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Clonal and seasonal genetic variation of major oil components of *Salvia fruticosa* Mill.

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Abstract: Anatolian sage (*Salvia fruticosa* Mill.) is widely used in many food, cosmetics, and pharmaceutical sectors. This study aimed to determine the differences in essential oil components and seasonal changes in the components of six clonally selected C-clones and one natural population in a randomized block design with three replications. Samples were collected monthly from C-clones for two years, and essential oil rates were determined. Clonal heritability was between 0.00 and 0.78. When the best clone was selected from six clones, the genetic gain changed from 12.4 to 44.8 for the essential oil components. Assessment of all clones revealed that the time of monthly harvests significantly affected essential oil components. The clone and harvest time interaction caused significant differences in essential oil components, and clones reached the highest values in different months. The correlation analysis showed a significant negative association between camphor and "β-pinene and β-caryophyllene" and a positive association between camphor and essential oil. High variation in the components, the differences in harvest times, and high correlations between components indicated that clones developed by selection have a significant production potential.

Keywords: anatolian sage; selection; harvest time; heritability; genetic gain

The genus *Salvia*, belonging to the Lamiaceae family, includes medicinal, aromatic plant species that have been used for a long time and have maintained their economic importance (Elmas et al. 2021). Regionally, in Anatolia or Asia Minor, *Salvia fruticosa* Mill., often known as Anatolian sage or sage, gray chalba, or apple sage, is an important sage species. Its leaves are consumed as tea, and the essential oil obtained from them is called 'apple oil,' which is good for respiratory tract infections, nervous diseases, and diarrhea and has a pain relief effect. In addition, Anatolian sage is used for essential oil production in Türkiye, and a significant part of the essential oil is exported (Dinçer et al. 2012; Elmas et al. 2021).

Chemical quality indicators have also gained importance recently for *Salvia* species. The type and ratio of components that determine essential oil quality can be used. *S. fruticosa*, which is widespread in the natural flora of Türkiye, Greece, the Peloponnese and has a lower thujone ratio that has a toxic effect over a specific dose, is more suitable for tea than *S. officinalis* (Papafotiou et al. 2023; Schmiderer et al.

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2023). Türkiye's medicinal and aromatic plants are mainly collected from nature, but the quality standardization of the products collected from nature is complex. In addition, the collecting these plants from nature should be supervised to conserve the natural flora, producers should be supported, and products that do not comply with the standards should not be marketed (Metin et al. 2012; Erol 2015). In addition, world markets and the pharmaceutical industry demand "standard" products with high amounts and quality active ingredients. Meeting growing demand and obtaining sufficient standard and quality products is impossible by collecting natural plants and can only be achieved by cultivating medicinal and aromatic plants obtained through selection and breeding studies (Bayram 2001; Leontaritou et al. 2020) directed to producer and market demand (Franzel et al. 1996). Thus, new cultivars have been developed by evaluating herb yield and essential oil ratio (Bayram 2001; Arslan et al. 2014; Lal et al. 2021). However, the research on the main component ratios of essential oils, clonal differences in essential oil components, harvest times', clone harvest time interactions, correlations between components, and genetic gain and heritability of essential oil are limited.

This study aimed to reveal (a) the differences in essential oil content, essential oil components of 6 C-clones (variety candidates) determined by the selection, (b) heritability and genetic gain essential oil component, (c) the effect of monthly harvest time on the clones, and (d) the interaction between clone components providing different options to the producer and the consumer regarding clones and harvest times.

MATERIAL AND METHOD

Six C-clones were selected among 1 250 clones from 15 different populations in the projects titled 'Selection Breeding in Some Sage (*Salvia* spp.) Species Growing in the Antalya Flora' and 'Identification of Genotypes with Superior Characteristics in the Anatolian Sage Populations from Antalya Flora.' In addition to six clones, the seedlings obtained from the seeds of a genotype were included as standard. (Table 1).

The summers are hot and dry, and the winters are warm and rainy in Antalya, where the field experiments were conducted. During the research (24 months), close to the long-term, the average temperature, precipitation, and relative humidity were 19 °C, 60 mm, and 73%, respectively.

A complete randomized block design with three replications was used in the experiment. The drip irrigation system was installed in the experimental field, and mulch was laid on the soil surface. Selected C-clones were planted in four rows, with ten plants for each row in 2017. Between and inter-row spacing in planting were 70 and 40 cm, respectively. All cultural practices were carried out during the growing seasons. The essential oil content and components were determined as three replications in the samples taken every month starting from June 2017 (three months after planting) for 24 months (Figure 1). Sampling was done in the form of 3 branches from each plant. It was dried with its branches at 40 °C for 48 hours. Dry leaves were used as samples. The essential oil ratio (%) was determined by the hydrodistillation method in the Clevenger apparatus. Distillation was carried out for three hours by adding 200 mL of distilled water to the 20 g dry leaf sample, and the essential oil ratio (mL/100 g dry sample, %) was calculated (Anonymous 2011).

The component ratios of essential oil were determined using a GC-MS/FID (Gas chromatography (Agilent 7890A)-mass detector (Agilent 5975C)/flame ionization detector) device and capillary column (HP Innowax Capillary; 60.0 m × 0.25 mm × 0.25 μm). The injection block temperature was set at 250 °C, column temperature was at 60 °C (10 minutes) and

Table 1. The locations of material used in the study

Population No.	Location	Altitude (m a.s.l.)	Coordinate
Fk3-16	Kemer	31	36 34 59 N 30 34 33 E
Ffk4-9	Kemer	137	36 32 06 N 30 32 35 E
Fk4-14	Kemer	137	36 32 06 N 30 32 35 E
Fk5-7	Kemer	20	36 40 96 N 30 33 43 E
Fd2-9	Demre	68	40 10 49 N 35 75 57 E
Fd4-13	Demre	5	40 10 18 N 35 75 49 E
Standard	Kumluca	57	36 14 11 N 30 24 41 E

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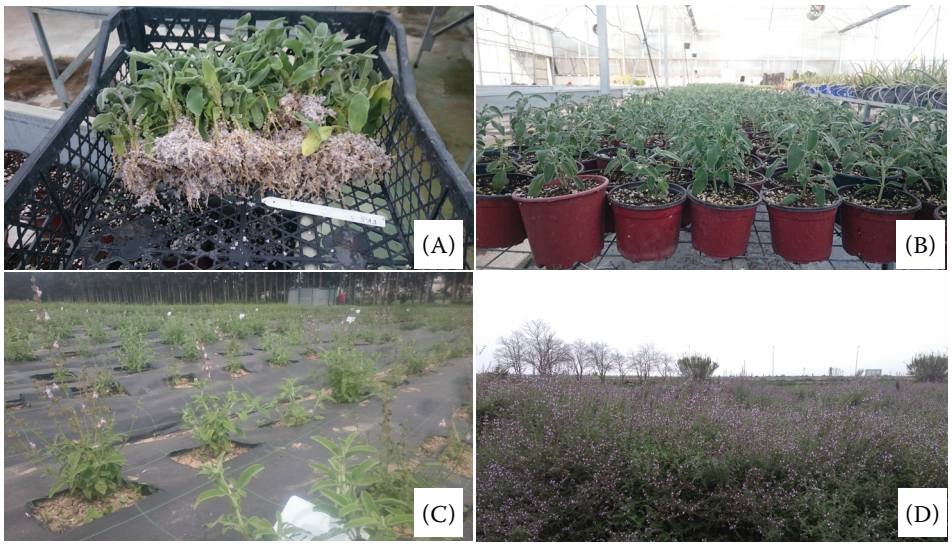

Figure 1. Images of the area where the study was carried out
(A) – rooted seedlings; (B) – seedlings ready for planting; (C) – 3 months after planting; (D) – 24 months after planting

increased from 60 °C to 220 °C at 4 °C/minute and kept at 220 °C for 10 minutes. MS detector data was used to identify the essential oil components, and Wiley7n and Oil Adams libraries were used for this purpose. In addition, the C8-40 alkane series data and the component retention index data were also used to identify the components. The component ratios also were determined by the Flame Ionization Detector (FID) (Özek et al. 2010). Images of laboratory studies are given in Figure 2.

The following mixed model was used to statistically analyse the measured and observed component data recorded during the two years of the experiment:

$$y_{ijkml} = \mu + T_i + P_j + B_k + C_l + CB_{kl} + e_{ijklm} \quad (1)$$

Where y_{ijkml} is observation on mth ramet, l^{th} clone, k^{th} block, j^{th} harvest time, i^{th} year, is overall mean, is the randomized effect of year, μ is overall mean, Ti is the randomized effect of year i (i = 1, 2), P_j is the fixed effect of j. harvest time (month) (j = 1,.. 12), B_k is the fixed effect of k. block (k = 1, .. 3), Cl is randomized effect of l. clone (genotype) (l = 1, .. 7), CPjl is interaction of clone and harvest time, CBkl is interaction of block and clone (plot), e_{ijklm} is the experimental error. Heritability and genetic gain equations are below:

$$H^2 = \frac{\sigma_c^2}{\sigma_c^2 + \sigma_{cp}^2 + \sigma_{cb}^2 + \sigma_e^2} \quad (2)$$

$$\Delta G = \frac{the\ best\ clone - overall\ mean}{overall\ mean} \quad (3)$$

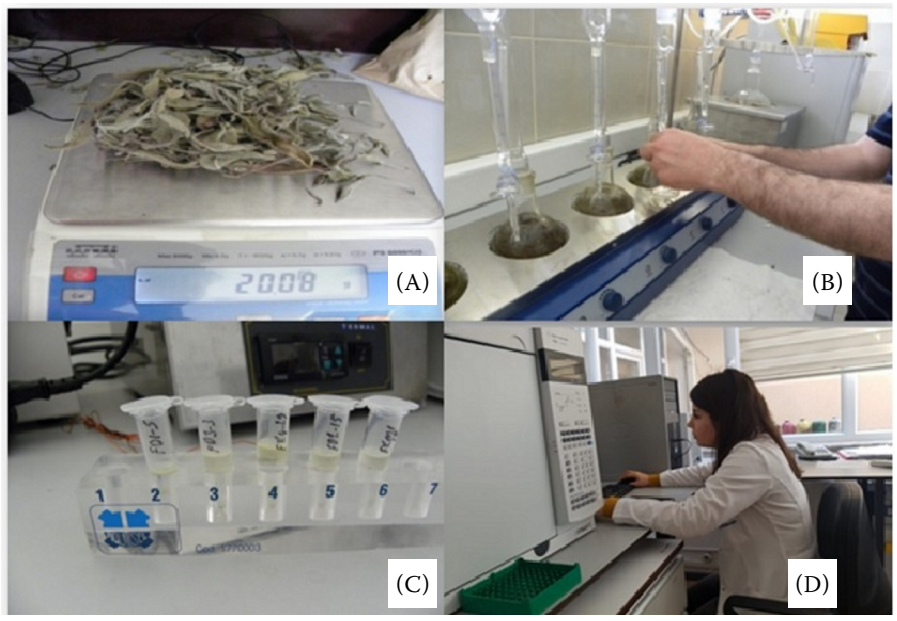

Figure 2. Images of laboratory studies
(A) – herbal sample; (B) – Clevenger apparatus; (C) – essential oils examples; (D) – GC-MS/FID (Gaschrom atography)

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Where H^2 is clonal heritability, σ_c^2 is clonal variance, σ_{cp}^2 variance of clone and harvest time interaction, σ_{cb}^2 is variance of clone and block interaction and σ_e^2 is error variance and ΔG is genetic gain.

After checking normal distribution, β-caryo phyllene, β-thujone, and Camphor were normalized by square root transformation. Student-Newman-Keuls (SNK) multiple comparison test was applied to statistically significant traits in variance analysis. The SAS 9.0 statistical software was used to analyse the data (SAS Institute Inc. 2002).

RESULTS AND DISCUSSION

Anatolian sage clones' mean essential oil content and main components of 1,8-cineol, camphor, β-pinene, β-caryophyllene and β-thujone were 3.09% 50.21%, 11.29%, 8.92%, 5.74% and 1.87%, respectively (Table 2). The values were compatible with those reported in other studies (Skoula et al. 2000; Leontaritou et al. 2020; Elmas et al. 2021).

The results of the variance analysis are given in Table 3. The differences between clones, harvest times, clone × harvest time interactions, and years were statistically significant, except for the essential oil in clones and β-thujone in years. The essential oil content and essential oil components of medicinal and aromatic plants may vary depending on ecological conditions, climate and soil characteristics, genotypic structure, part of the plant used and harvest time (Saharkhix et al. 2009). The clones show significant differences ($P < 0.01$) in all traits except for essential oil content, indicating the importance of genetic structure despite identical environmental conditions.

The comparisons of clones, genetics gain, and clonal heritabilities for traits were given in Table 4. The difference between the lowest and the highest ratio in the β-pinene was nearly two-fold, while the difference between these values was over three-fold for β-thujone. Regarding differences, genetic gains were between 15.0 and 44.2 from the standard and between 12.4 and 44.8 % from the overall mean, revealing the importance of clonal selection.

Clonal heritability varied from 0.00 to 0.68 in 2018 and 0.00 to 0.78 in 2019. The heritability was high in the β-thujone but low or moderate in other essential oil components. High and moderate heritabilities of essential oil, like β-thujone, cineol, and β-pinene, showed that successful breeding could be carried on sage's essential oil components. On the other hand, Sánchez-Vioque et al. (2022) found higher heritability for β-pinene (0.73), 1.8 cineole (0.88), camphor (0.87), and essential oil (0.69) in *Salvia lavandulifolia* Vahl. They found relatively higher heritabilities in *Salvia lavandulifolia* than in *Salvia fruticosa* in our study due to different statistical models and materials, including population. Another reason for the difference in heritabilities could be sourced from the fact that they used wild populations in contrast to our research.

Many researchers carried out studies on several aspects of Anatolian sage (Skoula et al. 2000; Dinçer et al. 2012; Aydın et al. 2019; Leontaritou et al. 2020). The essential oil ratios and main components obtained from the current study were like those mentioned research. However, we used clones due to more convenience for "standard" products, but the mentioned ones used Anatolian sage produced from seeds, not clones.

The mean essential oil ratios of the different clones essential oil ratios at the harvest times are given in Table 5. The highest essential oil ratio (3.93%) was obtained in August, while the lowest (2.10% and 2.11%) was recorded in February and March. β-Thujone, a toxic substance, had the highest value in March (1.05%) and April (1.25%). On the other hand, the 1,8-Cineol was the lowest (44.05 and 44.78%) in August and September when the essential oil was high; on the contrary, camphor, which was stated to have a toxic effect by Narayan and Singh (2012), had the highest ratio (21.36%) in August.

Table 2. Mean, minimum, maximum values and standard deviations of essential oil components (%) for clonal level

Parameter/Traits	Essential oil (%)	1.8 cineol (%)	Camphor (%)	β-pinene (%)	β-caryophyllene (%)	β-thujone (%)
Mean	3.07 ± 0.04	50.29 ± 0.3	9.86 ± 0.4	8.78 ± 0.1	5.39 ± 0.2	1.83 ± 0.06
Minimum	1.67	34.69	0.44	3.66	0.55	0.32
Maximum	6.00	67.85	33.29	18.40	15.68	8.18
Standard deviation	0.76	5.81	7.51	2.77	2.77	1.24
Coefficient of variation (%)	24.58	11.56	66.51	31.04	52.74	66.09

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Table 3. Variance analysis for all traits

Traits	Source of variation	Degrees of freedom	Mean squares	*F*-value	*P*-value
	Clone	6	0.20	0.60	0.7247
	Harvest time (month)	11	14.89	105.18	< 0.0001
	Clone × Harvest time	66	0.14	1.63	0.0030
Essential oil	Year	1	16.34	182.50	< 0.0001
	Block	2	0.17	0.62	0.5536
	Clone × Block	12	0.25	3.15	0.0003
	Error	326	0.09		
	Clone	6	540.04	76.87	< 0.0001
	Harvest time (month)	11	373.51	14.12	< 0.0001
	Clone × Harvest time	66	27.74	2.23	< 0.0001
1,8-cineol	Year	1	491.67	30.60	< 0.0001
	Block	2	16.59	2.28	0.1339
	Clone × Block	12	6.94	0.46	0.8739
	Error	326	12.41		
	Clone	6	2.73	5.17	0.0008
	Harvest time (month)	11	37.79	54.50	< 0.0001
	Clone × Harvest time	66	0.73	2.59	< 0.0001
Camphor	Year	1	2.22	7.87	0.0053
	Block	2	0.17	1.71	0.2058
	Clone × Block	12	0.90	0.26	0.9947
	Error	326	0.28		
	Clone	6	158.39	23.94	< 0.0001
	Harvest time (month)	11	91.09	13.40	< 0.0001
	Clone × Harvest time	66	7.26	4.34	< 0.0001
β-pinene	Year	1	20.75	12.49	0.0005
	Block	2	0.63	0.52	0.5927
	Clone × Block	12	1.10	0.67	0.7831
	Error	326	1.66		
	Clone	6	2.20	9.56	0.0002
	Harvest time (month)	11	9.12	73.58	< 0.0001
	Clone × Harvest time	66	0.13	1.37	0.0416
β-caryophyllene	Year	1	17.40	187.55	< 0.0001
	Block	2	0.08	0.41	0.6692
	Clone × Block	12	0.20	2.14	0.0145
	Error	326	0.09		
	Clone	6	6.60	42.18	< 0.0001
	Harvest time (month)	11	0.44	4.70	< 0.0001
	Clone × Harvest time	66	0.10	2.23	< 0.0001
β-thujone	Year	1	0.15	3.33	0.0691
	Block	2	0.10	1.01	0.3896
	Clone × Block	12	0.10	2.30	0.0079
	Error	326	0.04		

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Table 4. Comparison of clone means; the letters show different groups, clonal heritability and genetic gain for each essential component content (%)

Clone	Essential oil (%)	1,8 cineol (%)	Camphor (%)	*β*-pinene (%)	*β*-caryophyllene (%)	*β*-thujone (%)
Fd4-13	3.00^{a}	**56.53^{a}**	8.28^{c}	**10.26^{a}**	4.20^{d}	1.61^{c}
Fd2-9	3.09^{a}	51.96^{b}	8.71^{c}	**10.36^{a}**	4.57^{cd}	1.03^{d}
Fk3-16	3.08^{a}	50.77^{b}	8.99^{bc}	**10.29^{a}**	4.84^{cd}	0.99^{d}
Ffk4-14	3.16^{a}	50.71^{b}	9.66^{b}	9.13^{b}	5.25^{bcd}	1.46^{c}
Standard	3.06^{a}	49.15^{b}	**12.02^{a}**	7.29^{d}	5.46^{bc}	1.81^{b}
Fk4-9	3.15^{a}	48.00^{d}	9.94^{b}	8.45^{c}	6.14^{b}	**3.57^{a}**
Fk5-7	2.97^{a}	44.94^{e}	**11.40^{a}**	5.68^{e}	**7.25^{a}**	2.34^{b}
Overall mean	3.07	50.29	9.86	8.78	5.39	1.83
H^2 (2018)	0.0 ± 0.0	0.58 ± 0.38	0.09 ± 0.09	0.54 ± 0.36	0.48 ± 0.33	0.68 ± 044
H^2 (2019)	0.0 ± 0.0	0.28 ± 0.25	0.01 ± 0.05	0.30 ± 0.26	0.09 ± 0.10	0.78 ± 0.65
Genetic gain (*ΔG*)*						
To standard (%)	NA	15.0	–31.1	41.3	32.7	–44.2
To mean (%)	NA	12.4	–25.6	17.3	34.5	–44.8

*Selected the best clone or clones (for example in *β*-pinene) and compared of standard or overall mean, minus value shows toxic essential oil estimating lowest value instead of highest value; $^{a–d}$ the same letter shows a different group and bold letters show the best one or the best group; H^2 – the clonal heritability; NA – nonsignificant and no gain

Hold et al. (2000) also reported the highest values for camphor (14.93%) and *β*-thujone (2.79%) in summer. Sarrou et al. (2016) conducted a study between the autumn and spring seasons. They found the highest ratio of *β*-pinene (14.07%) and in *β*-caryophyllene (7.17%) in the spring, similar to our research. The relationships between harvest time and essential oil content were associated with temperature and light intensity, which caused a decrease in the essential oil ratio (Kargiolaki et al. 1994). In summary, the differences in components according to harvest time were underlined in our study. On the other hand, as seen in Table 3, the interaction of clone and harvest time that changed essential oil amount for each harvest time was statistically significant for all traits. Therefore, harvest time might be chosen to obtain the highest essential oil.

A correlation was estimated among essential oil components (Table 6). A negative correlation was recorded between camphor and *β*-pinene (–0.80) and between essential oil and *β*-caryophyllene (–0.72). Astani and Schnitzler

Table 5. Means differences of harvest time for main components of essential oil

	Essential oil	1,8-cineol	Camphor	*β*-pinene	*β*-caryophyllene	*β*-thujone
January	2.36^{h}	51.22^{c}	5.31^{f}	10.18^{b}	**7.95^{a}**	1.89^{ab}
February	2.10^{i}	51.74^{bc}	4.02^{f}	10.60^{b}	**8.09^{a}**	1.68^{ab}
March	2.11^{i}	52.65^{abc}	2.44^{g}	**12.33^{a}**	**7.88^{a}**	1.05^{c}
April	2.44^{h}	53.80^{ab}	4.05^{f}	10.53^{b}	**7.75^{a}**	1.25^{c}
May	3.72^{bc}	**54.71^{a}**	10.37^{d}	7.68^{d}	5.15^{c}	1.68^{ab}
June	3.37^{ef}	52.69^{abc}	13.21^{c}	7.69^{d}	4.37^{d}	1.63^{b}
July	3.60^{cd}	48.48^{d}	18.55^{ab}	6.85^{d}	2.45^{f}	1.94^{ab}
August	**3.93^{a}**	44.05^{e}	**21.36^{a}**	6.90^{d}	2.46^{f}	1.81^{ab}
September	3.82^{ab}	44.78^{e}	19.68^{ab}	7.29^{d}	2.66^{f}	1.96^{ab}
October	3.48^{de}	46.86^{d}	17.55^{b}	7.68^{d}	3.65^{f}	**2.12^{a}**
November	3.30^{f}	51.06^{c}	8.64^{e}	9.26^{c}	6.28^{b}	1.89^{ab}
December	2.80^{g}	50.94^{c}	5.60^{f}	10.96^{bc}	**8.20^{a}**	1.80^{ab}

$^{a–g}$letter shows a different group and bold ones show the best one or the best group.

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Table 6. Correlation between components

Trait	Essential oil	1.8-cineol	Camphor	*β*-pinene	*β*-caryophyllene
Essential oil	1				
1.8-cineol	–0.23 < 0.0001	1			
Camphor	0.69 < 0.0001	–0.62 < 0.0001	1		
β-pinene	– 0.50 < 0.0001	0.54 < 0.0001	–0.80 < 0.0001	1	
β-caryophyllene	–0.72 < 0.0001	0.06 0.193	–0.67 < 0.0001	0.34 < 0.0001	1
β-thujone	0.20	–0.29	0.26	–0.47	–0.02

(2014) reported that *β*-pinene has an antiviral effect and reduces viral infectivity by 100%. Koyama et al. (2019) determined the flavouring and wound-healing properties of *β*-caryophyllene. The results revealed that sage clones could be determined according to the production of targeted components, and the essential oil yield and the ratio of components can be increased together or selected only for desired essential oil components. Therefore, production planning should be carried out considering the correlations determined in our study for the compounds.

CONCLUSION

Our study differs from most previous studies because it uses clonal material instead of seeds. The study has revealed that the component ratios vary according to the clones, the component contents of clones differ according to the harvest time, and significant correlations between the contents. In addition, the clones were rich in some components and low in some toxic components. On the other hand, the heritabilities and genetic gains of essential oil components for clones indicated that breeding activities in *S. fruticosa* are indispensable. The findings also showed that fd4-13 and fd2-9 clones could be used for new varieties in further breeding studies due to higher 1.8 cineol and *β*-pinene levels and lower *β*-thujone and camphor levels.

The combinations between harvest time and essential oil components of clones and different correlations between oil contents in our study offer the opportunity to evaluate various options for producers and consumers. Proper use of options by the producers will supply more sustainable production with considerable genetic gain, and consumers can be introduced to alternative consumption of sage components at different times. Due to the positive or negative correlations between the oil contents, a clone can also be used for multiple purposes.

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