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Article

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Abstract: Wild aromatic plants present high intra- and interpopulation chemical polymorphisms in response to the contrasted environmental and geographical conditions. Here, we have studied *Teucrium marum* L. (Lamiaceae), a western Mediterranean island endemic plant, very well-known from the phytochemical point of view, but scarcely investigated regarding the ecological factors that influence phytochemical diversity within and among populations. Specific aims were to: 1) define the chemical composition variability of the essential oils; 2) determine the inter- and intrapopulation chemical variability; and 3) evaluate if the elevation and/or the soil substrate determined the phytochemical variability of *T. marum* along a gradient from coastal to mountain wild populations in Sardinia island (Italy). *Teucrium marum* fresh aerial parts were randomly collected from ten individuals in six different localities in Sardinia: three coastal and three mountainous. Dried leaf samples were hydrodistillated using a classical Clevenger apparatus to obtain the corresponding essential oils. The composition of each essential oil was chemically characterized and analysed by gas chromatography coupled to mass spectrometry. Ninety compounds were identified in the essential oils of *T. marum* from the six studied wild Sardinian populations. Statistical analysis showed significant differences in the phytochemical essential oil composition among and within the studied populations, which clustered following a geographical pattern rather than a simple climatic or edaphic grouping.

Keywords: germander; cat thyme; aromatic plants; chemical variability; GC and GC–MS analysis; elevation; essential oils; inter- and intrapopulation variability.

1. Introduction

Plant secondary metabolites like terpenes and phenolic compounds are known to have multiple ecological roles, notably to protect plants against herbivores, pathogens and abiotic stresses, but also to modulate plants-plants or plants-animals interactions [1-5]. Their production depends on the physical and chemical properties of the ecosystem, like precipitation, temperature, edaphic conditions but also the elevation [2-5]. Therefore, wild aromatic plants present high chemical polymorphisms in response to the contrasted environmental and geographical conditions of their different populations leading to an important intra- and interpopulation chemical variability [6,7]. In the same way, the variability of essential oils (EOs) composition of natural populations is related also to various environmental, geographical and genetic factors, which influence, in combination or independently, the EOs quantitative and qualitative composition and properties [2,5,8-18]. Of the 153 genera (from 49 families) of vascular plants that accumulate EOs, 90 occur in Mediterranean type ecosystems [5], characterized by a climate regime with mild wet winters and warm and dry summers, and 90 % or more of the annual precipitation typically occurring in the 6 months around winter [19]. The Mediterranean basin, one of the five Mediterranean areas of the world, has a high geographical and ecological heterogeneity which caused and still causes original patterns of vascular plant evolution at the population, community and landscape levels [5]. For this reason, it has been recently defined as a complex, multi-hierarchical system of islands-within-islands [20]. In this context, the combinations of several evolutionary factors, such as richness in microhabitats and geographical isolation over long periods, have promoted further diversification among and within species, giving rise to a huge contingent of Mediterranean exclusive (endemic) species particularly abundant in harsh environments [21], where secondary compounds evolved mainly as a defence against herbivores [5]. Even if there are some well documented studies showing evidence of differentiation of morphologic, genetic and phytochemical aspects among and within populations of endemic aromatic plants growing in Mediterranean harsh environments [15,22,23], however the ecological factors influencing these patterns still need to be revealed.

In this research, therefore, we consider as a model species *Teucrium marum* L. (cat thyme, Lamiaceae), a western Mediterranean endemic dwarf shrub, very well-known under the phytochemical point of view, but scarcely investigated regarding the ecological factors that influence phytochemical diversity within and among populations. In fact, although the phytochemical composition of its essential oil (EO) was previously investigated in both Sardinia and Corsica, it is worth to be noted that several researches from Sardinia were done on only one or few populations [24-28], whereas the main investigation realized on Corsica, even if carried on 25 populations, was mainly focused on analytical procedures and cannot be used for ecological conclusions [29].

Therefore, the general purpose of our paper is to give valuable information on the effects that ecological factors have on the intra- and inter-population phytochemical diversity of this species. Specific aims were to: 1) define the chemical composition variability of EOs; 2) determine the inter- and intrapopulation chemical variability; and 3) evaluate if the elevation gradient and/or soil substrate determine the phytochemical variability of *T. marum* from different coastal and mountain wild populations in Sardinia island (Italy).

2. Results

2.1. Phytochemical analysis

Overall, 90 compounds were identified (Table 1) in the EOs of *T. marum* from six different wild Sardinian populations.

The phytochemical analyses (Table 1) showed that the main components detected in the EOs of *T. marum* obtained from the Cagliari population were β-Bisabolene (18.26 %), Dolichodial (15.65 %) and (*E*)-β-Caryophyllene (8.69 %); from the Alghero population Dolichodial (34.38 %), 2-Methylbutyl-2-methylbutyrate (5.65 %) and 2-Methylbutyl-butyrate (5.76 %); from the Costa Paradiso population (*E*)-β-Caryophyllene (12.54 %), Dolichodial (11.25 %) and β-Bisabolene (8.23 %); from the Limbara population (*E*)-β-Caryophyllene (19.99 %), β-Bisabolene (12.03 %) and β-Sesquiphellandrene (9.53 %); from the Osilo population (*E*)-β-Caryophyllene (12.84 %), 2-Methylbutyl-butyrate (9.54 %) and 2-Methylbutyl-2-methylbutyrate (7.58 %); and finally the principal compounds from the Urzulei population were β-Bisabolene (13.24 %), (*E*)-β-Caryophyllene (13.22 %) and β-Sesquiphellandrene (11.58 %).

The SIMilarity PERcentage (SIMPER) analysis (see supplementary materials Table S1) showed that the volatile compounds listed above with four other compounds were also important in population chemical differences namely the unidentified compound 1508 (4.7 % contribution to the average population dissimilarity), Limonene (7.3 %), 2-Undecanyl acetate (4.6 %) and (*E*)-α-Bergamotene (3.8 %) of the average population dissimilarity and thus to the inter-population chemical differences. In total these ten volatile compounds explained 90 % of the phytochemical diversity observed in the six studied Sardinian populations. High percentage of Dolichodial was found in Alghero (34.4 %), Cagliari (15.6 %) and Costa Paradiso (11.3 %) populations, while a very lower quantity in the population of Limbara (0.5 %) and modest quantities in the populations of Urzulei (5.7 %) and Osilo (5.3 %). Contrarily to Dolichodial, (*E*)-β-Caryophyllene was found in higher percentages in Limbara (20 %), Urzulei (13.2 %), and Osilo (12.8 %) populations than Costa Paradiso (12.5 %), Cagliari (8.7 %) and Alghero (4.4 %) ones. β-Bisabolene was found in higher percentages in Cagliari (18.3 %), Urzulei (13.2 %) and Limbara (12 %) populations, in modest quantities in Costa Paradiso (8.2 %) and Osilo (6.3 %) populations and in lower quantity in Alghero (2.7 %). High quantities of unidentified compound 1508 was found only in Cagliari (13 %) and only traces in Alghero (0.05 %) ad Osilo (0.15 %) populations. Instead Limonene was found only in Cagliari population (6.16 %).

Table 1. List of the components identified in essential oils obtained from six different wild Sardinian populations of *Teucrium marum* L. For each compound, the mean relative amount (%) per population from ten specimens is reported. CAG (Capo S. Elia – Cagliari, CA), ALG (Lazzaretto – Alghero, SS), CP (Costa Paradiso - Trinità d'Agultu e Vignola, SS), LIMB (Limbara - Tempio Pausania, SS), OSL (Osilo, SS) and URZ (Genna Silana – Urzulei, NU).

No. Compound	RI (ap.) *	RI (p.) **	Compound name	CAG	ALG	CP	LIMB	OSL	URZ
1	718	1199	2-Methylbutanol	0.04	0.07	0.06	-	0.25	0.08
2	773	1078	Hexanal	0.12	0.13	0.72	0.26	0.24	0.29
3	824	1218	2-Hexenal	0.07	0.03	0.01	0.16	0.14	0.21
4	846	1345	Hexanol	0.02	-	0.25	0.08	0.17	0.14
5	847	1336	2-Methylpropyl-propionate	-	0.15	-	-	-	-
6	857	1117	3-Methylbutyl acetate	0.59	1.00	1.02	0.21	0.78	1.36
7	930	1584	3-Heptenol	-	-	-	-	-	0.08
8	936	1266	2-Methylpropyl-butanoate	-	0.40	0.19	0.15	0.62	0.04
9	952	1183	3-Methylbutyl-propanoate	-	0.72	0.19	0.18	0.89	0.04
10	960	1334	6-Methyl-5-hepten-2-one	1.15	0.82	1.73	0.95	0.68	1.14
11	975	1265	Butyl-butyrate	-	0.02	0.03	0.02	0.04	-
12	977	1233	2-Pentyl-furan	-	-	-	0.03	0.03	0.02
13	981	1152	Myrcene	0.07	0.01	-	-	0.02	0.01
14	985	1314	(*Z*)-3-Hexenyl acetate	-	0.06	0.01	0.05	0.05	0.10
15	988	1265	2-Methyl-propyl-2-methylbutyrate	-	0.30	0.12	0.05	0.33	0.02
16	993	1268	Hexyl acetate	0.35	1.19	4.54	1.66	0.59	1.52
17	999	1436	4-Methylanisole	0.04	0.06	0.01	0.02	-	-
18	1001	1280	3-Methylbutyl-2-methylpropanoate	-	0.18	0.05	0.02	0.19	-
19	1021	1203	Limonene	6.16	-	-	-	-	-
20	1025	1230	(*E*)-β-Ocimene	0.45	0.06	0.02	0.02	0.10	0.01
21	1040	1258	2-Methylbutyl-butyrate	0.04	5.76	6.56	2.55	9.54	1.25
22	1070	1449	Octanol	0.03	-	0.01	0.01	0.02	0.02
23	1081	1424	Nonanal	0.01	-	0.02	0.11	0.18	0.07
24	1082	1542	Linalool	0.40	0.53	0.62	0.17	2.07	0.71
25	1086	1378	Hexyl propanoate	-	0.40	1.44	0.89	0.57	0.11
26	1088	1415	2-Methylbutyl-2-methylbutyrate	-	5.95	5.08	1.29	7.58	0.61
27	1092	1374	Octen-3-yl acetate	0.37	0.35	1.81	0.86	0.37	1.11
28	1123	1527	(*Z*)-Tagetone	-	0.01	-	0.01	0.05	0.03
29	1130	1475	Citronellal	1.73	0.70	0.39	1.12	1.14	0.49
30	1131	1733	1.4-Dimethoxy-benzene	0.09	0.17	0.21	0.19	0.10	0.06
31	1137	1493	3-Methylbutyl-pentanoate	-	0.03	0.02	0.01	0.14	0.01
32	1157	1671	Nonanol	-	0.01	0.01	0.01	0.02	0.01
33	1165	1662	(*E*)-3-Hexenyl-butyrate	-	0.05	0.02	0.09	0.15	0.11
34	1169	1674	α-Terpineol	-	0.01	-	-	0.09	0.03

35	1172	1664	Estragole + Hexyl butyrate	1.19	4.51	5.92	5.45	5.15	5.40
36	1199	1234	Isopentyl-hexanoate	0.04	-	0.01	-	0.14	0.05
37	1203	1800	3-4-Dimethoxytoluene	0.20	0.20	0.02	0.02	0.13	0.14
38	1207	1763	Citronellol	0.17	0.04	0.01	0.40	0.24	0.08
39	1211	1674	Neral	-	0.12	0.06	0.04	0.34	0.52
40	1213	1427	(*Z*)-3-Hexenyl-2-methylbutanoate	-	0.04	0.02	0.02	0.13	0.07
41	1220	1416	Hexyl-2-methylbutanoate	-	0.33	2.19	1.60	1.03	0.86
42	1222	1809	Phenylethyl acetate	0.18	0.38	0.45	0.13	0.11	0.86
43	1232	1840	Geraniol	0.04	0.05	0.03	0.02	0.70	0.15
44	1234	1475	2-Methylbutyl-hexanoate	0.02	0.09	0.07	0.08	0.44	0.28
45	1239	1724	Geranial	0.45	0.29	0.15	0.15	0.75	0.99
46	1253	1920	Dolichodial	15.65	34.38	11.25	0.51	5.32	5.77
47	1264	1937	Epidolichodial	2.54	4.76	1.15	0.02	0.83	1.12
48	1270	1592	2-Undecanone	0.20	0.07	0.22	0.05	0.20	0.31
49	1308	1807	6-Undecanol	0.01	-	-	-	0.03	0.01
50	1312	1819	Benzyl-butyrate	-	0.03	-	0.01	0.02	0.01
51	1325	2158	Eugenol	0.06	0.08	0.03	0.01	0.03	0.07
52	1331	1656	Citronellyl acetate	0.21	2.33	0.85	5.04	1.15	0.47
53	1357	1751	Geranyl acetate	3.29	3.45	2.49	2.20	3.44	2.13
54	1366	2113	Methyleugenol	0.07	0.19	0.20	0.27	0.37	0.65
55	1385	1402	Dodedacanal	-	-	0.01	-	0.02	0.03
56	1412	1588	(*E*)-β-Caryophyllene	8.69	4.42	12.54	19.99	12.84	13.22
57	1414	1654	2-Undecanyl acetate	-	0.02	-	-	0.02	6.18
58	1425	1847	(*E*)-Geranylacetone	0.07	0.23	0.21	0.32	0.30	0.17
59	1428	1579	(*E*)-α-Bergamotene	7.13	4.12	5.01	6.61	6.41	1.31
60	1431	1583	Sesquisabinene	2.51	-	-	0.01	0.01	0.04
61	1434	1635	β-Barbatene	0.02	0.05	0.30	0.36	0.21	0.31
62	1443	1661	(*E*)-β-Farnesene	0.46	0.64	0.10	0.40	0.21	0.25
63	1444	1659	α-Humulene	1.25	0.32	2.58	4.17	2.85	5.31
64	1458	1988	Isopentyl-phenylacetate	0.02	0.18	0.21	0.21	0.30	0.28
65	1470	1692	(*Z.Z*)-α-Farnesene	0.15	0.03	0.11	0.15	0.10	0.38
66	1478	1718	(*Z.E*)-α-Farnesene	0.21	0.77	0.35	0.69	0.99	0.39
67	1482	1691	α-Zingiberene	-	-	-	0.01	0.02	0.06
68	1488	1725	Cuparene	-	-	0.02	0.01	0.01	0.02
69	1496	1719	β-Bisabolene	18.26	2.72	8.23	12.03	6.33	13.24
70	1508	1767	Unidentified compound 1508	12.99	0.05	-	-	0.15	-
71	1510	1761	β-Sesquiphellandrene	-	2.02	6.28	9.53	5.04	11.58
72	1518	1751	(*E*)-γ-Bisabolene	-	-	0.03	0.04	0.01	0.07
73	1538	2083	(*Z*)-3-Hexenyl-benzoate	0.02	-	0.05	-	0.19	0.07
74	1543	2033	(*E*)-Nerolidol	0.13	0.28	0.17	0.24	0.40	0.17
75	1563	1966	Caryophyllene-oxide	2.90	0.85	4.08	5.12	4.27	5.16
76	1568	1977	Germacrenol-B	-	-	0.03	-	0.02	0.08
77	1584	2180	Fokienol	0.17	0.03	0.07	0.06	0.04	0.25
78	1586	2228	Tetradecanal	-	-	0.13	-	0.54	-

79	1587	2073	Humulene-epoxide II	-	0.09	0.43	0.54	-	0.87
80	1588	2006	(*Z*)-α-Bergamotol	0.30	0.01	-	-	0.11	-
81	1595	1975	Hexyl-phenylacetate	-	-	0.06	-	-	-
82	1614	2329	Caryophylladienol II	-	-	0.08	0.01	0.03	0.20
83	1623	2178	Delta-9-10-Eremophilen-11-ol	0.34	0.29	0.23	0.14	0.24	0.35
84	1660	2175	α-Bisabolol	0.63	0.14	0.33	0.41	0.30	0.76
85	1666	2012	Dodecenyl acetate	0.40	0.60	0.38	0.31	0.25	0.34
86	1787	2369	Tetradecyl acetate	0.08	0.17	0.03	0.01	0.10	0.17
87	1824	2297	Heptadecadienone	0.01	0.01	0.05	0.11	0.27	0.07
88	1931	2214	α-Springene	0.46	1.39	1.65	3.01	1.94	1.55
89	1947	2239	b-Springene	0.28	1.10	1.14	2.83	1.37	1.21
90	2183	2497	Octadecyl acetate	0.04	0.22	0.73	0.90	0.33	0.37

Retention Indices on apolar RI (ap.) * and on polar RI (p.) ** columns determined relative to the retention times of a series of *n*-alkanes (C_7 - C_{28}) with linear interpolation. An example of the chromatograms analysed can be found in the Supplementary Materials (see Figure S2).

2.2. Inter- and intrapopulation chemical variability

The EOs obtained from aerial parts of *T. marum* collected in six different Sardinian locations presented different chemical compositions (Figure 1).

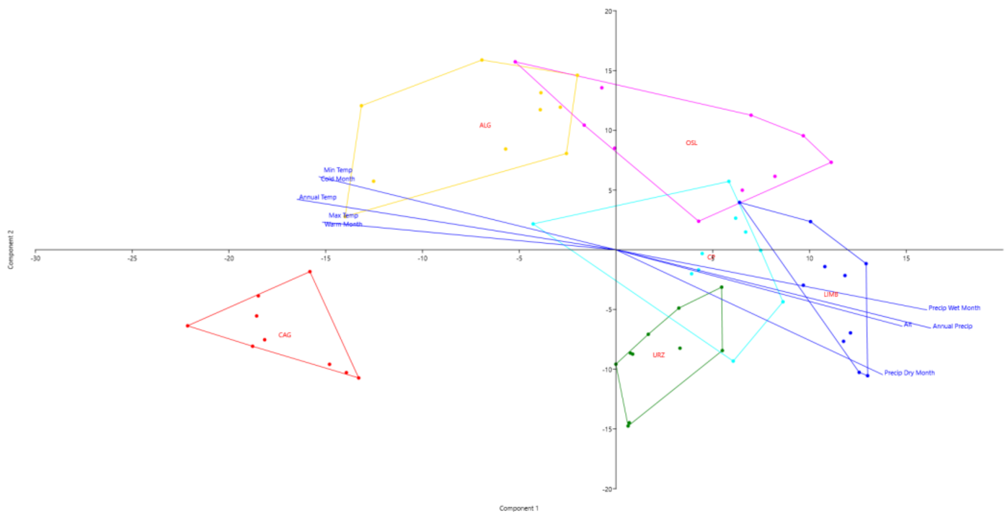


Figure 1. Principal Component Analysis (PCA - axis 1: 16 %; axis 2: 11,8 % of the variance explained) of clr-transformed (Center logratio) chemical compositions of *Teucrium marum* L. from six different natural Sardinian populations showing the environmental factors as supplementary variables - CAG (Capo S. Elia – Cagliari, CA), ALG (Lazzaretto – Alghero, SS), CP (Costa Paradiso - Trinità d'Agultu e Vignola, SS), LIMB (Limbara - Tempio Pausania, SS), OSL (Osilo, SS) and URZ (Genna Silana – Urzulei, NU).

The Principal Component Analysis (PCA) of the EOs samples composition (Figure 1) showed that overall all the populations are different from each other despite the similarity of some individuals belonging to different populations. Indeed, a few overlapping population areas shown on the PCA corresponded to one individual of the Osilo population that shared the same phytochemical composition with one individual of Alghero, as well as another individual of Osilo had a similarity in chemical composition with one sample from Costa Paradiso and another one from Limbara. Interestingly, the Cagliari and Urzulei populations were the only ones not sharing any individual with the other populations, the Urzulei population been above all the other populations on the third axis of the PCA (Figure not shown). The size of the area enclosing a given population also shows the compositional chemical variability of a given population. Alghero, Osilo and Costa Paradiso in fact possess a wider compositional variety than the populations of Cagliari, Limbara and Urzulei. The population of Osilo is the more variable with individuals "connecting" (though marginally) the three populations of Alghero, Costa Paradiso and Limbara. All the other populations have different chemical compositions with no overlapping. The PCA also showed that the distribution of the populations of Cagliari and Alghero is mainly correlated with abiotic factors related to temperature, while the populations of Limbara, Urzulei and partially Costa Paradiso with the precipitation gradient.

The diversity between populations is also confirmed by the non-parametric test ANalysis Of SIMilarity (ANOSIM), that showed a significant difference in the phytochemical EO composition among the studied populations (ANOSIM: mean rank within: 320.7; mean rank between: 952.4; R = 0.738, $p = 10^{-4}$). Moreover, the EO composition was significantly different among all the populations according to the multiple pairwise population comparisons ($p \leq 0{,}003$, Bonferroni-corrected *p*-values). These population differences in EO composition were significantly explained partly by both the different elevation and the soils substrate (two-way PERMANOVA: elevation $F_{5,35} = 4.36$, $p = 0.0001$; substrate $F_{3,35} = 4.91$, $p = 0.0001$; interaction (elevation x soil) $F_{15,35} = 0.98$, $p > 0.1$).

The dendrogram (see supplementary materials Fig. S1) obtained by Cluster Analysis (CA) showed that, on the basis of their EOs' chemical composition, the six studied Sardinian populations of *T. marum* were grouped in three main clusters: the first showed a major homogeneity and was composed only by the Cagliari population, the second showed a moderate heterogeneity and included the Alghero and Osilo populations with only one individual from the Costa Paradiso population; the last showed a marked heterogeneity and was composed by Limbara, Costa Paradiso and Urzulei populations.

3. Discussion

The high chemical variability of *T. marum* EOs found in the studied Sardinian populations was also found in other samples which had been analysed by other authors in previous studies [24,26-31]. To avoid that the singularity of the volatile compounds we found, with respect to other reports, was influenced by the sampling period and different extraction methods, we realized our field sampling over a short period of time during the flowering stage, and a standard extraction method for EO was used [29-31]. An interesting fact that has emerged in our EOs samples is the presence of Dolichodial: this compound has also been found in samples from Balearic islands [24,30] and in some samples from Sardinia island [24,26], together with other typical main compounds such as Caryophyllene oxide, α-Bergamotene, β-Bisabolene, (***E***)-β-Caryophyllene, β-Sesquiphellandrene, Estragole, Geranyl acetate, Epidolichodial, α-Humulene [27]. Anyway, in other Sardinian [28] and Corsican [29,31] samples, the above-mentioned compound Dolichodial, has not been found. However, although this compound is a typical production of the plant, as reported by several studies, it's not always detected in the extraction of essential oils [26,30]. Several factors could influence the chemical composition of the EOs, however, as regards the presence / absence of Dolichodial in the essential oil of *T. marum*, an explanation is reported by [29]. Indeed, the Corsican samples compound was found as the main volatile component with the HS-SPME solid phase extraction process while it was never detected in essential oils obtained by hydrodistillation (HD). The researchers hypothesize that Dolichodial was probably lost by chemical transformation under thermal conditions and acid pH in hydrodistillation. During the hydrodistillation step, the aldehyde group from the hydrate and the dihydrate forms of the Dolichodial, were partially transferred to the hydrolated aqueous phase due to its greater affinity with water. As Dolichodial was found in much higher quantities in Sardinia than in Corsican samples, this result may reflect regional differences of its abundance as probably only a (small) fraction is "lost" in this way. The percentage of Dolichodial in our samples was higher from coastal samples of Cagliari and Alghero, the lowest in Limbara and intermediate in Osilo and Urzulei. This could be related to altitude and covarying climatic factors which would explain how environmental factors strongly influence the EO phytochemical fingerprint, as reported by previous studies [2,5,8-14,16-18]. Another important aspect was that in our samples 2-Methylbutyl-2-methylbutyrate and 2-Methylbutyl butyrate compounds were the main components in some samples, while in other Mediterranean samples these compounds were not detected. Furthermore, our study of *T. marum* EOs evidenced a similar chemical composition of the main compounds such as *(E)*-β-Caryophyllene, β-Bisabolene and β-Sesquiphellandrene, commonly found in other samples analysed from Sardinia and other Mediterranean islands such as Corsica and Balearic islands [24,26-31].

Variation in secondary compounds composition among individuals and populations of the same species is a quite common feature of aromatic Mediterranean vascular plants, and can result from a genetic polymorphism in the production of EOs [5]. Geographic variation in the composition of EO is well documented not only in typical aromatic dwarf shrubs like *Salvia rosmarinus* Spenn. [22,32] and *Lavandula latifolia* Medik. [33], but also in evergreen sclerophyllous trees like *Quercus ilex* L. [34] and *Pinus pinaster* Aiton [35]. In the endemic dwarf *Helichrysum italicum* (Roth) G. Don subsp. *tyrrhenicum* (Bacch., Brullo & Giusso) Herrando, J.M. Blanco, L. Sáez & Galbany from Sardinia, a geographic variation in EO composition was related to population genetic structure but also to elevation [15,16]. Local environmental factors (like morphology which influences winter minimum temperatures) explained the phytochemical diversity among *Thymus vulgaris* L. populations at short spatial scales [36].

The phytochemical diversity among the studied populations of *T. marum* (see supplementary materials Fig. S1), seems to be biogeographically structured [37]. Each Sardinian population appeared to have a significant different EO composition resulting partly from unique combinations of elevation and soil substrate. Here the elevation is used as a proxy of climatic variables as they are highly correlated (as shown by PCA). These environmental factors are not probably the only explaining the EO composition differences among populations but they were significant. Further studies on characterizing the genetic divergence of *T. marum* populations as performed on *Thymus* spp. [9,10] or *Helichrysum italicum* [15] would be of great interest. The southernmost population from Cagliari is clearly isolated from the others (as it is also confirmed being the only with Limonene and the unidentified compound 1508), since it grows on a fossil island surrounded by alluvial plains mostly unsuitable for the species. Then, the populations from the western side of the island (Alghero and Osilo) are more similar than the eastern populations (Costa Paradiso, Limbara and Urzulei), even if some individuals do not fit within the population of provenance. However, the role played by climatic factors in shaping the observed pattern of phytochemical heterogeneity, as highlighted by the PCA (Fig. 1), is more evident when considering the compounds mainly characterizing the EOs of the studied populations: in particular, Dolichodial and (*E*)-β-Caryophyllene, follow opposite patterns determined by elevation (and, consequently, by bioclimate). The first compound is much more abundant in coastal populations (Alghero, Cagliari and Costa Paradiso) than in mountainous ones (Osilo, Limbara and Urzulei), whereas the second follows the opposite pattern. Although Dolichodial's role in the chemical defence of *T. marum* is well documented [38] and its biosynthesis is well known since a long time [39,40], our findings strongly support a significant difference in Dolichodial production between coastal and mountainous populations of the cat thyme. Considering the key role of this compound in the anti-insect defence of *T. marum* [38], it is possible that coastal populations produce higher amounts of Dolichodial to limit insect damages in the hottest and driest period of the year (summer). On the other hand, mountainous populations growing at higher elevations may suffer lower risks from insect damages in summer, because of the lower temperatures and consequent lower drought stress. Differences in EOs composition related to aridity gradients are well documented also in another Mediterranean dwarf Lamiaceae, as the Tunisian *Thymbra capitata* (L.) Cav. [41]. (*E*)-β-Caryophyllene is a natural sesquiterpene hydrocarbon present in hundreds of plant species [42], and is documented to (directly) inhibit bacterial growth in the model species *Arabidopsis thaliana* (L.) Heynh. [43]. It is therefore possible that higher amounts of this compound are more useful for populations of *T. marum* growing at higher elevations than for populations living in coastal environments, because moister climates generally promote bacterial and fungal development.

4. Materials and Methods

4.1 *Study area*

Sardinia, the second largest island of the Mediterranean Basin with an area of 24089 km^2 represents one of the 10 - 11 Mediterranean biodiversity hotspots [44-49]. Its climate is characterised by Mediterranean pluvi-seasonal oceanic macrobioclimate and forty-three detailed iso-bioclimates [50,51]. Its flora is composed of ca. 2300 native vascular plant taxa of which ca. 15 % are endemic of the Sardinian-Corsican biogeographic province and surrounding Tyrrhenian areas, therefore highlighting the originality of the islands' genetic and geological history [49,52-55].

In this context, *T. marum* is one of the most characteristic species of the dwarf shrub vegetation (garrigues) often derived by human induced degradation of pristine evergreen forests through fire, grazing and/or clearing. This endemic chamaephyte has been chosen as a guide species to name the phytosociologic alliance *Teucrion mari* Gamisans &

Muracciole 1984 [56] including several Sardinian-Corsican communities dominated by dwarf perennial plants.

Teucrium marum fresh aerial parts were collected during their flowering period (May and June 2022) in six different localities in Sardinia: three coastal and three mountainous (Table 2, Figure 2). For each station, ten individual plants were randomly sampled at a minimum distance of 20 m between each other, in order to avoid sampling siblings.

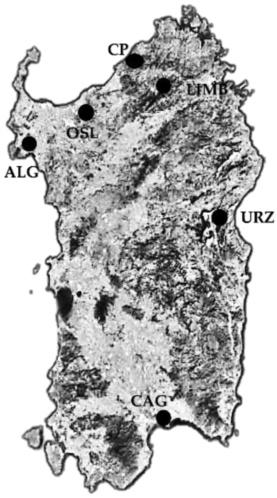
Table 2. Wild Sardinian populations of *Teucrium marum* L. data.

Population code	Site name and Municipality (Province)	WGS84 coordinates	Elevation (m a.s.l.)	Soil substrate	Bioclimate*
CAG	Capo S. Elia – Cagliari (CA)	39.1855475 N 9.1475736 E	41	Carbonate	2 - Lower thermo-Mediterranean, lower dry, strong euoceanic
ALG	Lazzaretto – Alghero (SS)	40.5839220 N 8.2482990 E	44	Dolostone	8 - Upper thermo-Mediterranean, upper dry, semi-hyperoceanic
CP	Costa Paradiso - Trinità d'Agultu e Vignola (SS)	41.0529210 N 8.9609560 E	174	Granites	9 - Upper thermo-Mediterranean, upper dry, strong euoceanic
LIMB	Mt. Limbara - Tempio Pausania (SS)	40.8485980 N 9.1646290 E	1263	Granites	40 - Lower supratemperate, lower humid, weak semi-continental
OSL	Mt. Tuffudesu - Osilo (SS)	40.7348590 N 8.6807020 E	704	Andesitic	28 - Upper meso-Mediterranean, upper subhumid, weak euoceanic
URZ	Genna Silana – Urzulei (NU)	40.1591710 N 9.5064150 E	1012	Carbonate	35 – Lower supra-Mediterranean, lower humid, weak semi-continental

*Numbers and bioclimate diagnosis refer to [50].

Figure 2. Sampled distribution around Sardinia in six different localities of *Teucrium marum* L. Costal localities: CAG (Capo S. Elia – Cagliari, CA), ALG (Lazzaretto – Alghero, SS) and CP (Costa

Paradiso - Trinità d'Agultu e Vignola, SS). Mountains localities: LIMB (Mt. Limbara - Tempio Pausania, SS), URZ (Genna Silana – Urzulei, NU) and OSL (Mt. Tuffudesu – Osilo, SS).



4.2 *Study species*

The highest diversification of Mediterranean endemic species rich in secondary compounds occurs in the families Apiaceae, Asteraceae and Lamiaceae [5]. Lamiaceae is a family of aromatic plants such as the genus *Teucrium* L. (germander) which includes 300 species distributed in Europe, North Africa and temperate areas of Asia, but mainly concentrated in the Mediterranean region [27,57]. The genus *Teucrium* is characterized by herbs or shrubs, with tubular or campanulate calyx, 2-lipped or actinomorphic, 5-toothed, the teeth equal or the upper the largest. Corolla is with one 5-lobed lip; tube without a ring of hairs inside, often included in the calyx. Nutlets are smooth or reticulate.

The studied species, *Teucrium marum* L., is a perennial aromatic suffruticose chamaephyte, characteristic of arid and stony environments (e.g. garrigues), from 0 to 1200 (rarely up to 1700) m a.s.l., endemic to the islands of Sardinia, Capraia, Montecristo and Gorgona in Italy, Corsica, Hyères, Murter in France and the Balearic archipelago in Spain. Cat thyme is an aromatic and medical plant, used in traditional medicine as diuretic, nervine, stimulant, stomachic, tonic, antibacterial, anti-inflammatory, antipyretic, cicatrizing properties, astringent and also to control haemorrhages [26-28,58]. The biological properties of single EO of cat thyme is determined, generally, by its main components [27,59].

4.3 *Plant Material and Essential Oil*

For each of harvested individual the first twenty cm of canopy (leaves and inflorescence parts) were collected. Samples were dried in a heating oven 100 % ventilated set to 40° C for 48 h in order to avoid any degradation and loss of EO.

Dried leaves of each sampled plant (average 47.1 g. max 71.7 - min 23.1 g) were introduced into a 2 L wide-neck flask reaction (QFR2LF. Quickfit®. Fisher Scientific. Pittsburgh. Pennsylvania. USA). One litre of distilled water was added and heated for two hours using a heating mantle (EM2000/CE. Electrothermal®. Vernon Hills. IL. USA). The essential oil was collected from each sampled plant using a classical Clevenger apparatus. The Clevenger apparatus was cooled at 6°C using a refrigerated fluid (mix of glycol–water) cooled and moved by a Minichiller® (C_{20}. Huber®. Freiburg. Germany). Then the EOs were diluted in 0.5 ml of pentane in the Clevenger due to low EO volume produced by some samples, transferred to a Gas Chromatography (GC) vial and 5 ml of pentane was added before its sealing. The GC vials were sealed and stored at 4° C before further analyses.

4.4 GC and GC–MS analysis

Gas Chromatography analyses were performed on a Perkin Elmer Clarus 500 gas chromatograph (FID. Perkin Elmer. Courtaboeuf. France) equipped with 2 fused silica gel capillary columns (50 m. 0.22 mm id. film thickness 0.25 μm): BP-1 (polydimethylsiloxane) and BP-20 (polyethylene glycol). The oven temperature was programmed from 60 to 220 °C at 2 °C/min and then held isothermally at 220 °C for 20 min. The injector temperature of 250 °C, detector temperature of 250 °C, carrier gas hydrogen (1.0 mL/min) and split of 1/60.

The EOs were also analysed with a PerkinElmer gas chromatography coupled with mass spectrometry (GC/MS).; TurboMass detector (quadrupole. PerkinElmer. Courtaboeuf. France) directly coupled with a PerkinElmer Auto system XL equipped with a fused silica gel capillary column (50 m. 0.22 mm id. film thickness 0.25 μm) (BP^{-1} polydimethylsiloxane). The analyses were performed with the following parameters. Helium was the carrier gas at 0.8 mL/min. The injection volume was 0.5 μL with a split of 1/75 at ; an injector temperature of 250 °C. The oven temperature was programmed from 60 to 220 °C with a 2 °C/min increase and finally held iso-thermally (20 min). The ion source temperature was 250 °C with an ionization energy of 70 eV. The electron ionization mass spectra were acquired over the mass range of 40-400 Da.

The GC retention indices (RIs) of each compound were determined on both polar and apolar columns relative to the retention times of a series of n-alkanes (C_7-C_{28}) with linear interpolation (‘Target Compounds’ software of PerkinElmer). The compounds were first identified by comparison of their two RIs with those of authentic compounds and literature data. These identifications were also confirmed by mass spectral comparison using a reverse match factor > 900 with commercial reference libraries (FFNSC2, Nist11W9, Perkin_ff2013, Wiley8). For each compound, the mean relative amount (%) per population, obtained from ten sampled specimens, is reported.

4.5 *Data analysis*

Statistical analyses were performed with Past 4.11 statistical software [60]. Raw data with compound percentages were clr-transformed (Center logratio) prior analysis to normalize the weight of all the compounds. Individual phytochemical compositions were visualized in a PCA with triplot for environmental factor, the compounds differentiating populations were determined with a SIMPER analysis, and finally the statistical population phytochemical differences were tested with an ANOSIM and the effect of environmental factors (elevation and soil substrate) by a PERMANOVA (with 9,999 replicates for each).

On the PCA analysis, environmental factors (extracted from [50,51]) and substrate (extracted from [62?]) were plotted as supplementary variables to visualize any correlation between phytochemical composition and environmental data as elevation, mean annual temperature, maximum temperature of the warmest month, minimum temperature of the coldest month, mean annual precipitation, precipitation of the wettest month, precipitation of the driest month.

SIMPER was applied for assessing which compounds were primarily responsible for observed differences between populations [61]. The Euclidean similarity measure was used for the SIMPER analysis. The overall significance of the difference was assessed by one-way ANOSIM and among population differences with a post-hoc test of multiple pairwise-population comparisons using Bonferroni-corrected p values. The factor effect (elevation, soil substrate (Table 1) and their interaction) was tested with a two one-way PERMANOVA. Differences with $p \leq 0.05$ between groups were considered significant.

5. Conclusions

The composition of Sardinian *T. marum* EOs was original from other Mediterranean known compositions, particularly for the high level of Dolichodial found in the coastal populations under study.

Within Sardinian populations we have found a significant effect of environmental/climatic factors on the EOs composition, with mountainous populations having an EO different from coastal ones.

Beyond the ecological factors determining intra- and inter-population phytochemical diversity, the data shown here give a scientific background to address the sustainable use of wild populations of aromatic plants. The Mediterranean basin is indeed a hotspot of medicinal and aromatic plants which have always been used by humans. But recently, their exploitation has dramatically increased and new scenarios are proposed for local development and sustainability in order to preserve this natural capital [62]. There is in fact growing awareness of the potential socio-economic benefits but also the environmental risks determined by the exploitation of wild populations of medicinal and aromatic plants, especially in Mediterranean islands [63,64]. Detailed data on the phytochemical characterization of wild populations of Mediterranean medicinal and aromatic plants, as presented here, is the first step to develop a correct approach for the possible use of natural products for human needs, as already done with other plants like *S. rosmarinus* used to control invasive alien plants expansion [65,66].

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Figure S1: Dendrogram; Table S1: SIMPER table analysis.

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