential oil has been found very different to the one from aerial parts and extremely rich in monoterpenoids, mainly, hydrocarbon monoterpenes and monoterpenic esters. As most of them are known by their allelopathic properties and *A. absinthium* is considered a typical invasive species, to know in detail its essential oil composition and seasonal variations may be a first step to study the release of monoterpenic allelopathic compounds in soil. This way, its hypothetical application as a source for natural herbicides would be borne in mind.

Keywords: Artemisia absinthium L., essential oil, roots, seasonal variations, monoterpenes, allelopathy.

Introduction

Artemisia absinthium L., known in Spain as “ajenjo”, is a yellow-flowering perennial undershrub widely extended in the Northern Hemisphere, mainly in Eurasia, but poorly represented in the Southern Hemisphere. Given that it has been cultivated for ages, its original distribution is not easy to specify and many of its populations may have a naturalized origin (1, 2). It grows in a wide variety of environmental conditions, often in anthropic habitats usually close to urban areas, such as wastelands, ditches, etc. and it can be considered as invasive species (1, 3). In the Iberian Peninsula, its optimal habitat is found in continental areas between 800 and 1500 m a.s.l. Although it requires an annual average precipitation higher than 400 mm, it resists droughts and frosts (3, 4). From the bioactivity point of view, both essential oil and different extracts of *Artemisia absinthium* L, are widely used because their medicinal properties and agricultural concerns, such as the acaricidal properties, antimicrobial, antifungical, insect repellent or allelopathic effect (5).

Any application of plant extracts bioactivity demands to standardize their chemical composition, both to obtain the most active composition and control the amount of phytotoxic compounds. For this reason, to know the sources of their chemical variability becomes indispensable. This variability may be due to genetic or environmental factors dependent on geographical and edaphoclimatic conditions, leading to the occurrence of different chemotypes as infraspecific chemical races. At the same time, seasonal and organ variations should be also considered.

Regarding to essential oil of aerial parts of *Artemisia absinthium* L., many different chemotypes have been characterized according their geographical origin (6). Nevertheless, almost all of them are based on four main compounds: (*Z*)-epoxyocimene, (*Z*)-chrysanthemyl acetate, sabinyl acetate and β-thujone. Each one of these compounds defines a “pure” chemotype, (4, 6). There are also different “mixed” chemotypes with balanced amounts of two or more of these compounds such as (*E*)-sabinyl acetate +

β–thujone (7), (*Z*)-epoxyocimene + β-thujone + (*E*)-sabinyl acetate (8) or (*Z*)-epoxyocimene + sabinyl acetate + chrysanthemyl acetate (9). As described by Ariño (4), seven pure or mixed chemotypes can be defined in the Iberian Peninsula from three of these compounds: (A) (*Z*)-epoxyocimene; (B) (*Z*)-epoxyocimene + (*Z*)-chrysanthemyl acetate; (C) (*Z*)-chrysanthemyl acetate; (D) (*Z*)-epoxyocimene+ β-thujone; (E) (*Z*)-epoxyocimene + (*Z*)-chrysanthemyl acetate + β-thujone; (F) (*Z*)-chrysanthemyl acetate +β-thujone and (G) β-thujone. Chemotypes (A) and (B) usually co-occur in the same populations and they are the most extended in north-eastern Iberian Peninsula, where Teruel is placed (4).

There are additional mixed chemotypes whose composition is characterized by one of these four mentioned constituents and other major ones: β-thujone + β-pinene (10); α and β–thujone + (*Z*)-chrysanthemol (11); β-thujone + β–myrcene + sabinene (12); β–myrcene + (*Z*)-chrysanthemyl acetate(13) and β–myrcene + β-thujone + (*E*)-sabinyl acetate (14). Moreover, as reported by other investigations, several chemotypes whose major compounds are not the four ones above mentioned have been also reported as for example: sabinene + β–myrcene (6), bornyl acetate (13), caryophyllene oxide + *p*-cymene + 1,8-cineol + (*Z*)-lanceol acetate (15), the same way that 1,8-cineol (6), neryl butanoate (6) or chamazulene (13) have been found as major compounds in other geographical origins .

Few data are available about yearly variation of aerial parts of chemotypes A and B (3, 16) in whose no statistical treatment was performed. These data lead to admit the no relevance of changes in oil composition due to seasonal variations or the different plant organs –leaves and flowers. Nevertheless, these studies have been conducted in locations whose winters are milder, in such a way that plants keep their activity over the whole year. In that respect, climatic conditions involving a certain shortness of yearly vegetative cycle could give rise to more marked seasonal variations and, therefore, to their significance.

Much more restricted data are available about the roots essential oil composition. Some researches give more or less detailed characterizations of *A. absinthium* root essential oil involving different chemotypes (8) highlighting the presence of monoterpenic esters and hydrocarbons. However, no available data are reported in the literature about seasonal variations.

Apart from the potential usefulness of roots essential oils themselves, (8, 17-19) an increasing attention is being paid to ecological role of volatile compounds occurrence in roots. They play complex and important roles in the rhizosphere, such as to regulate the soil microbial activity, changing the soil chemical and physical properties and inhibiting the growth of other competing species, accounting for the invasive behavior of plants such as *A. absinthium*.

Regarding to sampling methodology, most studies based on the chemical characterization of *A. absinthium* essential oil have been performed by means of samples made up by material randomly collected from an indeterminate number of plants. However, when it comes to consider the significance of organ or seasonal variations, the individual variability should be taken into account. Some researchers (4, 12) emphasize the high intrapopulational heterogeneity of *A. absinthium*, which makes difficult to interpret the influence of the mentioned factors. For this reason, in order to account for this individual variability, they argue for carrying out these studies by means of individual monitoring (12)

Thus, the objectives of this research are the following:

(1) To study how the seasonal variations are affected by a shorter vegetative cycle, bearing in mind the intrapopulational variability.

For this purpose, a wild population growing in a supramediterranean climate (Teruel, Spain) has been studied. In this place, despite the perennial nature of *A. absinthium*, it survives over the winter as small shoots (from november up to april aproximately, according the yearly special climatic characteristics). Only a previous study about seasonal variations in one of the typical *A. absinthium* chemotypes occurring in Iberian Peninsula, (*Z*)-epoxyocimene + (Z)-chrysanthemyl acetate, has been carried out, but with different climatic conditions -Haro (La Rioja) and Tarazona (Zaragoza)- (as is shown in table 1) and a fewer number of samplings and individuals (16).

(2) To describe the chemical composition of roots essential oil and its seasonal variation taking into account the co-occurrence of two different *A. absinthium* chemotypes typical in Iberian Peninsula (A and B, in the population). These data could contribute to consider the allelopathic potential of roots and aerial parts of *A. absinthium* and, therefore, its hypothetical activity as a source for natural herbicides.

Table 1. Climate conditions in the area of study (Calamocha) and locations of a similar study (Haro and Tarazona)

Experimental

Plant Material

Location and sampling

The investigation was carried out in a population of A. absinthium growing spontaneously in a wasteland on the outskirts of Calamocha, Teruel, Spain, (latitude: 40º 25' 46 " N and longitude: 6º 42' 43" O). Voucher specimens are lodged in the herbarium at the Mediterranean Agroforestal Institute (Universitat Politècnica de València, Spain).

With respect to sampling methodology, to monitor the same individuals over vegetative cycle has been not obviously possible in this study, given that the analysis of roots involves a destructive sampling. This way, it has been carried out by collecting 5 whole individual plants (aerial parts and roots, separately) randomly selected in the population throughout their successive phenological stages: (1) early vegetation (05/06/2012); (2) full vegetation (06/26/2012); (3) full blooming (08/18/2012); (4) full blooming-beginning of fruit maturation (09/15/2012); (5) advanced fruit maturation (10/14/2012); (6) winter shoots (01/22/2013). Reserve samples were collected in order to process at least two samples belonging to each chemotype.

Aerial parts

The plant material was cleaned and the most lignified stems were removed. It was frozen at -40ºC up to isolation of essential oil by hydrodistillation method using a Clevenger apparatus. After 2 hours of distillation the oil samples were collected, dried with anhydrous sodium sulphate and stored in glass vials at -18º C in the absence of light until GC analysis. The essential oil yield was determined on a volume to fresh weight basis. The average values for essential oil content of the five individuals from each sampling were calculated (table 2).

Roots

Each of one of the selected plants was pulled up carefully to obtain the root material. This was gently washed with distilled water, dried at room temperature and frozen up to

-40ºC. Before extraction, it was cut into pieces smaller than 0.5 cm. The isolation of essential oil was carried out by means of a SDE Likens-Nickerson apparatus for 2 h using 5 mL of dichloromethane as solvent. The extracts were dried with anhydrous Na_2SO_4 and kept in a sealed vial at -18ºC until chromatographic analysis.

Meteorological data

Daily precipitations and average temperature over the experimental period have been reported from a weather station placed in Calamocha (Teruel). According the registered data (fig.1), the climatic behaviour was typical of the location, with a quite mild and wet fall (a total precipitation of 337.6 mm over the experimental period).

Analisis of the essential oils

The analysis of samples was carried out by GC-FID and GC-MS. A Clarus 500 GC (Perkin-Elmer Inc.) chromatograph equipped with an FID detector and capillary column ZB-5 (30 m x 0.25 mm i.d. x 0.25 μm film thickness) was used for quantitative analysis. The injection volume was 1 μL. The GC oven temperature was programmed from 50ºC 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 250ºC. The percentage composition of the essential oil was computed from GC peak areas without correction factors by means of the software Total Chrom 6.2 (Perkin-Elmer Inc., Wellesley, PA)

Analysis by GC-MS was performed using a Clarus 500 GC-MS with the same capillary column, carrier and operating conditions above described for GC analysis. Ionization source temperature was set at 200˚C and 70 eV electron impact mode was employed. MS spectra were obtained by means of total ion scan (TIC) mode (mass range m/z 45-500 uma). The total ion chromatograms and mass spectra were processed with the software Turbomass 5.4 (Perkin-Elmer Inc.).

Retention indices were determined by injection of C8–C25 *n*-alkanes standard (Supelco) under the same conditions. The essential oil components were identified by comparison of their mass spectra with those of computer library NIST MS Search 2.0 and available data in the literature (8, 23). Identification of the following compounds was confirmed by comparison of their experimental RI with those of authentic reference standards (Sigma-Aldrich): α-pinene, β-pinene, camphene, myrcene, limonene, *(Z)*-β-ocimene, camphor, terpinolene, β-thujone, borneol, terpinen-4-ol, bornyl acetate, geraniol and linalool.

Statistical analysis

The statistical analysis was carried out by means of an analysis of variance (ANOVA) using Statgraphics 5.1. Software. As the raw data were expressed as % peak areas, they were subjected to arcsin[square root(%/100)] transformation and previous homocedasticity test. Then, Tukey's HSD multiple-range test at $P<0.05$ was used to consider significant differences in average values of the components among the sampling periods.

Table 2a. Yield and composition of the essential oil Artemisia absinthium aerial parts throughout the vegetative period.

Table 2b. Composition of essential oil of Artemisia absinthium roots throughout the vegetative period.

Figure 1. Meteorological data over the experimental period

Figure 2. Evolution of major compounds for chemotype A ((*Z*)-epoxyocimene). Values having different letters are significantly different from each other according Tukey's HSD test ($P<0.05$)

Figure 3. Evolution of major compounds for chemotype B ((*Z*)-epoxyocimene +(*Z*)-chrysanthemyl acetate). Values having different letters are significantly different from each other according Tukey's HSD test ($P<0,05$)

Table 3. Major compounds in roots for each one of the aerial parts chemotypes

Results and Discussion

The composition of aerial parts and roots of the studied population are shown in tables 2a and 2b, respectively. The first point that should be outlined is its great individual variability taking into account the standard deviation values. This fact has been also reported in early investigations (4, 16). If the population is considered as a whole, the amounts of major compounds show the following range: 49.3%-71.5% for (*Z*)-epoxyocimene and 7.6%-19.0% for (*Z*)-chrysanthemyl acetate. Nevertheless, when individual data about this last compound are examined, there are two groups with a well-defined range of variation: 15.4-33.1% and 0-1.4 %. This fact can be accounted for the co-occurrence in this population of the two most characteristic chemotypes of *A. absinthium* in North-Eastern Iberian Peninsula: chemotype A, with (*Z*)-epoxyocimene as the main component, and B, with (*Z*)-epoxyocimene and *(Z)*-chrysanthemyl acetate as major ones. As reported in previous researches (4), the following ranges were found for these chemotypes in Iberian Peninsula: chemotype A: 40-77% of (*Z*)-epoxyocimene and < 0.6% of (*Z*)-chrysantemyl acetate; chemotype B: 7-65% and 5-70%, for both components, respectively. The rest of major compounds has been found oxygenated monoterpenes: linalool (0.4%-10.4%), camphor (tr-5.3%), borneol (0.7%-7.1%). These last compounds were not major ones in samples from Haro and Tarazona; regarding to linalool, the amount was similar in the Haro population (4).

Bearing in mind the co-occurence of chemotypes A and B in the studied population, a more reliable description of seasonal variation for major compounds can be achieved if separate data for each chemotype are considered (Fig. 2 and 3, chemotypes A and B respectively). (*Z*)-epoxyocimene shows similar trends for both chemotypes. An initial increase takes place during the vegetative growing (stronger and statistically meaningful for chemotype B), whereas (*Z*)-chrysanthemyl acetate shows the opposing variation. The same trend can be observed over the rest of period: (*Z*)-epoxyocimene keeps the lower rate after flowering to seeds ripening whereas (*Z*)-chrysanthemyl acetate shows a smooth and no significant increase at the end of flowering. Although no information about phenological stages and climate data are provided in early studies (16), their results are quite similar if the foreseeable delay in spring shooting owing to harder winter in Teruel is borne in mind.

Linalool variation is not affected by the chemotype and show a significant maximum at the end of flowering for chemotype A, and a similar and smoother but no significant one for B. Taking into account the climate differences and the hypothetical correspondence between phenological stages and dates, these results are similar to those reported in the above mentioned study (16). In the same way, no significant differences were found in the rest of major compounds when a preliminary statistical analysis was performed in order to test hypothetical differences in composition between chemotypes A and B.

The sesquiterpenic fraction shows germacrene-D (0.1%-7.1%) and β-caryophyllene (0.5%-1.6%) as main components. These data are very similar to ones from literature with respect to order of magnitude. Nevertheless, the seasonal variation shows noticeable differences when data are compared. Whole sesquiterpenes show a significant decrease from the beginning up to end of vegetative cycle. β-caryophyllene reaches a higher concentration from June to October (seeds ripening) with a minimum at the beginning of the study period (spring shooting). The maximum amount of germacrene-D was found at the beginning, keeping a similar amount from June (vegetative growth) to October (advanced fruit maturation) and falls in January (winter shoots). These variations do not match with the ones reported for Haro and Tarazona populations (4).

The EO yield (Fig.4) shows a minimum both in spring and winter shoots. The highest level is registered in full blooming and, after a little but significant decrease up to end of flowering, increases again and show a significant decrease throughout winter.

Regarding to root essential oil composition, it was found quite similar to what was reported in literature for a sample belonging to the β-thujone chemotype (8). The main bulk of constituents from roots was composed of hydrocarbon monoterpenes accounting for a 43.8-55.1% with β-myrcene (20.2-31. 9%) and α-fenchene (12.4-23.6%) as the major components, whereas 35.6% of hydrocarbon monoterpenes was reported in the above mentioned research (8), being α-fenchene (23.3%) the main compound. Both studies agree in the high level of monoterpenic esters: 63.5 % (12) and 37.2%-45.7% in the present work. The composition of this fraction shows some relevant differences: 59.3% are acyclic esters –linalyl, mainly- (8), whereas the cyclic monoterpenic esters predominate in the present study (27.1-38.0%). Sesquiterpenic compounds, mainly caryophyllene oxide, hardly reach a maximum amount of 2.1 % at the first sampling. They were not detected in the referred previous research (8).

With regard to EO roots composition in other Artemisia species, the sesquiterpenic fraction was the major one (55.7 %) in *Artemisia vulgaris* L., whose aerial parts contain sabinene, 1,8-cineole and beta-thujone as a major compounds (8). In the same way, the sesquiterpenic fraction in *Artemisia Annua* roots was the most important: cis-artennuic alcohol, b-caryophyllene, beta-farnesene and caryophyllene oxide were found as a major compounds (17). A sesquiterpenoid (beta-cedren-9-ona) was found the major compound in *Artemisia persica* Boiss. (76,7 %) whereas the major monoterpenoids (cis-ocimenone, ascaridol and alpha terpinene) reached a 76,7 % (18). Regarding to *Artemisia tridentata* Nutt., the monoterpenic fraction was found the most important (45,5 % and 39,8 % in roots and shoots, respectively) with camphor, artemisia triene and 1,8-cineol as main compounds. The sesquiterpenic fraction reached a 8.8 % in roots and only traces in shoots (19).

About seasonal variations, no significant differences can be observed regarding the hydrocarbon monoterpenes and groups of compounds based on their chemical nature (except for monoterpenic oxygenated no esters). Among the major compounds, only bornyl acetate shows a significant difference from the spring shoots to middle winter ones, keeping its level stable over growing, flowering and ripening stages. Likewise, neryl isovalerate reaches a significant higher level at the beginning of vegetative cycle. Nevertheless, it might be worthy to consider some minor but no negligible compounds (camphor, borneol, cis-geraniol and geranyl acetate) which show a significant increase throughout the most active period of vegetative cycle, from full vegetative growing up to seeds ripening.

This relative higher stability in roots seasonal variations with respect the aerial parts ones can be interpreted taking into account the just mentioned great intra-populational

variability reported for aerial parts which also become patently clear for roots in this study. On the other hand, the more stable conditions of temperature and moisture in soil can be taken into account. Moreover, according to the rainfall distribution (Fig.1), with wet spring and fall, a relative high soil moisture level could be expected throughout the whole vegetative cycle.

A key point in this discussion is to relate the invasive nature of *A. absinthium* with the allelopathic potential of secondary metabolites which can be released from its aerial parts and roots. These compounds may be released in soil by three ways: roots exudates, leaching from stems and leaves, and litter decomposition. Given that the monoterpenic fraction is the main one both in aerial parts and roots, the literature concerning its hypothetical influence on germination and seedling growth of other plants should be examined, mainly those involved compounds found in *A. absinthium.*

The effect of monoterpenes on germination and seedling growth has been extensively reported in a number of studies under laboratory conditions (24). As reported by Zahed et al. (25), the essential oil of *Schinus molle* L. fruits and leaves, whose main components are α and β-phellandrene, β-myrcene and α-pinene, has been found active on wheat germination and radicle elongation. In another study related to biological activity of 30 monoterpenes on seed germination and seedling growth of *Amaranthus retroflexus* L., *Chenopodium album* L. Bosc ex Moq. *and Rumex crispus* L., the most active ones were found alcohols, aldehydes and ketones as bornyl acetate, geranyl acetate and linalyl acetate (26). Limonene, camphor and bornyl acetate were also found as the major compounds in leaves and fresh litter leachate of *Juniperus ashei* Buchholz, which showed inhibitory activity on *Bouteloua curtipendula* (Michx.) Torr., both in laboratory and field conditions (27). Regarding the allelopathic activity of monoterpenic compounds released from roots, camphor, 1,8-cineol and neryl isovalerate released from roots of *A. tridentata* in hydroponic cultures were found active in seedling growth bioassays of *Nicotiana attenuate* Torr. ex S.Watson (19).

These investigations agree in attributing the higher activity to water soluble compounds (28). Nevertheless, as pointed out by Abrahim, D. (29) in a study concerning camphor, 1,8-cineol, limonene and α-pinene effects on germination, primary root growth and mitocondral respiration of maize, the hypothesis that monoterpenes can interfere with germination should not be excluded. Despite their low water solubility, a small but constant flux into the soil can lead to a continuous uptake by seeds. This way, the potential activity of the high proportion of hydrocarbon monoterpenes found in *A. absinthium* roots should be not underestimated.

Worthy of mention is the significant increase of the compounds with higher allelopathic potential (oxigenated monoterpenes no esters) during the most active period in vegetative cycle, when the plants need to defense against competing ones. Regarding the aerial parts, this is particularly evident in the linalool variation throughout the vegetative cycle, the same way as it happens with other minor compounds as borneol and α–terpineol. Likewise, the progressive increasing of camphor from the beginning up to full blooming can be interpreted in the same way. Concerning essential oil roots composition, leaving aside the allelopathic potential of major and more hydrophobic compounds (hydrocarbons and monoterpenic esters), oxygenated monoterpenes no esters show also a significant increase from vegetative growing up to seeds ripening.

Conclusions

Chemical composition of essential oils from aerial parts and roots of chemotypes A and B of *A. absinthium*. were found considerably different. Oxygenated monoterpenes with acyclic and pinane skeletons compounds predominate in chemotypes A and B of aerial parts, respectively. Monoterpenic hydrocarbons and esters with acyclic, camphene and menthane molecular skeletons were found the major components in roots essential oil. Sesquiterpenic compounds can be considered as minor ones both in aerial parts and roots. Only β-caryophyllene and germacrene-D reach appreciable amounts in aerial parts, as well as caryophyllene oxide in roots. These data can be together interpreted with those from other chemotypes of *A. absinthium* (6) or other *Artemisia* species (8, 17), taking into account the specificity of metabolic pathways in aerial parts and roots.

Despite the high intra-populational variability already referred in literature, significant seasonal variations have been found concerning major compounds, even more, when chemotypes A and B (2) are independently considered. Consequently, to collect material in any time of vegetative cycle could not be indifferent. Anyway, if seasonal variations of aerial parts and roots are compared, a higher stability can be appreciate in last ones, mainly, with respect to monoterpenic hydrocarbons. The appearance of these statistically significant differences with regard to previous studies (4, 16) could be due to the higher shortness of vegetative cycle. Indeed, despite its perennial character, *A. absinthium* plants remain in the studied population as a little shoots from end of fall up to middle spring.

The invasive behavior of *A. absinthium* can be explained owing to the occurrence of a number of potential allelopathic compounds which could be released in soil. Given that the noteworthy differences in monoterpenic composition between aerial parts and roots, further studies to determinate their concentration in soil should be conducted in order to clarify the role of monoterpenic compounds in allelopathic activity of *A. absinthium*. As a consequence from its high proportion, a particular attention should be paid to *cis*-epoxyocimene –the main compound in chemotypes A and B of aerial parts- which can be expected an active allelopathic compound.

In summary, the results of this study can lead to pose several questions which may open new research perspectives in order to a successful application in field conditions of products based on *A. absinthium* essential oils. Firstly, (1) to determinate what compounds from aerial parts or roots of *A. absinthium* can be found in rizhosphere, (2) to test the possible interactions between essential oil components and soil material, (3) which plants share their habitat with *A. absinthium* and which are present in the population vicinity but does not inside, in order to know the possible target species in later bioassays.

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Display quotations of over 40 words, or as needed.

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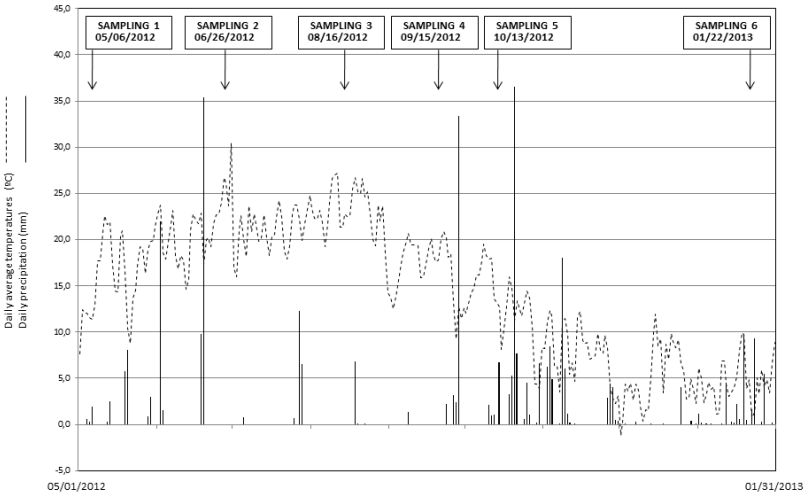
Heading 4: create the heading in italics. Run the text on after a punctuation mark.

1. This is a note. The style name is Footnotes, but it can also be applied to endnotes.


SAMPLING 1
05/06/2012
SAMPLING 2
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10/13/2012
SAMPLING 6
01/22/2013
Daily average temperatures (°C)
Daily precipitation (mm)
45,0
40,0
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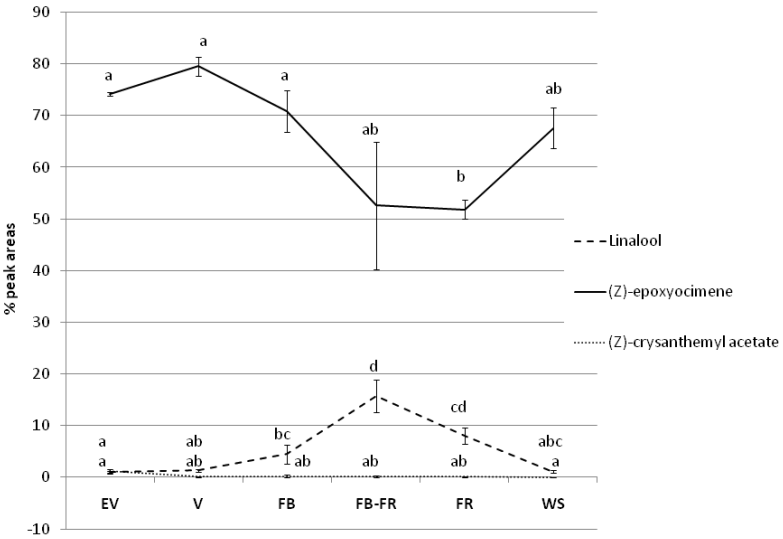
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Linalool
(Z)-epoxyocimene
(Z)-crysanthemyl acetate
EV
V
FB
FB-FR
FR
WS

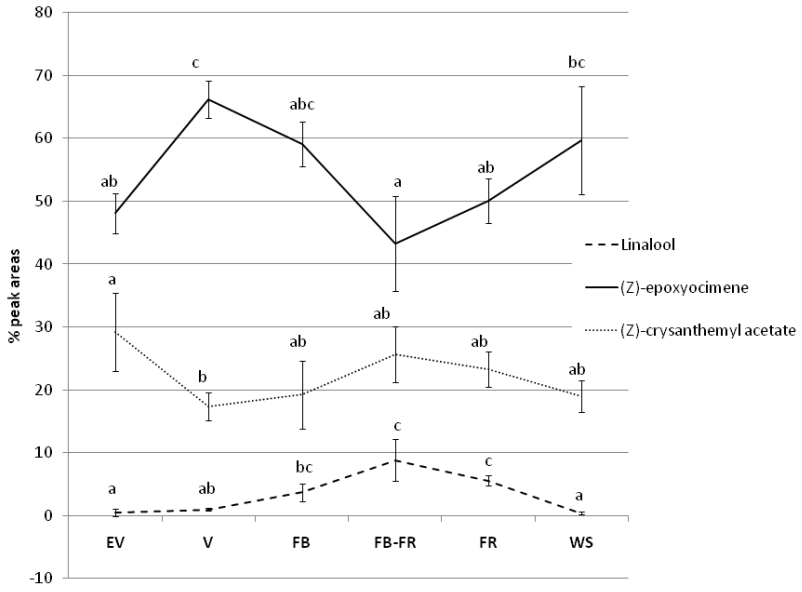
% peak areas
Linalool
(Z)-epoxyocimene
(Z)-crysanthemyl acetate
EV
V
FB
FB-FR
FR
WS

Table 1

Location	Haro[1] (Rioja)	Tarazona[2] (Zaragoza)	Calamocha[1] (Teruel)
Altitude (m.a.s.l.)	479	480	884
Average yearly temperatura (ºC)	12.7	12.8	10.2
Average yearly precipitation (mm)	646	465	414
Simple continentality index[3](Ic)	15.6	18.7	17.7
Simple continentality index (compensated by altitude) (Ic')[4]	18.5	21.6	23.0
Thermicity index (It)[5]	223	247	155

Notes: [1]Values obtained from literature (20); [2]Values calculated from database (21);
[3]$I_c = T_{max}-T_{min}$, (T_{max}: Average temperature of hottest month, T_{min}: Average temperature of coldest month) (22);
[4]$I_c' = I_c$ + [altitude (0.6/100)] (22); [5]$I_t = (T + M + m)\ 10$, (T: average yearly temperature; M: average highest temperatures of coldest month, m: average lowest temperatures of coldest month) (22)

Table 2a

				Phenological stage[1]					
				EV	V	FB	FB-FR	FR	WS
	Yield[2]			0.2 ±0.04a	0.5±0.15bc	0.6±0.16c	0.4±0.07b	0.5±0.07bc	0.2±0.08a
COMPOUND[3]	RI[4]	RI[5]	ID method[6]						
(*E*)-2-hexenal	856	855	MS, RI	-	tr[7]	tr	-	tr	-
α-pinene	938	939	MS, RI, std	0.3±0.45a	0.8±0.29a	0.6±0.38a	0.8±0.28a	0.8±0.21a	0.6±0.49a
camphene	953	954	MS, RI, std	tr	0.1±0.16a	0.3±0.19a	0.2±0.16a	0.2±0.16a	0.2 ±0.00a
sabinene	974	975	MS, RI	tr	tr	0.2 ±0.23	tr	tr	-
β-pinene	979	979	MS, RI, std	0.7±0.91a	1.1±1.09a	-	0.4±0.82a	0.3±0.26a	0.8±0.46a
octan-3-ol	990	991	MS, RI	-	tr	-	-	-	-
myrcene	994	990	MS, RI, std	0.2±0.14a	0.3±0.14a	0.3±0.17a	0.6±0.99a	0.2±0.11a	0.2±0.57a
limonene	1031	1029	MS, RI, std	tr	tr	0.1±0.00a	tr	tr	0.1±0.00a
(*Z*)-β-ocimene	1040	1037	MS, RI, std	0.7±0.26ab	1.2±0.48ab	1.9±0.88b	0.9±0.76ab	0.5±0.10a	0.5±0.42a
(*E*)-β-ocimene	1051	1050	MS, RI	-	tr	tr	-	-	-
terpinolene	1085	1088	MS, RI, std	tr	tr	tr	-	-	tr
α-pinene epoxide	1096	1099	MS, RI	0.3±0.12ab	0.2±0.04a	0.2 ±0.08a	tr	0.1±0.05a	0.4±0.06b
linalool	1107	1096	MS, RI, std	0.7±0.50a	1.1±0.27a	4.2±1.55b	10.4±3.80c	6.5±1.66bc	0.8±0.35a
1-octen-3-ol acetate	1111	1112	MS, RI	0.1±0.25a	0.40 ±0.06a	-	0.7 ±1.61a	tr	-
β-thujone	1116	1114	MS, RI, std	0.1±0.05a	-	0.2±0.18a	0.3±0.19a	0.3±0.16a	0.1±0.07a
(Z)-β-epoxyocimene population	1142	1132	MS, RI	58.5±14.45ab	71.5±7.67b	66.1±7.24ab	49.3±12.30a	50.8±2.86a	64.4±2.39ab

chemotype A				74.2±0.28a	79.5±1.82a	70.8±4.05a	52.6±12.31ab	51.9±1.83b	67.6±3.88ab
chemotype B				48.1±3.20a	66.2±3.00b	59.1±3.52ab	43.3±7.59a	50.0±3.56ab	59.7±8.63ab
(*E*)-β-epoxyocimene	1149	1142	MS, RI	2.1±0.58a	2.3±0.55a	2.5±0.99ab	3.0±1.54ab	3.3±1.49ab	4.7±0.16b
camphor	1145	1146	MS, RI, std	0.8±1.75a	1.4±2.05a	5.0±3.19a	3.1±3.46a	4.6±3.04a	5.3±0.00a
(*Z*)-chrysanthemol	1167	1164	MS, RI	tr	0.1±0.03a	0.1±0.05a	0.2±0.11a	0.1±0.09a	-
borneol	1173	1169	MS, RI, std	0.9±1.32a	0.7±0.46a	2.4±3.13a	1.6±1.49a	7.1±6.15a	2.8±5.76a
terpinen-4-ol	1182	1177	MS, RI, std	0.3±0.22a	tr	0.3±0.22a	0.1±0.14a	0.6±0.40a	0.2±0.10a
(*Z*)-3-hexenyl butanoate	1188	1186	MS, RI	-	-	tr	-	tr	-
α-terpineol	1195	1188	MS, RI, std	0.4±0.22a	0.4±0.20a	0.6±0.16a	0.7±0.56a	0.4±0.41a	tr
(*Z*)-geraniol	1231	1229	MS, RI, std	tr	-	-	tr	0.3 ±0.44a	0.2 ±0.60a
(*E*)-chrysanthemyl acetate	1238	1238	MS, RI	tr	-	tr	-	-	-
(*Z*)-chrysanthemyl acetate									
population	1267	1265	MS, RI	18.0±15.96a	10.4±9.57a	7.8±10.76a	17.1±13.63a	14.0 ±12.83a	7.6±12.16a
chemotype. A				1.1±0.40a	tr	0.2±0.20a	0.2±0.11a	0.1±0.14a	-
chemotype B				29.2±6.19a	17.3±2.21b	19.2±5.43ab	25.6±4.45ab	23.2±2.80ab	19.0±2.52ab
bornyl acetate	1285	1288	MS, RI, std	0.2 ±0.26a	-	-	-	-	0.2±0.11a
α-copaene	1374	1376	MS, RI	tr	tr	-	-	-	-
β-bourbonene	1381	1388	MS, RI	0.1±0.10	-	-	-	tr	tr
(*Z*)–jasmone	1390	1392	MS, RI	-	tr	-	-	-	-
β-caryophyllene	1416	1419	MS, RI	0.7±0.16ab	1.2±0.42abc	1.1±0.63abc	1.5±0.43bc	1.6±0.52c	0.5±0.04a
α-humulene	1453	1454	MS, RI	tr	0.1±0.02	tr	tr	-	-
(Z)-muurola-4(14), 5-diene	1465	1466	MS, RI	tr	-	-	-	tr	-

(*E*)-cadina-1(6),4-diene	1474	1476	MS, RI	-	-	tr	-	-	0.8±0.03
germacrene-D	1480	1485	MS, RI	2.5±0.41a	1.3±0.37b	0.7±0.17bc	0.8±0.22bc	0.6±0.27c	0.1±0.15d
β-selinene	1486	1490	MS, RI	-	tr	0.4±0.23a	0.4±0.61a	tr	-
valencene	1492	1496	MS, RI	-	0.2±0.13a	-	-	0.3±0.00b	0.3±0.16ab
α-selinene	1496	1498	MS, RI	tr	tr	-	-	-	-
γ-cadinene	1512	1513	MS, RI	0.5±0.41a	tr	tr	tr	-	0.3±0.34a
hymachalene <alpha-dehydro-ar>	1518	1517	MS, RI	tr	0.1±0.13a	tr	0.3±0.26a	tr	-
(*E*)-nerolidol	1561	1563	MS, RI	0.1±0.10a	-	0.1±0.09a	0.1±0.17a	0.1±0.18a	tr
neryl, 2-methylbutanoate	1572	1579	MS, RI[8]	0.3±0.18a	-	-	0.1±0.19a	0.1±0.20a	0.2±0.38a
caryophyllene oxide	1577	1583	MS, RI	0.1±0.08a	0.1±0.05a	0.3±0.20ab	0.3±0.20ab	0.5±0.27b	-
geranyl isovalerate	1605	1607	MS, RI	0.8±0.53a	0.1±0.06b	0.3±0.09ab	0.2±0.12b	0.1±0.16b	0.4±0.28ab
β–cedrene epoxide	1620	1622	MS, RI	0.2±0.34a	tr	-	0.2±0.31a	-	-
(*Z*)-methyl jasmonate	1642	1649	MS, RI	0.2±0.18a	0.2±0.17a	0.4±0.08a	0.4±0.46a	0.3±0.24a	tr
intermedeol	1661	1666	MS, RI	0.9±1.04a	0.5±0.26	0.4±0.42a	0.6±0.51a	0.8±0.24a	1.5±0.53
α-bisabolol	1684	1685	MS, RI	0.4±0.25a	0.2±0.11a	0.1±0.12a	0.2±0.20aa	0.2±0.12a	tr
chamazulene	1729	1731	MS, RI	1.9 ±1.98a	1.3 ±0.71a	1.1 ±1.53a	1.1 ±1.20a	0.5 ±0.5a	2.3 ±0.04a
(*Z*)-nuciferyl propanoate	1890	1893	MS, RI[8]	tr	-	-	-	tr	-
(*E*)-nuciferyl butanoate	2010	2012	MS, RI[8]	0.8±0.14a	0.4±0.09a	0.5±0.08a	0.9±0.75a	0.8±0.40a	0.6±0.37a
TOTAL IDENTIFIED				**92.0**	**97.4**	**98.2**	**96.5**	**96.0**	**96.1**
MONOTERPENES				**1.9**	**3.5**	**3.4**	**2.9**	**2.0**	**2.4**
OX. MONOTERPENES				**81.4**	**88.2**	**89.8**	**86.2**	**88.4**	**87.3**
SESQUITERPENES				**5.7**	**4.1**	**3.3**	**3.8**	**3.0**	**4.3**

OX. SESQUITERPENES	**2.4**	**1.2**	**1.3**	**2.2**	**2.3**	**2.1**
OTHER	**0.3**	**0.6**	**0.4**	**1.1**	**0.3**	**0.0**

Notes:

1. EV: Early vegetation (05/06/2012); V: Vegetation (06/26/2012); FB: Full bloom (08/18/2012); FB-FR: Full bloom-beginning of fruit maturation (09/15/2012); FR: Advanced fruit maturation (10/14/2012); WS: Winter shoots (01/22/2013)
2. % -V(mL) essential oil/ m(g) of fresh material-. Values within a row for each compound having different letters are significantly different from each other according Tukey's HSD test ($P<0.05$).
3. Compounds are listed in order of their elution from a ZB-5 column.
4. Kovats retention indices as determined on ZB-5 column using homologous series of n-alkanes.
5. Kovats retention indices from literature (23), except for those marked with[8]
6. Methods of identification: MS, by comparison of the mass spectrum with those of the computer mass libraries; RI, by comparison of RI with those from the literature; std, by co-injection of an authentic sample.
7. tr: traces (<0.1%)
8. Kovats retention indices from literature (8)

Table 2b. Composition of essential oil of *Artemisia absinthium* roots throughout the vegetative period

				Phenological stage[1]					
				EV	V	FB	FB-FR	FR	WS
COMPOUND[2]	RI[3]	RI[4]	ID method[5]						
santene	890	888	MS, RI	0.4±0.21a[6]	0.5±0.21a	0.4±0.13a	0.5±0.47a	0.7±0.35a	tr[7]
santolina triene	910	908	MS, RI	0.2±0.03a	0.2±0.03a	0.10 ±0.08a	tr	0.1±0.08a	-
tricyclene	924	926	MS, RI	tr	0.1±0.08a	0.1±0.08a	tr	0.2±0.04a	tr
α-pinene	935	939	MS, RI, std	1.5±0.33a	1.9±0.45a	2.0±0.64a	1.9±0.34a	2.1±0.49a	2.0 ±0.69a
α-fenchene	952	952	MS, RI	20.4±4.48a	23.7±7.80a	13.9±9.24a	12.4±4.42a	14.2±6.47a	20.3±11.39a
camphene	954	954	MS, RI, std	1.2±0.78a	1.9±1.48a	2.4±1.59a	1.8±1.22a	2.9±0.78a	0.9±1.09a
sabinene	974	975	MS, RI	0.3±0.03a	0.3±0.05a	0.4±0.13a	0.5±0.11a	0.4±0.21a	0.5±0.77a
β-pinene	979	979	MS, RI, std	1.4±0.22a	1.3±0.34a	1.7±0.39a	2.4±1.16a	2.3±1.69a	1.0±0.73a
β-myrcene	994	991	MS, RI, std	29.2±0.95a	22.1±4.90a	21.9±10.98a	31.9±4.10a	25.6±9.02a	20.2±7.61a
α–phellandrene	1006	1002	MS, RI	-	-	0.3±0.27a	0.2±0.23a	0.5±0.58a	-
p-cymene	1025	1024	MS, RI, std	-	-	tr	-	tr	tr
limonene	1031	1029	MS, RI, std	0.8±0.29a	0.9±0.41a	1.1±0.30a	1.2±0.12a	0.8±0.67a	0.4±0.36a
(Z)-β-ocimene	1040	1037	MS, RI, std	tr	-	-	-	-	tr

linalool	1099	1096	MS, RI, std	-	-	0.1±0.17a	-	-	0.2±0.32a
(*Z*)-β−epoxyocimene	1131	1132	MS, RI	-	0.3±0.28a	3.0 ±3.54b	tr	tr	tr
camphor	1145	1146	MS, RI	tr	0.2 ±0.19a	0.6 ±0.26a	0.3±0.12a	0.42 ±0.27a	tr
borneol	1173	1169	MS, RI std	0.2 ±0.12a	0.6 ±0.35ab	1.5±1.34ab	1.4±0.79b	2.1±0.71b	0.1±0.13a
(Z)-geraniol	1229	1227	MS, RI, std	tr	0.2±0.20ab	1.4±1.26ab	1.1 ±0.64ab	1.6±1.16b	tr
methyl camphenoate	1249	1257[8]	MS, RI	0.2±0.14a	0.3±0.21a	0.4±0.28a	0.5±0.28a	0.6±0.31a	tr
(Z)-chrysamthemyl acetate	1257	1262	MS, RI	0.3±0.21a	tr	0.2±0.22ab	tr	-	0.3±0.54a
bornyl acetate	1288	1288	MS, RI, std	14.8 ±2.73a	21.1±2.09bc	19.2±2.47abc	17.8±2.69ab	20.1±2.37abc	25.8±6.41c
carvacrol	1302	1303	MS, RI, std	0.3±0.17a	0.2±0.14a	0.3±0.25a	0.5±0.33a	0.5±0.42a	tr
myrtenyl acetate	1321	1326	MS, RI	0.3±0.13a	0.2±0.20a	0.6±0.10a	0.4±0.31a	0.4±0.26a	0.30±0.49a
dihydrocarveol acetate iso	1332	1329	MS, RI	tr.	tr.	tr	-	tr.	-
β-terpinyl acetate	1347		MS	10.6±4.83a	5.8±4.48a	14.2±2.42a	9.9±6.63a	10.1±7.15a	10.1 ±13.10a
dihydrocarveol acetate neoiso	1361	1359	MS, RI	tr	-	0.1±0.12a	0.3±0.06a	0.3±0.20a	-
linalyl isobutanoate	1371	1375	MS, RI	0.1±0.08a	0.2±0.13a	0.1±0.16a	-	tr	n.d.
geranyl acetate	1375	1381	MS, RI	3.8±0.19ab	5.4±1.41b	3.5±1.62ab	5.8±1.76b	5.1±1.05b	1.8±1.54a
(*E*)-myrtanol acetate	1380	1386	MS, RI	tr	tr	0.2±0.24a	0.1±0.18a	0.3±0.28a	-
β-caryophyllene	1416	1419	MS, RI	0.2±0.12a	0.3±0.09a	0.3±0.18a	0.4±0.13a	-	-

α -terpinyl isobutanoate	1466	1473	MS, RI	tr	tr	tr	0.1±0.12a	0.2±0.15a	-
isobornyl n-butanoate	1480	1475	MS, RI	1.1±0.77 a	1.7±1.41 a	3.1±1.63 a	1.8±0.84 a	2.9±2.74 a	1.8±1.73 a
neryl isobutanoate	1485	1490	MS, RI	0.5±0.43 a	0.7±0.42 a	0.1±0.10 a	0.3±0.18 a	0.1±0.18 a	0.4±0.27 a
geranyl isobutanoate	1508	1514	MS, RI	0.2±0.18	0.3±0.19	tr	0.4±0.06	0.3±0.19	0.1±0.11
neryl isovalerate	1578	1582	MS, RI	4.5±1.86a	0.2±0.03b	0.1±0.08b	tr	0.1±0.08b	tr
caryophyllene oxide	1586	1583	MS, RI	1.9±1.39 a	1.6±1.25 a	0.5±0.43 a	0.4±0.44 a	0.5±0.35 a	0.4±0.52 a
geranyl-2-methylbuthanoate	1603	1601	MS, RI	tr	0.1±0.12 a	0.1±0.17 a	0.3±0.50 a	0.1±0.16 a	-
geranyl isovalerate	1605	1607	MS, RI	0.7±0.73a	0.9±0.66a	-	tr	-	tr
hexadecanoic acid	1950	1960	MS, RI	2.6±0.42a	0.8±0.58bc	0.1±0.19c	tr	0.1±0.13c	1.9±2.62ab
monoterpenes			**55.4**	**52.9**	**44.3**	**52.8**	**49.8**	**45.3**	
ox. monoterpenes (no esters)			**0.7**	**1.8**	**7.3**	**3.8**	**5.22**	**0.3**	
monoterpenic esters			**36.9**	**36.6**	**41.5**	**37.2**	**40**	**40.6**	
sesquiterpenoids			**2.1**	**1.9**	**0.8**	**0.8**	**0.5**	**0.4**	
others			**2.6**	**0.8**	**0.1**	**0**	**0.1**	**1.9**	
Total identified (%)			**97.7**	**94.0**	**94.0**	**94.6**	**95.62**	**88.5**	

1. EV: Early vegetation (05/06/2012); V: Vegetation (06/26/2012); FB: Full bloom (08/18/2012); FB-FR: Full bloom-beginning of fruit maturation (09/15/2012); FR: Advanced fruit maturation (10/14/2012); WS: Winter shoots (01/22/2013)
2. Compounds are listed in order of their elution from a ZB-5 column.
3. Retention indices as determined on ZB-5 column using homologous series of n-alkanes.
4. Retention indices from literature (23)
5 Methods of identification: MS, by comparison of the mass spectrum with those of the computer mass libraries; RI, by comparison of RI with those from the literature; std, by injection of an authentic sample.
6. Values within a row for each compound having different letters are significantly different from each other according Tukey's HSD test ($P<0.05$).
7. tr: traces (<0.1%);

Table 3. Major compounds in roots for each one of the aerial parts chemotypes

Major compounds in roots	Chemotype A (% peak areas)	Chemotype B (% peak areas)
α−fenchene	17.7±10.35	18.4±6.39
β−myrcene	25.6±6.24	23.5±9.30
bornyl acetate	19.5±4.18	20.2±5.04
terpinyl acetate	10.6±7.77	9.4±6.03
geranyl acetate	4.0±1.31	4.0±2.00
geranyl isovalerate	17.7±10.35	18.4±6.39

Note: no significant differences were found by means of Tukey test ($P<0.05$) in any case.