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Temperature effect on a high stearic acid sunflower mutant

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Abstract

Vegetable oil with elevated saturated fatty acid content may be useful for producing solid fat without hydrogenation or transesterification. Under the nutritional point of view stearic acid is preferred to other saturated fatty acids because of its neutral effect on serum cholesterol lipoproteins. Selection of a very high stearic acid sunflower (*Helianthus annuus* L.) line (CAS-14), with up to a 37.3% of stearic acid in the seed oil, and the relationship between the expression of this character and the growth temperature are presented. The mutant was selected from the M_2 progeny of 3000 mutagenized seeds (4 mM sodium azide mutagenesis treatment) by analysing the fatty acid composition of half-seed by gas liquid chromatography. In order to genetically fix the mutant character, plants were grown at high day/night temperatures during seed formation. We found that temperatures higher than 30/20 °C are required for good expression of the phenotype, the maximum stearic acid content being obtained at 39/24 °C. This behaviour is totally opposed to that observed in normal and previously isolated high-stearic acid sunflower lines that contain more stearic acid at low temperature. Thus, a new type of temperature regulation on the stearate desaturation must occur. This line is the sunflower mutant with the highest stearic acid content reported so far.

Keywords: *Helianthus annuus*; Asteraceae; Sunflower; Temperature; Mutant; Fatty acid desaturation; Lipid; Stearic acid

1. Introduction

The properties of a vegetable oil are determined by the fatty acid composition of its lipids, mainly triacylglycerols, which constitute the oil. In temperate oil crops, the main fatty acids are the unsaturated oleic and linoleic acids (Padley et al., 1994). These fact make those oils normally liquid and thus not suitable for many food industry purposes, where fats that are solid at room temperature are needed. To overcome this problem, hydrogenation or transesterification of oils has been widely used, although both methods negatively modify the nutritional properties of vegetable oils. Transesterification increases the saturated content at position *sn*-2 of triacylglycerols, thus increasing its atherogenic effect (Kritchevsky et al., 1995). Hydrogenation produces several types of unhealthy *trans* isomers (Willet and Ascherio, 1994). The best way to avoid these chemical modifications is to increase the total amount of saturated fatty acids in those oils through the generation of genetic variability after mutagenesis or the introduction of new enzymatic activities by genetic engineering. Among the possible saturated fatty acids, stearic acid is the preferred saturated fatty acid because its neutral effect on serum lipoprotein cholesterol (Pearson, 1994).

Mutant sunflower (*Helianthus annuus* L.) lines with increased content of palmitic acid, like CAS-5 with 25.0% in the seed oil, or stearic acid, like CAS-3 with 26.0% of stearic acid in the seed oil, have been isolated (Osorio et al., 1995). In soybean (*Glycine max* L.), the mutant line A6 was isolated (Graef et al., 1985). This line has up to 28% of stearic acid in its seed oil. Also, a high palmitic acid soybean line with a 17.0% of palmitic acid has been selected (Fehr et al., 1991). The introduction of genes from plants capable of producing high saturated fatty acids content into traditional crops through genetic engineering has been very useful. A canola (*Brassica napus* L.) line with 28.0% of stearic acid has been developed after the introduction of an antisense mRNA of the stearate desaturase (Knutzon et al., 1992), or through the introduction of a new thioesterase from high stearic acid plants, like the one of *Garcinia mangostana* (Facciotti et al., 1999).

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In sunflower and other oil seed crops, growth temperature influences the fatty acid composition, mainly regulating the ratio of oleic and linoleic acid. This effect not only occurs in the normal lines, but also in some mutants like the high oleic sunflower (Garcés et al., 1989). In soybean, growth temperature influences the stearic acid content of the A6 mutant, accumulating more stearic acid at high temperature (Rennie and Tanner, 1989). Whereas the high stearic acid sunflower mutant CAS-3 is slightly influenced by growth temperature, medium stearic acid CAS-4 and CAS-8 lines, and also normal sunflower lines increase the stearic acid content at low temperature (Martínez-Force et al., 1998). This is in opposition to the behaviour of the A6 mutant of soybean. These new lines are very useful in order to study the regulation of the fatty acid biosynthesis in seeds as well as the effect of the main environmental factor, the temperature, affecting the fatty acid biosynthesis in seed oils.

In this work, we selected a temperature dependent high stearic acid mutant sunflower line and studied the relationship between this new character and the growth temperature.

2. Results and discussion

In the analysis of the progeny of the mutagenized seeds, a putative mutant seed with a high content of stearic acid in its oil was found in one capitulum. The half seed analysis of all seeds from this capitulum showed that 35 seeds had more than 20% of stearic acid, eight of them containing more than 30%. The rest of the seeds had a stearic acid content below 15% (Fig. 1). The normal stearic acid content in the parental line is ca. 7%. To genetically fix the mutant genes the half-seeds with the higher content of stearic acid (more than 30%) were selected. These half-seeds were grown and self-pollinated, selecting again those with highest stearic acid content, sowing the seeds in the field at the end of winter with a day/night temperature of ca. 22–24 and 12–14 °C, respectively. To fix the new charater this procedure was repeated for 3 consecutive years (Table 1). During these 3 years, the average stearic acid content in the seeds increased from 13.8% in the first year, to 21.5% in the second year and then to 27.7% in the third. The range of the stearic acid content of analysed half-seeds from different capitula was similar, but we always had some seeds with low stearic acid content, hence, the normal selection procedure was not efficient in order to fix the high stearic character. The next generation from selected plants always generated low and high stearic acid content seeds in each capitulum, so that, although seeds with around 30% of stearic acid were obtained, we also obtained seeds with a low stearic acid content (Table 1). We then realized that the highest stearic acid seeds were obtained from the plants that produced the seeds during the period of maximum summer temperatures. Those temperatures were between 35 and 40 °C at daylight and 20 and 25 °C at night. In order to genetically fix this line, the plants were sown three months before the maximal annual temperatures, the flowering and seed formation taking place during those maximal temperatures. By using this strategy, the mutant CAS-14 was fixed (Table 2). This new mutant line has a very high stearic acid content, up to 37.3% depending on the growth conditions, and also a slightly bigger quantity of palmitic acid (8.4%) than the parental line CAS-10 (7.8%). This mutant line has more stearic acid than the previous high stearic acid sunflower mutants (Osorio et al., 1995), where the CAS-3 mutant had 26.0%, or in soybean (Graef et al., 1985), where the A6 mutant line has 28.0% of stearic acid in the seed oil. The increase on stearic acid was at the expense of oleic acid and some linoleic acid.

When plants were cultivated in growth chambers with elevated growth temperature, the stearic acid seed content was increased (Table 3). At temperatures below 25/

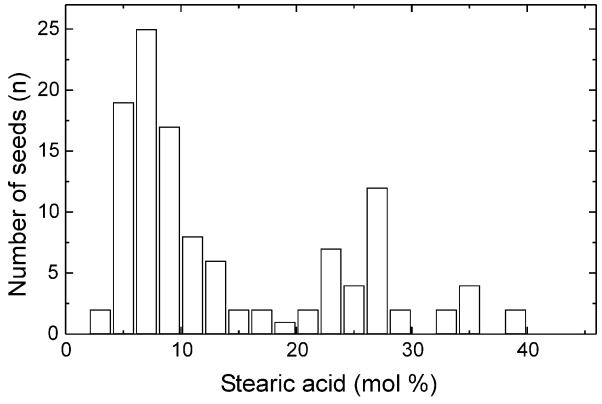

Fig. 1. Stearic acid content of individual M_2 half-seeds from the M_1 original capitulum of the CAS-14 mutant line and number of seeds in each class. Plants were grown in the field (Marchena) with an ca. temperature of minimum 15–25 °C and maximum 30–40 °C.

Table 1
Fatty acid composition of seeds from the mutant sunflower line CAS-14 during its fixation in the field[a]

Year		Fatty acid composition (%)			
		Palmitic	Stearic	Oleic	Linoleic
1995	Mean±S.D.	9.6±2.0	13.8±9.8	26.5±13.5	50.0±9.1
	Range	5.4–14.6	3.5–36.5	2.1–54.8	25.9–62.6
1996	Mean±S.D.	9.3±0.9	21.5±6.2	28.1±10.4	41.0±4.7
	Range	7.4–10.6	12.1–28.7	14.2–38.9	31.2–44.9
1997	Mean±S.D.	11.1±1.2	27.7±9.5	14.9±8.7	46.3±5.2
	Range	8.1–12.6	6.3–37.8	3.2–29.8	31.9–59.7
Control	Mean±S.D.	7.3±0.6	6.6±1.6	31.0±3.8	55.1±3.1
	Range	6.1–8.4	4.0–9.9	21.7–37.7	49.4–62.1

[a] These data are the mean±S.D. resulting from at least 36 half-seeds from three different capitula per year.

Table 2
Fatty acid composition of mutant sunflower line CAS-14 and the control parental line CAS-10 grown at 39/24 °C during the period of seed formation[a]

		Fatty acid composition (%)					
		Palmitic	Stearic	Oleic	Linoleic	Arachidic	Behenic
CAS-10	Mean±S.D.	7.8±0.5	7.5±1.1	59.4±3.5	24.5±2.5	0.4±0.5	0.4±0.6
	Range	7.2–8.5	6.1–9.7	53.1–64.6	20.7–28.6	0–1.2	0–1.4
CAS-14	Mean±S.D.	8.4±1.1	37.3±3.2	12.4±4.9	38.0±3.5	2.2±0.4	1.8±0.7
	Range	6.2–10.5	27.4–40.7	7.4–26.2	30.6–44.2	1.6–2.8	0.2–2.6

[a] These data are the mean±S.D. resulting from the analysis of 18 seeds from three different capitula.

Table 3
Fatty acid composition of CAS-6 (control) and CAS-14 sunflower seeds grown in a growth chamber at different temperatures[a]

T (°C)			Fatty acid composition (%)					
			Palmitic	Stearic	Oleic	Linoleic	Arachidic	Behenic
20/10	CAS-6	a	6.1±0.4	7.4±0.7	22.7±1.5	62.4±1.8	0.5±0.1	0.9±0.3
		b	5.7–6.5	6.6–8.2	21.5–24.8	60.6–64.8	0.4–0.6	0.7–1.3
	CAS-14	a	5.6±0.5	7.8±1.1	26.2±4.1	58.6±3.6	0.5±0.3	1.3±0.1
		b	4.8–6.3	4.6–9.2	15.9–32.2	52.9–66.9	0.3–1.5	1.0–1.5
25/15	CAS-6	a	5.7±0.2	7.5±0.4	28.8±2.9	56.7±3.4	0.4±0.1	0.8±0.1
		b	5.4–5.9	6.9–8.1	25.9–34.0	50.5–59.8	0.4–0.5	0.7–1.0
	CAS-14	a	5.3±0.5	6.7±1.6	33.9±6.7	52.0±5.8	0.7±0.4	1.8±0.6
		b	4.7–6.6	4.4–9.0	22.0–45.4	39.2–62.5	0.3–1.5	1.0–2.8
30/20	CAS-6	a	4.6±0.2	5.8±1.0	53.4±2.4	35.0±2.9	0.4±0.1	0.8±0.1
		b	4.4–5.1	4.1–7.5	47.1–56.7	30.6–42.1	0.2–0.7	0.5–1.0
	CAS-14	a	6.7±1.1	14.2±7.6	36.5±11.1	40.5±9.0	0.8±0.4	1.2±0.5
		b	5.1–9.4	5.2–33.4	12.5–53.8	28.7–56.8	0.4–1.8	0.4–1.8
35/25	CAS-6	a	5.3±0.2	5.6±0.2	56.9±2.5	31.3±2.5	0.2±0.1	0.7±0.1
		b	5.0–5.5	5.3–6.1	51.7–59.7	29.1–36.6	0.1–0.4	0.6–0.8
	CAS-14	a	8.8±0.9	34.0±5.2	24.1±8.6	29.1±3.6	2.1±0.5	2.0±0.3
		b	7.4–10.6	25.8–43.0	8.6–40.6	22.9–35.0	1.5–3.2	1.7–2.8

[a] These data are the mean±S.D. (a) and range (b) from the analysis of 18 seeds of three different capitula.

15 °C, there was no increased accumulation of stearic acid in the seed oil. When the temperatures were higher than 30/20 °C, the character was expressed. At 35/25 °C, the stearic acid content was 34.0%. At the same time, the palmitic acid was slightly increased, and the contents of oleic and linoleic acids were reduced, probably due to the normal effect of temperature on the ratio oleic/linoleic (Garcés et al., 1992). In order to test whether this relationship between growth temperature and stearic acid content had a similar behaviour when plants were not cultivated in growth chambers, plants were grown at several temperatures in the field and in the greenhouse during summer time at very high temperatures, measuring the average day/night temperature during seed formation. To analyse the stearic acid seed content we selected three different high temperatures, and seeds from these plants were collected for fatty acid half-seed analysis. As expected, the stearic acid content increased with temperature, mainly from 30/20 °C to 35/22 °C (14.2 and 34.0%, respectively), reaching the maximum stearic acid content of 36.9% at the highest temperature (Fig. 2). A similar effect was found in the contents of the other saturated fatty acids: palmitic, arachidic, and behenic. The relationship between the growth temperature and the stearic acid content found in CAS-14 was the opposite to that previously found in normal sunflower oils, in which there was more stearic acid at low temperature (Lajara et al., 1990). These authors found, in a study covering 33 field locations in Spain, that the stearic acid content decreased progressively from 6.0 to a 3.2% from Northern Spain (colder weather) to Southern Spain (warmer weather). Also, in the medium stearic acid mutants CAS-4 and CAS-8, a negative relationship between growth temperature and the stearic acid content has been described. At 30/20 °C, these mutants contain 11–12% stearic acid, whereas at 20/10 °C they contain 18–20% stearic acid (Martínez-Force et al., 1998). The soybean mutant line A6 has normal stearic acid content (2.4%) at 15/12 °C that is elevated (22.3%) at 28/22 °C, but decreases a little at

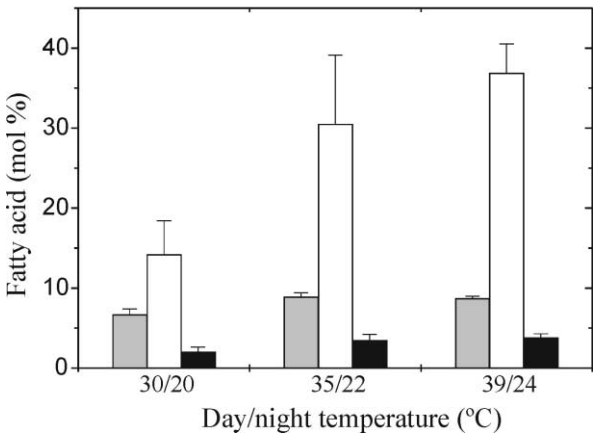

Fig. 2. Temperature effect on the saturated fatty acids content of the mutant sunflower line CAS-14 grown in a greenhouse at three high day/night temperatures. Data are the mean plus error bar from at least 24 individual half-seeds. ■ = palmitic acid; □ = stearic acid; ■ = arachidic plus behenic acids.

higher temperatures, having 18.2% at 40/30 °C (Rennie and Tanner, 1989). This relation is partially similar to the CAS-14 mutant, where the concentration of stearic acid increased with growth temperature. These results coincide with the enzymatic regulation of the stearate desaturase activity by temperature in soybean (Cheesbrough, 1990). This author found that the activity was reduced under high temperatures, making the accumulation of stearic acid possible. The CAS-14 mutant should have a reduced stearate desaturation activity at high temperature, reducing the amount of oleic acid produced by the fatty acid biosynthesis pathway. This high stearic sunflower mutant has the highest stearic acid content described so far (35.0%). However, a high growth temperature is required for the expression of the character. This temperature must always be higher than 30/20 °C.

3. Conclusions

After a chemical treatment with the plant mutagen sodium azide, it has been possible to generate genetic variability in the seed fatty acid content. From the progeny of the mutated seeds a very high stearic acid content mutant was selected. The character has been found to depend on growth temperature, for good phenotype expression temperatures higher than 30/20 °C day/night were needed. Under these conditions the stearic content was around 15%, whereas the maximum stearic content, higher than 35%, was found when growth temperatures were near 40 °C during the daylight and 25 °C at night. This increment was at the expenses of oleic acid. The level of linoleic acid also showed some modifications but was more related to the temperature than to the mutant character.

4. Experimental

4.1. Plant material

The sunflower seeds used in this work were from the lines CAS-6 and CAS-10 with a normal fatty acid composition. These lines have an oil with standard high linoleic fatty acid composition. The plants, including the selected mutant CAS-14, were cultivated in growth chambers at the indicated day/night temp. with 16 h photoperiod and 300 μmol m^2 s^{-1} light intensity or in spring sowing in the field on Marchena (Seville, Spain). To study the effect of the growth temperature in the expression of the character, the plants were cultivated in growth chambers with different temp. from 20/10 to 39/24 °C. Seeds were harvested after maturity was completed around 40 days after flowering.

4.2. Mutagenesis treatment

Chemical mutagenesis was carried out with sodium azide (4 mM; 0.1 M K-Pi buffer, pH 3; Nilan et al., 1973), following the method described in Osorio et al. (1995). The mutagenesis treatment was carried out with 3000 dry seeds exposed to the mutagen solution for 1 h at room temperature. Mutagenised seeds were sown in the field on Marchena (Seville). M_1 plants were self-pollinated by covering them with a paper bag and M_2 seeds were collected for mutant selection. Two seeds were taken from each individual capitulum and their fatty acid composition was determined by the half-seed method. The distal portion of cotyledons was used for lipid analysis (half seed method) and the rest of the seed containing the embryo was stored at room temperature. If the half-seed analysed showed an interesting fatty acid composition, the stored part of the seed containing the embryo was germinated and growth to obtain the next seed generation, always by self-pollination by covering the capitulum with a paper bag.

4.3. Lipid analysis

To determine the fatty acid composition of seed samples the lipids were extracted, transmethylated and purified by the one-step method of Garcés and Mancha (1993) with some modifications. Half-seed samples were heated at 80 °C for 2 h in MeOH:toluene:dimethoxypropane:H_2SO_4:heptane (33:14:2:1:50; by vol.) and, after cooling, the fatty acid methyl esters were recovered in the upper phase. Analysis of the fatty acid methyl esters composition was developed in a 5890A gas chromatograph (Hewlett-Packard, Palo Alto, CA) with a SP-2380 capillary column (30 m length; 0.32 mm i.d.; 0.20 μm film thickness) of fused silica (Supelco, Bellefonte, PA) and quantified by hydrogen flame ionization detection (FID). Hydrogen was used as carrier gas, the

linear gas rate being 28 cm/s. The injector and detector temp. was 220 °C, and the oven temp. was 170 °C. Samples (1 μl) were injected with a split ratio of 1/50. Fatty acids were identified by comparison with known standards (Sigma, S. Louis, MI).

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