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RESEARCH

# Novel Safflower Germplasm with Increased Saturated Fatty Acid Content

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**ABSTRACT**

Safflower (*Carthamus tinctorius* L.) oil with high concentration of saturated fatty acids has potential applications in the food industry. So far only germplasm with increased stearic acid content (50–120 g kg$^{-1}$), controlled by alleles at the locus *St*, has been developed. The objectives of the present research were to evaluate safflower germplasm for saturated fatty acid content, to isolate lines with increased levels of palmitic acid and stearic acid, to study the inheritance of both traits, and to evaluate the feasibility of recombining them. Germplasm evaluation and further selection led to the isolation of the line CR-50 with increased palmitic acid content (98.2 ± 7.9 g kg$^{-1}$ vs. 64.0 ± 3.4 g kg$^{-1}$ in the check) and the line CR-13 with increased stearic acid content (92.8 ± 9.2 g kg$^{-1}$ vs. 22.2 ± 3.4 g kg$^{-1}$ in the check). Inheritance studies including evaluation of $F_1$, $F_2$, and $F_3$ seed generations from crosses with the nuclear male-sterile line CL1, with conventional fatty acid profile, suggested that increased palmitic acid content was determined by additive alleles at a single locus *Pa*, whereas stearic acid content was controlled by partially recessive alleles at a single locus, probably *St*. Recombination of *pa* and *st* alleles produced transgressive segregants with 211.3 ± 14.6 g kg$^{-1}$ saturated fatty acids, compared with 149.6 ± 14.0 g kg$^{-1}$ in CR-50, 159.7 ± 8.0 g kg$^{-1}$ in CR-13, and 89.3 ± 4.9 in the line CL1. These are the highest levels of saturated fatty acids reported so far in safflower.

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Vegetable oils with high concentration of saturated fatty acids have important applications in the food industry, especially for the production of shortenings, margarines, and spreads. These food products are usually produced by partial hydrogenation of vegetable oils that are liquid at room temperature, a process that produces *trans* and positional fatty acid isomers that have been associated with a number of detrimental effects on human health (Katan, 1998). Increasing the natural saturated fatty acid level in the vegetable oil increases its viscosity, forming a semisolid fat at room temperature, which reduces or eliminates the need for hydrogenation (Liu et al., 2002).

Germplasm with increased levels of saturated fatty acids has been developed in several oilseed crops, for example high palmitic acid (>250 g kg$^{-1}$) in sunflower (*Helianthus annuus* L.) (Osorio et al., 1995; Fernández-Martínez et al., 1997) and soybean [*Glycine max* (L.) Merr.] (Fehr et al., 1991), or high stearic acid ( > 250 g kg$^{-1}$) in sunflower (Osorio et al., 1995; Fernández-Moya et al., 2002), soybean (Bubeck et al., 1989), rapeseed (*Brassica napus* L.) (Knutzon et al., 1992), and cottonseed (*Gossypium* spp.) (Liu et al., 2002).

Conventional safflower (*Carthamus tinctorius* L.) seed oil contains 60 to 80 g kg$^{-1}$ palmitic acid, 20 to 30 g kg$^{-1}$ stearic acid, 160 to 200 g kg$^{-1}$ oleic acid, and 710 to 750 g kg$^{-1}$ linoleic acid (Knowles, 1989). Ladd and Knowles (1970) reported increased stearic acid levels (50–120 g kg$^{-1}$) in several germplasm accessions. High stearic acid content was determined principally by alleles at a single locus *St* (Ladd and Knowles, 1970).

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The objectives of the present research were to evaluate safflower germplasm for saturated fatty acid content, to isolate lines with increased levels of palmitic acid and stearic acid content, to study the inheritance of both traits, and to evaluate the feasibility of recombining them.

# MATERIALS AND METHODS

## Evaluation of Germplasm and Selection

One hundred thirty-two safflower accessions were provided by the Western Regional Plant Introduction Station of the U.S. Department of Agriculture. The accessions were selected on the basis of the information on their fatty acid profiles available at the Germplasm Resources Information Network (GRIN) (www.ars-grin.gov/npgs/), based on research conducted by Johnson et al. (1999). Sixteen $S_0$ half seeds from each accession were analyzed for fatty acid composition as described below.

$S_0$ half seeds showing increased levels of either palmitic acid or stearic acid were selected and the corresponding $S_0$ plants were grown in pots under open-air conditions in 1999. The seeds were planted in January and the plants were harvested in July. Plants of the Spanish cultivar Rancho, with conventional fatty acid profile, were used as a check in this and subsequent evaluations. The heads of the plants were covered with paper bags before beginning of flowering to ensure self-fertilization. Twenty-four $S_1$ half seeds from each $S_0$ plant were analyzed for fatty acid composition. Selected $S_1$ half seeds were germinated and the corresponding $S_1$ plants were grown in pots in 2000 and self-pollinated as in the previous generation. Twenty-four $S_2$ half seeds from each $S_1$ plant were analyzed for fatty acid composition, which confirmed the genetic stability of the traits. Differences between means were evaluated with the Student–Newman–Keuls test (Steel and Torrie, 1980).

## Genetic Study

A genetic study was conducted using the lines CR-50 and CR-13, with increased levels of palmitic acid and stearic acid, respectively, developed in this research, and the nuclear male-sterile line with conventional fatty acid profile CL1, isolated from the USDA-ARS germplasm accession PI 560161, which in turn derived from the germplasm line UC-148 (Heaton and Knowles 1980). Twenty-four half seeds of CL1, CR-50, and CR-13 were analyzed for seed oil fatty acid profile. The seeds were germinated, and after 15 d in a growth chamber (25/15°C [day/night] with 16-h daylength), the plants were transplanted into the field in January 2005. Heads of all the plants were covered with paper bags before flowering to avoid contamination with external pollen. Male-sterile heads of CL1 plants were pollinated with fresh pollen from plants of CR-50 and CR-13. $F_1$ seeds as well as seeds of the parents were analyzed for fatty acid composition and the corresponding plants were grown in the field in 2006. $F_1$ plants were self-pollinated to obtain $F_2$ seeds, which were analyzed for fatty acid composition. One population of 154 $F_2$ plants from the cross CL1 × CR-50 and another one of 96 $F_2$ plants from the cross CL1 × CR-13 were grown in the field in 2007 to evaluate fatty acid profile at the $F_3$ seed level. Twenty-four $F_3$ individual half seeds from each male-fertile $F_2$ plant were analyzed for fatty acid composition to determine presence or absence of segregation for palmitic acid (CL1 × CR-50) or stearic acid content (CL1 × CR-13).

The chi-square test was used to evaluate proposed segregation ratios. Means were compared using independent *t* tests. The *t* test was also applied to determine differences between $F_1$ means and the midparent value.

## Recombination of High Palmitic Acid and High Stearic Acid

Flowers from CR-50 plants grown in the field in 2005 were emasculated and their stigmas pollinated with fresh pollen from CR-13 plants following the procedure reported by Knowles (1980). $F_1$ seeds as well as seeds of the parents were analyzed for fatty acid composition and the corresponding plants were grown in the field in 2006 to obtain the $F_2$ seed. $F_2$ seeds were analyzed for fatty acid profile and seeds combining high palmitic acid and high stearic acid content were selected and the corresponding $F_2$ plants were grown in the field in 2007. Twenty-four $F_3$ half seeds were analyzed from each $F_2$ plant.

## Analysis of Fatty Acids by Gas–Liquid Chromatography

The fatty acid composition of the oil in cotyledon tissues from individual seeds was analyzed by simultaneous extraction and methylation (Garcés and Mancha, 1993) followed by gas–liquid chromatography using a PerkinElmer Autosystem gas–liquid chromatograph (PerkinElmer Corporation, Norwalk, CT). A 2-m-long column packed with 3% SP-2310/2% SP-2300 on Chromosorb WAW (Supelco Inc., Bellefonte, PA) was used. The oven, injector, and flame ionization detector were held at 185, 275, and 250°C, respectively.

# RESULTS

## Isolation of Lines with Increased Levels of Palmitic Acid or Stearic Acid

The analysis of the original $S_0$ seed received from the seed bank showed average palmitic acid contents from 40.0 to 78.7 g $kg^{-1}$ and average stearic acid contents from 11.0 to 80.3 g $kg^{-1}$ in the 132 safflower accessions. At the single seed level, palmitic acid and stearic acid contents ranged from 34.4 g $kg^{-1}$ to 101.7 g $kg^{-1}$ and from 7.9 to 99.1 g $kg^{-1}$, respectively. Twenty-five half seeds from 17 accessions were selected using an arbitrary cut-off of 75 g $kg^{-1}$ palmitic acid and 27 half seeds from 10 accessions were selected using an arbitrary cut-off of 40 g $kg^{-1}$ stearic acid. Germination of the selected seeds and plant survival were very poor, which resulted in only 12 mature plants from the initially selected 52 $S_0$ seeds. The analysis of the $S_1$ seeds from these plants showed maximum palmitic and stearic acid averages of 95.1 ± 4.2 g $kg^{-1}$ (mean ± standard deviation; plant CR-50, accession PI 306686) and 95.0 ± 11.8 g $kg^{-1}$ (plant CR-13, accession PI 198990), respectively. The check line Rancho showed average values of 64.0 ± 3.6 g $kg^{-1}$ palmitic acid and 22.4 ± 3.2 g $kg^{-1}$

stearic acid. Three other $S_0$ plants showed increased stearic acid averages of 40.0 ± 3.0 g kg$^{-1}$ (plant CR-58, accession PI 311738), 67.1 ± 7.9 g kg$^{-1}$ (plant CR-69, accession PI 387821), and 71.7 ± 4.0 g kg$^{-1}$ (plant CR-65, accession PI 343778), in all cases below the stearic acid content of CR-13. In addition to the increased palmitic acid content, the seeds of the plant CR-50 also showed a slightly increased stearic acid content of 32.5 ± 6.0 g kg$^{-1}$.

$S_1$ plants from CR-50 and CR-13 were evaluated to confirm the genetic stability of the increased palmitic acid and stearic acid content, respectively. Palmitic acid content in CR-50 $S_2$ seeds averaged 98.2 ± 7.9 g kg$^{-1}$, with a range of variation from 85.5 to 126.2 g kg$^{-1}$. Stearic acid content in CR-13 $S_2$ seeds averaged 92.8 ± 9.2 g kg$^{-1}$, with a range of variation from 68.3 to 113.8 g kg$^{-1}$. Seeds of the cultivar Rancho from plants grown in the same environment averaged 64.0 ± 3.4 g kg$^{-1}$ palmitic acid and 22.2 ± 3.4 g kg$^{-1}$ stearic acid, with ranges of variation from 57.8 to 76.2 g kg$^{-1}$ for palmitic acid, and from 12.5 to 29.0 g kg$^{-1}$ for stearic acid. The overall fatty acid profile of $S_2$ seeds from CR-13, CR-50, and the check line Rancho is presented in Table 1.

## Genetic Study of Increased Palmitic Acid Content

Seeds of the nuclear male-sterile line CL1 used as female parent averaged 67.3 ± 6.2 g kg$^{-1}$ palmitic acid. Seeds of the line CR-50 averaged 114.9 ± 13.5 g kg$^{-1}$ palmitic acid. $F_1$ seeds from the cross CL1 × CR-50 averaged 90.8 ± 6.7 g kg$^{-1}$ palmitic acid. The $F_1$ palmitic acid content was not significantly different from the midparent value ($t$ = 0.44, $P > 0.05$), suggesting intermediate inheritance of increased palmitic acid content.

Palmitic acid content in $F_2$ seeds fell into three classes comprising palmitic acid values from 55.3 to 71.1 g kg$^{-1}$ (average 62.0 g kg$^{-1}$; $n$ = 39), 76.3 to 96.3 g kg$^{-1}$ (average 88.2 g kg$^{-1}$; $n$ = 98), and 98.0 to 124.6 g kg$^{-1}$ (average 109.7 g kg$^{-1}$; $n$ = 55) (Fig. 1). The number of individuals in the three classes followed a 1:2:1 distribution ($\chi^2$ = 2.75, $P$ = 0.25), corresponding to the segregation of additive alleles at a single locus. The analysis of the $F_3$ seed generation confirmed monogenic inheritance. From an initial population of 154 $F_2$ plants, 114 of them were male fertile and produced seed under isolation. The proportion of male-fertile and male-sterile plants (3:1, $\chi^2$ = 0.08, $P$ = 0.78) corresponded to the segregation of the recessive *Ms* gene controlling male sterility in safflower (Heaton and Knowles, 1982). From the 114 male-fertile $F_2$ plants, 21 of them showed conventional palmitic acid content, 68 of them segregated for increased palmitic acid content, and 25 of them had all the $F_3$ seeds expressing increased palmitic acid content, which did not differ significantly from the expected 1:2:1 distribution ($\chi^2$ = 4.53, $P$ = 0.10).

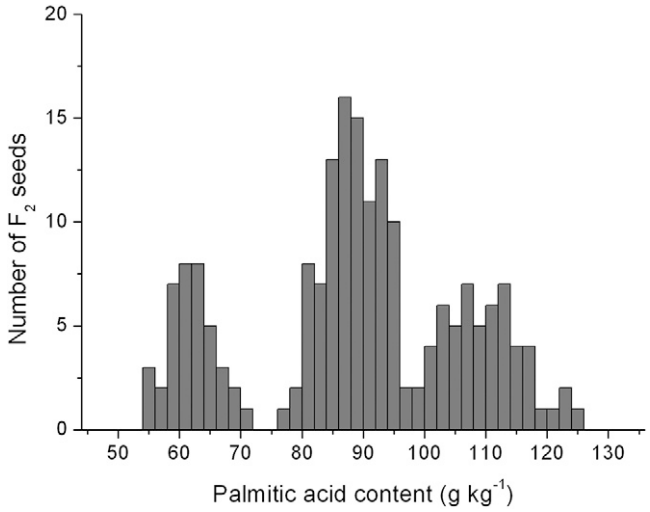


Figure 1. Palmitic acid content in $F_2$ seeds from the cross between the safflower lines CL1, with conventional seed fatty acid profile, and CR-50, with increased palmitic acid content.

**Table 1. Fatty acid profile (g kg$^{-1}$) in $S_2$ seeds of the safflower lines CR-13, with increased stearic acid content; CR-50, with increased palmitic acid content; and the cultivar Rancho grown as a check in the same environment. The data are presented as mean ± standard deviation.**

| Line | $n$† | Palmitic acid | Stearic acid | Oleic acid | Linoleic acid |
|---|---|---|---|---|---|
| CR-13 | 288 | 60.2 ± 1.6a‡ | 92.8 ± 9.2c | 120.9 ± 10.0a | 726.1 ± 15.4b |
| CR-50 | 288 | 98.2 ± 7.9c | 35.5 ± 4.4b | 151.1 ± 18.8b | 715.2 ± 24.7a |
| Rancho | 288 | 64.0 ± 3.4b | 22.2 ± 3.4a | 155.6 ± 20.6c | 758.1 ± 22.7c |

†Twenty-four individual seeds from 12 $S_1$ (CR-13 and CR-50) or check (Rancho) plants.

‡Values in a column followed by the same letter are not significantly different according to Student–Newman–Keuls test at $\alpha$ = 0.05.

## Genetic Study of Increased Stearic Acid Content

Seeds of the nuclear male-sterile line CL1 averaged 20.2 ± 3.8 g kg$^{-1}$ stearic acid. Seeds of the line CR-13 averaged 99.4 ± 12.3 g kg$^{-1}$ stearic acid. $F_1$ seeds from the cross CL1 × CR-13 averaged 33.0 ± 8.5 g kg$^{-1}$ stearic acid. $F_1$ stearic acid content was significantly greater than that of CL1 ($t$ = 15.7, $P < 0.01$), but lower than the midparent value ($t$ = 30.13, $P < 0.01$), suggesting partial dominance of conventional over increased stearic acid content.

No clear-cut classes could be identified in the segregation of stearic acid content in the $F_2$ seed generation (Fig. 2). However, the analysis of the $F_3$ seed generation allowed the separation of $F_2$ genotypes. The initial population of 96 $F_2$ plants segregated for the male-sterility *Ms* gene, which produced 70 male-fertile and 26 male-sterile plants, fitting the expected 3:1 ratio ($\chi^2$ = 0.22, $P$ = 0.64). The analysis of individual $F_3$ seeds showed 20 $F_{2:3}$ families with conventional stearic acid content that did not segregate for increased stearic acid levels, because they showed low average stearic acid content and low standard deviation; 37 $F_{2:3}$ families that segregated from conventional to increased stearic acid

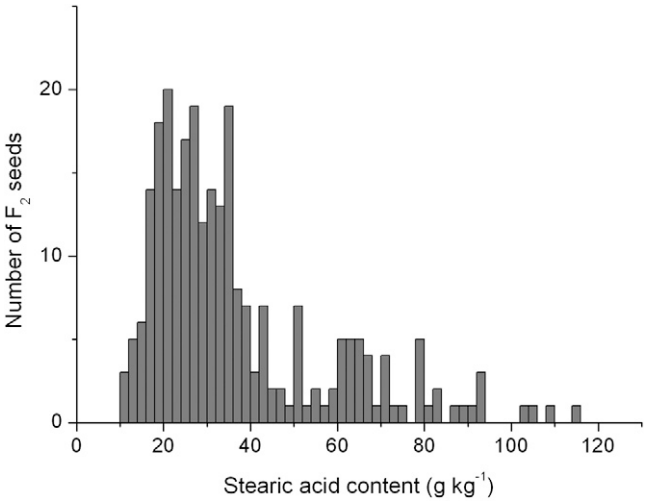


Figure 2. Stearic acid content in $F_2$ seeds from the cross between the safflower lines CL1, with conventional seed fatty acid profile, and CR-13, with increased stearic acid content.

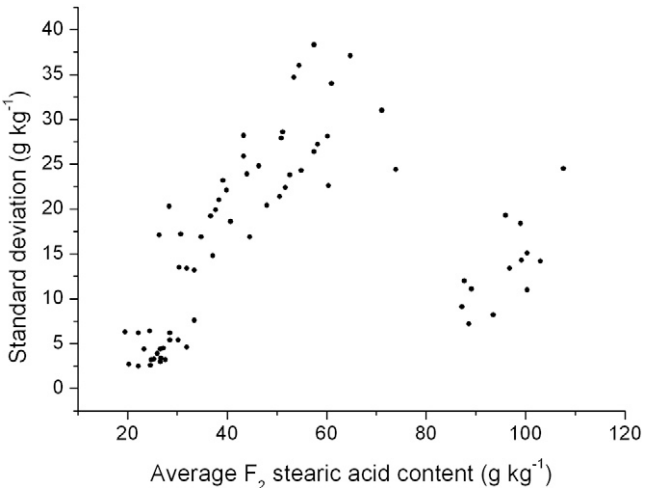


Figure 3. Average stearic acid content and standard deviation in $F_{2:3}$ families (individual $F_3$ seeds analyzed) from the cross between the safflower lines CL1, with conventional seed fatty acid profile, and CR-13, with increased stearic acid content.

content, as revealed by an increased standard deviation; and 13 $F_{2:3}$ families with all the $F_3$ seeds having increased stearic acid content (Fig. 3). The three phenotypic classes fitted a 1:2:1 (conventional/segregating/increased stearic acid content) ratio ($\chi^2 = 1.63$, $P = 0.44$) corresponding to the segregation of alleles at a single gene. Average stearic acid content in the three phenotypic classes was 26.0, 47.0, and 96.0 g kg$^{-1}$, respectively.

### Recombination of Increased Levels of Palmitic Acid and Stearic Acid

Seeds from the line CR-50 averaged 114.9 ± 13.5 g kg$^{-1}$ palmitic acid and 40.1 ± 6.5 g kg$^{-1}$ stearic acid, which represented an average saturated fatty acid content of 154.9 ± 15.5 g kg$^{-1}$. Seeds from the line CR-13 averaged 58.3 ± 1.9 g kg$^{-1}$ palmitic acid and 99.4 ± 12.3 g kg$^{-1}$ stearic acid, totaling 157.8 ± 11.5 g kg$^{-1}$ saturated fatty acids. $F_1$ seeds from the cross CR-50 × CR-13 averaged 85.5 ± 7.7 g kg$^{-1}$ palmitic acid and 60.9 ± 7.7 g kg$^{-1}$ stearic acid, with the sum of both saturated fatty acids being 146.4 ± 3.9 g kg$^{-1}$.

$F_2$ seeds showed transgressive segregation for total saturated fatty acid content, from 92.8 to 243.5 g kg$^{-1}$, compared with 137.1 to 201.1 g kg$^{-1}$ in CR-50 and 134.5 to 196.4 g kg$^{-1}$ in CR-13 seeds from plants grown in the same environment as $F_1$ plants. The highest saturated fatty acid levels in $F_2$ seeds were produced by the simultaneous increase of both palmitic and stearic acid content (Fig. 4). $F_2$ seeds with transgressive high saturated fatty acid content were selected and the $F_2$ plants were grown together with the parents to confirm the recombination of the traits and the subsequent transgressive saturated fatty acid content. Palmitic acid and stearic acid contents in $F_3$ seeds, the parents CR-50 and CR-13, and the line CL1 with conventional fatty acid profile are shown in Fig. 5. Seeds of CL1, CR-50, and CR-13 averaged 89.3 ± 4.9, 149.6 ± 14.0, and 159.7 ± 8.0 g kg$^{-1}$ saturated fatty acids, respectively whereas the $F_3$ seed generation averaged 211.3 ± 14.6 g kg$^{-1}$ saturated fatty acids as the sum of 97.8 ± 8.7 g kg$^{-1}$ palmitic acid (compared with 111.6 ± 11.2 g kg$^{-1}$ in CR-50) and 113.5 ± 13.7 g kg$^{-1}$ stearic acid (compared with 94.7 ± 17.3 g kg$^{-1}$ in CR-13).

## DISCUSSION

Ladd and Knowles (1970) reported the identification of increased stearic acid content (50 to 120 g kg$^{-1}$) in two safflower accessions from Israel and Russia, respectively. They found that the increased stearic acid levels were produced by alleles at one locus *St*, with the allele for conventional low levels of stearic acid being partially dominant to the allele for increased levels. The line CR-13 developed in the present research derived from the USDA germplasm accession PI 198990, donated from Israel. Even though there is no indication in the GRIN database (www.ars-grin.gov/npgs/) that the accession PI 198990 was the same or derived from that studied by Ladd and Knowles (1970), both the increased stearic acid levels (e.g., 68.3–113.8 g kg$^{-1}$ in the $S_2$ seed generation) and the mode of inheritance of the trait, monogenic with partial dominance of conventional over increased stearic acid content, suggest that the line CR-13 probably has the genotype *stst*.

There are no previous reports of genetically stable increased palmitic acid content in safflower. In previous evaluations of accessions from the USDA safflower germplasm collection, Fernández-Martínez et al. (1993) reported maximum palmitic acid contents at the single seed level of up to 119 g kg$^{-1}$, whereas Johnson et al. (1999) reported a maximum average palmitic acid content of 69 g kg$^{-1}$. In both cases, no further research was conducted to determine whether genetically stable increased palmitic acid levels could be isolated. The selection and inheritance study conducted in the present research has demonstrated that

increased palmitic acid levels in safflower are the result of alleles with additive effect at a single locus. We have named this locus *Pa* to follow previous nomenclature in this species for genes controlling modified levels of stearic acid (*St*), oleic acid (*Ol*), and linoleic acid (*Li*) contents (Knowles, 1989). The line CR-50 with increased palmitic acid content derived from the USDA germplasm accession PI 306686 collected in Israel. Interestingly, the increased palmitic acid content of CR-50 seeds was accompanied by a slight increase in stearic acid content, although of smaller magnitude to that identified in the line CR-13. It is noteworthy that both the accession PI 306686 as well as the abovementioned accession PI 198990 are part of the USDA core collection of safflower (www.ars-grin.gov/npgs/).

Recombination of the *pa* and *st* alleles enabled a practically complete recovery of the increased palmitic acid and stearic acid levels from CR-50 and CR-13, respectively. The resulting recombinants showed an average saturated fatty acid content (sum of palmitic acid and stearic acid) of 211 g $kg^{-1}$, which represents an increase of more than 50 g $kg^{-1}$ in relation to the saturated fatty acid content of the individual lines CR-50 and CR-13. The recombinants showed even a greater stearic acid content than the line CR-13, which was attributed to the contribution to increased stearic acid content by the *pa* alleles, as deduced from the stearic acid levels observed in the line CR-50, already discussed above. In sunflower, Pérez-Vich et al. (2000) studied the recombination of the high palmitic acid content of the CAS-5 mutant and the high stearic acid content of the CAS-3 mutant. They found that the loci controlling high palmitic acid content in CAS-5 exerted an epistatic effect over the loci controlling high stearic acid content in CAS-3, in such a way that no intermediate or high stearic acid levels were expressed in combination with high palmitic acid levels.

The present research has led to higher saturated fatty acid levels in safflower than those previously reported in this crop. Such increased levels are only marginally lower those available in other crops such as sunflower (Osorio et al., 1995) or soybean (Bubeck et al., 1989; Fehr et al., 1991). It is interesting to note that in both crops increased saturated fatty acid levels have been achieved through mutagenesis. Even in other crops such as cotton (Liu et al., 2002) and rapeseed (Knutzon et al., 1992), increased saturated fatty acid content has been only obtained following transgenic approaches. Conversely, the increased saturated fatty acid content in safflower is the result of genetic variation already available in germplasm collections. This reinforces the importance of safflower germplasm for the development of novel seed quality traits,

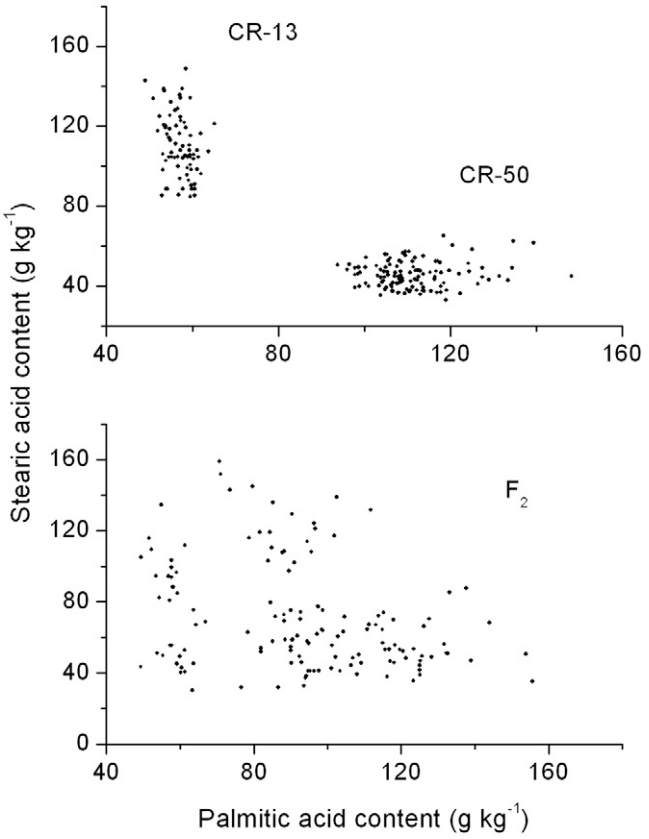


Figure 4. Scatter plot of palmitic acid and stearic acid contents in the seeds of the safflower lines CR-50, with increased palmitic acid content; CR-13, with increased stearic acid content; and $F_2$ seeds from a cross between them.

including a wide range of different fatty acid profiles (Knowles, 1989) or modified tocopherol content (Velasco and Fernández-Martínez, 2004) and profile (Velasco et

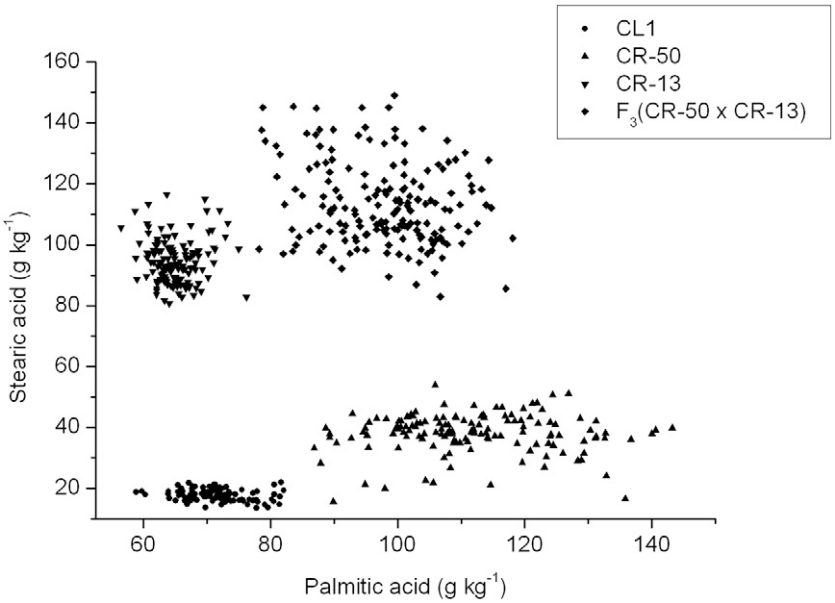


Figure 5. Scatter plot of palmitic acid and stearic acid contents in the seeds of the safflower lines CL1, with conventional seed oil fatty acid profile; CR-50, with increased palmitic acid content; CR-13, with increased stearic acid content; and $F_3$ seeds from the cross between CR-50 and CR-13.

al., 2005). Further advances in increasing saturated fatty acid content in safflower may be expected by further germplasm evaluation and line isolation, and through recombination of different genetic sources of increased saturated fatty acid content.

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