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Unravelling the impact of seed fatty acid profiles on spinach seed germination under temperature stress

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Abstract: Limited knowledge exists regarding the fatty acid profiles of spinach (*Spinacia oleracea* L.) seeds and their correlation with germination. This study aims to address this gap by thoroughly investigating these profiles and their relationship with seed germination. Therefore, the objectives include assessing the impact of different temperatures on seed germination in spinach cultivars, identifying cultivar(s) tolerant to temperature stress during germination, and exploring the relationship between fatty acid profiles and seed germination under varying temperature conditions. Nine spinach cultivars ('Matador-1, 2, and 3', 'Ranchero F_1', 'Aras F_1', 'El Tajin', 'Alreal F_1', 'Catrina F_1', and 'Poyraz F_1') were used as seed material. The study employed simple correlation tests, stepwise multiple regression tests, and principal component analyses (PCA) to determine these relationships. The fatty acid contents significantly varied among cultivar seed samples. C18:2n-6, C18:1n-9, C16:0, and C18:0 exhibited the highest concentrations, followed by C20:1n9 at less than 3%, with other fatty acids at even lower contents (<1%) in spinach cultivars. These analyses revealed that the optimal temperature for spinach seed germination is 20 °C. Above 20 °C, germination percentages decreased, with a sharp decline at 35 °C. 'Catrina F_1' demonstrated notable heat tolerance, with a 62.04% germination rate at 32 °C and 9.10% at 35 °C, indicating its potential as a valuable heat-tolerant cultivar for spinach breeding programs. C17:1, C18:1n9, and monounsaturated fatty acids (MUFA) displayed negative correlations with germination percentage, while positive correlations were observed between C18:3n-6, C20:1n9, and germination percentage at 30 °C and 32 °C. Based on the results of simple correlation tests, stepwise multiple regression tests, and PCA, it has been revealed that C16:1n7, C17:1, C18:1n9, C18:2n6, C18:3n-6, C20:1n9, C22:0, MUFA, and n-6 PUFA have great potential to predict the germination capacity of spinach seeds under stress temperatures.

Key words: *Spinacia oleracea* L., fatty acid, germination, spinach, temperature stress

1. Introduction

The economic significance of spinach (*Spinacia oleracea* L.) is steadily increasing on a global scale due to its high nutritive content and vital role in human nutrition. European spinach production alone exceeds 690,000 tons, contributing to the substantial worldwide production of approximately 31 million tons in 2020 (Bhattarai and Shi, 2021; Akan, 2022).Spinach cultivation is a year-round possibility, contingent upon regional temperature conditions (Koike et al., 2011; Chitwood et al., 2016). It is a cool-season, annual crop with an optimal growth temperature range of 15–20 °C, a minimum at around 5 °C, and a maximum at 32 °C. While spinach can germinate at soil temperatures above 1.7 °C, the most favorable germination rates occur at approximately 21 °C. Nevertheless, germination vigor significantly diminishes at temperatures exceeding 30 °C, making early spring or late fall more conducive for cultivation (Chitwood et al., 2016; Welbaum, 2015). Atherton and Farooque (1983) reported that spinach seeds germinate at soil temperatures between 5 °C and 30 °C, with the highest germination rate at 20°C. The germination rate sharply declines between 25 °C and 30 °C. Leskovar and Esensee (1999) stated that spinach seeds do not germinate at 35 °C; however, Chitwood et al. (2016) suggested that if a spinach genotype can germinate at high temperatures like 35 °C and maintain a high germination percentage, it may indicate heat tolerance. To achieve high yields in both field and greenhouse conditions, spinach seeds must germinate rapidly and uniformly, particularly at high temperatures, in addition to optimum temperatures. Due to growing demand, extending the production season and developing heat-tolerant cultivars are essential for year-round spinach production. Understanding temperature tolerance in spinach is crucial for prolonging its growing season and developing heat-tolerant cultivars. Germination is a key

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factor in assessing heat tolerance in spinach, as genotypes with high germination rates at elevated temperatures can contribute to year-round production (Chitwood, 2016; Katzman et al., 2001).Fatty acid β-oxidation, fueled by acetyl coenzyme A and β-oxidase, provides ATP energy and plays a vital role in germination. Additionally, seed oils undergo quantitative changes in structural oils due to membrane formation during germination, especially under temperature-related abiotic stress (Huang and Grunwald, 1990; Wanasundara et al., 1999; Copeland and McDonald, 2001; Kaymak, 2012, Kaymak, 2014a; Kaymak, 2014b). Moreover, fatty acid profiles can serve as valuable biochemical markers in plant breeding (Barthet, 2008; Kaymak, 2015). However, there is limited knowledge regarding the fatty acid profiles of spinach seeds, with only Lee (2015) providing partial information on a single spinach cultivar. The present study aims to fill this research gap by comprehensively examining the fatty acid profiles of spinach seeds and their responses to temperature stress. Analyzing fatty acid profiles and their responses to temperature stress will aid in the initial selection of temperature-stress-resistant cultivars or genotypes in breeding studies.

Fatty acid β-oxidation, occurring during seed germination, induces structural alterations in seed oils, particularly under temperature-related abiotic stress. These alterations could potentially influence the germination capabilities of diverse spinach varieties, making them critical factors in selecting temperature-stress-resistant varieties or genotypes.

As such, the objectives of this study are twofold: first, to assess the influence of various temperatures on seed germination in different spinach cultivars; secondly, to identify cultivars demonstrating tolerance to temperature stress during germination. Additionally, the study aims to explore the correlation between fatty acid profiles and seed germination under varying temperature conditions.

2. Materials and methods

2.1. Materials and treatments

This study, conducted at Atatürk University in 2019 and 2020, aimed to assess the potential impact of fatty acid composition on the seed germination of nine spinach (*Spinacia oleracea* L.) cultivars: 'Matador-1, 2, and 3', 'Ranchero F_1', 'Aras F_1', 'El Tajin', 'Alreal F_1', 'Catrina F_1', and 'Poyraz F_1', across low, optimum, and high temperatures. The seeds utilized were one-year-old spinach seeds, selected from these cultivars due to their popularity and frequent use in Turkish production, sourced from various Turkish seed companies.

Nine temperature treatments were implemented: 2.5, 5, 10, 15, 20, 25, 30, 32, and 35 °C. The previously established optimum germination temperature for spinach is 20 °C, with inhibition observed at 30 °C and complete cessation at 35 °C. To explore the impact of heat stress, three temperatures above the optimum (30, 32, and 35 °C) were chosen based on previous studies (Atherton and Farooque, 1983; Leskovar and Esensee, 1999; Katzman et al., 2001; Chitwood et al., 2016). Considering the limited research on spinach seed germination at low temperatures, 2.5, 5, and 10 °C were selected to represent low-temperature stress conditions.

Germination tests were conducted with four replicates of 50 seeds each, disinfested in 1% sodium hypochlorite for 15 min to eliminate seed-borne microorganisms. The seeds from each cultivar were placed in 9-cm Petri dishes in the dark and subjected to a seed germination unit (BINDER, D-78532, Tuttlingen, Germany) for 21 days following ISTA guidelines (2011), at 2.5, 5, 10, 15, 20, 25, 30, 32 and 35 °C. The seeds were incubated between two filter papers saturated with water containing Benomyl (Methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate, 50%) at a concentration of 1 g L^{-1} to prevent fungal pathogen growth.

2.2. Measurement of germination parameters

Visible-radicle protrusion (≥2 mm) served as the germination criterion, as established by Kaymak (2012). Germination progress was monitored at 24-hour intervals over a 21-day period, with germinated seeds recorded, and the results expressed as a germination percentage. Simultaneously, the length of the radicle was measured using a caliper.Germination speed (GS) was determined using the formula introduced by Kaymak et al. (2009): GS = Germination Percentage in the 1st day/1 + … + Germination Percentage in the 21st day/21. Here, 1 and 21 represent the mean values for the first and last days during the germination test.

Mean germination time (MGT) was computed using the formula MGT = Σ (ni × ti / ni), as cited by Alsaeedi et al. (2017). MGT represents the mean germination time, 'ni' denotes the number of germinated seeds on each germination day, and 'ti' signifies the number of days within the germination period (spanning from 0 to 21 days).

2.3. Fatty acid analysis

The lipid extraction from seeds, approximately 1 g in quantity, followed the procedure outlined by Folch et al. (1957). Seed samples were homogenized in a mixture of chloroform and methanol (2:1 v/v), supplemented with 0.01% (w/v) butylated hydroxytoluene (Sigma, ≥99.0% gas chromatography, B1378) as an antioxidant, at a ratio of 20 vol. (w/v) for 1 min. The homogenization process was carried out on ice, while subsequent steps such as filtration and incubation were conducted at temperatures between 20 and 22 °C. Following this, the organic solvent was evaporated using a stream of nitrogen, and the lipid content was determined gravimetrically.

To produce fatty acid methyl esters (FAMEs) from the lipids, the crude lipid extract was saponified with NaOH in methanol. Subsequently, FAMEs were synthesized through transmethylation using boron trifluoride (BF3) in methanol, following the method of Metcalfe and Schmitz (1961). The analysis of fatty acid methyl esters was conducted using a "Hewlet Packard" Agilent 6890 N model gas chromatograph, equipped with a flame ionization detector and a DB 23 capillary column (60 m in length, 0.25 mm inner diameter, and 0.25 µm particle size). The temperature program for the chromatograph initiated at 190 °C for 35 min and then increased at a rate of 30 °C per minute until it reached 220 °C, where it was held for 5 min. Hydrogen gas (at a flow rate of 2 mL min^{-1}) served as the carrier gas. Identification of specific fatty acids was achieved by comparing their retention times and peaks with a standard mixture of fatty acids (Supelco 37 component FAME mix, catalog No. 47885-U), and their quantities were determined in accordance with the method outlined by Kaymak (2015).

2.4. Statistical analysis

The germination test was carried out following a completely randomized block design with four replications. The resulting data were subjected to one-way analysis of variance (ANOVA), and mean comparisons were conducted using Duncan's multiple range test. Prior to statistical analysis, an arcsine transformation was applied to the germination percentage data.

Furthermore, stepwise multiple regression and correlation coefficients (r) between the fatty acid profile and germination percentage, along with principal components (Principal Component Analyses, PCA), were determined for all temperatures. Fatty acid data were presented as mean ± standard deviation (SD) and analyzed through one-way analysis of variance (ANOVA). Significant means were compared using Duncan's multiple range tests at a significance level of $\alpha = 0.05$ ($n = 4$).

3. Results and discussion

Variations in germination percentage and speed among the nine spinach cultivars were determined under the tested temperatures (Table 1). With the exception of Matador-1, all other cultivars exhibited the highest germination percentages at 15 and 20 °C. Conversely, deviations from these temperatures, whether increasing or decreasing, resulted in a decline in germination percentage. El-Tajin demonstrated the highest germination percentage at 10 °C, ranging from 62.04% (Poyraz F_1) to 88.72% (El-Tajin). At 25 °C, Aras F_1, Alreal F_1, Matador 1 and 2, Catrina F_1, and Poyraz F_1 displayed intermediate germination percentages. Notably, germination percentages at 5 °C for all cultivars, except for Poyraz F_1, exceeded those at 30 °C. At 32 °C, Ranchero F_1 and Matador-3 exhibited no germination, while other cultivars showed percentages ranging from 11.31% (Poyraz F_1) to 62.04% (Catrina F_1). Interestingly, Matador-2 and Catrina F_1 maintained germination percentages above 50% at 32 °C. However, none of the cultivars, except Matador-1 (9.10%) and Catrina F_1 (9.10%), germinated at 35 °C. Ultimately, it was observed that high-temperature stress had a more unfavorable impact than low-temperature stress on the germination of the tested spinach cultivars.

Table 1 also illustrates the seed germination speed of the nine spinach cultivars across temperatures ranging from 2.5 °C to 35 °C. Statistically significant differences in germination speeds at different temperatures were observed for all cultivars ($p = 0.01$). The highest germination speeds among the nine cultivars were recorded at 15 °C, with speeds ranging between 0.28% (Matador-1, 35 °C) and 14.06% (El-Tajin, 15 °C). Increasing the germination temperature from 2.5 °C to 15 °C corresponded to an improvement in germination speed. However, raising the temperature from 20 °C to 35 °C resulted in a decrease in germination speed across cultivars. Furthermore, temperatures between 10 °C and 20 °C exerted a more significant effect on germination speed than low and high temperatures, with a decline observed at values below and above the specified range.

The decline in spinach seed germination under heat stress has been consistently documented by several researchers (Wahid et al., 2007; Chitwood et al., 2016; Neto et al., 2020). Magnee et al. (2020) also reported the high sensitivity of spinach seed germination to temperature fluctuations. This decrease in germination is attributed to the induction of dormancy by elevated temperatures (Neto et al., 2020). Among various environmental factors, high temperatures are identified as the most critical factor inhibiting radicle growth during spinach seed germination (Ashraf and Foolad, 2005). The optimal germination temperature for spinach falls within the range of 15–20 °C, with germination rates declining outside this temperature range (Ting et al., 2012; Chitwood et al., 2016). Similarly, Imran et al. (2021) reported that spinach seeds exhibit optimal germination between 15 and 24 °C. Beyond 20 °C, germination starts to decrease, reaching rates below 50% at 30 °C and ceasing entirely at 35 °C (Røeggen, 1984; Leskovar et al., 1999). Previous studies have specified the limited germination of spinach seeds at temperatures below 12.3 °C and above 23.3 °C, with complete cessation at 35 °C (Wilcox and Pfeiffer, 1990; Leskovar and Esensee, 1999). In the present research, a significant and substantial decrease in germination percentage was observed at temperatures exceeding 20 °C across all cultivars. While there was a decline in germination percentages at temperatures below 20 °C, it was not as pronounced as at higher temperatures. Moreover, germination did not occur in cultivars other

Table 1. Germination percentages (%) and speeds (%) of nine spinach cultivars at various temperatures.

					Cultivars					
	Aras F_1	Alreal F_1	El-Tajin	Matador-1	Matador-2	Ranchero F_1	Catrina F_1	Poyraz F_1	Matador-3	Mean
Temperature	Germination percentage (%)									
2.5 °C	59.35 f	65.67 d	60.69 c	66.45 d	57.11 e	66.04 d	58.71 e	1.28 h	42.70 d	53.11 E
5 °C	71.58 c	75.32 c	72.57 b	76.02 c	70.68 d	69.97 c	72.99 c	43.85 e	60.02 c	68.11 D
10 °C	81.09 b	80.40 b	88.72 a	83.97 b	74.76 c	76.55 b	79.46 b	62.04 c	66.90 b	77.10 C
15 °C	88.72 a	88.72 a	88.72 a	88.72 a	79.46 b	88.72 a	88.72 a	72.10 b	72.97 a	84.09 B
20 °C	88.72 a	88.72 a	88.72 a	74.69 c	88.72 a	88.72 a	88.72 a	88.72 a	72.61 a	85.37 A
25 °C	68.88 d	72.61 c	26.54 d	61.01 e	60.02 e	31.62 e	71.20 c	56.17 d	20.67 e	52.08 E
30 °C	62.04 e	32.58 e	22.55 e	32.25 f	56.25 e	12.72 f	65.32 d	40.39 f	18.28 e	38.04 F
32 °C	16.90 g	14.69 f	19.81 f	26.52 g	50.77 f	1.28 g	62.04 de	11.31 g	1.28 f	22.73 G
35 °C	1.28 h	1.28 g	1.28 g	9.10 h	1.28 g	1.28 g	9.10 f	1.28 h	1.28 f	3.02 H
	Germination speed (%)									
2.5 °C	5.69 f	5.19 d	4.87 c	6.07 c	5.15 e	5.47 d	4.70 d	-	2.97 c	5.01 E
5 °C	9.22 e	9.61 c	9.35 b	9.77 b	9.62 c	7.95 c	9.37 c	4.09 d	7.21 b	8.46 C
10 °C	12.96 b	12.21 ab	13.78 a	13.34 a	11.68 b	9.69 b	12.86 a	8.08 b	10.18 a	11.64 B
15 °C	14.04 a	13.49 a	14.06 a	13.96 a	12.54 ab	13.84 a	13.74 a	12.02 a	11.75 a	13.27 A
20 °C	12.92 b	11.62 b	13.22 a	10.40 b	13.29 a	9.50 b	13.41 a	12.42 a	8.33 b	11.68 B
25 °C	11.49 c	10.19 c	2.69 d	9.25 b	9.95 c	2.95 e	11.61 b	7.14 b	1.23 c	7.39 D
30 °C	10.29 d	4.52 d	1.81 de	3.72 d	9.68 c	0.43 f	11.44 b	5.37 c	1.21 c	5.39 E
32 °C	0.87 g	0.81 e	1.32 e	2.45 d	8.19 d	-	10.12 c	0.47 e	-	3.46 F
35 °C	-	-	-	0.28 e	-	-	0.29 e	-	-	0.29 G

Capital and small letters indicate statistical significance at the p = 0.01 level for each temperature specified in the table column.
The symbol '-' signifies that seeds did not germinate, indicating uncollectable data.

than Matador-1 and Catrina F_1 at 35 °C. Correspondingly, Masuda and Konishi (1993) and Masuda et al. (2005) noted a significant reduction in the germination percentage of spinach seeds at temperatures exceeding 25 °C. Previous studies have highlighted the ability of spinach seeds to germinate within the temperature range of 5 °C to 30 °C, with a sharp decline between 25 °C and 30 °C, and optimal germination rates at 15 °C and 20 °C (Atherton and Farooque, 1983). Additionally, Chitwood et al. (2016) determined that the germination percentage of spinach seeds varies based on both varieties and temperatures.

In this study, variations in germination speed were observed across different varieties and temperatures. Notably, an increase in germination speed was noted at temperatures ranging from 10 °C to 20 °C, while a decrease was observed at temperatures below and above this range. The germination speed of seeds, in general, can be affected by stress factors such as temperature, drought, salinity, and seed coat impermeability. Additionally, variations in germination speed may arise due to differences in species and cultivars. The balance of growth regulators within the seed or shell can also play a role in affecting germination speed. In the case of spinach, germination inhibition may occur as germination inhibitors are secreted from the pericarp at temperatures below and above the optimum range (Suganuma and Ohno, 1984), impacting the overall germination speed. Balkaya (2004) highlighted that germination speed can vary based on species and cultivars, with the seed coat and temperature exerting an influence.

Suganuma and Ohno (1984) stated that spinach seeds exhibit rapid germination at 20 °C, with both germination percentage and speed decreasing as temperatures rise. Chen et al. (2010) also found that spinach seeds germinate quickly at 15 °C and 20 °C, with the highest germination rates observed at these temperatures. The findings of this study regarding germination percentage and speed align with and support the results of previous research.

The impact of germination temperature on the MGT of spinach cultivars' seeds was evident (Table 2). The highest MGT was observed at low temperatures, such as 2.5 °C and 5 °C, while lower MGT values were associated with temperatures where germination was highest. At low temperatures, spinach seed germination occurred over a more extended time period and at a higher rate (Tables 1 and 2). Interestingly, MGT exhibited lower values at high temperatures, such as 32 °C and 35 °C, where germination rates were low. Germination at high temperatures (32 °C and 35 °C) mainly occurred within the first 7–10 days, with no germination observed in the later period. This phenomenon likely contributed to the low MGT at high temperatures. For instance, the shortest MGT (7.0 days) was observed in Catrina F_1 and Matador-1 at a temperature of 35 °C. Similar MGT values were noted at temperatures of 15 °C and 20 °C, considered optimum conditions. If spinach seeds can germinate at high temperatures, but this occurs within the first 10 days, it suggests that the seeds may subsequently perish due to the adverse effects of high temperatures in the later stages.

The effect of temperatures (ranging from 2.5 °C to 35 °C) on MGT varies across cultivars, and significant differences were observed between the means at the 1% level. Under conditions of salinity, drought, and heat stress, vegetable seed germination percentages tend to decrease, while MGT increases. Although MGT can differ based on species and cultivars, it is considered a reliable indicator of seed vigor. Various priming applications, such as those involving KNO3 and KH2PO4, have been shown to affect the MGT of spinach seeds. For instance, after applications with KNO3 and KH2PO4, the MGT was found to be 10.4 days in the control (at 5 °C), whereas it decreased to 5.4 days and 4.7 days at 15 °C and 25 °C, respectively (Orhan, 2013).

The radicle length results indicate substantial variations among spinach cultivars and tested temperatures (Table 2). Radicle length was more extended at 15 °C and 20 °C compared to low (2.5 °C) and high temperatures (32 °C and 35 °C). This suggests that the inhibitory effect of low and high temperatures on radicle length is more pronounced than that of optimum temperatures. This trend is consistent across all cultivars, with Catrina F_1 and Matador-1 showing the strongest response at high temperatures. Additionally, the highest radicle length was consistently observed at 15 °C across all cultivars, ranging from 3.3 mm (35 °C) to 62.0 mm (15 °C).

Table 3 presents the major fatty acids in the seeds of the nine spinach cultivars. Significant differences were found in the fatty acids contents among the cultivars' seed samples. Linoleic (C18:2n-6), oleic (C18:1n-9), palmitic (C16:0), and stearic acid (C18:0) were the predominant fatty acids, followed by C20:1n9 at less than 3%, with other fatty acids present at even lower concentrations (<1%) in spinach cultivars.

Total SFA, MUFA, n-6 PUFA, and total oil contents varied, as detailed in Table 4. PUFA ranged from 41.30% (El-Tajin) to 62.52% (Matador-3) of total fatty acids across all cultivars. Monounsaturated and saturated fatty acids contributed to 23.02% (Catrina F_1), 27.41% (Ranchero F_1), 13.77% (Matador-3), and 30.65% (El-Tajin) of total fatty acids in the tested spinach cultivars, respectively. Seeds of all tested cultivars exhibited high concentrations of oil content, with Matador-3 having the highest (12.12%) and Poyraz F_1 the lowest (9.97 Although the total oil content of small-seeded vegetables varies between 7% (*Spinacia oleracea* L.) and 50% (Cucurbits) (Al-Khalifa, 1996; Taylor, 1997),), specific lipid contents for pepper, radish, and cabbage are 22%, 36%, and 38%, respectively (Taylor,

Table 2. Mean germination time (MGT) in days and radicle length in millimeters for nine spinach cultivars at each of the nine temperatures.

					Cultivars					
	Aras F_1	Alreal F_1	El-Tajin	Matador-1	Matador-2	Ranchero F_1	Catrina F_1	Poyraz F_1	Matador-3	Mean
Temperature	Mean germination time (days)									
2.5 °C	13.5 a	14.6 a	15.9 a	13.8 a	16.3 a	16.1 a	14.9 a	-	15.7 a	15.1 A
5 °C	10.2 b	10.4 b	10.3 b	9.9 b	9.8 b	12.3 b	10.1 b	13.1 a	10.8 b	10.8 B
10 °C	7.8 cde	8.2 cd	7.4 d	7.5 ef	7.8 c	9.8 c	8.0 c	9.7 b	8.5 c	8.3 D
15 °C	7.2 e	7.5 d	7.2 d	7.2 ef	7.5 c	7.4 d	7.3 c	9.2 bcd	8.0 c	7.6 E
20 °C	8.2 cd	9.1 bcd	7.9 cd	8.1 d	7.9 c	10.5 bc	7.8 c	8.4 bcd	10.8 b	8.7 D
25 °C	8.4 c	9.2 bcd	9.3 bc	8.8 c	8.0 c	11.1 bc	8.3 c	9.3 bc	11.4 b	9.3 C
30 °C	7.6 cde	9.4 bc	8.2 cd	7.6 de	7.5 c	11.7 b	7.2 c	7.6 d	7.6 c	8.3 D
32 °C	7.4 de	7.6 d	7.9 cd	7.6 de	7.3 c	-	7.5 c	7.8 cd	-	7.6 E
35 °C	-	-	-	7.0 f	-	-	7.0c	-	-	7.0 F
	Radicle length (mm)									
2.5 °C	6.1 f	5.4 e	6.4 f	5.3 fg	5.2 f	4.6 e	5.1 e	-	3.7 e	5.2 H
5 °C	26.4 c	19.2 d	23.2 c	21.9 d	23.7 c	12.9 d	19.5 b	17.0 c	24.6 c	20.9 D
10 °C	44.5 b	31.6 b	38.8 b	37.7 b	35.2 b	28.9 b	28.0 a	29.7 a	31.9 b	34.0 B
15 °C	62.0 a	45.2 a	51.2 a	46.1 a	39.9 a	48.9 a	29.2 a	31.4 a	34.7 a	43.2 A
20 °C	29.1 c	23.8 c	35.8 b	28.2 c	24.2 c	24.4 b	18.2 b	28.4 a	22.9 c	26.1 C
25 °C	20.6 d	21.4 d	17.2 d	22.3 d	12.9 d	19.6 c	12.9 c	23.7 b	16.8 d	18.6 E
30 °C	13.0 e	5.0 e	12.2 e	11.3 e	10.8 de	5.2 e	10.7 d	13.7 cd	5.8 e	9.7 F
32 °C	9.0 ef	2.5 f	4.7 f	8.0 f	8.8 e	-	9.3 d	10.5 d	-	7.5 G
35 °C	-	-	-	3.3 g	-	-	3.6 e	-	-	3.4 I

Statistical significance at the p = 0.01 level for each temperature is denoted by capital and small letters in the respective column of the table.
The symbol '-' indicates that seeds did not germinate, meaning data could not be collected.

Table 3. The fatty acid composition of seeds from nine spinach cultivars.

Cultivars	C 14 : 0	C 15 : 0	C 16 : 0	C 16 : 1 n7	C 17 : 0	C 17 : 1
Aras F_1	0.50 ± 0.17 cd	0.19 ± 0.04 b	14.64 ± 1.88 bc	0.67 ± 0.25 b	-	0.14 ± 0.03 b
Alreal F_1	0.34 ± 0.01 d	0.19 ± 0.03 b	13.33 ± 1.88 bc	0.31 ± 0.01 cd	0.31 ± 0.13 b	0.22 ± 0.01 ab
El-Tajin	1.45 ± 0.30 a	0.34 ± 0.09 a	21.55 ± 5.98 a	1.20 ± 0.21 a	0.82 ± 0.56 a	-
Matador-1	0.94 ± 0.23 b	0.19 ± 0.07 b	17.07 ± 3.49 b	0.75 ± 0.28 b	0.36 ± 0.01 b	-
Matador-2	0.36 ± 0.08 d	0.17 ± 0.03 b	13.14 ± 0.26 bc	0.27 ± 0.02 d	0.11 ± 0.09 b	0.15 ± 0.01 b
Ranchero F_1	0.60 ± 0.05 c	0.12 ± 0.01 b	15.67 ± 0.07 bc	0.73 ± 0.03 b	0.34 ± 0.11 b	-
Catrina F_1	0.42 ± 0.05 cd	0.18 ± 0.09 b	14.43 ± 0.23 bc	0.35 ± 0.08 cd	-	-
Poyraz F_1	0.62 ± 0.04 c	0.20 ± 0.04 b	15.44 ± 0.20 bc	0.53 ± 0.09 bc	0.33 ± 0.04 b	0.19 ± 0.01 ab
Matador-3	0.13 ± 0.01 e	0.12 ± 0.02 b	11.91 ± 0.06 c	0.23 ± 0.04 d	-	0.25 ± 0.12 a
	C 18 : 0	C 18 : 1n9	C 18 : 2n6	C 18 : 3 n6	C 20 : 1n9	C 22 : 0
Aras F_1	5.61 ± 0.23 a	23.72 ± 1.97 ab	51.81 ± 3.78 c	0.63 ± 0.02 b	2.03 ± 0.05 b	0.26 ± 0.07 bc
Alreal F_1	2.71 ± 1.05 b	23.86 ± 0.10 ab	56.56 ± 2.95 b	0.62 ± 0.04 b	1.55 ± 0.22 c	-
El-Tajin	6.13 ± 0.39 a	24.81 ± 2.91 a	40.94 ± 3.69 e	0.37 ± 0.03 c	2.03 ± 0.14 b	0.37 ± 0.02 a
Matador-1	7.42 ± 4.28 a	24.88 ± 1.50 a	46.24 ± 6.72 d	0.39 ± 0.02 c	1.62 ± 0.21 c	0.16 ± 0.05 e
Matador-2	2.41 ± 0.60 b	21.79 ± 1.05 bcd	58.45 ± 0.35 ab	0.87 ± 0.36 a	2.12 ± 0.06 b	0.20 ± 0.06 de
Ranchero F_1	6.12 ± 0.70 a	25.23 ± 0.51 a	49.31 ± 0.25 cd	0.44 ± 0.06 bc	1.45 ± 0.08 c	-
Catrina F_1	3.02 ± 0.58 b	20.03 ± 0.39 d	58.17 ± 1.07 ab	0.45 ± 0.05 bc	2.64 ± 0.09 a	0.31 ± 0.05 b
Poyraz F_1	5.61 ± 0.88 a	23.04 ± 1.43 abc	51.71 ± 0.45 c	0.45 ± 0.07 bc	1.64 ± 0.02 c	0.24 ± 0.03 cd
Matador-3	1.61 ± 0.33 b	21.16 ± 0.52 cd	62.00 ± 0.56 a	0.53 ± 0.03 bc	2.07 ± 0.19 b	-

Fatty acid composition of seeds, including miristic acid (C14:0), palmitic acid (C16:0), palmitoleic acid (C16:1n-7), margaric acid (C17:0), stearic acid (C18:0), oleic acid (C18:1n-9), linoleic acid (C18:2n-6), arachidic acid (C20:0), gadoleic acid (C20:1n-9), behenic acid (C22:0); different letters in each row indicate significant differences at p = 0.05.

Table 4. Concentrations of saturated fatty acids (SFA), mono-unsaturated fatty acids (MUFA), poly-unsaturated fatty acids (n-6 PUFA), and total oil content in seeds across nine spinach cultivars.

Cultivars	SFA	MUFA	n-6 PUFA	Total oil
Aras F_1	21.07 ± 1.71 b-e	26.49 ± 2.09 ab	52.45 ± 3.80 c	11.09 ± 0.48 bcd
Alreal F_1	16.88 ± 3.10 def	25.94 ± 0.11 abc	57.18 ± 2.99 b	10.70 ± 0.32 de
El-Tajin	30.65 ± 6.52 a	27.25 ± 3.75 a	41.30 ± 3.68 e	9.98 ± 0.28 f
Matador-1	26.13 ± 8.13 ab	27.24 ± 1.43 a	46.63 ± 6.70 d	11.22 ± 0.08 bc
Matador-2	16.34 ± 0.98 ef	24.34 ± 0.99 bcd	59.33 ± 0.01 ab	11.49 ± 0.53 b
Ranchero F_1	22.84 ± 0.94 bc	27.41 ± 0.62 a	49.75 ± 0.31 cd	10.44 ± 0.01 e
Catrina F_1	18.36 ± 0.72 c-f	23.02 ± 0.40 d	58.62 ± 1.12 ab	10.89 ± 0.06 cde
Poyraz F_1	22.44 ± 1.12 bcd	25.41 ± 1.51 a-d	52.16 ± 0.38 c	9.97 ± 0.27 f
Matador-3	13.77 ± 0.29 f	23.56 ± 0.42 cd	62.52 ± 0.54 a	12.12 ± 0.20 a

Concentrations of saturated fatty acids (SFA), poly-unsaturated fatty acids (n-6 PUFA), and monounsaturated fatty acids (MUFA) across different spinach cultivars. Significantly different means within each column are denoted by different letters at $p = 0.05$.

1997). Additionally, Lee et al. (2015) reported a 4.5% total oil content for spinach seeds. In our study, the determined total oil contents of spinach seeds were higher than those reported by Taylor (1997) and Lee et al. (2015). However, this disparity could be attributed to variations in spinach cultivars, as seed oil content is influenced by factors such as harvest time, maturity level, seasonal fluctuations, drying conditions, cultivar type, variety, soil conditions, and storage conditions (De Mello, 2000; Kaymak, 2012; Kaymak, 2014a).

The number of in-depth studies on the fatty acid profile of spinach seeds is scarce. In one of the limited studies on this subject, it was reported that palmitic, stearic, oleic, and linoleic acid in spinach seeds constituted 22.6%, 1.7%, 15.4%, and 49.3%, respectively (Lee et al., 2015). According to the findings of this research, the fatty acid profiles of the seeds exhibited significant variations among cultivars. For instance, linoleic acid ranged from 40.94% (El Tajin) to 62.00% (Matador-3), oleic acid varied from 20.03% (Catrina F_1) to 25.23% (Ranchero F_1), palmitic acid ranged from 11.91% (Matador-3) to 21.55% (El Tajin), and stearic acid ranged from 1.61% (Matador-3) to 7.42% (Ranchero F_1). Previous studies have shown that the fatty acid profiles of vegetable seeds, such as summer and winter squash, cucumber, melon, watermelon, and gherkin, vary based on both species and cultivars within the species (Kaymak, 2012; Kaymak, 2014a; Kaymak, 2014b, Kaymak et al., 2022). It is well-established that the fatty acid profile differs based on production region, temperature, harvest period, genetic structure, breeding period, and ecological factors of the region (Baydar, 2000; Kaymak, 2015). As far as our knowledge extends, this research represents a pioneering effort that significantly contributes to the existing scientific knowledge. It addresses a notable gap in the research domain by being the first study to comprehensively examine the fatty acid profiles of spinach seeds across nine distinct cultivars.

Cluster analysis was employed to gain a deeper understanding of the fatty acid contents of the cultivars and to reveal differences between them (Figure 1). The dendrogram resulting from the cluster analysis categorized cultivars into two main groups based on fatty acids content, namely Cluster 1 and Cluster 2. Cluster 1 includes Aras F_1, Poyraz F_1, Ranchero F_1, Matador-1, and El-Tajin cultivars, while Cluster 2 comprises Alreal F_1, Matador-2, Matador-3, and Catrina F_1 cultivars. According to the cluster analysis results, it can be inferred that Aras F_1 and Poyraz F_1, as well as Matador-2 and Matador-3 spinach cultivars, share similarities in terms of the examined characteristics (Figure 1).

The correlation coefficients between the fatty acid profile, total oil, and seed germination percentage at various temperatures for all spinach cultivars are presented in Tables 5 and 6. Significant correlations were observed between 10 °C and 32 °C for seed germination and fatty acid contents of spinach seeds at various temperatures. However, no significant correlations were identified between the fatty acid contents of seeds and germination percentage at 2.5, 5, and 35 °C. Significant correlations were determined between fatty acids and germination percentage at 30 °C and 32 °C, considered stress temperatures, where the germination percentage decreases considerably. Although fatty acids C17:1, C18:1n9, and MUFA were negatively correlated with germination percentage, there were positive correlations between C18:3n-6, C20:1n9, and germination percentage. Other significant correlations at 10 °C, 15 °C, 20 °C, and 25 °C are clearly presented in Table 5.

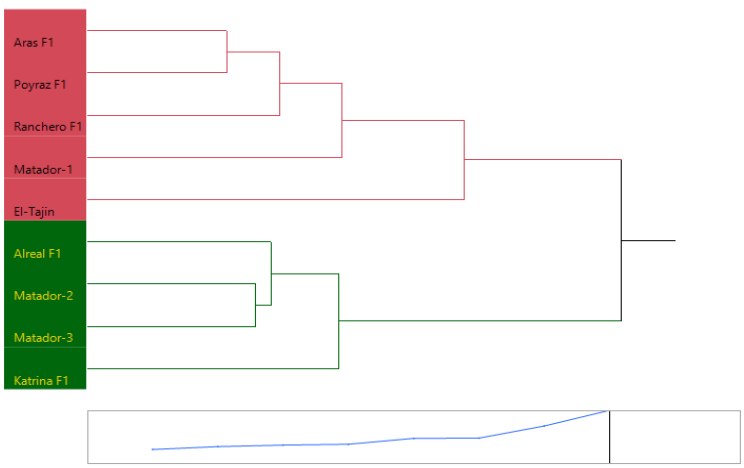

Figure 1. The dendrogram resulting from cluster analysis based on fatty acids.

Table 5. Correlation coefficients (r) illustrating the relationship between fatty acid profiles and germination percentages at different temperatures.

	Germination percentage at								
Fatty acids	2.5 °C	5 °C	10 °C	15 °C	20 °C	25 °C	30 °C	32 °C	35 °C
C 14 : 0	0.126	0.171	0.544**	0.362*	0.112	–0.249	–0.252	–0.030	0.122
C 15 : 0	0.012	0.099	0.386*	0.180	0.245	–0.047	0.028	0.115	–0.046
C 16 : 0	0.089	0.122	0.437**	0.311	0.136	–0.235	–0.209	–0.034	0.080
C 16 : 1n7	0.165	0.164	0.516**	0.394*	0.139	–0.318	–0.299	–0.206	–0.018
C 17 : 0	0.088	0.108	0.375	0.255	0.069	–0.493*	–0.351	–0.114	–0.070
C 17 : 1	–0.102	–0.129	–0.328	–0.042	–0.413	–0.452*	–0.644**	–0.435	–0.069
C 18 : 0	0.029	0.032	0.296	0.295	0.026	–0.045	–0.158	–0.205	0.159
C 18 : 1n9	0.214	0.182	0.344*	0.339*	0.071	–0.133	–0.440**	–0.447**	–0.181
C 18 : 2n6	–0.132	–0.150	–0.499**	–0.401*	–0.134	0.202	0.294	0.197	–0.047
C 18 : 3n6	0.079	0.080	–0.129	–0.213	0.181	0.283	0.387*	0.238	–0.310
C 20 : 1n9	0.066	0.132	0.095	0.020	0.086	0.129	0.594**	0.675**	0.319
C 22 : 0	0.016	0.018	0.276	0.263	0.576**	–0.472*	–0.106	–0.013	–0.227
SFA	0.069	0.097	0.434**	0.336*	0.114	–0.185	–0.214	–0.101	0.118
MUFA	0.218	0.197	0.360*	0.354*	0.091	–0.089	–0.340*	–0.359*	–0.126
n-6 PUFA	–0.129	–0.146	–0.497**	–0.402*	–0.127	0.208	0.301	0.201	–0.054
Total oil	0.231	0.191	–0.155	–0.221	–0.619**	–0.023	0.114	0.114	0.134

* – significant at p = 0.05, ** – significant at p = 0.01 and the other are not significant.

Additionally, similar results were obtained in stepwise multiple regression analysis (Table 6), indicating the indirect effects of fatty acids in addition to their direct effects on germination percentage. The regression equations (Y), R square, and other details are shown in Table 6 for germination percentage. As observed in the correlation analysis, most of the fatty acids' indirect effects are clearly stated in the related table.

The germination process heavily relies on free fatty acid β-oxidation, acetyl coenzyme A, and β-oxidase, all of which significantly contribute to energy production in the form of ATP. Furthermore, it is a well-established fact that the oils stored within seeds are metabolized during germination to supply the required energy for high-energy activities, particularly under adverse abiotic conditions like temperature stress. Additionally, the composition of structural oils undergoes quantitative alterations as new membranes are formed (Huang and Grunwald, 1990; Wanasundara et al., 1999; Copeland and McDonald, 2011; Kaymak, 2012, Kaymak, 2014a; Kaymak, 2014b). Germination of cultivars at various temperatures is closely related to extra or deficient endogenous fatty acids. The results of the present study support previous research, confirming that triglycerides, a major form of stored lipids in seeds, are hydrolyzed by lipases to diglycerides, monoglycerides, and then to glycerol and fatty acids (Krist et al., 2005). Similarly, Salisbury and Ross (1985) determined that since oils provide more energy than sugars, carbohydrates are converted into fats and provide more energy for seed germination. Fatty acids and high total oil content can prevent dormancy caused by high temperatures and increase germination. Furthermore, fatty acids are essential in plant breeding, as the fatty acids in seeds can be used as biochemical markers (Barthet, 2008; Kaymak, 2015). Therefore, these research results, the first in spinach, are predicted to be a key and provide convenience when determining spinach cultivars or genotypes resistant to heat stress.

Barthet (2008) determined that the studied species can be classified by using statistical similarities or differences of C18:1(n-7) / (n-9) ratios for chemotaxonomy in cruciferous crops. Previous research, such as Kaymak (2015), emphasized the importance of palmitoleic acid in synthesizing long-chain fatty acids and proposed using (n-7) / (n-9) ratios of erucic acid as biochemical markers for assessing variations among radish varieties. Similar findings emerged from previous studies on various vegetable species, employing correlation and Stepwise Multiple Regression tests. For instance, Kaymak (2012) established statistically significant relationships between total oil, fatty acid composition, and the germination rate and speed of cucurbit seeds, identifying C14:0, C18:2n-6, and C20:0 as influential factors. Additionally, simple correlation coefficients and stepwise multiple regression analyses suggested that palmitic acid (C16:0), oleic acid (C18:1n-9), linoleic acid (C18:2n-6), stearic acid (C18:0), myristic acid (C14:0), palmitoleic acid (C16:1n-7), margaric acid (C17:0), arachidic acid (C20:0), eruic acid (C22:1n-9), behenic acid (C22:0), MUFA, n-6 PUFA, and total oil significantly affect seed germination in pepper, eggplant, radish, and cabbage at different temperatures (Kaymak, 2014a). In line with these findings, Kaymak (2014b) reported similar results in the germination rates of pepper cultivars under different temperature conditions. These observations suggest that fatty acid levels, whether high or low, play a major role in the germination of pepper cultivars at diverse temperatures, with linoleic acid (C18:2n-6) standing out as particularly influential.

In addition to simple correlation coefficients and stepwise multiple regression analysis, principal component analysis (PCA) was performed to determine the relationship between fatty acids and germination. Different analyses were employed to comprehensively evaluate the subject and provide a clearer understanding. The applicability of PCA in the dataset was assessed, and the correlation coefficient matrix's identity matrix nature was examined using Bartlett's sphericity test. Bartlett's sphericity test yielded a value of 341.395 ($p < 0.001$), indicating that the correlation matrix is not an identity matrix, suggesting significant correlations among some variables. Therefore, PCA is deemed applicable to the dataset.

The results of principal component analysis are presented in Table 7. The eigenvalues of each principal component and the proportion of total variance explained by these eigenvalues are provided. Principal components with eigenvalues greater than 1 were considered significant. The first five components explain a total variance of 92.859% of the dataset. Specifically, the first component explains 46.496% of the total variance, the second component explains 19.53%, the third component explains 12.677%, the fourth component explains 8.251%, and the fifth component explains 5.904%. The total variance explained by the other three components is 7.141%, and these components were not considered significant.

Upon examining Table 8 and Figure 2, it can be observed that the variables contributing the most to the first principal component are, in descending order, n-6 PUFA (8.403), C18:2n6 (8.393), SFA (8.193), and C16:1n7 (8.033). Similarly, the second principal component is primarily influenced by GP 32 °C (16.194), GP 30 °C (11.212), C20:1n9 (9.944), and GP5 °C (8.550). The third principal component's major contributors include GP2.5 °C (19.262), C22:0 (14.940), GP 5 °C (14.663), and GP 15 °C (7.902). Moving on to the fourth component, GP 20 °C (27.556), C18:3n6 (15.402), GP 35 °C (15.399), and GP 25 °C (10.222) variables have the most substantial impact.

Table 6. Stepwise multiple regression analysis between fatty acid profile and germination percentage at various temperatures.

		Unstandardized coefficients		Standardized coefficients		
Temperatures		B	Std. Error	Beta	t	p-values
2.5 °C	Constant	147.958	3.709		39.893	0.000
	C 22 : 0	–601.815	17.073	–0.998	–35.249	0.000
R square = 0.997 Y = 147.958 + (–601.815 × C 22 : 0)						
5 °C	Constant	27.913	1.688		16.532	0.000
	C 18 :3 n6	35.44	2.162	0.993	16.394	0.000
R square = 0.985 Y = 27.913 + (35.44 × C 18 :3 n-6)						
10 °C	Constant	54.064	1.411		38.307	0.000
	C 18 :3 n6	17.747	1.807	0.98	9.821	0.001
R square = 0.960 Y = 54.064 + (17.747 × C 18 :3 n-6)						
15 °C	Constant	87.147	3.482		25.028	0.000
	C 22 : 0	–61.744	16.029	–0.888	–3.852	0.018
R square = 0.788 Y = 87.147 – (61.744 × C 22 : 0)						
20 °C [z]						
25 °C	Constant	27.909	3.24		8.613	0.001
	C 18 : 2n6	0.546	0.06	0.977	9.096	0.001
R square = 0.954 Y = 27.909 + (0.546 × C 18 : 2n6)						
30 °C	Constant	–86.007	10.747		–8.003	0.001
	n-6 PUFA	2.423	0.197	0.987	12.324	0.000
R square = 0.974 Y = –86.007 + (2.423 × n-6 PUFA)						
32 °C	Constant	114.328	4.835		23.646	0.000
	C 22 : 0	–422.776	22.256	–0.995	–18.996	0.000
R square = 0.989 Y = 114.328 + (–422.776 × C 22 : 0)						
35 °C [z]						

[z]: Y and *R* square could not be computed as there was no difference between the germination percentages at 20 °C and 35 °C (Table 1).

Table 7. The results of principal component analysis (PCA).

	F1	F2	F3	F4	F5	F6	F7	F8
Eigenvalue	11.624	4.883	3.169	2.063	1.476	0.816	0.575	0.395
Variability (%)	46.496	19.530	12.677	8.251	5.904	3.263	2.299	1.579
Cumulative (%)	46.496	66.026	78.703	86.955	92.859	96.122	98.421	100.00

Table 8. According to PCA, the contribution of the variables (%).

	F1	F2	F3	F4	F5
C14: 0	7.789	0.085	1.105	0.790	1.148
C15: 0	4.319	1.017	5.531	0.362	9.827
C16: 0	7.838	0.093	1.437	0.751	1.492
C16: 1 n7	8.033	0.031	0.175	0.179	0.822
C17: 0	6.360	1.084	0.378	0.085	4.410
C17: 1	4.097	4.393	0.317	4.267	1.704
C18: 0	6.173	0.233	0.007	0.051	13.660
C18: 1n9	5.437	2.630	3.925	3.875	1.243
C18: 2n6	8.393	0.077	0.099	0.000	0.222
C18: 3 n6	3.206	0.691	0.328	15.402	4.270
C20: 1n9	0.964	9.944	4.035	5.019	7.694
C22: 0	1.364	6.244	14.940	0.090	0.458
SFA	8.193	0.000	0.840	0.396	0.311
MUFA	6.019	1.474	3.203	3.611	1.564
n-6 PUFA	8.403	0.087	0.105	0.007	0.259
Total Oil	4.016	0.267	6.121	6.100	1.465
GP 2.5 °C	0.521	5.436	19.262	0.081	3.755
GP 5 °C	0.617	8.550	14.663	0.246	2.561
GP 10 °C	3.567	7.085	5.119	0.174	3.260
GP 15 °C	2.644	6.765	7.902	1.684	0.291
GP 20 °C	0.318	1.535	4.054	27.556	0.166
GP 25 °C	0.388	7.158	0.073	10.222	19.305
GP 30 °C	0.997	11.212	4.283	3.536	3.660
GP 32 °C	0.290	16.194	1.840	0.117	0.024
GP 35 °C	0.055	7.713	0.256	15.399	16.427

GP: Germination percentage

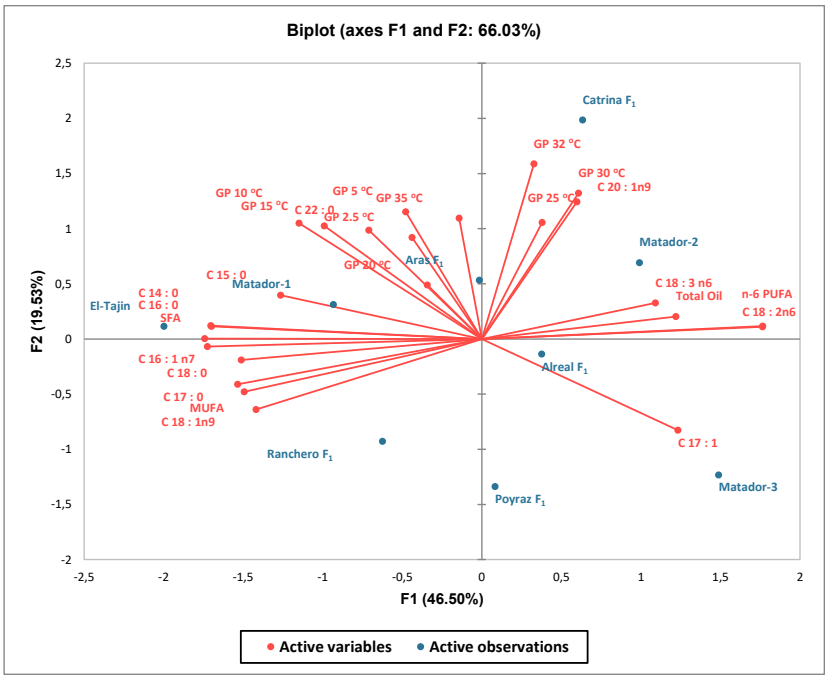

Figure 2. Principal component analysis illustrating the relationships between fatty acids and the germination of spinach cultivars under different temperature conditions.

Finally, the fifth component's primary contributors are GP 25 °C (19.305), GP 35 °C (16.427), C 18:0 (13.660), and C15:0 (9.827).

The principal component analysis (PCA) conducted in this study has provided valuable insights into the complex interplay between temperature stress and fatty acid profiles during spinach seed germination. The results indicate that the first five principal components collectively capture an impressive 92.859% of the total dataset variance, signifying their significance in explaining the relationships within the data. These findings align with our initial hypothesis that temperature stress significantly influences fatty acid profiles during seed germination. Notably, the first principal component, accounting for 46.496% of the total variance, is predominantly influenced by n-6 PUFA, C18:2n6, SFA, and C16:1n7, emphasizing the pivotal role these fatty acids play in mediating the impact of temperature stress on seed germination. The subsequent components, each contributing substantial portions of the variance, elucidate additional key variables in this intricate relationship. Our results reveal a nuanced connection between temperature stress and fatty acid composition. The exclusion of nonsignificant components underscores the importance of focusing on these significant components in future research to decipher the specific mechanisms governing this relationship. This study not only advances our understanding of the temperature-fatty acid-seed germination nexus but also lays the foundation for future investigations aimed at unraveling the specific metabolic pathways and molecular processes through which temperature stress modulates fatty acid profiles during spinach seed germination.

4. Conclusion

The optimal temperature ranges for spinach seed germination, as determined by this study, fall between 15 and 20 °C. Beyond 20 °C, germination percentages decline significantly, with a sharp drop observed at 35 °C, except for the Matador-1 and Catrina F_1 cultivars. Most spinach varieties, excluding 'Aras F_1,' 'Catrina F_1,' and 'Poyraz F_1,' experienced a substantial decrease in germination percentages, dropping to less than 30% above 25 °C. 'Catrina F_1' demonstrated notable heat tolerance, with a 62.04% germination rate at 32 °C and 9.10% at 35 °C, making it a potential candidate for heat-tolerant spinach breeding. The variability among spinach cultivars suggests the feasibility of breeding for heat tolerance.

The study identified significant correlations between fatty acid profiles and germination percentages at stress temperatures, particularly at 30 °C and 32 °C, where germination percentages substantially decrease. Negative correlations were found between C17:1 and C18:1n9 with germination percentage, while positive correlations were observed between C18:3n-6 and C20:1n9 with

germination percentage at stress temperatures exceeding 30 °C. Furthermore, stepwise multiple regression and principal component analysis indicated that the levels of certain fatty acids, including C16:1n7, C18:2n6, C18:3n6, C22:0, SFA, and PUFA, play a pivotal role in germination at various temperatures in the tested cultivars.

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Conflict of interest
The authors declare no conflicts of interest.

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