# Oleic acid variation and marker-assisted detection of Pervenets mutation in high- and low-oleic sunflower cross

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**Abstract:** *High-oleic sunflower oil is in high demand on the market due to its heart-healthy properties and richness in monounsaturated fatty acids that makes it more stable in processing than standard sunflower oil. Consequently, one of sunflower breeder's tasks is to develop stable high-oleic sunflower genotypes that will produce high quality oil. We analyzed variability and inheritance of oleic acid content (OAC) in sunflower, developed at the Institute of Field and Vegetable Crops, by analyzing $F_1$ and $F_2$ progeny obtained by crossing a standard linoleic and high-oleic inbred line. $F_2$ individuals were classified in two groups: low-oleic with OAC of 15.24-31.28% and high-oleic with OAC of 62.49-93.82%. Monogenic dominant inheritance was observed. Additionally, several molecular markers were tested for the use in marker-assisted selection in order to shorten the period of detecting high-oleic genotypes. Marker F4-R1 was proven to be the most efficient in detection of genotypes with Pervenets (high-oleic acid) mutation.*

**Key words**: *Helianthus annuus L., fatty acids, molecular marker, breeding.*

## INTRODUCTION

Sunflower (*Helianthus annuus* L.) is the fourth most important source of edible vegetable oil in the world after palm, soybean and canola oil, contributing up to 12% of the edible oil produced globally (Rauf et al. 2017). Sunflower oil has multiple uses in both food and non-food industries (biofuel, lubricants, surfactants, polymer synthesis). Standard sunflower oil is naturally rich in polyunsaturated linoleic acid that makes up about 70% of the total sunflower oil content, and the second most abundant is monounsaturated oleic acid contributing with 20% (Cvejić et al. 2014a). The expanding and ever growing market demands not only standard oils, but also vegetable oils with altered properties. Creation of the first sunflower cultivar Pervenets (Soldatov 1976) with elevated oleic acid content (OAC) in the late 20th century allowed the expansion of the sunflower breeding programs and, ultimately, enabled sunflower breeders to comply with the market demands. Pervenets was obtained by treating the seed of VNIMK 8931 variety with 0.5% DMS solution. In the M3 generation, Soldatov selected single plants containing over 70% oleic acid, developing the Pervenets variety with 80-90% oleic acid in oil (Soldatov 1976, Lacombe et al. 2004, Cvejić et al. 2014b). Later on, more cultivars with altered fatty acid content were developed

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(Andrich et al. 1992, Osorio et al. 1995, Fernández-Martinez et al. 1997, Škorić et al. 2007, Velasco et al. 2008, Leon et al. 2013a, b, Alberio et al. 2016, Cvejić et al. 2016). However, when it comes to the development of sunflower genotypes with the elevated OAC, the Pervenets remain the most commonly used source of the increased OAC.

The advantages of high-oleic oils are numerous, not only for the human consumption, but also in food processing. Due to its high oxidative stability, high-oleic sunflower oil is more stable in the frying process and exposure to high temperatures. Lack of trans fatty acids renders it healthier. In addition, high-oleic oil has reduced rancidity and extended shelf life. In processing, high-oleic oil has reduced cleaning costs and it is easier to transport and store (Vannozzi 2006). Furthermore, a diet rich in high-oleic sunflower oil and margarine has positive effects on blood lipids and factor VII coagulant activity (factor VIIc) (Allman-Farinelli et al. 2005). All the benefits mentioned make high-oleic sunflower oil in high demand by both the industry and the consumers. Consequently, significant attention is paid not only to the creation of high-oleic sunflower lines and hybrids, but also to shedding light on the mechanism(s) that cause the significant OAC increase.

Inheritance of OAC has widely been investigated. The results reported so far differ, but there is a common consensus that OAC is greatly affected by the genetic background of the recipient genotype (Schuppert et al. 2006, van der Merwe et al. 2013, Ferfuia et al. 2015), and by the environment (Izquierdo and Aguirrezábal 2008, Hlisnikovský et al. 2015, Regitano Neto et al. 2016). Urie (1984) reported the dominant mode of inheritance for high OAC, while Fick (1984) reported the partially dominant mode of inheritance. Joksimović et al. (2006) showed that additive gene action plays a more important role in OAC than the non-additive gene action. In addition, there were different reports describing the presence of a modifier gene and one or more genes influencing OAC (Fernández et al. 1999, Lacombe et al. 2004, Berville 2010). Ferfuia and Vannozzi (2015) reported that the high-oleic trait is influenced by at least three loci and reported a significant maternal effect on OAC. Since the high-oleic trait is such a complex one, these authors expressed the need for conducting further testing in different sunflower crossings and in different locations under different temperature and field conditions, in order to get a better insight into the genetic system controlling oleic acid levels and the effect of environment on OAC.

On the molecular level, it was reported that the increased OAC in Pervenets was caused by the partial duplication of the *FAD2-1* allele, which had caused silencing of the *FAD2-1* gene (Lacombe et al. 2002, Schuppert et al. 2006). Therefore, both the standard and the high-oleic genotypes contain the *FAD2-1* sequence. However, in the high-oleic genotype there is an addition of the intergenic region (IGR) separating common *FAD2-1* sequence and the truncated intron and exon that make up the duplicated sequence designated as *FAD2-1D* (Schuppert et al. 2006). This *FAD2-1* duplication is termed *Ol* mutation.

*FAD2-1* encodes FAD2 (oleoyl-phosphatidyl choline desaturase), an enzyme that catalyzes synthesis of linoleic acid from oleic acid. In sunflower genome, there are three FAD genes: *FAD2-1*, *FAD2-2*, *FAD2-3* (Hongtrakul et al. 1998, Martínez-Rivas et al. 2001). While *FAD2-2* and *FAD2-3* are weakly expressed, *FAD2-1* is strongly expressed in developing seeds of standard type sunflower (Martínez-Rivas et al. 2001). The seed-specific *FAD2-1* gene has unequivocally been associated with the $Ol_1$ gene (Hongtrakul et al. 1998). Different authors mapped $Ol_1$-*FAD2-1* locus on LG14 (Lacombe and Berville 2001, Pérez-Vich et al. 2002, Schuppert et al. 2006). Some of the first molecular studies conducted by Dehmer and Friedt (1998) found RAPD PCR fragments AC10-765 and F15-690 at 7.2 cM and 7.0 cM from $Ol_1$, respectively. Later on, Pérez-Vich et al. (2002) reported a QTL describing 84.5% of the phenotypic variation in C18:1 content. These authors concluded that the detected QTL was most likely one of the *Ol* genes controlling high OAC. Schuppert et al. (2006) developed dominant INDEL markers for detection of *Ol* mutation (presence or absence of tandem *FAD2-1* repeats) and identified almost 50 SNPs and several INDELs downstream of *FAD2-1*. Lacombe et al. (2009) developed two types of markers; the first was a codominant SSR marker located in the intron of the putative *FAD2-1* gene and tightly linked to the mutation, and the second group comprised the dominant markers specific for the mutation. In the last several years, identification of the appropriate and easy-to-use molecular markers for the detection of high-oleic genotypes has intensified owing to an increasing interest of breeding companies to offer a greater variety of sunflower high-oleic genotypes. Several authors have worked on the development of the methods for *Ol* gene detection with molecular markers (Nagarathna et al. 2011, Singchai et al. 2013, Bilgen 2016, Dimitrijević et al. 2016), but the obtained methods and results showed the need for further validation in different sunflower populations and genetic backgrounds (Singchai et al. 2013, Bilgen 2016).

In the present work, the high-oleic line Ha-26-Ol was crossed with the standard linoleic line RAJ-SIN-IMI, both developed at the Institute of Field and Vegetable Crops. The goal was twofold: (1) to analyze the variability of OAC in these genotypes and (2) to analyze and identify easy-to-use molecular markers that will be the most efficient in the detection of *FAD2-1D* in sunflower, distinguishing sunflower genotypes with elevated OAC.

## MATERIAL AND METHODS

### Plant material

High-oleic B line Ha-26-Ol and low-oleic line SIN-RAJ-IMI were used as the parental lines. Ha-26-Ol is a near isogenic line developed from Ha-26 (Škorić and Jocić 1998), with average OAC exceeding 80%. SIN-RAJ-IMI is a standard linoleic imidazoline-tolerant line created at the Institute of Field and Vegetable Crops with 20% OAC on average. Ha-26-Ol and SIN-RAJ-IMI were crossed to produce $F_1$ progeny. $F_2$ generation, consisting of 86 individuals, was produced by self-fertilization of a single $F_1$ plant.

Parental lines Ha-26-Ol and SIN-RAJ-IMI, $F_1$ and $F_2$ were grown in the field. Leaf samples for molecular analysis were collected at two leaf-stage, and seeds for oleic acid content analysis at physiological maturity on R9 (Schneiter and Miller 1981) from ten plants/heads of each parental line and $F_1$ progeny, as well as from all of 83 $F_2$ plants.

### Oleic acid content

Fatty acid composition was analyzed by gas chromatography (GC). Samples for GC analysis were prepared in a hydraulic press (Sirio, Mikodental 10-tons strength, cc 400 bars). Two grams of seeds were pressed to yield approximately 0.5 ml of oil that was used for GC analysis. In the reaction vial 270 µL of TMSH (transesterification agent) was added to exactly 30 µL of oil, well shook in the vortex, and kept at room temperature for an hour.

Oleic acid was identified by use of a reference mixture of fatty acids methyl esters (FAME). Multi-standard (FAME RM-1, Cat. no. O7006, Supelco) containing the methyl esters of palmitic, stearic, oleic, linoleic, linolenic and arachidic fatty acids was used to confirm the retention times, as well as to confirm that the peak areas reflected the actual composition of these mixtures.

Agilent 5890 gas chromatograph equipped with flame ionization detector (FID) was used for the analysis of fatty acid methyl esters. Fused silica capillary column with polyethylene stationary phase (HP-INNOWAX, 30 m × 0.25 mm i.d. and 0.25 µm film thickness) was used for the separation. The sample volume injected was 1 µL and split ratio was 1:50. Helium was used as a carrier gas at a constant pressure of 53 kPa at 50 °C. The injector and detector temperatures were set at 250 °C and 280 °C, respectively, and the initial temperature of 50 °C was held for 1 min, then increased to 200 °C at a rate of 25 °C $min^{-1}$, followed by another increase to 230 °C at a rate of 3°C/min, and then maintained for 18 min. The results were processed by the ChemStation software and expressed as the percentage of individual fatty acids in the oil sample. Oleic acid content (OAC) is shown as the percentage of the content of the total fatty acids.

### Molecular analysis

Leaves of parental lines (bulk sample – 10 plants per sample), $F_1$ (bulk sample – 10 plants), and individual samples of 86 $F_2$ plants were used for the molecular analysis. Samples were immediately put in liquid nitrogen and later stored at -70°C until DNA extraction.

DNA was extracted from leaves using the modified CTAB protocol (Permingeat et al. 1998). The following markers were used for the detection of *FAD2-1D* or *FAD2-1* sequence: F13-R5 and F4-R1 (F 13 – 5'-TCAACAGCCTCTTCCTCCTCAG-3'; R5 – 5'-GTAGTTTTGGAAAGCTAGAGACC-3'; F4 – 5'-GTAACGTCTGCGCGCTTGCAGACATCA-3'; R1 – 5'-GGTTTTGCATGAGGGACTCGATCGAGTG-3') (Schuppert et al. 2006) and Fsp-b-R1 (Fsp-b 5'-AGAAGAGGGAGGTGTGAAG-3'; R1 – 5'-AGCGGTTATGGTGAGGTCAG-3') (Lancombe et al. 2009). PCR with primers F13-R5 and F4-R1 was performed as described by Schuppert et al. (2006), and with Fsp-b-R1 as described by Lancombe et al. (2009) in the PCR reaction described by Dimitrijević et al. (2010). Products of PCR amplification were run on 2% agarose gels stained with ethidium-bromide and visualized with the BIO-Print system (Vilber Lourmat, Marne-La-Vallée, France).

## RESULTS AND DISCUSSION

### Oleic acid content

GC analysis of parental lines showed that high-oleic parental line Ha-26-Ol contained 86% of oleic acid, and the standard linoleic line SIN-RAJ-IMI contained OAC 17.89%. The average OAC of the $F_1$ progeny was 88.44% indicating the dominant mode of inheritance of OAC, while the OAC of the $F_2$ progeny ranged between 15.24% and 93.82%. Analysis of distribution of OAC in the tested $F_2$ individuals showed two groups: the first group in which OAC varied between 15.24% and 31.28% (low-oleic group), and the second group where OAC ranged between 62.49% and 93.82% (high-oleic group) (Figure 1). None of the $F_2$ individuals had OAC in between these two groups. Therefore, on the



**Figure 1.** Distribution of oleic acid content (%) in examined $F_2$ sunflower plants, $P_1$ is high-oleic parental line – Ha-26-Ol and $P_2$ is standard parental line RAJ-SIN-IMI

basis of phenotype, the threshold for high OAC between the two groups could be set at 60%. High OAC threshold is an important parameter in breeding and seed production since oils with OAC above a certain threshold receive a prime over the regular price in today's market (Angeloni et al. 2016). Furthermore, the distribution of OAC of $F_2$ individuals and threshold set allows breeders to classify the individuals in low-, normal-, mid-, or high-oleic group and evaluate whether the parental lines used for crossing should be used in future breeding. This is especially important since OAC is highly dependent on both the genotype and its interaction with the environment (van der Merwe et al. 2013), and *Ol* locus exhibits genetically unstable expression (Demurin and Škorić 1996).

There is no general consensus on the threshold value, and it is set for each cross individually due to the influence of genetic background and conditions in which the plants were grown. The threshold set in our research is within the range of previously reported thresholds for high OAC, which were set between 55% and 70% (Lacombe and Bervillé 2001, Lacombe et al. 2002, Bilgen 2016). The analysis of $F_2$ generation showed grouping of the individuals in two groups: 65 plants were in the high-oleic group and 21 plants in the low-oleic group. The ratio 65:21 fits 3:1 ratio, which is in agreement with monogenetic dominant inheritance (2 alleles at one locus) ($\chi^2$, p>0.05). Monogenic dominant mode of inheritance was also reported by Urie (1984), Lacombe et al. (2000), Lacombe and Bervillé (2001). In addition, OAC of $F_1$ progeny was similar to the OAC of the high-oleic parent, thus further supporting the dominant monogenetic inheritance in the cross performed in our study. OAC of $F_1$ progeny can be a good initial parameter for determination of OAC inheritance in sunflower. This is in agreement with the results of Varès et al. (2002) who detected that OAC of $F_1$ obtained by crossing high-oleic and linoleic lines had similar OAC as high-oleic parental line in the dominant inheritance, while in intermediate inheritance OAC of $F_1$ was lower than OAC of high-oleic parental line.

Within the high-oleic group, two sub-groups were observed: 43 plants in the 61-80% OAC and 22 plants in the 80-95% OAC sub-group. The reason for this sub-grouping could be the number of copies of *FAD2-1D* alleles: 22 plants had two *FAD2-1D* alleles (homozygous plants; *OlOl*), and 43 plants had one *FAD2-1D* (heterozygous plants; *Olol*). If observed through the segregation ratio of $F_2$ progeny, it fits 1:2:1 ratio (22(*OlOl*):43(*Olol*):21(*olol*)) for the dominant monogenic inheritance of high-oleic acid content trait ($\chi^2$, p>0.01). Bearing in mind that 2 g of seeds of each $F_2$ plant was used for GC analysis, when homozygous $F_2$ plant was analyzed all the seeds within one head were homozygous for high-oleic trait, while heterozygous $F_2$ plants contained two alleles in three different combinations: homozygous dominant (*OlOl*), homozygous recessive (*olol*) and heterozygous (*Olol*) which influenced OAC content. This means that heterozygous $F_2$ plants had a certain variation in OAC in the seeds from a single head, which is supported by the results reported by Demurin et al. (2000) who found great variation in OAC in $F_2$ progeny plants that varied from 24% to 68% in a single sunflower head due to the segregation in the two parent phenotypic classes.

### Molecular analysis

Two markers used in this study enabled the discrimination between high-oleic parental line Ha-26-Ol and standard linoleic line SIN-RAJ-IMI, while one marker was monomorphic (Figure 2). Marker F13-R5 (Schuppert et al. 2006) was developed as codominant INDEL for *FAD2-1* detecting minimum 8 alleles in several confectionary and oilseed sunflower
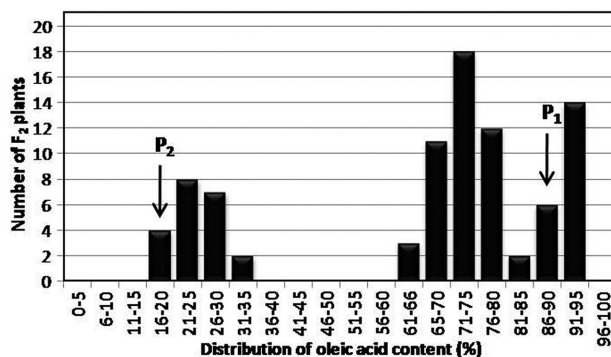
lines, open-pollinated cultivars and sunflower wild populations. It was shown to be monomorphic between the parental lines used in this study (Figure 2A). This marker amplified a band of approximately 340 bp in both parental lines and $F_1$ progeny.

Markers F4-R1 (Schuppert et al. 2006) and Fsp-b-R1 (Lacombe et al. 2009) enabled the discrimination between parental lines; however, neither of them enabled discrimination between homozygous and heterozygous genotype (Figure 2 B, C, respectively). Molecular marker Fsp-b-R1, also known as N1-3F/N2-1R (Bervillé et al. 2009), was created to amplify high-oleic specific element and it amplifies a band of approximately 891 bp. In our study, Fst-b-R1 amplified a band approximately 900 bp in high-oleic line and $F_1$ progeny, while this band was absent from the standard linoleic line. In addition, this primer amplified several common bands between parental lines and $F_1$. The forward primer of the F4-R1 marker is located in intergenic DNA sequences upstream of *FAD2-1D* making it a *FAD2-1D* specific marker. In our study, F4-R1 amplified a single band approximately 650 bp in high-oleic line Ha-26-Ol and in $F_1$ progeny. The absence of this band was recorded in SIN-RAJ-IMI line. Similarly, Schuppert et al. (2006) amplified a 653 bp band in mutant lines (high-oleic lines), while this band was absent in standard sunflower lines.

Due to clearer profiles obtained by using F4-R1, this primer was used for molecular analysis of *FAD2-1D* in $F_2$ individuals (Figure 2D). It amplified a band of the expected length, approximately 650 bp, in 65 $F_2$ plants (that had OAC higher than 61%), while this band was not amplified in 21 $F_2$ plants (in which OAC was lower than 32%). Similarly, Bilgen (2016) was able to discriminate between high-oleic (60-92%) and low-oleic (below 60%) sunflower $F_3$ individuals by the use of two types of molecular markers developed by Bervillé et al. (2009). The first marker was HO PCR specific fragment-marker (N1-3F/N2-1R) that amplified a 870 bp fragment in genotypes carrying Pervenets mutation and codominant SSR (N1-1F/N1-1R) that amplified a 249 bp fragment in homozygous low-oleic genotype and 246 bp fragment in homozygous high-oleic genotype. Nagarathna et al. (2011) and Singchai et al. (2013) successfully discriminated between the high-oleic and low-oleic genotypes by using N1-3F/N2-1R. This primer amplified a band 800 to 900 bp in high-oleic genotypes, which was absent in low- and mid-oleic genotypes. In addition, Singchai et al. (2013) identified 9 additional polymorphic SSRs between high- and low-oleic sunflower lines, while 27 SSRs were monomorphic. Lacombe et al. (2002) used probes (molecular hybridization) for discrimination between genotypes with OAC higher than 65 and 70% (higher OAC for both homozygous and heterozygous plants) and lower OAC (homozygous for *ol*). The probe revealed 8 kb and 15 kb *HindIII* fragment in low-oleic and high-oleic genotype, respectively. Lacombe et al. (2002) found 2 exceptions in which two genotypes with low-oleic phenotype carried the mutation that should have led to the increase in OAC.
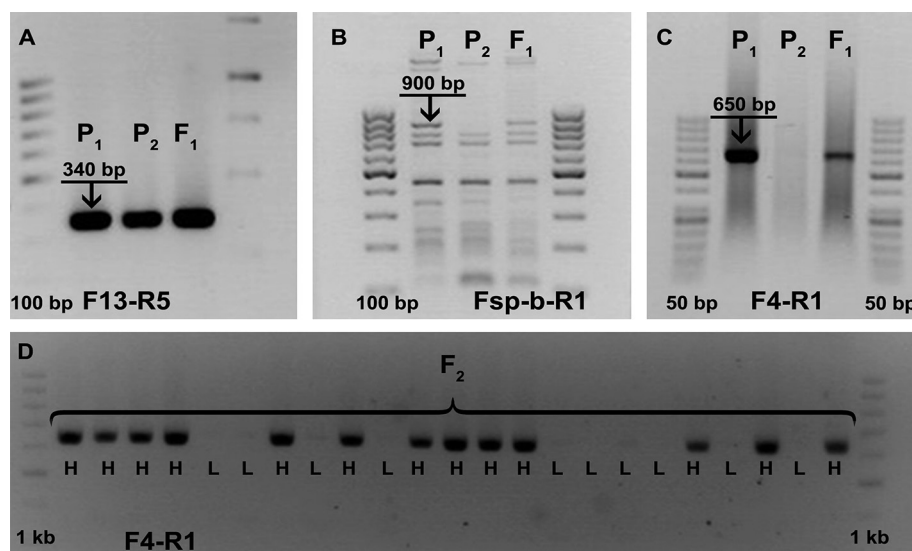


*Figure 2*. Molecular profiles of parental lines – high-oleic Ha-26-Ol ($P_1$), standard line (RAJ-SIN-IMI) ($P_2$), $F_1$ obtained by use of different molecular markers: A) F13-R5 (Schuppert et al. 2006); B) Fsp-b-R1 (Lacombe et al. 2009); C) F4-R1 (Schuppert et al. 2006); D) profiles of individuals of $F_2$ population obtained by amplification with F4-R1 (Schuppert et al. 2006) (DNA ladder 50 bp, 100 bp and 1 kb, Thermo Scientific)

Due to the well-known influence of genetic background on OAC (van der Merwe et al. 2013, Ferfuia et al. 2015,) certain markers in different sunflower populations should be validated (Bilgen 2016, Singchai et al. 2013). Similarly, Imerovski et al. (2014) validated SSR markers for downy mildew resistance in sunflower. Marker F4-R1 was previously tested in numerous sunflower genotypes (Schuppert et al. 2006, Dimitrijević et al. 2016) and now it was validated in $F_2$ sunflower population. It is easy to use and requires use of only agarose gels. The advantage of using molecular markers is that the genotyping results can be obtained before the sunflower growth is completed. Consequently, breeders can discard genotypes without Pervenets mutation before flowering saving both time and money on maintaining the unwanted genotypes.

## ACKNOWLEDGMENTS

## REFERENCES

Alberio C, Izquierdo NG, Galella T, Zuil S, Reid R, Zambelli A and Aguirrezábal LA (2016) A new sunflower high oleic mutation confers stable oil grain fatty acid composition across environments. **European Journal of Agronomy 73**: 25-33.

Allman-Farinelli MA, Gomes K, Favaloro EJ and Petocz P (2005) A diet rich in high-oleic-acid sunflower oil favorably alters low-density lipoprotein cholesterol, triglycerides, and factor VII coagulant activity. **Journal of the American Dietetic Association 105**: 1071-1079.

Andrich G, Balzini S, Zinnai A, Fiorentini R, Baroncelli S and Pugliesi C (1992) The oleic/linoleic ratio in achenes coming from sunflower lines treated with hard X-rays. In **Proceedings of the 13th international sunflower conference**. ISA, Pisa, p. 1544-1549.

Angeloni P, Echarte MM, Irujo GP, Izquierdo N and Aguirrezábal L (2016) Fatty acid composition of high oleic sunflower hybrids in a changing environment. **Field Crops Research 202**: 146-157.

Berviлlé A, Lacombe S, Veillet S, Granier C, Leger S and Jouve P (2009) Method of selecting sunflower genotypes with high oleic acid content in seed oil. **United States patent application** US 11/587,956.

Berviлlé A (2010) Oil composition variations. In Hu J, Seiler G and Kole C (eds) **Genetics, genomics and breeding of sunflower**. Science Publisher, Boca Raton, FL, p. 253-277.

Bilgen BB (2016) Characterization of sunflower inbred lines with high oleic acid content by DNA markers. In Kaya Y and Hasancebi S (eds) **Proceedings of the 19th international sunflower conference**. ISA, Edirne, p. 662-668.

Cvejić S, Jocić S, Dimitrijević A, Imerovski I, Miladinović D, Jocković M and Miklič V (2016) An EMS mutation altering oil quality in sunflower inbred line. In Kaya Y and Hasancebi S (eds) **Proceedings of the 19th international sunflower conference**. ISA, Edirne, p. 422-430.

Cvejić S, Jocić S, Miladinović D, Jocković M, Imerovski I, Sakač Z and Miklič V (2014b) Development and utilization of sunflower genotypes with altered oil quality. **Journal of Processing and Energy in Agriculture 18**: 191-195.

Cvejić S, Miladinović D and Jocić S (2014a) Mutation breeding for changed oil quality in sunflower. In Tomlekova NB, Kozgar MI, Wani MR (eds) **Mutagenesis: exploring genetic diversity of crops**. Wageningen Academic Publishers, Wageningen, p. 77-96.

Dehmer KJ and Friedt W (1998) Development of molecular markers for high oleic acid content in sunflower (*Helianthus annuus* L.). **Industrial Crops and Products 7**: 311-315.

Demurin Y and Škorić D (1996) Unstable expression of *Ol* gene for high oleic acid content in sunflower seeds. In **Proceedings of the 14th international sunflower conference.** ISA, Beiging/Shenyang, p. 12-20.

Demurin YA, Škorić D, Verešbaranji I and Jocić S (2000) Inheritance of increased oleic acid content in sunflower seed oil. **Helia 23**: 87-92.

Dimitrijević A, Imerovski I, Miladinović D, Jocković M, Cvejić S, Jocić S, Zeremski T and Sakač Z (2016) Screening of the presence of ol gene in NS sunflower collection. In Kaya Y and Hasancebi S (eds) **Proceedings of the 19th international sunflower conference**. ISA, Edirne, p. 661-667.

Dimitrijević A, Imerovski I, Miladinović D, Tancić S, Dusanić N, Jocić S and Miklič V (2010) Use of SSR markers in identification of sunflower isogenic lines in late generations of backcrossing. **Helia 33**: 191-198.

Ferfuia C and Vannozzi GP (2015) Maternal effect on seed fatty acid composition in a reciprocal cross of high oleic sunflower (*Helianthus annuus* L.). **Euphytica 205**: 325-36.

Ferfuia C, Turi M and Vannozzi GP (2015) Variability of seed fatty acid composition to growing degree-days in high oleic acid sunflower genotypes. **Helia 38**: 61-78.

Fernández H, Baldini M and Olivieri AM (1999) Inheritance of high oleic acid content in sunflower oil. **Journal of Plant Breeding and Genetics 53**: 99-103.

Fernández-Martinez JM, Osorio J, Mancha M and Garces R (1997) Isolation of high palmitic mutants on high oleic background. **Euphytica 97**: 113-116.

Fick GN (1984) Inheritance of high oleic acid in the seed oil of sunflower. In **Proceedings of the 6th sunflower research workshop**. Sunflower Association, Bismarck, p. 9.

Hlisnikovský L, Kunzová E, Hejcman M, Škarpa P, Zukalová H and Menšík L

(2015) The effect of climate, nitrogen and micronutrients application on oiliness and fatty acid composition of sunflower achenes. **Helia** 38: 221-39.

Hongtrakul V, Slabaugh MB and Knapp SJ (1998) A seed specific D12 oleate desaturase gene is duplicated, rearranged, and weakly expressed in high oleic acid sunflower lines**. Crop Science 38**: 1245-1249.

Imerovski I, Dimitrijević A, Miladinović D, Jocić S, Dedić B, Cvejić S and Šurlan-Momirović G (2014) Identification and validation of breeder-friendly DNA markers for Pl arg gene in sunflower. **Molecular Breeding 34**: 779-788.

Izquierdo NG and Aguirrezábal LA (2008) Genetic variability in the response of fatty acid composition to minimum night temperature during grain filling in sunflower. **Field Crops Research 106**: 116-25.

Joksimović J, Atlagić J, Marinković R and Jovanović D (2006) Genetic control of oleic and linoleic acid contents in sunflower. **Helia 29**: 33-40.

Lacombe S and Berville A (2001) A dominant mutation for high oleic acid content in sunflower (*Helianthus annuus* L.) seed oil is genetically linked to a single oleate-desaturase RFLP locus. **Molecular Breeding 8**: 129-137.

Lacombe S, Guillot H, Kaan F, Millet C and Berville A (2000) Genetic and molecular characterization of the high oleic content of sunflower oil in Pervenets. In **Proceedings of the 15ᵗʰ international sunflower conference**. ISA, Toulouse, p. 12-15.

Lacombe S, Kaan F, Griveau Y and Berville A (2004) The pervenets high oleic mutation: methodological studies. **Helia 27**: 41-54.

Lacombe S, Leger S, Kaan F, Berville A and Sas M (2002) Genetic, molecular and expression features of the Pervenets mutant leading to high oleic acid content of seed oil in sunflower. **Oléagineux, Corps gras, Lipides 9**: 17-23.

Lacombe S, Souyris I and Berville AJ (2009) An insertion of oleate desaturase homologous sequence silences via siRNA the functional gene leading to high oleic acid content in sunflower seed oil. **Molecular Genetics and Genomics 281**: 43-54.

Leon AJ, Zambelli AD, Reid RJ, Morata MM and Kaspar M (2013a) Nucleotide sequences mutated by insertion that encode a truncated oleate desaturase protein, proteins, methods and uses. **WIPO Patent** WO/2013/004281, Jan 10.

Leon AJ, Zambelli AD, Reid RJ, Morata MM, Kaspar M, Martinez-Force E, Garcés R, Salas JJ and Venegas-Caleron M (2013b) Isolated mutated nucleotide sequences that encode a modified oleate destaurase sunflower protein, modified protein, methods and uses. **WIPO Patent** WO/2013/004280, Jan 10.

Martínez-Rivas JM, Sperling P, Luehs W and Heinz E (2001) Spatial and temporal regulation of three different microsomal oleate desaturase genes (FAD2) from normal-type and higholeic varieties of sunflower (*Helianthus annuus* L.). **Molecular Breeding 8**: 159-168.

Nagarathna TK, Shadakshari YG and Ramanappa TM (2011) Molecular analysis of sunflower (*Helianthus annuus L.*) genotypes for high oleic acid using microsatellite markers. **Helia 34**: 63-68.

Osorio J, Fernández-Martinez JM, Mancha M and Garces R (1995) Mutant sunflower with high concentration in saturated fatty acid in the oil. **Crop Science 35**: 739-742.

Pérez-Vich B, Fernández-Martínez JM, Grondona M, Knapp SJ and Berry ST (2002) Stearoyl-ACP and oleoyl-PC desaturase genes cosegregate with quantitative trait loci underlying high stearic and high oleic acid mutant phenotypes in sunflower. **Theoretical and Applied Genetics 104**: 338-349.

Permingeat HR, Romagnoli MV and Vallejos RH (1998) A simple method for isolating high yield and quality DNA from cotton (*Gossypium hirsutum* L.) leaves. **Plant Molecular Biology Reported 16**: 1-6.

Rauf S, Jamil N, Ali Tariq S, Khan M and Kausar M (2017) Progress in modification of sunflower oil to expand its industrial value. **Journal of the Science of Food and Agriculture**. Doi: 10.1002/jsfa.8214.

Regitano Neto A, Miguel AMRDO, Mourad AL, Henriques EA and Alves RMV (2016) Environmental effect on sunflower oil quality. **Crop Breeding and Applied Biotechnology** 16: 197-204.

Schneiter A and Miller JF (1981) Description of sunflower growth stages. **Crop Science 21**: 901-903.

Schuppert GF, Tang S, Slabaugh MB and Knapp SJ (2006) The sunflower high-oleic mutant Ol carries variable tandem repeats of FAD2-1, a seed-specific oleoyl-phosphatidyl choline desaturase. **Molecular Breeding 17**: 241-256.

Singchai A, Muangsan N and Machikowa T (2013) Evaluation of SSR markers associated with high oleic acid in sunflower. **International Journal of Biological, Food, Veterinary and Agricultural Engineering 7**: 631-634.

Škorić D and Jocić S (1998) Good combining ability for productivity of line Ha-26. In Turkulov J (ed) **Proceedings of the 39ᵗʰ oil industry conference**. Faculty of Techology, Novi Sad, p. 53-59.

Škorić D, Jocić S, Lečić N and Sakač Z (2007) Development of sunflower hybrids with different oil quality. **Helia 30**: 205-212.

Soldatov KI (1976) Chemical mutagenesis in sunflower breeding. In **Proceedings of the 7ᵗʰ international sunflower conference**. ISA, Krasnorar, p. 352-357.

Urie AL (1984) Inheritance of very high oleic acid content in sunflower. In **Proceedings of the 6ᵗʰ sunflower research workshop**. Sunflower Association, Bismarck, p. 8-9.

van der Merwe R, Labuschagne MT, Herselman L and Hugo A (2013) Stability of seed oil quality traits in high and mid-oleic acid sunflower hybrids. **Euphytica 193**: 157-68.

Vannozzi GP (2006) The perspectives of use of high oleic sunflower for oleochemistry and energy raws. **Helia 29**: 1-24.

Varès D, Lacombe S, Griveau Y, Berville A and Kaan F (2002) Inheritance of oleic acid content of F₁ seed in a complete diallel cross between seven sunflower lines. **Helia 25**: 105-12.

Velasco L, Pérez-Vich B and Fernández-Martínez JM (2008) A new sunflower mutant with increased levels of palmitic acid in seed oil. **Helia 31**: 55-60.