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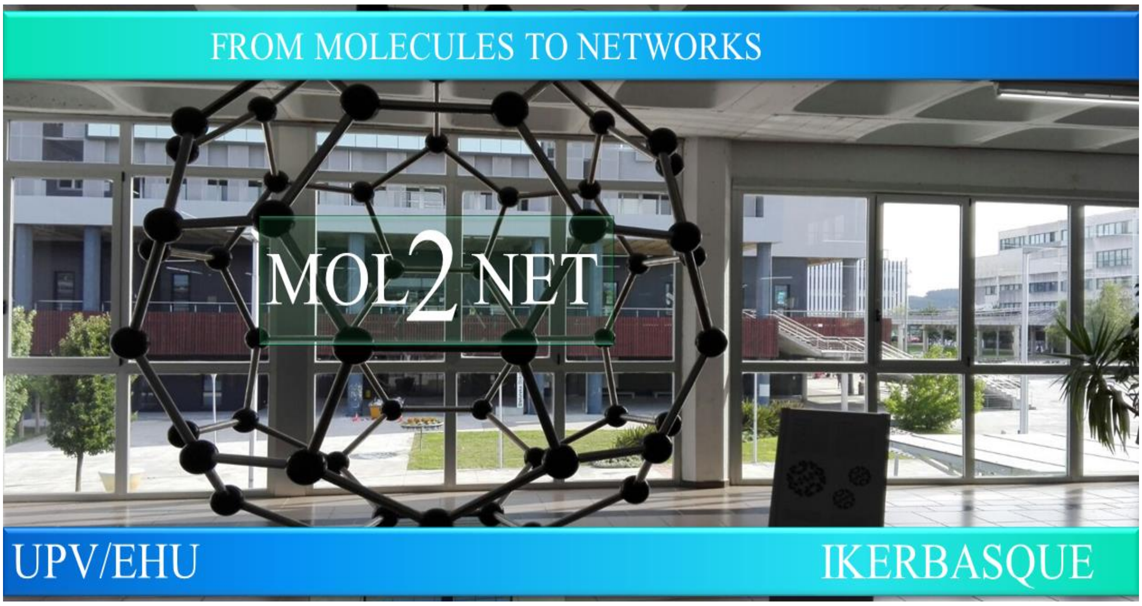

Volatile composition of the critically endangered plant *Thymus webbianus* Rouy from the Natural Park "Penyal d'Ifach" (Calp, Spain)

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Abstract.

<u>Thymus webbianus</u> Rouy (Lamiaceae) is an endemic plant known from only two populations located in the Natural Park "Penyal d'Ifac" (Calp), and in the Natural Park of Serra Gelada (Benidorm-Alfàs del Pi) in Spain. At the Spanish state level, it has been listed as "Critically Endangered", and "Vulnerable" in the Valencian Region. Moreover, its taxonomic position remains unclear, and further research on this species is needed. Therefore, this work was aimed to characterize the chemical composition in volatiles from four subpopulations located in the Natural Park "Penyal d'Ifach" through HS-SPME-GC-MS. An overall of 53 compounds were identified in these samples. Three major compounds (representing more than 5 % in peak area) were identified in all samples: β-caryophyllene (S1= 9.23 ± 0.19, S2= 6.29 ± 0.20, S3= 11.25 ± 0.74, and S4= 11.26 ± 0.34), D-limonene, (S1=5.01 ± 0.21, S2= 7.95 ± 0.34, S3= 10.54 ± 1.17, and S4= 9.22 ± 0.51), and bicyclogermacrene (S1= 5.21 ± 0.19, S2= 7.24 ± 0.79, S3= 7.07 ± 0.4, and S4= 5.37 ± 0.43). The other compounds were presented in lower amounts, and/or in a more reduced number of samples. The richness in β-caryophyllene, D-limonene, and bicyclogermacrene might be used as a marker, to separate T. webbianus from T. vulgaris. Also, the lack of thymol (a widespread terpene within the genus Thymus) could be characteristic of T. webbianus. However, given the large amount of chemotypes described for T. vulgaris this idea has to be treated with caution, and further studies on the phytochemistry of T. webbianus (including samples from T. vulgaris in the nearby, as well as samples from the natural hybrid T. ×valentinus) should be performed in order to clarify whether the chemical profile may be useful for the taxonomical arrangement of the species, and to establish its conservation status.

Keywords: *Gas Chromatography-Mass Spectrometry*, *threatened plants, <u>Thymus webbianus</u>, volatiles*

Introduction

Sea cliffs show a great heterogeneity in microenvironments, where many of them act as refuges for exclusive plant taxa (Costa, 1999; Strumia et al., 2020; Barnard et al., 2021). For this reason, it is not surprising that a large number of rare, and endemic plant species are found in these ecosystems, and restricted to a very limited territory (Laguna et al. 1998; Bañares et al., 2010; Buira et al., 2016). *Thymus webbianus* Rouy (Lamiaceae) is a good example of these endemic plants, and it grows in calcareous maritime rocks in the Southern Valencian Region of Spain (Crespo et al., 2003; Moreno et al., 2012). To date, its presence is only known in two populations located in the Natural Park "Penyal d'Ifac" (Calp, Alicante province), and in the Natural Park of Serra Gelada (Benidorm-Alfàs del Pi, Alicante province). Overall, 300 individuals were counted a decade ago (Iriondo et al., 2009; Aguilella et al. 2010). However, the current demographic situation of this species is unknown. At the Spanish state level, it has been listed as "Critically Endangered" (Bañares et al., 2010), and as "Vulnerable" in the Valencian Region (Order 2/2022, of February 16). However, no information is available at higher geographical levels (Eurpean, Mediterranean, or global scopes) since its European conservation assessment is still lacking in specialized reports (Bilz et al. 2011), or in reference datasets such as the Red List of the International Union for Nature Conservation, IUCN (https://www.iucnredlist.org/).

On the other hand, the taxonomical position of this species remains controversial. It has been considered as a separated species from closely related *Thymus* (*T. vulgaris* L.) due to the presence of rigid hairs on leaf margins and/or petioles, glabrescent abaxial side of leaves, bigger corollas, and often decumbent habit (Laguna et al., 1998; Mateo and Crespo 2014). However, a more synthetic proposal within the genus *Thymus* has been placed it within the natural variability of the widely-distributed *T. vulgaris* (Morales, 1986; 2010; Ferrer-Gallego et al. 2013). This controversy is also motivated due to the remarkable morphological variability of *T. webbianus* individuals in the known populations, which is also increased due to the fact that the natural hybrid between *T. vulgaris*, and *T. webbianus* (*T.* ×*valentinus*) is also presented in the distribution area (Crespo et al., 2003; Iriondo et al. 2009).

According to Morales (2010), the *T. webbianus* populations from Penyal d'Ifach are morphologically different to the other populations screened of *T. vulgaris*. These populations show the characters stated in the original description of the species that motivated its consideration as a separated taxon from *T. vulgaris* (Rouy, 1988). In this context of uncertainty, Morales (2010) stated that further research on this species is needed in order to clarify the taxonomical position of *T. webbianus* within the complex of *T. vulgaris*. Detailed information on the taxa identity, including results from the chemophenetic, molecular, and morphological fields, is necessary in order to design accurate plant conservation strategies (Richard and Evans, 2005; Laguna et al., 2004; Heywood 2007; Rouhan and Gaudeul, 2014; Ely et al. 2017). This information is particularly important for megadiverse groups such *Narcissus* (Berkov et al. 2020), *Hieracium*, and *Pilosella* (Willer et al. 2021), or *Thymus* (Laguna et al. 1998). Early studies have been characterized flavonoids, and phenolics from *T. webbianus* (Blázquez et al. 1990; 1994; Zafra-Polo et al. 1990). The most abundant compound of the essential oil was germacrene B, followed by terpinen-4-ol, β-caryophyllene, 1,8-cineole, and borneol. However, a more in depth research on its chemical composition, and the variability present among the natural populations is certainly interesting for a better characterization of the species.

Therefore, the aim of this study was to characterize the chemical profile of *T. webbianus* in four subpopulations of the Natural Park "Penyal d'Ifach" in order to provide new data on its taxonomical identity and, in turn, to provide more accurate insights into the conservation needs of this critically endangered endemic plant from the Iberian Peninsula.

Materials and Methods

Plant collection

A field campaign was developed during the last week of April 2022 in the distribution area of the species. A screening on those subpopulations showing the distinctive characters described for *T. webbianus* (presence of rigid hairs on leaf margins and/or petioles, glabrescent abaxial side of leaves, big corollas, and decumbent habit) was done in the subpopulations present in the Natural Park "Penyal d'Ifach" (municipality of Calp, Valencian Region, Spain), and selected flowering individuals were harvested. Then, the plant samples were dried in the dark at room temperature conditions (≈ 22 ºC) until extraction for chemical analyses.

Plant extraction, and chemical characterization

After drying, samples (0.02 g) from the plant material were subjected to chemical analyses. A Solid Phase Micro-Extraction (SPME) was performed, and a further analysis by Gas Chromatography coupled to Mass Spectrometry (GC-MS) using the Headspace (HS) technique was done in order to determine the presence of volatile compounds, and estimate their relative content (percentage of peak area). The identification of volatile compounds was carried out using the spectrum library present in the equipment's software (NIST Chemistry WebBook). The analyses were carried out by triplicate.

Results and Discussion

An overall of 53 compounds were identified through HS-SPME-GC-MS in *T. webbianus* samples. Three major compounds (representing more than 5 % in peak area) were identified in all samples (Table 1): β-caryophyllene (S1= 9.23 ± 0.19, S2= 6.29 ± 0.20, S3= 11.25 ± 0.74, and S4= 11.26 ± 0.34), D-limonene, (S1=5.01 ± 0.21, S2= 7.95 ± 0.34, S3= 10.54 ± 1.17, and S4= 9.22 ± 0.51), and bicyclogermacrene (S1= 5.21 ± 0.19, S2= 7.24 ± 0.79, S3= 7.07 ± 0.4, and S4= 5.37 ± 0.43). 13 compounds are presented in more than 2% of peak area in, at least, 3 of the samples screened. These compounds include: sabinene, β-myrcene, α-terpinene, β-ocimene, γ-terpinene, cis-sabinene hydrate, linalool, camphor, p-cymene, endo-borneol, aromadendrene, eudesma-1,4(15),11-triene, and spathulenol (Table 1). The other 40 compounds are generally presented in lower amounts. The results obtained in the present work show certain variability of the volatile composition in the sampled subpopulations. When compared to the previous work made by Zafra-Polo et al. (1988) we found that terpinen-4-ol, β-caryophyllene, and borneol were also present, in remarkable amounts, as happens in our samples too. However, germacrene B, the most abundant compound detected in Zafra-Polo et al. (1988), as well as 1,8-cineole, where not detected in our work in none of the four samples screened. Also, thymol was not observed in our samples, neither in Zafra-Polo et al. (1988). As thymol is present (often in high amounts) in most of the species screened of *Thymus*, especially in Western-Mediterranean populations of *T. vulgaris* (Arraiza et al., 2009; Figuereido et al.

2010; Stahl-Biskup and Saez, 2002) the lack of this compound could be characteristic of *T. webbianus*.

Similarly, the richness in β-caryophyllene, D-limonene, and bicyclogermacrene might be used as a marker, to separate *T. webbianus* from *T. vulgaris*. However, given the large amount of chemotypes described for *T. vulgaris* (Satyal et al. 2016; Torras et al. 2007) this idea has to be treated with caution. Therefore, further studies on the phytochemistry of *T. webbianus* (including samples from *T. vulgaris* in the nearby, as well as samples from *T. ×valentinus*) should be performed in order to clarify whether the chemical composition of these populations corresponds to a particular chemotype of *T. vulgaris*, or (on the contrary) it can correspond to a separated taxon. Ideally, these works would gain accuracy in case of combining the chemical characterization, with morphological, and molecular data, in order to establish the taxonomical identity of *T. webbianus*, and finally its conservation needs.

Compounds	**RT (min)**	**CAS**	**Sample 1**	**Sample 2**	**Sample 3**	**Sample 4**
β-thujene	8,360	28634-89-1	1.80 ± 1.56	3.78 ± 0.20	0.83 ±0.04	2,03 ± 0.06
camphene	9,078	79-92-5	0.21 ± 0.02	0.18 ± 0.01	0.22 ± 0.04	0.24 ± 0.01
sabinene	9,984	3387-41-5	2.25 ± 0.07	3.49 ± 0.06	0.87 ± 0.01	2.08 ± 0.09
1-octene-3-ol	10,246	3391-86-4	0.41 ± 0.02	0.29 ± 0.02	0.04 ± 0.01	0.00 ± 0.00
β-myrcene	10,669	123-35-3	3.39 ± 0.30	0.75 ± 0.15	7.42 ± 0.71	3.53± 0.57
α-phellandrene	11,078	99-83-2	2.33 ± 0.13	2.66 ± 0.13	1.30 ± 0.10	1.81 ± 0.08
α-terpinene	11,513	99-86-5	4.55 ± 0.21	6.18 ± 0.26	1.63 ± 0.15	3.60 ± 0.06
p-cymene	11,766	99-87-6	2.46 ± 0.30	3.49 ± 0.53	2.13 ± 0.38	2.13 ± 0.03
D-limonene	11,934	5989-27-5	5.01 ± 0.21	7.95 ± 0.34	10.54 ± 1.17	9.22 ± 0.51
trans-β-ocimene	12,281	3779-61-1	0.66 ± 0.06	0.33 ± 0.02	0.38 ± 0.04	0.32 ± 0.05
β-ocimene	12,655	13877-91-3	6.63 ± 0.60	1.87 ± 0.34	2.01 ± 0.35	2.16 ± 0.26
γ-terpinene	12,982	99-85-4	5.64 ± 0.26	7.70 ± 0.25	2.52 ± 0.14	4.61 ± 0.08
cis-sabinene hydrate	13,247	15537-55-0	4.78 ± 0.19	5.59 ± 0.12	0.78 ± 0.04	2.80 ± 0.08
terpinolene	13,928	586-62-9	2.17 ± 0.12	2.75 ± 0.06	0.87 ± 0.17	1.51 ± 0.02
trans-sabinene hydrate	14,227	17699-16-0	1.93 ± 0.11	2.76 ± 0.08	1.32 ± 0.09	1.95 ± 0.03
linalool	14,376	78-70-6	6.13 ± 0.18	1.15 ± 0.02	4.39 ± 0.27	2.18 ± 0.18
1,2,4,5-tetramethylbenzene (durene)	14,908	95-93-2	0.30 ± 0.12	0.10 ± 0.01	0.16 ± 0.04	0.23 ± 0.00
cosmene	15,266	460-01-5	1.36 ± 0.07	0.31 ± 0.02	0.37 ± 0.04	0.31 ± 0.08
camphor	15,625	76-22-2	2.79 ± 0.07	1.00 ± 0.03	2.24 ± 0.21	2.08 ± 0.16
endo-borneol	16,241	507-70-0	1.32 ± 0.17	2.75 ± 0.12	2.63 ± 0.14	4.82 ± 0.19
terpinen-4-ol	16,558	562-74-3	2.75 ± 0.09	6.86 ± 0.28	0.83 ± 0.05	1.86 ± 0.05
α-terpinol	16,864	98-55-5	0.99± 0.01	1.31 ± 0.02	0.54 ± 0.00	0.67 ± 0.02
bornyl formate	17,696	7492-41-3	0.24 ± 0.04	0.06 ± 0.02	0.07 ± 0.02	0.07 ± 0.03

bornyl acetate	18,844	76-49-3	0.27 ± 0.28	0.24 ± 0.03	0.28 ± 0.01	0.17 ± 0.04
carvacrol	19,145	499-75-2	0.18 ± 0.04	0.00 ± 0.00	0.03 ± 0.00	0.04 ± 0.01
elixene / γ-elemene	19,776	3242-08-8/ 29873-99-2	1.85 ± 0.14	2.75 ± 0.28	2.18 ± 0.22	1.66 ± 0.06
α-cubebene	19,980	-	0.00 ± 0.00	0.00 ± 0.00	0.36 ± 0.02	0.24 ± 0.01
carvacrol acetate	20,373	6380-28-5	0.12 ± 0.01	0.22 ± 0.05	0.24 ± 0.09	0.19 ± 0.01
α-copaene	20,418	95910-36-4	0.08 ± 0.01	0.00 ± 0.00	0.52 ± 0.03	0.30 ± 0.01
(-)-β-bourbonene	20,573	5208-59-3	0.16 ± 0.01	0.15 ± 0.01	0.20 ± 0.01	0.17 ± 0.01
β-elemene	20,657	515-13-9	0.59 ± 0.05	0.17 ± 0.01	0.49 ± 0.01	0.51 ± 0.01
1,1,4,7-tetramethyl-1a,2,3,4,6,7,7a,7b-octahydro-1H-cyclopropa[e]azulene	20,748	405112-35-8	0.32 ± 0.02	0.64 ± 0.03	0.40 ± 0.04	0.30 ± 0.03
isocaryophillene	20,907	NIST 140072	0.29 ± 0.01	0.29 ± 0.04	0.51 ± 0.09	0.45 ± 0.02
β-maaliene	20,957	489-29-2	0.60 ± 0.02	0.67 ± 0.01	1.34 ± 0.08	0.68 ± 0.07
β-caryophyllene	21,139	87-44-5	9.23 ± 0.19	6.29 ± 0.20	11.25 ± 0.74	11.26 ± 0.34
β-vatirenene	21,224	27840-40-0	1.03 ± 0.06	1.09 ± 0.03	1.41 ± 0.02	1.21 ± 0.01
α-maaliene	21,334	489-28-1	0.61 ± 0.02	0.94 ± 0.19	0.70 ± 0.06	0.59 ± 0.03
aromandendrene	21,400	489-39-4	3.40 ± 0.09	4.86 ± 0.15	3.90 ± 0.40	2.97 ± 0.26
selina-5,11-diene	21,441	52026-55-8	0.99 ± 0.04	1.39 ± 0.08	1.48 ± 0.05	1.11 ± 0.03
D-germacrene	21,972	-	0.00 ± 0.00	2.38 ± 0.05	1.10 ± 0.13	0.85 ± 0.05
eudesma-1,4(15),11-triene	22,022	212394-95-1	2.51 ± 0.19	0.00 ± 0.00	2.98 ± 0.20	2.61 ± 0.13
bicyclogermacrene	22,193	24703-35-3	5.21 ± 0.19	7.24 ± 0.79	7.07 ± 0.40	5.37 ± 0.43
γ-cadinene	22,403	-	0.00 ± 0.00	0.00 ± 0.00	1,74 ± 0.14	0.99 ± 0.04
aromadendrene, dehydro-	22,494	NIST 156125	1.75 ± 0.10	1.73 ± 0.08	5.03 ± 2.14	4.24 ± 0.27
6-epi-shyobunol	22,561	-	0.00 ± 0.00	0.00 ± 0.00	2.11 ± 0.22	1.27 ± 0.10
α-cadinene	22,699	-	0.00 ± 0.00	0.00 ± 0.00	1.02 ± 0.07	0.77 ± 0.03
elemol	22,852	639-99-6	4.04 ± 0.20	0.00 ± 0.00	0.00 ± 0.00	2.94 ± 0.36
spathulenol	23,241	6750-60-3	2.19 ± 0.04	2.94 ± 0.28	3.41 ± 0.23	2.85 ± 0.32
α-eudesmol	24,160	473-16-5	1.71 ± 0.09	0.91 ± 0.02	1.74 ± 0.14	2.38 ± 0.54
thujopsenal	24,502	470-41-7	1.00 ± 0.09	1.05 ± 0.10	1.91 ± 0.30	1.92 ± 0.37
shyobunol	24,607		0.00 ± 0.00	0.00 ± 0.00	1.53 ± 0.23	0.93 ± 0.07
azulol	25,537	489-84-9	0.51 ± 0.02	0.63 ± 0.02	0.84 ± 0.07	0.69 ± 0.07
1,1'-biphenyl,2,4,6-trimethyl-	26,134		0.07 ± 0.01	0.08 ± 0.00	0.12 ± 0.02	0.11 ± 0.01

Table 1.- Volatile composition of four *Thymus webbianus* Rouy samples collected in the Natural Park "Penyal d'Ifach" (Alicante, Spain). The analyses were performed by Solid Phase Micro-Extraction (SPME), and a further analysis by Gas Chromatography-Mass Spectrometry (GC-MS) using the Headspace (HS) technique. Data are presented as means ± standard deviations.

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