The Effect of Geographic Location and Seed Storage Time on the Content of Fatty Acids in Stone Pine (*Pinus pinea* L.) Seeds

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The fatty acids content in pine seeds is important because it affects germination success and commercial value. In this study, the amount of fatty acids in the seeds of stone pine (*Pinus pinea* L.) was determined in seeds collected from different geographical locations and after two-year storage. The pine seeds were collected from different locations where peanut pine is densely located in the Western Black Sea Region of northern Turkey in 2019. The results showed that the content of some fatty acids (C18:1n-9, ΣMUFA, C18:2n-6, and ΣPUFA) in the seeds collected from different geographical locations did not differ significantly, but the content of others (C16:0, C18:0, C20:0, ΣSFA, and C18:3n-3) was dependent on the locations. The difference among locations was up to 40% ($p < 0.05$). In general, the lowest values were obtained from the seeds of Bartın Karaçaydere, and the highest values were obtained from the seeds of Bartın Çakraz and Bartın Avara locations. After two years of storage, the content of all the fatty acids in the seeds decreased based on the location, and a decrease of more than 25% in the content of some fatty acids was observed.

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INTRODUCTION

Turkey is home to very valuable pure and mixed forest stands consisting of a large number of tree species, with the effect of different environmental conditions that have emerged with the effect of different geographical conditions. While these tree species have an important production value, such as providing wood raw materials, they also have value in the production of non-wood by-products (pine nut, laurel, linden, chestnut, *etc*.). In this sense, many species in Turkey have the feature and value of being widely used, especially in agroforestry activities. Stone pine (*Pinus pinea* L.) is one of these species. The seeds of stone pine play an important role in the Mediterranean diet due to their exquisite flavor (Evaristo *et al*. 2010). Turkey is among the top three countries in the world ranking in terms of pine nut production. According to the statistical figures announced, the pine nut export made by Turkey in 2013 was approximately 500 thousand tons (Kılcı *et al.* 2014). This export amount reached 1.1 million tons in 2015, and its contribution to the national economy of our country is 40.3 million dollars (Kurt *et al.* 2016). To ensure the continuity of pine nut production and export and continue its contribution to the national economy, it is necessary to protect the pine species and increase the forest area in suitable ecological conditions with afforestation. To achieve this goal, it is important to produce, collect, and use healthy and improved pine seeds in sapling production studies. Thus, collecting seeds from suitable locations and keeping them within the appropriate storage time interval is important in terms of producing improved seedling material (Ürgenç 1998). In this case, necessary actions in terms of seed physiology and sapling development must be carried out under natural processes. Fatty acids play an important role in the germination of seeds and seedling development, both physiologically and anatomically, as a critical factor in the realization of these processes (Fenner and Thompson 2005).

Fatty acids are carboxylic acids with an aliphatic chain of variable length, usually with an even number of carbons. Fatty acids are divided into 4 groups according to chain length: short carbon chains from C2: 0 to C6: 0, medium chains from C6: 0 to C12: 0, long chains C12: 0 to C18: 0, and very long chains over 18 carbons. The main fatty acids in plants are palmitic acid (C16: 0) and 18-carbon fatty acids (El Omari *et al.* 2021). The fatty acids found in *Pinus pinea* seeds are a good source of dietary phytosterols. These are components of plant cell membranes that have been reported to inhibit intestinal absorption of cholesterol, thereby lowering total plasma cholesterol and LDL (low-protein diet) levels. These compounds have anti-inflammatory, anti-atherogenic, and antioxidant activities as well as other beneficial properties, including anticancer effects (Lutz *et al.* 2017).

Understanding the effects of geographical location and seed storage time on the amount of fatty acids may provide information on obtaining improved seed material, especially by evaluating the changes caused by these factors on seed physiology and germination. The commercial value of seeds is proportional to the amount of fatty acids. In the studies carried out to date, there are no studies on how the fatty acid contents of stone pine seeds, which have a very high commercial value, change depending on the location and the change in fatty acids depending on storage.

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In this study, the effects of location and storage time on the content of fatty acids in pine seeds, collected from 7 different geographical locations, were investigated.

EXPERIMENTAL

Seed Samples

The pine seeds used in this study were collected from seven different locations (Karaçaydere, Amasra, Çakraz, Avara, Kurucaşile, Cide, and Çaycuma) in the Western Black Sea Region of Turkey, with five repetitions (2 kg each) and each one from the dominant pine trees of the study area in July of 2019. The locations where the samples were collected are areas where the same climate type prevails, and they are in the very humid climate class according to both Lang and DeMartone climate classifications (URL-1). The average annual temperature throughout the region is 12.8 °C, and the total annual precipitation is around 1050 mm (URL-2).

Samples were dried at 70 °C for 48 h. Dried samples were ground to pass through a 1-mm sieve (Kökten and Özel 2020).

Seed Storage

To determine the effects of seed storage period on fatty acids content, pine seeds were stored in sterile storage bags at 4 °C and 65% humidity for 2 years according to Gamlı and Hayoğlu (2007).

Oil Extraction and Preparation of Fatty Acid Methyl Esters (FAME)

Seeds were ground into powder using a mill. The milled samples were homogenized in 10 mL of hexane-isopropanol (3:2), centrifuged at 5000 rpm for 10 min (Kaplan *et al.* 2019); after 2.5 mL of 2% methanolic sulfuric acid was added, the mixture was vortexed. This mixture was incubated for 15 h at 50 °C, cooled to room temperature, and vortexed after adding 2.5 mL of 5% NaCl. The fatty acid methyl esters (FAME) formed in the tubes were extracted with 2.5 mL of hexane, and the hexane phase was taken with a Pasteur pipette and treated with 2.5 mL of 2% $KHCO_3$. The phases were allowed to separate for 1 h. The solvent of the mixture containing methyl esters was evaporated under nitrogen at 45 °C. It was dissolved in 1 mL of hexane and analyzed by gas chromatography-mass spectrometry (GC-MS; Agilent 7890A/5970 C, Santa Clara, CA, USA). The upper part was removed by filtration (Kökten and Özel 2020).

Capillary GLC

The fatty acids in the lipid extracts were converted to methyl esters in methanol with 2% sulfuric acid (v/v) (Christie 1993). The fatty acid methyl esters were extracted with n-hexane. The methyl esters were separated and quantified by gas chromatography and flame ionization detection (FID). Chromatography was performed with a capillary column (SGE Analytical BPX90, 100 m × 0.25 mm × 0.25 µm column, Ringwood, Victoria, Australia) using helium as carrier gas (flow rate 1 mL/min), H_2 and air for FID detector, and N_2 for makeup. The other chromatographic conditions were as follows: initial temperature 120 °C, increasing by 5 °C/min to 200 °C, 4 min hold time, then 5 °C/min until the final temperature reaches 260 °C, 8 min hold time, total analysis time 40 min, and injection volume 1 µL. Identification of the individual compound was performed by frequent comparison with authentic standard mixtures analyzed under the same conditions (Kökten and Özel 2020).

Statistical Analysis

The data obtained in the study were evaluated with the help of SPSS 22.0 package program. Analysis of variance was applied to the data in order to determine whether there was a statistically significant ($p<0.05$) difference between the factors in the study. Homogeneous groups were formed by applying the Duncan test to the data for the factors with statistically significant differences, and the groups formed as a result of the Duncan test were coded with letters and are shown in the tables.

RESULTS AND DISCUSSION

The composition of some fatty acids extracted from the seeds collected from different locations used in the study is given in Table 1.

Table 1. Fatty Acids Composition (%) of Seeds Collected from Different Locations

Fatty Acid	Year	Bartın Karaçaydere	Bartın Amasra	Bartın Çakraz	Bartın Avara	Bartın Kurucaşile	Kastamonu Cide	Zonguldak Çaycuma	F-value
C16:0	2019	4.92 a	5.44 bc	5.75 de	5.95 e	5.60 cd	5.19 ab	5.15 ab	15.514***
	2021	4.37 ab	4.28 a	4.73 d	4.54 bcd	4.43 abc	4.65 cd	4.49 abcd	4.132*
	F value	24.214**	97.340**	64.355**	122.053***	93.583**	20.297*	33.168**	
C18:0	2019	2.81 a	3.07 bc	3.63 e	3.14 cd	3.25 d	3.26 d	2.94 ab	24.132***
	2021	2.12 a	2.85 c	3.05 d	2.92 cd	2.87 c	2.42 b	2.15 a	73.992***
	F value	142.830	9.185*	49.960**	8.963*	26.631**	153.132***	160.026***	
C20:0	2019	0.53 a	0.62 c	0.69 e	0.65 cd	0.66 de	0.57 b	0.56 ab	26.357***
	2021	0.45 a	0.48 b	0.57 d	0.57 d	0.54 c	0.49 b	0.49 b	29.188***
	F value	38.400**	117.600***	54.000**	24.000**	54.000**	38.400**	29.400**	
ΣSFA	2019	8.26 a	9.13 bc	10.07 e	9.74 de	9.51 cd	9.02 bc	8.65 ab	16.012***
	2021	6.94 a	7.61 b	8.35 d	8.03 cd	7.84 bc	7.56 b	7.13 a	14.248***
	F value	51.214**	55.862**	60.348**	64.282**	61.309**	52.908**	63.790**	
C18:1n-9	2019	36.92	38.11	39.36	39.12	38.89	37.97	37.45	1.916 ns
	2021	34.68	35.84	36.87	36.95	36.61	36.08	35.43	1.803 ns
	F value	6.691 ns	6.451 ns	7.287 ns	5.577 ns	6.257 ns	4.465 ns	5.292 ns	
ΣMUFA	2019	36.92	38.11	39.36	39.12	38.89	37.97	37.45	1.916 ns
	2021	34.68	35.84	36.87	36.95	36.61	36.08	35.43	1.803 ns
	F value	6.691 ns	6.451 ns	7.287 ns	5.577 ns	6.257 ns	4.465 ns	5.292 ns	
C18:2n-6	2019	44.05	45.09	46.19	46.44	45.87	44.61	44.18	1.590 ns
	2021	43.55	43.43	43.67	43.79	43.57	43.06	43.67	0.102 ns
	F value	.224 ns	2.426 ns	5.380 ns	5.943 ns	4.516 ns	2.148 ns	0.231 ns	
C18:3n-3	2019	0.23 a	0.26 b	0.32 d	0.35 e	0.30 c	0.22 a	0.25 b	70.857***
	2021	0.17 a	0.23 b	0.26 c	0.28 d	0.24 b	0.19 a	0.19 a	53.053***
	F value	72.250**	13.500**	54.000**	73.500**	54.000**	13.500*	54.000***	
ΣPUFA	2019	44.28	45.35	46.51	46.79	46.17	44.83	44.43	1.730 ns
	2021	43.72	43.66	43.93	44.07	43.81	43.25	43.86	0.122 ns
	F value	.278 ns	2.476 ns	5.591 ns	6.174 ns	4.746 ns	2.197 ns	0.286ns	

ns: p>0.05 *: p<0.05 **: p<0.01 ***: p<0.001 a,b,c: Groups formed after Duncan test. Each letter denotes groups for that factor. The indications in Table 1 "a" "ab" "b" "bc" *etc*. relate to the comparison of the content of individual fatty acids between different locations or the content of individual fatty acids and the storage time.

The analysis of variance results indicated that the amount of fatty acids (C18:1n-9, ΣMUFA, C18:2n-6, and ΣPUFA) in seeds collected from seven locations were not significantly different ($p > 0.05$). At the same time, the difference between the amounts of these acids in the seeds collected in different years was statistically insignificant. The amount of other fatty acids in the seeds collected from different locations and in different years was significantly different ($p < 0.05$). Thus, location and storage time significantly affects the amount of these fatty acids in the seeds. While all values of fatty acids in the seeds collected from Bartın Karaçaydere location for both in 2019 and 2021 were the lowest as a result of the Duncan test, the seeds collected from Bartın Çakraz and Bartın Avara locations were the highest. The highest values of fatty acids were accepted as 100%, and their proportional changes based on location are given in Table 2.

Table 2. Proportional Change of Fatty Acids Based on the Location (%)

Fatty Acid	Year	Bartın Karaçaydere	Bartın Amasra	Bartın Çakraz	Bartın Avara	Bartın Kurucaşile	Kastamonu Cide	Zonguldak Çaycuma
C16:0	2019	82.69	91.43	96.64	100	94.12	87.23	86.55
	2021	92.39	90.49	100	95.98	93.66	98.31	94.93
C18:0	2019	77.41	84.57	100	86.5	89.53	89.81	80.99
	2021	69.51	93.44	100	95.74	94.10	79.34	70.49
C20:0	2019	76.81	89.86	100	94.2	95.65	82.61	81.16
	2021	78.95	84.21	100	100	94.74	85.96	85.96
ΣSFA	2019	82.03	90.67	100	96.72	94.44	89.57	85.9
	2021	83.11	91.14	100	96.17	93.89	90.54	85.39
C18:1n-9	2019	93.80	96.82	100	99.39	98.81	96.47	95.15
	2021	93.86	97.00	99.78	100	99.08	97.65	95.89
ΣMUFA	2019	93.80	96.82	100	99.39	98.81	96.47	95.15
	2021	93.86	97.00	99.78	100	99.08	97.65	95.89
C18:2n-6	2019	94.85	97.09	99.46	100	98.77	96.06	95.13
	2021	99.45	99.18	99.73	100	99.50	98.33	99.73
C18:3n-3	2019	65.71	74.29	91.43	100	85.71	62.86	71.43
	2021	60.71	82.14	92.86	100	85.71	67.86	67.86
ΣPUFA	2019	94.64	96.92	99.4	100	98.67	95.81	94.96
	2021	99.21	99.07	99.68	100	99.41	98.14	99.52

When the proportional changes of the fatty acids in the seeds were examined, the amount of C16:0, which is one of the fatty acids found to be significant in the seeds collected from Bartın Karaçaydere, was 82.69% of the seeds collected from Bartın Avara in 2019, and the amount in the seeds obtained from Bartın Amasra was 90.49% of the seeds gathered from Bartın Çakraz in 2021. Similarly, the amount of C18:0 found in the seeds collected from Bartın Karaçaydere was 77.41% of the seeds taken from Bartın Çakraz in 2019, and this rate was decreased to 69.51% in 2021. While the highest values of C20:0

and ΣSFA were obtained in seeds collected from Bartın Çakraz, the lowest values were obtained in seeds gathered from Bartın Karaçaydere. The amount of C20:0 determined in the seeds collected from Bartın Karaçaydere was 76.81% and 78.95% of the seeds collected from Bartın Çakraz in 2019 and 2021, respectively. While this rate was 82.03% for ΣSFA in seeds collected in 2019, it was calculated as 83.11% in seeds analyzed in 2021. The highest values in terms of C18:3n-3 were obtained in the seeds taken from Bartın Avara, while the lowest values were obtained in the seeds collected from Kastamonu Cide in 2019 and Bartın Karaçaydere in 2021. The lowest values are as low as 62.86% in seeds collected in 2019 and 60.71% in seeds collected in 2021. These results show that the variation that depended on the location. The amount of some fatty acids varied by almost 40%.

The amount of fatty acids obtained from the stored seeds was lower than the amount determined from the new seeds, indicating that two-year storage caused a significant decrease in the amount of fatty acids. The proportional changes in the fatty acids based on the location are given in Table 3.

Table 3. Proportional Change of Fatty Acids Due to Storage Time (%)

Fatty Acid	Bartın Karaçaydere	Bartın Amasra	Bartın Çakraz	Bartın Avara	Bartın Kurucaşile	Kastamonu Cide	Zonguldak Çaycuma
C16:0	88.82	78.68	82.26	76.30	79.11	89.60	87.18
C18:0	75.44	92.83	84.02	92.99	88.31	74.23	73.13
C20:0	84.91	77.42	82.61	87.69	81.82	85.96	87.50
ΣSFA	84.02	83.35	82.92	82.44	82.44	83.81	82.43
C18:1n-9	93.93	94.04	93.67	94.45	94.14	95.02	94.61
ΣMUFA	93.93	94.04	93.67	94.45	94.14	95.02	94.61
C18:2n-6	98.86	96.32	94.54	94.29	94.99	96.53	98.85
C18:3n-3	73.91	88.46	81.25	80.00	80.00	86.36	76.00
ΣPUFA	98.74	96.27	94.45	94.19	94.89	96.48	98.72

As presented in Table 3, the amount of all fatty acids that was significantly different based on the location was decreased as a result of 2-year-storage. The proportion between stored and new seeds was between 76.30% and 89.60% for C16:0, 73.13% and 92.99% for C18:0, 77.42%-87.69% for C20:0, 82.43% and 84.02% for ΣSFA and 73.91%-88.46% for C18:3n-3. Therefore, it can be said that the amount of some fatty acids decreased up to 26.87% after two-year storage.

The composition of some fatty acids (C18:1n-9, ΣMUFA, C18:2n-6, and ΣPUFA) in *Pinus pinea* seeds collected from different locations were not significantly different ($p > 0.05$), and the composition of C16:0, C18:0, C20:0, ΣSFA, and C18:3n-3 was significantly different between locations ($p < 0.05$). The difference was almost 40% based on the location. Considering that the lowest values were obtained from the seeds of Bartın Karaçaydere and the highest values were obtained from the seeds located in Bartın Çakraz and Bartın Avara within the scope of the study, it can be said that the values obtained in locations close to the sea were higher. As it is known, environmental and especially climatic conditions affect all phenotypic characteristics in plants, and this also affects the chemical content of organs (Savas *et al.* 2021; Karacocuk *et al.* 2022; Varol *et al.* 2022). In addition, studies have shown that micro-environmental conditions are more effective than the main climate type (Cetin *et al.* 2018; Yigit *et al.* 2021). Therefore, the lower fatty acid values obtained in the seeds of Karaçaydere origin, which are farther from the sea, although they are located in the area where the same main climate type prevails, can be explained by the change in micro-ecological conditions.

The amounts of fatty acids vary significantly between species. In a study comparing the fatty acids of *Pinus halepensis, Pinus brutia* and *Pinus pinaster* seeds, it was found that the seeds of *P. halepensis* and *P. brutia* were significantly richer than those of *P. pinaster* in terms of α-tocopherol, γ-tocopherol, γ-tocotrienol, and δ-tocopherol. The same study revealed that while *P. halepensis* seeds had the highest α-tocopherol content, *P. brutia* seeds contained a higher amount of γ-tocopherol than other species (Khouja *et al.* 2021). However, the composition of fatty acids may differ between individuals of the same species grown in different locations. For example, the UFA content of *P. pinea* L. seeds grown in Turkey and Tunisia were determined as 47.6% and 47.28%, respectively (Nergiz and Donmez 2004; Nasri *et al.* 2005). The composition of fatty acids in *Pinus pinea* seeds also differs in seeds collected from different macrozones. It was determined that C16:0 was between 6.16 and 6.50%, C18:0 was between 4.17 and 4.49%, C20 was between 0.77 and 0.90%, C18:1n-9 varied between 37.13 and 40.92%, C18:2n-6 varied between 47.64 and 50.72% and ΣPUFA ranged from 48.01 to 51.08% in seeds collected from different macrozones (Lutz *et al*. 2017).

The fluctuation of fatty acids is related to plants growing conditions. Plant phenotypic characters are shaped under the mutual interaction of genetic structure and environmental conditions, and location directly affect the genetic structure, and the growing environment directly affects environmental conditions (Ozel *et al.* 2021; Varol *et al.* 2021; Sevik *et al.* 2021). The composition of fatty acids in pine nuts differs depending on geographical and climatic conditions, and therefore it is stated that the fatty acid composition of seeds collected from different climatic conditions could be at different levels (Savage 2001; USDA 2015; Lutz *et al.* 2017).

The fat content of most of the nuts ranges from 440 to 740 g/kg (Ryan *et al.* 2006; Sabaté and Wien 2010). This value reaches approximately 500 g/kg for pine nuts (Evaristo *et al.* 2010). While most tree seed oils have a high content of monounsaturated fatty acids, primarily oleic acid (18:1n-9), *Pinus pinea* seed oil is rich in terms of polyunsaturated (PUFA) oils, particularly linoleic acid (18:2n). Although *Pinus pinea* seeds have high-fat content and are energy-dense foods, they are considered to have a healthy fatty acid profile. Furthermore, pine nut consumption has been associated with reduced body mass index and is recommended in healthy diets (Lutz *et al.* 2017).

Due to these properties, *Pinus pinea* seeds are commercially extremely valuable seeds, and these values are related to the content of fatty acids. Therefore, it is important to determine how fatty acid content changes as a result of storage time. The results of the study show that at the end of a two-year storage period, all of the fatty acid content in the seeds presents a decrease, the decrease is depended on the location and can be more than 25% for some fatty acids. Similar results have been reported by Singh *et al*. (1992) conducted on *Pinus gerardiana* seeds. According to the results of the study, the deterioration in the seeds was reached to a maximum in the second half of the one-year storage period. While the free fatty acid content, saponification, and peroxide values increased, moisture content, gross weight, iodine value, and oil content of the seed decreased significantly with storage. Total nitrogen, protein, non-reducing sugar, and total sugar of the seeds were decreased significantly towards the end of the storage period.

The amount of fatty acids in the seeds also affects germination success. It is stated by Marquez-Millano *et al*. (1991) that as the amount of fatty acid in the seeds decreases, the germination success decreases. In general, the integration of low humidity conditioning and near-freezing temperature storage is a viable way to preserve postharvest quality and extend the shelf life of pine nuts (Cai *et al.* 2013).

In the study conducted by Mahdhi *et al*. (2021) on *Pinus halepensis* seeds, it was presented that not only seed storage but also seed drying methods affect the amount of fatty acid. The total phenol content of the seeds was calculated as 14.63 ± 0.05 mg and 12.69 ± 0.07 mg GAE/g DW in sun-dried seeds and seeds dried by convection methods, respectively. The results of the study also showed that the fatty acids present in the oils are mainly UFAs for 89.74% in oilseeds obtained after convection-drying method and 90.10% for oilseeds obtained after sun-drying method.

CONCLUSIONS

1. The proportion of fatty acids in *Pinus pinea* seeds differed by up to 40% depending on locations. The amount of fatty acid is of great importance in terms of both the success of seed germination and the commercial value of the seed. Therefore, it is important to find out the locations growing seeds having high fatty acid content and to use these seeds in plantations in terms of both increasing the success of afforestation and producing more commercially valuable seeds. Consequently, it is necessary to carry out studies taking into account the seed transfer regions.
2. The highest fatty acid ratios were obtained in seeds collected from Bartın Çakraz and Bartın Avara. It will be more suitable to collect seeds from these two locations for the production of seedlings needed for afforestation studies in the region. In the case of obtaining an insufficient amount of seeds from Bartın Çakraz and Bartın Avara locations, seeds can be obtained from other locations determined to have high fatty acid ratios as a result of the study (for example Bartın Kurucaşile).
3. After a two-year storage period, the content of fatty acids decreased by more than 25%. In this case, it is recommended to sell commercially produced seeds as soon as possible before they lose quality Seeds that need to be saved should be kept in suitable conditions, as stated in the literature, they should be stored in low humidity conditions and at temperatures close to freezing. If storage is really necessary, it is recommended to choose the seeds that presented the least decrease in the content of fatty acids (for example Bartın Karaçaydere).

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