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Spatial Variation in Yield, Chemical Composition, and Phytotoxic Activity of *Cistus ladanifer* Essential Oils

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Cistus ladanifer L. (rockrose) is a widespread shrub species of the Mediterranean region with products highly valued by the perfume and cosmetics industry. In this research, the variability in yield, chemical composition and phytotoxic activity of *C. ladanifer* essential oils collected from 12 plots belonging to four natural populations and settled on two different types of edaphic substrates were evaluated. The essential oils were analyzed by GC-MS. The essential oil content ranged from 0.19 to 0.42 mL/100 g. The volatile profiles were found to be rich in oxygenated sesquiterpenes and oxygenated monoterpenes. PCA analysis clustered the samples into two groups that were mainly attributed to the type of substrate on which the plants grow. Furthermore, CCA and correlation analysis revealed that soil organic matter was the most effective edaphoclimatic driver accounting for these high levels of variation in essential oil yield and composition. Finally, *C. ladanifer* essential oils showed strong phytotoxic activity on *R. sativus* seedlings, indicating its potential use as a natural bio-herbicide in agriculture. The results showed that the effect associated to local edaphoclimatic conditions not only impacted on the quality and quantity of the essential oil, but also on the industrial uses derived from its biological activities.

Introduction

Cistus plants, also known as rockroses, belong to a Mediterranean native genus of shrubs belonging to the Cistaceae family.[1] This genus reaches the highest diversity in the Iberian Peninsula with 12 species, among which *Cistus ladanifer* L. stands out because of its wide distribution (2 million hectares).[2] The young leaves and stems of this plant secrete a sticky oleoresin, called labdanum, highly demanded by the perfume industry due to its fragrant and fixative properties.[3] *C. ladanifer* grows in different subtypes of Mediterranean climate and it tolerates drought, continuous solar irradiance, and extreme temperatures.[4] The flowering period is from March to May, and full fruit maturation occurs in late summer. This shrub is considered a pyrophytic species that colonize open areas after a fire, as well as abandoned fields, and marginal areas.[5] In addition, by releasing allelopathic compounds which inhibit the growth of other plants,[6,7] it can reduce competition and become a colonizing species.[8] Its ability to colonize disturbed sites, also implies rapid fuel accumulation and severe fire hazards.[2]

Wildfires cause severe damage to Mediterranean ecosystems every year, whose recurrence and severity are expected to increase under the predicted climate scenarios.[9] Therefore, novel forest and shrubland management strategies are needed to take these scenarios into account and reduce risks.[10] Some of the alternatives proposed to mitigate these effects focus on fuel reduction through brush clearing.[11] However, fuel treatments are costly and need to be carried out periodically.[12] Developing high-added value bioproducts such as essential oils while preventing fires may be a cost-efficient strategy in the context of a green and circular economy.[13]

Essential oils (EOs) are complex mixtures of volatile compounds derived from the secondary metabolism of plants, which generally contain two or three main components in fairly high concentrations (20–70%) as compared to other components present in trace amounts.[14] The main groups of compounds in essential oils are terpenes and terpenoids, associated with plant protection functions against herbivores, pathogens, and stressful conditions, as well as pollinator attraction.[15] EOs have been reported as strong natural bioherbicidal agents and their bioactive potential derives from a complex interaction of major and minor volatile components.[16]

In *C. ladanifer*, although labdanum gum has been the product traditionally demanded by the perfume industry, other products including essential oil have received increased interest in recent years.[17] In turn, as an alternative use to other unsustainable synthetic chemical molecules, several biological activities such as antioxidant,[18] antimicrobial[19,20] and phytotoxic[21] have been attributed to *C. ladanifer* essential oils.

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From a chemical point of view, some studies have shown that α-pinene, viridiflorol, (*E*)-pinocarveol, *p*-cymene, camphene, bornyl acetate, and ledol are the major compounds quantified in the essential oil of *C. ladanifer*.[4,18] However, the published data suggest that the chemical composition of essential oil from wild-grown *C. ladanifer* plants varies widely among different regions and countries.[17,22–24] In turn, Mariotti et al. (1997)[25] also found a high degree of intraspecific variability in 20 *C. ladanifer* individual plants originating from Spain and cultivated in Corsica. It is well established that diverse environmental, geographic, genetic, and physiological factors influence the high variations in essential oil quantity and quality.[26] Furthermore, these phytochemical variations within the same species regularly correspond the variability of biological activities.[27]

To the best of our knowledge, there are no previous studies on the inter- and intra-population variability of *C. ladanifer* essential oils and their biological activity according to environmental factors. In this study the following specific questions are addressed: (1) Are there intra- and inter-population variations in the yield and chemical composition of *C. ladanifer* essential oil? (2) Is this variation associated with environmental factors? (3) Does such variability influence the phytotoxic activity of essential oil?

Results and Discussion

Essential oil yield

The essential oil yield ranged from 0.19 to 0.42 mL/100 g of dry plant material (Figure 1). These results agree with those reported in *C. ladanifer* from Portugal and Morocco under similar conditions (0.2–0.3 mL/100 g).[19,22] Fluctuations observed in essential oil yield have often been associated with environmental or genetic factors under which plants grow.[28]

Regarding the lithic material on which the plants grow, the nANOVA analysis showed to be statistically significant ($F_{1,48} = 68.52$; $P < 0.001$) for the two types of substrate tested, with granitic substrate presenting higher values than slate substrate. In addition, the nANOVA showed a significant effect at the population level ($F_{2,48} = 25.74$; $P < 0.001$) and also for the different plots within the same population ($F_{8,48} = 4.63$; $P < 0.001$). The Villasbuenas de Gata population presented a significantly higher yield of essential oil (0.33 mL/100 g d.w) than the other populations studied. In contrast, the Valverde del Fresno population showed the lowest concentration of oil (0.22 mL/100 g d.w). The inter-plot variability in essential oil yield within the same population was lower than that observed among populations. However, plot A2 of the Pozuelo de Zarzón population exhibited significant differences in essential oil yield compared to plots A1 ($t = -3.48$; $P = 0.045$) and A3 ($t = 4.18$; $P = 0.006$). On the other hand, plot D1 of the Villasbuenas de Gata population was also statistically different from plots D2 ($t = -3.89$; $P = 0.01$) and D3 ($t = -3.97$; $P = 0.01$). In line with our

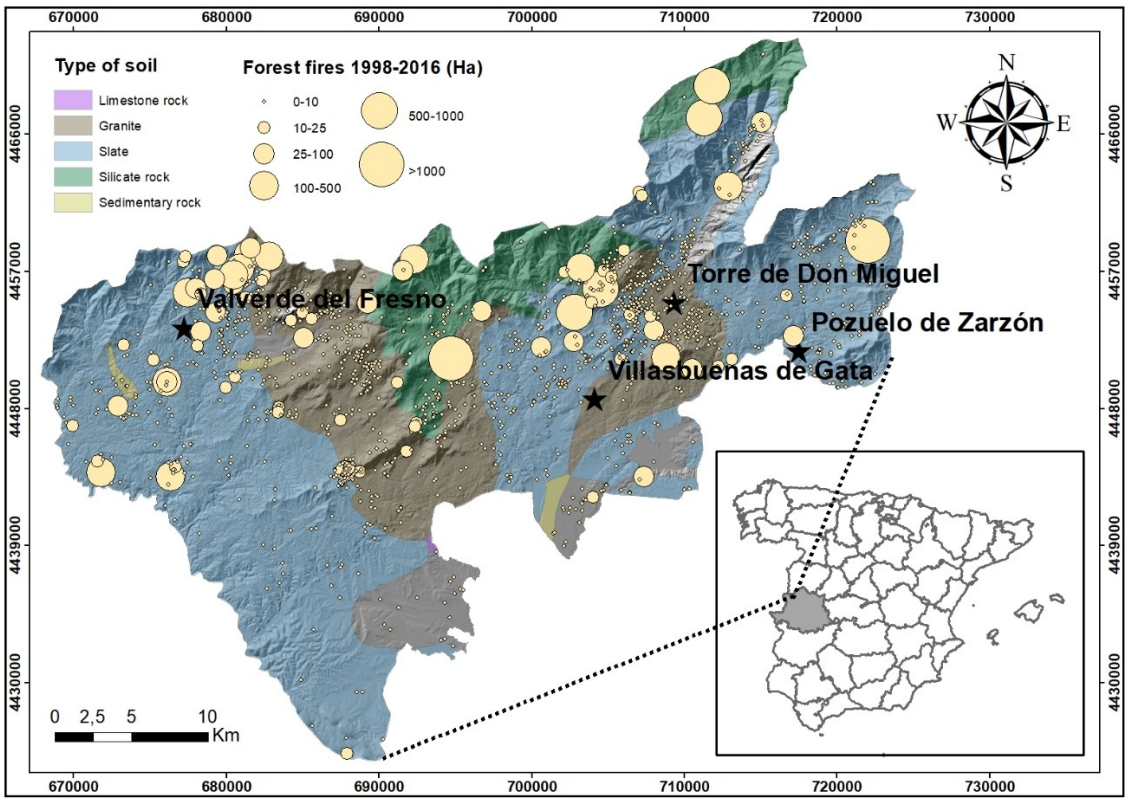

Figure 1. Geographical distribution of the four *C. ladanifer* populations studied.

results, Angelopoulou et al. (2001) reported inter-population differences in the essential oil content of *Cistus parviflorus* L. plants collected from nine populations on the Greek island of Crete. Likewise, in a study with *Cistus albidus* plants growing on two types of soil, Robles and Garzino (1998)[29] concluded that those on siliceous areas presented higher essential oil yields than those on calcareous areas. However, another study conducted by Robles and Garzino (2000)[30] on *Cistus monspeliensis* showed, conversely, that plants grown on calcareous soils presented higher oil yields than those on siliceous soils. These variations detected in essential oil yields have often been linked to the influence of specific edaphoclimatic variables.[31,32]

In contrast to the strong effect observed of edaphic factors on yield, microclimatic conditions do not seem to play a consistent role in explaining variations on yield, see data shown in Table S1. Therefore, these results indicate that the EO yielded can be significantly influenced by specific edaphic variables. A multiple linear regression model was used to relate the amount of essential oil and the observed edaphic variables. The final model fitted ($F_{1,10} = 16.74$; $P = 0.002$) explained about 58.9% of the observed variability in the response, and only the organic matter content was selected as a significant predictor. Consequently, those plots with a higher yield also presented a higher organic matter content in the soil (Spearman's $r = 0.86$, $P < 0.001$) (Table 3). As in our study, Fernández-Sestelo and Carrillo (2020)[33] also reported the highest yields of *Lavandula latifolia* essential oils in soils with a higher organic matter content. In this respect, soil organic matter improves the physical, chemical and biological characteristics of the soil and, hence, increases soil fertility and the water status,[34] which can derive in both more growth and a greater accumulation of secondary metabolites (Figure 2).

Essential oil composition

The GC-MS analysis of the *C. ladanifer* essential oil samples allowed the identification of a total of 103 compounds, representing 78.38% to 81.77% of the total essential oil. The results showed that the chemical composition of the samples was similar in qualitative terms (number of compounds) but that there were differences in the relative percentage (%) of the essential oil components (Table S2). All identified compounds were grouped into six groups: monoterpene hydrocarbons, monoterpene oxygenates, sesquiterpene hydrocarbons, sesquiterpene oxygenates, diterpene hydrocarbons and "others". In turn, the 20 major compounds quantified have been included for statistical analysis. These major components and groups of compounds of the essential oils are listed in Table 1. At the species level (average of the 12 plots), the groups with oxygenated components were in all cases predominant in the oil, with a higher fraction of oxygenated sesquiterpenes (36.1%), followed by oxygenated monoterpenes (15.7%). The minor volatile fractions were represented in decreasing order by hydrocarbon sesquiterpenes (9.7%), hydrocarbon monoterpenes (8.8%) and hydrocarbon diterpenes (2.4%). In agreement with the present results, in a study with *C. ladanifer* essential oil from Guadarrama mountain range (Madrid, Spain), Verdeguer et al. (2012)[21] also reported significantly higher concentrations of both oxygenated monoterpenes (59.7%) and oxygenated sesquiterpenes (19.4%) compared to their hydrocarbon counterparts (6.9 and 3.7%, respectively). By contrast, another study located in Portugal, approximately 40–60 km from our study area, Tavares et al. (2020)[18] described an essential oil in which monoterpenes (54.5%) represented a considerably higher percentage than sesquiterpenes (11.6%). The main constituents of the EOs were viridiflorol (19.9%), ledol (9.6%), α-pinene

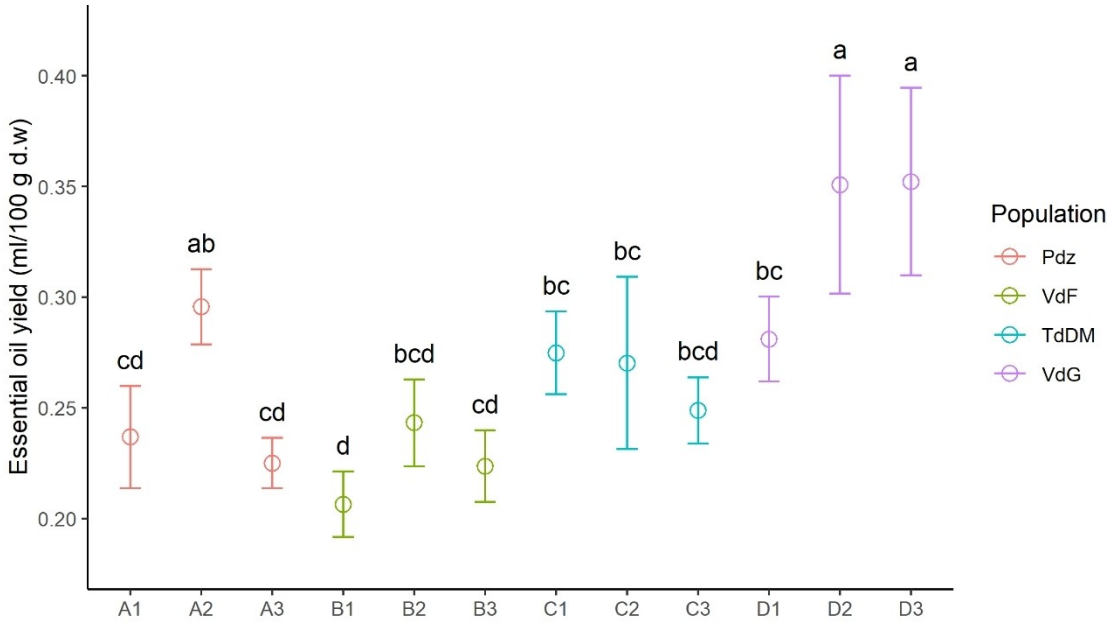

Figure 2. Essential oil yield from different *Cistus ladanifer* collection plots. The values were calculated as volume (mL) of essential oil per 100 g of dry plant matter. Values are expressed as mean ± standard deviation (n = 5). Different letters indicate significant differences ($P < 0.05$) in Tukey's test (HSD).

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Table 1. Major compounds and groups of compounds (%) of *Cistus ladanifer* essential oils from 12 plots distributed in four populations and two substrate types.

Compounds [a]		Slate						Granite						*p*-value
		Pozuelo de Zarzón			Valverde del Fresno			Torre de Don Miguel	Villasbuenas de Gata					
	RI	A1	A2	A3	B1	B2	B3	C1	C2	C3	D1	D2	D3	
α-pinene	**936**	**4,10abcd**	**5,51ab**	**3,20cd**	**2,49d**	**3,32bcd**	**2,74cd**	**5,58ab**	**4,89abcd**	**5,69ab**	**4,47abcd**	**6,63a**	**5,88a**	<0,001
camphene	951	0,76 ab	0,75 ab	0,43 b	0,88 ab	1,34 a	1,03 ab	0,47 b	0,69 ab	0,83 ab	1,00 ab	0,98 ab	0,93 ab	0,709
p-cymene	1026	1,12 ab	1,28 a	1,15 ab	0,77 d	0,94 bcd	0,80 cd	1,19 ab	1,16 ab	1,26 a	1,03 abcd	1,21 ab	1,18 ab	<0,001
2,2,6-trimethyl-cyclo-hexanone	1037	2,61 a	2,38 a	2,68 a	2,19 a	2,37 a	2,59 a	2,43 a	2,05 a	2,50 a	2,21 a	2,45 a	2,29 a	0,595
(*E*)-pinocarveol	**1139**	**3,74bcd**	**5,15ab**	**2,97cd**	**2,72d**	**2,92cd**	**2,35d**	**4,51ab**	**5,30a**	**5,11ab**	**4,43abc**	**4,57ab**	**4,81ab**	<0,001
pinocarvone	1162	1,37 bcd	1,79 ab	0,95 d	1,00 d	1,12 cde	0,81 de	1,64 ab	1,87 ab	1,94 a	1,57 abc	1,67 ab	1,66 ab	<0,001
borneol	1166	1,16 a	1,09 a	0,93 a	1,22 a	1,25 a	1,28 a	0,86 a	1,33 a	1,16 a	1,21 a	1,01 a	1,05 a	0,548
myrtenol	1195	0,83 bc	1,07 ab	0,81 bc	0,63 c	0,62 c	0,61 c	1,02 ab	1,16 a	1,05 ab	0,97 ab	0,91 ab	1,00 ab	<0,001
bornyl acetate	1285	2,97 ab	3,13 ab	2,57 b	3,20 ab	3,44 ab	3,73 a	2,51 b	2,99 ab	2,94 ab	3,18 ab	3,25 ab	3,61 a	0,497
Alloaromaden-drene	1454	1,06 a	1,08 a	1,06 a	1,22 a	1,36 a	1,10 a	1,05 a	1,07 a	1,14 a	1,12 a	1,32 a	1,21 a	0,384
viridiflorene	1488	1,98 ab	2,05 ab	2,37 a	2,20 ab	1,83 ab	2,21 ab	1,52 b	1,62 ab	1,65 ab	1,93 ab	1,92 ab	1,96 ab	<0,001
palustrol	1559	1,01 bc	0,95 c	1,13 ab	1,13 ab	1,08 abc	1,19 a	0,96 c	0,95 c	0,93 c	1,01 bc	1,01 bc	1,02 bc	<0,001
spathulenol	1570	1,42 a	1,38 a	1,58 a	1,65 a	1,76 a	1,85 a	1,53 a	1,66 a	1,47 a	1,47 a	1,58 a	1,48 a	0,007
viridiflorol	**1585**	**20,96abc**	**17,68d**	**22,51a**	**21,36abc**	**21,91ab**	**20,46abcd**	**18,32 d**	**18,90cd**	**19,84bcd**	**19,27cd**	**19,83bcd**	**19,35cd**	<0,001
ledol	**1594**	**9,14ab**	**8,91ab**	**10,47ab**	**11,28a**	**10,23ab**	**10,42ab**	**10,08ab**	**9,65ab**	**8,65b**	**9,83ab**	**8,27b**	**8,45b**	<0,001
1-epi-cubenol	1621	1,11 bc	1,24 abc	0,97 c	1,43 ab	1,61 a	1,36 ab	1,38 abc	1,30 abc	1,27 abc	1,48 ab	1,12 bc	1,26 abc	0,836
ß-eudesmol	1641	0,91 a	1,12 a	0,87 a	0,99 a	1,05 a	0,93 a	1,03 a	1,03 a	0,89 a	1,15 a	0,91 a	0,98 a	0,900
cadalene	1668	1,32 abc	1,41 abc	1,49 abc	1,90 a	1,75 ab	1,58 abc	1,44 abc	1,21 bc	1,09 c	1,33 abc	1,18 bc	1,29 abc	<0,001
15-nor-labdan-8-ol	1961	1,92 ab	1,31 abc	1,88 ab	1,90 ab	1,41 abc	1,94 a	1,27 c	1,30 c	1,45 bc	1,28 c	1,18 c	1,14 c	<0,001
16-kaurene	2020	0,99 a	0,81 a	1,02 a	1,15 a	1,00 a	1,14 a	0,79 a	0,90 a	0,98 a	0,99 a	0,92 a	0,65 a	0,015
Total represented (%)		60,09	61,05	61,27	62,29	60,12	59,59	61,02	61,82	60,93	61,91	61,21	60,09	
Total Identified (%)		79,78	81,30	79,36	78,82	80,40	78,38	80,77	80,49	81,77	80,54	81,77	81,43	
Monoterpene hydrocarbons		8,34 abcde	10,2 abc	6,97 cde	5,90 e	7,49 bcde	6,40 de	9,60 abcd	9,34 abcd	10,32 ab	8,83 abcde	11,31 a	10,52 ab	<0,001
Oxygenated monoterpenes		15,19 bcd	18,41 ab	13,07 cd	12,00 d	12,40 d	12,07 d	17,17 ab	19,00 a	18,22 ab	16,02 abc	16,61 ab	17,08 ab	<0,001
Sesquiterpenes hydrocarbons		9,00 a	9,71 a	9,51 a	10,68 a	10,67 a	9,96 a	9,63 a	8,81 a	8,67 a	10,01 a	9,50 a	9,87 a	0,014

Table 1. continued

Compounds [a]		Slate					Granite					*p*-value
		Pozuelo de Zarzón			Valverde del Fresno		Torre de Don Miguel	Villasbuenas de Gata				
	RI	A1	A2	A3	B1	B2	B3	C1	C2	C3	D1	D2	D3	
Oxygenated sesquiterpenes		36,08 abc	32,70 c	39,16 a	39,56 a	39,37 a	37,91 ab	35,01 bc	35,00 bc	34,39 bc	35,87 abc	34,14 bc	33,98 bc	< 0,001
Diterpenes		2,91 a	2,12 ab	2,90 a	3,04 a	2,41 ab	3,08 a	2,06 ab	2,19 ab	2,42 ab	2,27 ab	2,10 ab	1,79 b	< 0,001
Others		8,26 a	8,15 a	7,76 a	7,64 a	8,06 a	8,74 a	7,29 a	7,44 a	7,75 a	7,53 a	8,10 a	8,20 a	0,243

[a] Compounds listed in order of elution on HP-5 apolar column; IR: Kovats retention indices relative to n-alkanes (C6-C17) on HP-5 apolar column; values are expressed as mean (n = 5) of relative percentage compounds; values ≥ 5 % for at least one sample in bold. The yield percentage was calculated as volume (mL) of essential oil per 100 g of dry plant matter. In each row, values followed by different letters indicate significant differences ($P < 0.05$) in Tukey's test (HSD).

(4.5 %), (*E*)-pinocarveol (4.1 %), and bornyl acetate (3.1 %). Additionally, other components noted for their fragrance importance such as 2,2,6-trimethylcyclohexanone (2.3 %) and 15-nor-labdan-8-ol (1.47 %) were identified in relatively lower proportions. In general, by comparing our results with those of nearby areas of Spain, the main compounds identified are similar to those reported by other authors, but the proportions of each one of them differed widely. Mediavilla et al. (2021)[17] reported α-pinene (48.2 %), viridiflorol (11.3 %), and ledol (3.2 %) as the main constituents of *C. ladafiner* essential oils from the north of Guadalajara. On the other hand, in another study which described two essential oils from Zamora and Huelva, Xavier et al. (2021)[35] also reported that the major compounds were α-pinene, viridiflorol, and ledol, accounting in each site for 19.3–42.5, 13.4–24.1, 4.06–6.94 % respectively. Finally, in our previous study in a neighbouring population,[20] similar proportions of the major compounds viridiflorol (18.6 %), ledol (7.1 %), bornyl acetate (4.8 %), and 2,2,6-trimethylcyclohexanone (2.8 %) were found, but a considerably higher proportion of α-pinene (14.8 %) was detected compared to the present study. Thus, the discrepancies between our results and those previously described by other authors could be linked not only to geographic, environmental or genetic factors but also to differences in harvesting period, plant parts, sample size, storage, drying or extraction method.[36]

Variations of essential oil composition among plots, populations and substrate types

The nAnovas showed significant variations ($P < 0.05$) in essential oils chemical composition among plots, populations and substrate types (Table 1). The highest and lowest α-pinene values were obtained in the Villasbuenas de Gata (plot D2, 6.63 %) and Valverde Del Fresno (plot B1, 2.49 %) populations, respectively. (*E*)-pinocarveol showed the highest percentage in plot C2 of Torre de Don Miguel (5.30 %), while the lowest was observed in plot B3 of Valverde del Fresno (2.35 %). For viridiflorol and ledol, the highest contents were recorded in plots A3 of Pozuelo de Zarzón (22.51 %) and B1 of de Valverde del Fresno (11.28 %), respectively, while the lowest contents for both compounds were observed in plot A2 of Pozuelo de Zarzón (17.68 y 8.91 %). Additionally, there was significant inter-population variation between plots A2 and A3 of Pozuelo de Zarzón for compounds α-pinene, camphene, (*E*)-pinocarveol, pinocarvone, palustrol, and viridiflorol. On the other hand, those samples collected in plots established on granitic substrate presented significantly higher percentages in the monoterpenes α-pinene, *p*-cymene, (*E*)-pinocarveol, pinocarvone and myrtenol than those plots established on a slate substrate (except plot A2). In contrast, the plots on a slate substrate (except A2 once again) presented significantly higher percentages than those on a granite substrate in the sesquiterpenes viridiflorene, palustrol, spathulenol, viridiflorol, ledol and cadalene, as well as in the diterpene 15-nor-labdan-8-ol (Table 1).

To evaluate the global variation and affinity relationships between the chemical compositions of the plots, a principal component analysis (PCA) was applied to the data matrix linking essential oil major compounds and groups of compounds for the individual samples. Figure 3 shows the distribution of variables and individuals in the bi-dimensional space for the first two components of the analysis (54.98% of the variation). The first principal component (43.01%) clustered individuals according to substrate type. Thus, all individuals from plots belonging to the slate substrate (except plot A2) were grouped in the positive part of axis 1 and were characterized by higher concentrations of the sesquiterpenes and diterpenes viridiflorene, palustrol, spathulenol, viridiflorol, ledol, cadalene, 15-nor-labdan-8-ol, and 16-kaurene. In turn, on the negative side of this axis 1 were clustered all the individuals of the plots on granitic substrate plus the individuals of plot A2. These plots were characterized by a higher richness in the monoterpenes α-pinene, *p*-cymene, (*E*)-pinocarveol, pinocarvone, and myrtenol. Finally, within these two groups, certain individual segregation associated with axis 2 (11.97%) was observed (Figure 3).

There are several reports describing the chemical composition of C. *ladanifer* essential oils from different Mediterranean countries such as Spain,[17,20] Portugal,[22,37] France[30] and Morocco,[4,24] but limited research revealing intra- and inter-population variations is reported.[25,35] Therefore, it has already been pointed out that chemical variation occurs mainly according to geographic and genetic characteristics. However, chemical distribution is not always concordant with these factors but also seems to be linked to local selective forces influencing chemical diversity.[38] Indeed, local biotic (insects, animals and associated plants) and/or abiotic (temperature, topography, average precipitation and edaphic factors) selective factors are known to act on the biosynthetic pathways of volatile compounds and contribute to the emergence of different chemical profiles.[39] Consequently, in our case, these variations in secondary metabolism compounds under different local edaphic conditions may be associated with diverse ecological adaptation mechanisms,[15] which could affect the distribution of different C. *ladanifer* ecotypes in space. However, further specific studies are needed to select chemotypes with desirable commercial characteristics for the perfume, cosmetic and agri-food industries.

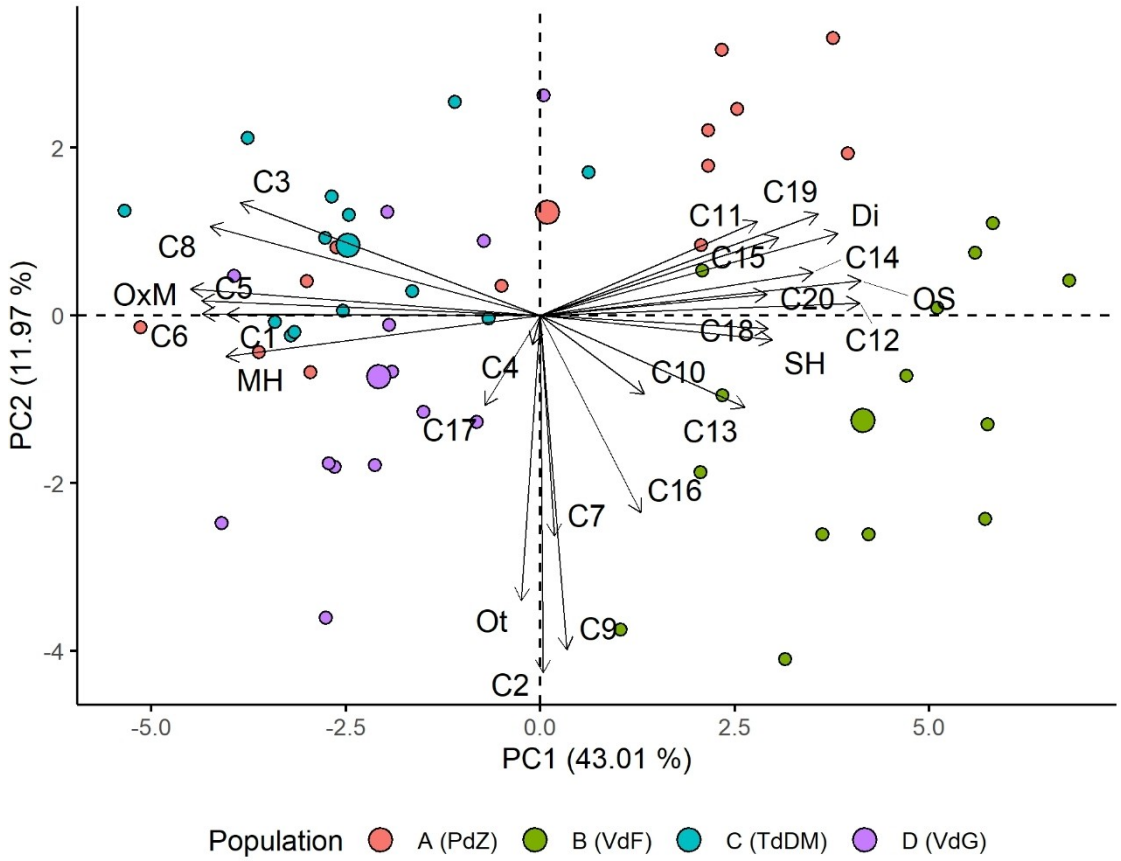

Figure 3. PCA analysis performed on the chemical composition of *Cistus ladanifer* essential oils of different plots. Identification of major compounds and groups of compounds analysed: α-pinene (C1), camphene (C2), *p*-cymene (C3), 2,2,6- trimethylcyclohexanone (C4), (*E*)-pinocarveol (C5), pinocarvone (C6), borneol (C7), myrtenol (C8), bornyl acetate (C9), Alloaromadendrene (C10), viridiflorene (C11), palustrol (C12), spathulenol (C13), viridiflorol (C14), ledol (C15), 1-epi-cubenol (C16), *ß*-eudesmol (C17), cadalene (C18), 15-nor-labdan-8-ol (C19), 16-kaurene (C20), Monoterpene hydrocarbons (MH), Oxygenated monoterpenes (OM), Sesquiterpenes hydrocarbons (SH), Oxygenated sesquiterpenes (OS), Diterpenes (Di) and Others (Ot).

Influence of soil characteristics on chemical composition variability

To estimate the influences of edaphic variable on chemical variability, a canonical correspondence analysis (CCA) based on major compounds, plots and edaphic characteristics was carried out (Table 2). Edaphic characteristics set included pH, electrical conductivity (EC), organic matter (OM), total nitrogen (N), cation exchange capacity (CEC), sand, silt, and clay. The first three canonical sets explained 81.89, 6.52 and 4.57% of the variation in chemical composition through soil characteristics, respectively, which together accounted for a total variance of 92.99%.

According to the CCA analysis (Table 2) and the correlation coefficients between chemical composition and edaphic characteristics (Table 3), soil organic matter content was positively correlated ($P < 0.05$) on the one hand, with the concetration α-pinene ($R = 0.92$), *p*-cymene ($R = 0.79$), (*E*)-pinocarveol ($R = 0.72$), pinocarvone ($R = 0.71$), and myrtenol ($R = 0.61$), and on the other hand, negatively ($P < 0.05$) to palustrol ($R = -0.69$), viridiflorol ($R = -0.69$), ledol ($R = -0.79$), cadalene ($R = -0.67$), 15-nor-labdan-8-ol ($R = -0.85$), and 16-kaurene ($R = -0.71$). Thus, the highest soil organic matter contents were observed in the plots located on granite substrate and again also in plot A2 of Pozuelo de Zarzon (slate). Therefore, as was previously shown by the PCA analysis, the first CCA component segregated the plots mainly into two groups linked to soil physicochemical characteristics. In addition, the second CCA component and the correlation analysis revealed that 2,2,6-trimethylcyclohexanone was positively correlated ($P < 0.05$) with electrical conductivity ($R = 0.74$) and silt content ($R = 0.66$), while borneol was negatively correlated ($P < 0.05$) with both parameters, electrical conductivity ($R = -0.81$) and silt content ($R = -0.63$).

Table 2. Canonical coefficients, eigenvalues, estimated and cumulative variance for the first three CCA sets between the major components of *C. ladanifer* essential oils and edaphic variables.

Traits	CCA1	CCA2	CCA3		CCA1	CCA2	CCA3
Compounds				Plots (Population)			
α-pinene	−2.24	−0.64	0.37	A1 (PdZ)	0.10	−0.80	0.64
camphene	0.20	0.86	0.60	A2 (PdZ)	−1.05	−0.03	−0.61
p-cymene	−0.99	−1.83	−0.00	A3 (PdZ)	1.23	−2.63	0.26
2,2,6-trimethylcyclohexanone	0.22	−1.04	0.09	B1 (VdF)	1.68	0.51	−0.86
(*E*)-pinocarveol	−1.93	0.32	−0.69	B2 (VdF)	1.11	1.20	0.40
pinocarvone	−1.93	0.62	−0.25	B3 (VdF)	1.65	0.96	0.75
borneol	0.32	3.15	−0.51	C1 (TdDM)	−0.72	−0.80	−2.38
myrtenol	−1.48	−0.60	−1.10	C2 (TdDM)	−0.78	0.90	−1.71
Bornyl acetate	0.02	2.28	1.94	C3 (TdDM)	−0.73	−0.10	0.80
Alloaromadendrene	0.05	0.72	1.42	D1 (VdG)	−0.18	1.05	−0.87
viridiflorene	0.47	−1.30	1.19	D2 (VdG)	−1.07	−0.55	2.01
palustrol	0.50	−0.18	0.30	D3 (VdG)	−0.98	0.31	1.49
Spathulenol	0.49	0.99	−0.50				
viridiflorol	0.49	−0.38	0.61				
ledol	0.62	−0.11	−1.70				
1-epi-cubenol	0.22	2.64	−0.91	**Eigen-value**	0,014	0,001	0,001
ß-eudesmol	−0.25	0.97	−0.98	**Variance (%)**	81.89	6.52	4.57
cadalene	0.93	0.09	−1.08	**Cumulative variance (%)**	81.89	88.42	92.99
15-nor-labdan-8-ol	1.25	−1.58	0.41				
16-kaurene	1.09	1.19	0.05				
Edaphic variables[a]							
pH	−0.32	−0.26	−0.03				
EC (dS/m)	0.03	−0.66	0.24				
OM (%)	−0,87	0.17	0.11				
N (% Total)	0.23	0.42	0.06				
CEC	−0,58	0.17	−0.14				
Sand (%)	0,13	0.49	−0.04				
Silt (%)	0.08	−0.66	−0.04				
Clay (%)	−0.27	0.42	0.11				

[a] OM: Organic matter; CEC: cation exchange capacity; N: total nitrogen; EC: electrical conductivity.

Table 3. Spearman's rank correlation coefficient (R) between essential oil major compounds or groups of compounds and edaphoclimatic variables.

Compounds	Edaphoclimatic characteristics[a]								
	pH	EC [dS/m]	OM [%]	N [% Total]	CEC	Sand [%]	Silt [%]	Clay [%]	Alt [m]
α-pinene	0.39	0.34	0.92 ***	−0.47	0.27	−0.15	0.15	0.09	−0.39
camphene	−0.56 *	−0.42	−0.07	0.35	−0.03	0.32	−0.45	0.22	0.05
p-cymene	0.42	0.49	0.79 **	−0.51	0.38	−0.39	0.42	−0.01	−0.42
2,2,6-trimethylcyclohexanone	−0.33	0.74 **	−0.09	−0.56 *	−0.30	−0.12	0.66 *	−0.55	−0.04
(*E*)-pinocarveol	0.48	0.15	0.72 **	−0.19	0.47	−0.21	−0.01	0.31	−0.20
pinocarvone	0.53	−0.01	0.71 **	−0.16	0.48	−0.17	−0.01	0.27	−0.02
borneol	−0.17	−0.81 **	−0.46	0.75 **	0.25	0.33	−0.63 *	0.44	0.52
myrtenol	0.62 *	0.14	0.61 *	−0.13	0.53	−0.13	0.00	0.24	−0.10
Bornyl acetate	−0.61 *	−0.27	−0.01	0.17	−0.13	0.16	−0.32	0.20	0.05
Alloaromadendrene	−0.36	−0.50	0.05	0.26	−0.17	−0.15	−0.30	0.29	−0.16
viridiflorene	−0.28	0.08	−0.55	0.20	−0.07	−0.33	0.14	0.03	−0.24
palustrol	−0.53	−0.08	−0.69 *	0.21	−0.48	0.03	−0.05	−0.10	−0.04
Spathulenol	−0.29	−0.44	−0.56	0.19	−0.37	0.38	−0.17	−0.17	0.56
viridiflorol	−0.41	−0.18	−0.69 *	0.23	−0.59 *	−0.23	0.01	−0.06	−0.01
ledol	−0.42	−0.09	−0.79 **	0.17	−0.48	0.25	0.03	−0.34	0.35
1-epi-cubenol	−0.42	−0.38	−0.14	0.26	−0.17	0.60 *	−0.44	−0.01	0.38
ß-eudesmol	−0.08	−0.27	0.14	0.31	0.33	0.20	−0.28	0.24	0.03
cadalene	−0.59 *	−0.02	−0.67 *	0.09	−0.45	0.02	0.20	−0.30	0.25
15-nor-labdan-8-ol	−0.15	−0.18	−0.85 ***	0.32	−0.10	−0.12	0.17	−0.15	0.45
16-kaurene	−0.58 *	−0.08	−0.71 *	0.04	−0.46	0.22	0.15	−0.48	0.58
Monoterpene hydrocarbons	0.39	0.30	0.90 ***	−0.42	0.30	−0.22	0.12	0.16	−0.37
Oxygenated monoterpenes	0.60 *	0.21	0.66 *	−0.20	0.54	−0.13	0.07	0.22	−0.07
Sesquiterpenes hydrocarbons	−0.68 *	−0.27	−0.32	0.29	−0.31	0.20	−0.29	0.05	−0.03
Oxygenated sesquiterpenes	−0.39	−0.29	−0.82 **	0.31	−0.53	0.17	−0.08	−0.23	0.27
Diterpenes	−0.37	−0.04	−0.78 **	0.17	−0.27	−0.02	0.22	−0.32	0.39
Others	−0.38	0.21	0.00	−0.12	−0.02	−0.30	0.18	0.13	0.07
Oil yield (% v/d.w)	0.14	0.27	0.86 ***	−0.28	0.27	0.04	−0.13	0.22	−0.50

[a] EC: electrical conductivity; OM: Organic matter; N: total nitrogen; CEC: cation exchange capacity; Alt: altitude; *, **, **: Significant at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

The effect of environmental factors on the chemical composition of essential oils extracted from several Mediterranean evergreen species such as *Pistacia lentiscus*,[16] *Mentha suaveolens*,[40] *Myrtus communis*,[41] and *Rosmarinus officinalis*[42] was previously determined. For our scenario, as described above for EO yield, all tested plots of *C. ladanifer* grew probably under similar microclimatic conditions (Table S1). Furthermore, previous research suggests that the edaphic characteristics of each type of soil may affect the distribution of ecotypes of the essential oil chemical compounds in other species of the genus *cistus*.[29,30] However, limited data are known about the relationship between soil characteristics and the essential oil composition of *C. ladanifer*.

Based on the CCA and correlation analysis, and in order of relevance, organic matter, electrical conductivity and silt content were found to be the most effective soil factors accounting for the variations in chemical composition. Fernández-Sestelo and Carrillo (2020)[33] also reported that soil organic carbon content and electrical conductivity affected the accumulation of the oxygenated monoterpenes 1,8-cineole and linalool in *Lavandula latifolia* essential oils from Spain. On the other hand, the silt quantity constituted the main edaphic variable underlying variations in the chemical composition of *Origanum compactum* essential oils from Morocco.[43] In contrast to our findings, Amzallag et al. (2005)[44] observed that the organic matter content correlated negatively with the relative percentage of the monoterpenes α-pinene, β-pinene, and 1,8-cineole in *Origanum dayi* essential oils, while it was positively correlated with the sesquiterpene β-caryophyllene oxide.

Phytotoxic activity

The phytotoxic potential of *C. ladanifer* essential oils from the 12 plots studied was determined. All samples presented a strong dose-dependent phytotoxic effect on Seed Germination

and Seedling Length (Table 4). ED50 values for the essential oil ranged from 0,025 ± 0,011 to 0,059 ± 0,016 µl/mL for Germination Inhibition and from 0,017 ± 0,004 to 0,053 ± 0,023 µl/mL for Seedling Length Inhibition. In addition, as shown in Figure 4, the results revealed a significant effect ($P < 0.05$) between populations and substrate type for both Germination Inhibition and Seedling Length Inhibition. Therefore, the plots on a slate substrate (except plot A2) presented lower ED50 values than those on granite, which indicates a higher phytotoxic activity.

Phytotoxic activity on Germination and Seedling Growth Inhibition of essential oils has been attributed to different classes of terpenes.[45] Previous studies have demonstrated that these compounds can affect physiological processes, such as cell viability, enzyme activity, chlorophyll synthesis and organelle reduction due to membrane disruption,[46] but also inhibit DNA and RNA synthesis.[47] In our research, the phytotoxic variation observed between plots reflected differences in the essential oils chemical composition. Table 5 reports the results of the correlation analysis between the essential oils major compounds and their phytotoxic activity expressed as ED50 values. Plots with higher percentages of the monoterpenes α-pinene, *p*-cymene, (*E*)-pinocarveol, pinocarvone, and myrtenol had significantly higher ED50 values, and hence less phytotoxic activity. On the contrary, the plots that showed stronger phytotoxicity (lower ED50 value) had higher percentages of the sesquiterpenes and diterpenes viridiflorene, palustrol, viridiflorol, and 15-nor-labdan-8-ol.

Few studies have been conducted on the phytotoxic activity of essential oils obtained from *C. ladanifer*. Furthermore, since these studies were carried out with a single essential oil sample, it was not possible to associate this phytotoxic effect with the major chemical compounds. For instance, Verdeguer et al. (2012)[21] and Benali et al. (2020)[19] reported the phytotoxic activity of *C. ladanifer* essential oil from two different geographical origins (Spain y Morocco, respectively) on weed and tomato seeds. In a previous study of ours,[20] a strong phytotoxic effect of *C. ladanifer* essential oil on *R. sativus* was also found. To our knowledge, several reports have shown that sesquiterpenes, especially oxygenates, are largely responsible for phytotoxic effects of plant essential oils.[48] However, other authors have found that monoterpenes are also biologically active compounds with phytotoxic potential.[45,49] Additionally, other minor phenolic compounds (*p*-cresol, 2-phenylethanol, 3-phenyl-1-propanol) present in the *C. ladanifer* essential oil have shown phytotoxic activity when evaluated individually.[50] Therefore, the potential phytotoxic effect of essential oils is not directly attributable to the major compounds, but to the synergistic or antagonistic effect resulting from the interaction among all the compounds.

Table 4. Phytotoxic activity of *Cistus ladanifer* essential oils of different plots.

Lithology	Population	Plot	Germination Inhibition ED_{50} [µl/mL][a]	seedling length inhibition ED_{50} [µl/mL]
Slate	PdZ	A1	0,025 ± 0,011	0,017 ± 0,004
		A2	0,044 ± 0,006	0,031 ± 0,009
		A3	0,033 ± 0,014	0,024 ± 0,007
	VdF	B1	0,033 ± 0,011	0,026 ± 0,014
		B2	0,034 ± 0,010	0,018 ± 0,011
		B3	0,028 ± 0,006	0,026 ± 0,004
Granite	TdDM	C1	0,055 ± 0,018	0,036 ± 0,008
		C2	0,059 ± 0,016	0,037 ± 0,008
		C3	0,049 ± 0,012	0,034 ± 0,013
	VdG	D1	0,040 ± 0,005	0,032 ± 0,007
		D2	0,048 ± 0,026	0,026 ± 0,012
		D3	0,051 ± 0,026	0,053 ± 0,023

[a] ED_{50}: effective dose 50 (logit analysis) in µl/mL of Petri plate headspace. Values are expressed as mean ± standard deviation (n = 5).

Table 5. Correlation coefficients (R) between essential oil major compounds or groups of compounds and the phytotoxic activity.

Compounds	Germination Inhibition ED_{50} [µl/mL][a]	Seedling length inhibition ED_{50} [µl/mL]
α-pinene	0.71 **	0.52 *
camphene	−0.33	−0.30
p-cymene	0.55 *	0.36
2,2,6-trimethylcyclohexanone	−0.17	−0.26
(*E*)-pinocarveol	0.80 **	0.70 *
pinocarvone	0.78 **	0.61 *
borneol	−0.22	−0.11
myrtenol	0.75 **	0.72 **
Bornyl acetate	−0.29	0.18
Alloaromadendrene	−0.30	−0.42
viridiflorene	−0.83 ***	−0.58 *
palustrol	−0.79 **	−0.64 *
Spathulenol	−0.23	−0.35
viridiflorol	−0.71 **	−0.75 **
ledol	−0.49	−0.35
1-epi-cubenol	0.08	0.16
ß-eudesmol	0.23	0.22
cadalene	−0.58 *	−0.50
15-nor-labdan-8-ol	−0.76 **	−0.61 *
16-kaurene	−0.47	−0.41
Monoterpene hydrocarbons	0.68 *	0.50
Oxygenated monoterpenes	0.80 **	0.69 *
Sesquiterpenes hydrocarbons	−0.53	−0.32
Oxygenated sesquiterpenes	−0.67 *	−0.62 *
Diterpenes	−0.78 **	−0.64 *
Others	−0.09	−0.09

[a] *, **, **: Significant at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

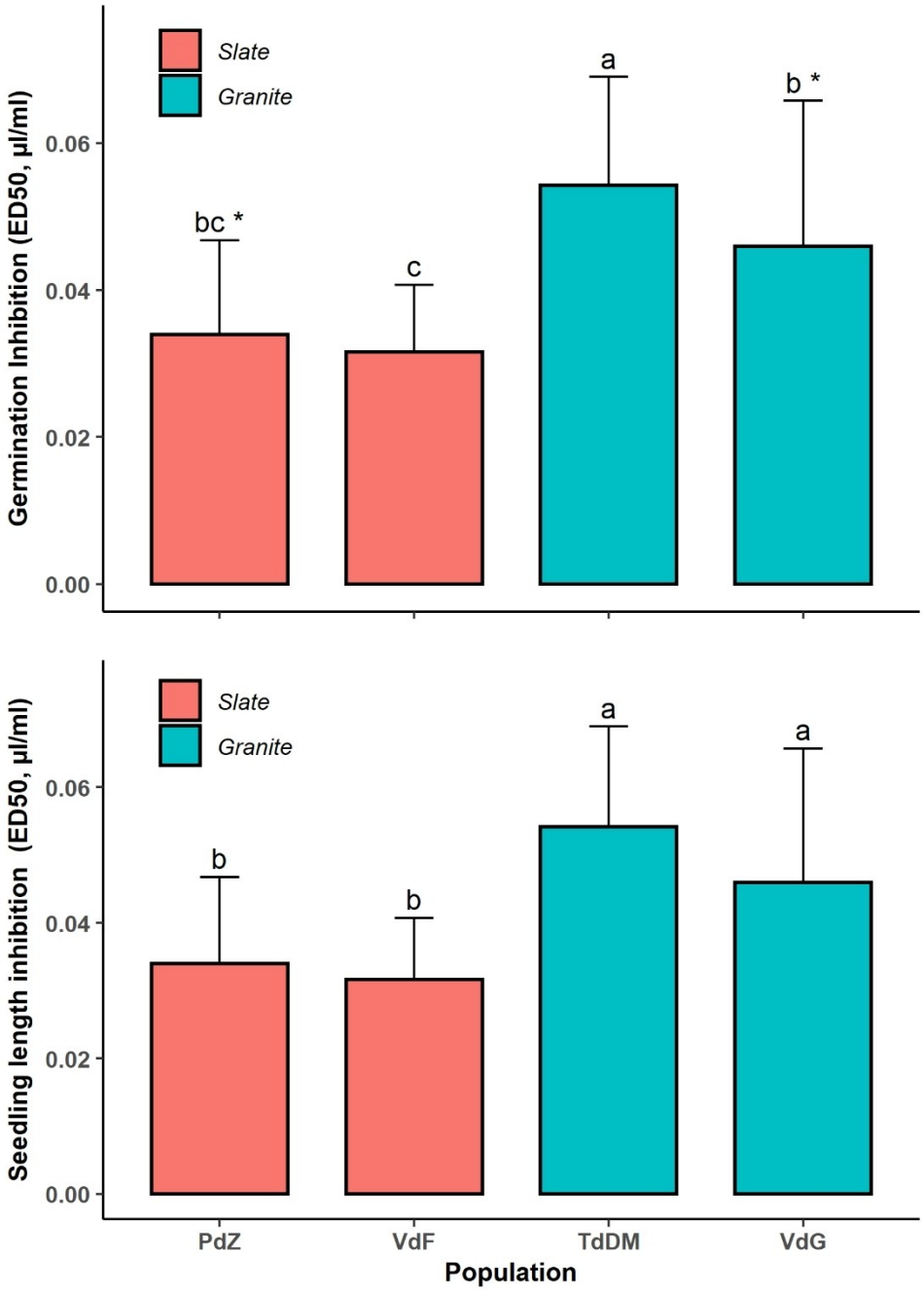

Figure 4. Phytotoxic activity of *Cistus ladanifer* essential oils on seed germination and seedling length of radish (*Raphanus sativus*). ED50: effective dose 50 (logit analysis) in µl/mL of Petri plate headspace. Values are expressed as means ± standard deviation (n = 15). For nANOVA analysis followed by Tukey's multiple range test, different letters or asterisks indicate statistically ($P < 0.05$) y marginally ($0.05 < P > 0.10$) significant differences, respectively.

Conclusions

The present research reveals a considerable inter- and intra-population variation in the yield, chemical composition and phytotoxic activity of *C. ladanifer* essential oils from plants growing on two different types of edaphic substrates. *C. ladanifer* essential oils were characterized by a high content of oxygenated sesquiterpenes and oxygenated monoterpenes. Chemical composition analyses grouped the plots according to the type of substrate on which the plants grow, indicating an important role of edaphic factors. Thus, variations in soil characteristics could act as an important force in the distribution of the best-adapted ecotypes to local habitats. In our study, organic matter content was identified as the most relevant edaphic variable accounting for these variations. Furthermore, *C. ladanifer* essential oils showed strong phytotoxic activity on germination and early growth of *R. sativus* seedlings, indicating their potential use as natural bio-herbicides in agriculture. The results point to the impact that environmental factors have not only on yield and chemical composition, but also on the

biological activity of essential oils and their possible industrial applications. However, terpene biosynthesis pathways are also determined by genetic factors and, therefore, further studies are required to identify specific chemotypes with desirable commercial characteristics for the perfume, cosmetic and agri-food industries.

Experimental Section

Plant material and site description

Plant material was harvested from four wild populations of *C. ladanifer* located within an area with a high fire risk in the northwest of Extremadura (Spain) (Figure 1). All populations fell within the supra-Mediterranean bioclimatic zone, with similar latitude and altitude conditions (Table S1). To address the effect of geological substrate, two populations were located in slate and two in granite. To analyse intra-population variability, three plots per population were established with a minimum distance of 500 m from each other. Five randomly distributed sampling points were selected in each plot. Aerial parts of a minimum of 20 randomly selected individual plants were collected from each sampling point in August 2018 (fruit maturation stage). The plants were identified by botanist Dr. Vázquez-Pardo M.C. according to the specimen HSS45679 deposited in the herbarium CICYTEX. The samples were air-dried at ambient temperature under shade for two weeks and stored in dark conditions until oil extraction.

The edaphic and climatic characteristics of the different test plots were determined. Temperature (°C) and precipitation (mm) data were obtained from the nearest meteorological stations of the Spanish Meteorological Agency (AEMET). A soil sample of about 1 kg per plot was taken at a depth of 30 cm (within the plant's rhizospheres). In turn, each of these soil samples (12 in total) was composed of five subsamples (one per sampling point) which were homogeneously mixed. Soil analysis was carried out by the Elemental and Molecular Analysis Service of the University of Extremadura. The physical and chemical soil properties analysed are listed below, together with the analytical techniques used in brackets: pH (electrometry), electrical conductivity (conductimetry), total nitrogen (Kjeldahl method), organic matter (Walkley-Black method), cation exchange capacity (1 N ammonium acetate method), assimilable phosphorus (Olsen method) and texture (Boyoucos method).

Essential oil extraction

Essential oils were isolated by hydrodistillation with a Clevenger-type apparatus for 3 hours from 200 g of dried aerial parts, according to the procedure described in the European Pharmacopoeia (2010). The essential oil obtained was dried with anhydrous Na_2SO_4 and stored in a glass vial at −18 °C until use. The essential oil content was calculated as volume (mL) of essential oil per 100 g of dried plant material.

Gas Chromatography-Mass Spectrometry (GC-MS) analyses

Essential oils were analysed using an Agilent 6890 N gas chromatograph (GC) (Palo Alto, CA, U.S.A.) equipped with an HP-5 column (30 m×0.25 mm i.d., 0.25 μm film thickness). Helium was used as a carrier gas, operating under constant pressure conditions (elution of β-ionone at 27.60 min) and the split ratio to 100:1. The GC was associated with an Agilent model 5972 inert mass spectrometry detector (Agilent, Palo Alto, CA, USA). The initial oven temperature was 60 °C; then it was increased to 155 °C at a rate of 2.5 °C/min; finally, it was raised to 250 °C at a rate of 10 °C/min. The Injection Port and the detector temperature were adjusted at 250 and 280 °C, respectively. The mass scan ranged from 50 to 550 m/z at 3.21 scan/s and the ionizing energy was 70 eV. The quadrupole temperature was 150 °C and the electron multiplier voltage was maintained at 1300 V (Jordán et al., 2009). For each sample, 0.1 μl of pure essential oil was injected.

Individual volatile compounds were identified by comparison of their Retention Indexes (RI) with those published in the literature,[51,52] and also by comparison of their mass spectra with those included in the NBS75 K library (U.S. National Bureau of Standards, 2002). The retention indexes were calculated according to a series of *n*-alkanes (C6-C17). The relative percentage (%) of each compound was determined according to the chromatographic peak areas using the total ion current (see Figure S1). Only compounds with a percentage $\geq$1 % were considered for analysis.

Phytotoxic activity

Radish (*Raphanus sativus L.*) was the species selected for the experiments because of its susceptibility to phytotoxic metabolites.[53] Sets of twenty previously disinfected seeds were placed in Petri plates of 9 cm diameter with two layers of Whatman no. 1 filter paper. The filter papers on the plates were wetted with 2 mL of distilled water and essential oils were deposited as a single drop in the center of the plates at different doses (0.022, 0.044, 0.088 and 0.178 μl/mL for 90 mL Petri plate headspace). Furthermore, Petri plates containing only distilled water were used as controls. Five replicates were prepared per essential oil sample and dose. The Petri plates were sealed with Parafilm and incubated at 25 °C in darkness. After 7 days, percentages of germination and seedling lengths (hypocotyl plus radicle) were recorded. Germination (GIP) and seedling length (LIP) inhibition percentages were calculated according to the following equation: GIP/LIP = (C–T/C)×100, where C and T correspond respectively to the control and the treatment (Benchaa et al., 2019). Finally, the results were expressed as ED50 (effective dose 50) values obtained by logit analysis with essential oil dose versus percentage inhibition of both seed germination and seedling length.[54] These analyses were run in R software using the drm function (drc library).[55]

Statistical analysis

Variation and drivers of essential oil yield, the relative percentage of major compounds or groups of compounds and phytotoxic activity (ED_{50} values, μl/mL) were evaluated by nested analysis of variance (nANOVA hereafter). Substrate type, population and plot were included as nested independent factors in hierarchical order. In each case, Shapiro-Wilks and Bartlett's tests were used to assess the normality and homoscedasticity of the data. All pairwise mean comparisons were conducted with Tukey's test (HSD) at a significance level of $P < 0.05$.

The relationship between essential oil yield and the edaphic variables measured was evaluated by multiple linear regression and correlation tests. Models obtained were compared using Akaike information criterion corrected for small sample size (AICc) and the model with the lowest AICc was retained.[56]

Among-plot variation of the essential oil composition was evaluated through Principal Component Analysis (PCA).[57] A canonical correspondence analysis (CCA) was conducted to assess the dominant relationships between plots, essential oil chemical composition and edaphic characteristics.[58] In addition, the correla-

tions between the major compounds or groups of compounds and the edaphic properties or phytotoxic activity were determined. All statistical analyses were performed with R Core Team (2022).[59]

Author Contributions

All authors contributed to the design of the study, setup of experiments, data collection and analysis. Carlos Pérez-Izquierdo drafted the first manuscript. All Authors contributed critically to the interpretation of the results and text revisions. All authors approved the final manuscript.

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Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: *Cistus ladanifer* · environmental factors · essential oil · GC-MS · phytotoxicity

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