# SCREENING OF THE PRESENCE OF OL GENE IN NS SUNFLOWER COLLECTION

***Aleksandra DIMITRIJEVIĆ[1]*, Ivana IMEROVSKI[1], Dragana MILADINOVIĆ[1], Milan JOCKOVIĆ[1], Sandra CVEJIĆ[1], Siniša JOCIĆ[1], Tijana ZEREMSKI[1], Zvonimir SAKAČ[1]***

[1]*Institute of Field and Vegetable Crops, Maksima Gorkog 30, 21000 Novi Sad, Serbia*

**aleksandra.dimitrijevic@nsseme.com*

## ABSTRACT

Providing high quality oil is of great interest for oil companies. When it comes to sunflower oil, there are two types of oil on the market: high linoleic and high oleic. High oleic oil is considered a healthier version of oil, since it rich in omega-9 fatty acids that are oxidative more stable than linoleic fatty acid (omega-6 fatty acid), dominant in common sunflower oil. Development of high oleic sunflower genotypes was enabled by the discovery of Pervenets mutant sunflower population. In the IFVCNS, there is a great collection of sunflower inbred lines with wide range of oleic acid content (OAC). From the collection, we have chosen 62 genotypes for determination of OAC. In addition we used molecular marker reported by Schuppert et al. (2006) to screen for presence of the mutation that led to increase in OAC. The OAC in lines in which the presence of the mutation was detected ranged between 36.48 and 88.61% (mutant lines derived from high oleic line L31 – 36.48 – 56.58 and standard inbred lines 58.25 – 88.61%); while in lines where OAC varied between 14.24 and 34.46% this mutation was not detected. These results will help in choosing the best parental lines in future breeding programs, while the marker used will enable quick detection of the mutation. In addition it showed that the mutation in mutant lines most likely did not affect the analyzed part of the FAD2-1D sequence.
**Key words:** Oleic acid, Helinathus annuus L., marker assisted selection

## INTRODUCTION

Sunflower (*Helianthus annuus* L.) is the third most important oilcrop in the world. Sunflower oil is naturally rich in polyunsaturated omega-6 fatty acid, linonelic acid, ranging between 50 and 70%, while the content of monounsaturated omega-9 fatty acid, oleic acid, ranges between 20 and 25% (Kabbaj et al., 1996). The creation of mutant sunflower cultivar Pervenets (Soldatov, 1976), obtained by dimethyl-sulfate (DMS) treatment lead to broadening of sunflower breeding programs and allowed creation of high oleic sunflower lines and hybrids. Today, the end user is dictating what type of oil is in high demand. Therefore, breeders create and conduct their breeding programs in compliance with the market demand.

In today's market, high oleic oil is considered to be healthier than high linoleic, since it rich in omega-9 fatty acids that are oxidative more stable than linoleic fatty acid. This trait is important in food industry since a lot of processing activities include higher temperature treatment or converting some unsaturated fats into saturated fats in order to achieve higher melting point. The conversion is important in margarine production because oil stays solid at room temperature. Bearing in mind benefits that high oleic oil has on human health and in industry due to their temperature stability, introduction of *Ol* gene in breeding programs originating from Pervenets became the basis for sunflower breeding for high oleic lines and hybrids, since Pervenets mutant has oleic acid content (OAC) greater than 65% (Soldatov, 1976; Lacombe and Bervillé, 2001, Lacombe et al., 2004).

There are different reports about the inheritance of OAC. Initially, Urie (1984) reported dominant mode of inheritance, while Fick (1984) reported partially dominant mode of inheritance. Later on, there were reports of existence of a modifier gene (Urie, 1985; Miller et al., 1987; Fernández et al., 1999) or one or more genes that influence OAC (Fernández-Martínez et al., 1989; Pérez-Vich et al., 2002; Velasco et al., 2000). In general, OAC varies depending on the genetic background of the recipient genotype.

The molecular change underlying the increase in OAC in Pervenets is the duplication of the *FAD2-1* allele. FAD2 (oleoyl-phosphatidyl choline desaturase) is an enzyme that catalyses synthesis of linoleic acid from oleic acid (Okuley et al., 1994). Three FAD genes are present in sunflower genome: *FAD2-1*, *FAD2-2*, *FAD2-3* (Hongtrakul et al., 1998; Martínez-Rivas et al., 2001). Of those three, only *FAD2-1* is strongly expressed in developing seeds (Hongtrakul et al., 1998). Partial duplication of this gene led to silencing of the *FAD2-1* gene, thus decreasing the activity of FAD enzyme leading to accumulation of oleic acid (Lacombe et al., 2002). *FAD2-1* was reported to cosegregate with *Ol* gene at LG14 (Lacombe and Bervillé, 2001; Pérez-Vich et al., 2002; Schuppert et al., 2006). Hongtrakul et al. (1998) and Schuppert et al. (2006) reported that duplicated sequence of *FAD2-1* does not differ from the corresponding wild type sequence.

So far, different molecular tools were used for analysis of *FAD2-1* gene (Hongtrakul et al., 1998; Dehmer, and Friedt, 1998; Lacombe and Bervillé, 2001; Lacombe et al., 2000; 2002; 2004; 2009, Schuppert et al., 2006). Some of these reports include identification or development of molecular markers for detection of *FAD2-1*. At the Institute of Field and Vegetable Crops there are 2 registered high oleic sunflower hybrids, however we are expanding our breeding program for creation of greater variety of high oleic hybrids.

In present work sunflower inbred lines were selected from a considerable sunflower collection developed at the Institute of Field and Vegetable Crops, Novi Sad (IFVCNS) and was screened for presence of *Ol* mutation by use of INDEL molecular marker reported by (Schuppert et al., 2006). This marker is developed to detect presence of *Ol* mutation since forward primer corresponds to the intergenic region present in *Ol* mutation and reverse primer is complementary to coding region of *FAD2-1*. To verify molecular results, OAC of chosen lines was analyzed by use of gas chromatography (GC). The main goal of present work was to evaluate the efficiency of the INDEL marker for marker assisted selection in IFVCNS lines and to identify the best high oleic parental lines for future crossings.

## MATERIAL AND METHODS

### Plant material

Chosen plant material for analysis includes lines that are used in current breeding program at the IFVCNS and vary in OAC (Table 1). Additionally, four mutant lines (M-1, M-2, M-3, M-4) derived from high oleic proprietary line developed at the IFVCNS were analyzed in order to try do detect changes on a molecular level that underlined decrease in OAC.

Plants were grown in growth chamber in Klasmann Deilmann Substrat 1 until reaching two leaf-pair stage when leaves were sampled for DNA extraction. Out of each examined sunflower line a bulk sample of 10 plants was formed and plant leaves were kept at -70ºC until DNA extraction.

### Oleic acid content

Oil samples were obtained by pressing of 2 grams of seeds in a hydraulic press (Sirio, Mikodental 10 tons stregth, cc 400 bars) to yield approximately 0.5 ml of oil available for GC analysis. In the reaction vial 270 µl of TMSH (transesterification agens) was added to exactly 30 µl of oil, well shaked in the vortex, and kept at room temperature for an hour.

**Table 1.** Tested sunflower genotypes, their oleic acid content and obtained molecular profiles (presence or absence of a part of the *FAD2-1D* sequence)

| Genotype - sunflower line | Oleic acid content in % | Presence of *FAD2-1D* mutation* | Genotype - sunflower line | Oleic acid content in % | Presence of *FAD2-1D* mutation * |
|---|---|---|---|---|---|
| L1 | 88.61 | + | L34 | 79.17 | + |
| L2 | 87.74 | + | L35 | 78.98 | + |
| L3 | 87.39 | + | L36 | 78.56 | + |
| L5 | 87.04 | + | L37 | 77.35 | + |
| L6 | 86.63 | + | L38 | 77.06 | + |
| L7 | 86.62 | + | L39 | 76.42 | + |
| L8 | 86.56 | + | L40 | 75.33 | + |
| L9 | 86.29 | + | L41 | 74.15 | + |
| L10 | 85.94 | + | L42 | 70.22 | + |
| L11 | 85.55 | + | L43 | 69.40 | + |
| L12 | 85.41 | + | L44 | 69.33 | + |
| L13 | 85.10 | + | L45 | 68.29 | + |
| L14 | 84.92 | + | L46 | 68.29 | + |
| L15 | 84.72 | + | L47 | 63.45 | + |
| L16 | 84.69 | + | L48 | 62.04 | + |
| L17 | 84.17 | + | L49 | 58.25 | + |
| L18 | 84.04 | + | M-4 | 56.58 | + |
| L19 | 83.63 | + | M-3 | 50.13 | + |
| L20 | 83.49 | + | M-1 | 49.93 | + |
| L22 | 83.45 | + | M-2 | 36.48 | + |
| L23 | 82.91 | + | L50 | 34.46 | - |
| L24 | 82.88 | + | L51 | 28.30 | - |
| L25 | 82.31 | + | L52 | 24.52 | - |
| L26 | 81.64 | + | L53 | 22.03 | - |
| L27 | 81.39 | + | L54 | 21.53 | - |
| L28 | 81.29 | + | L55 | 21.47 | - |
| L31 | 80.57 | + | L56 | 17.31 | - |
| L30 | 80.37 | + | L57 | 17.03 | - |
| L32 | 79.51 | + | L58 | 14.24 | - |
| L33 | 79.47 | + | | | |

* presence of amplified band (+), absence of amplified band (-)

The oleic acid was identified using a reference mixture of fatty acids methyl esters (FAME). A multi-standard from Supelco (FAME RM-1, Cat. no. O7006) 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 actual composition of these mixtures.

Oleic acid content analysis was performed on Agilent 5890 gas chromatograph equipped with flame ionization detector (FID) and split/splitless injector (split ratio of 1:50). The separation was performed on a fused silica capillary column (HP-INNOWAX, 30m×0.25mm i.d., and 0.25µm film thickness). Helium was used as carrier gas at a constant pressure of 53kPa at 50°C min). The temperature program was as follows: initial temperature of 50°C was held for 1 min, increased to 200°C at a rate of 25°C/min, then increased to 230°C at a rate of 3°C/min, and and hold for 18 min. The injector and detector temperatures were set at 250 and 280°C respectively. The sample volume injected was 1 µl. The results were processed using ChemStation software and expressed as the percentage of individual fatty acids in the oil sample.

**Molecular analysis**

DNA was extracted from leaves by use modified CTAB protocol (Permingeat et al., 1998). For detection of *FAD2-1D* sequence primer pair F4-R1 was used (Schuppert et al., 2006). PCR was performed as described by Schuppert et al. (2006) in mix described by Dimitrijević et al. (2010). Products of PCR amplification were run on 2% agarose gels and visualized with the BIO-Print system (Vilber Lourmat, Marne-La-Vallée, France).

## RESULTS AND DISCUSSION

Oleic acid content varied between tested lines, ranging from 14.24 to 88.61% (Table 1, Figure 1). Thirty one sunflower line (L1-L31) had OAC higher that 80% , 22 lines (L32-L49 and M-1, M-2, M-3, M-4), had OAC ranging between 36 and 80% and 9 lines (L50-L58) had less than 36% OAC. Even though mutant lines, (M-1, M-2, M-3, M-4) originate from high oleic line, GC analysis showed significant decrease in OAC (Table 1).

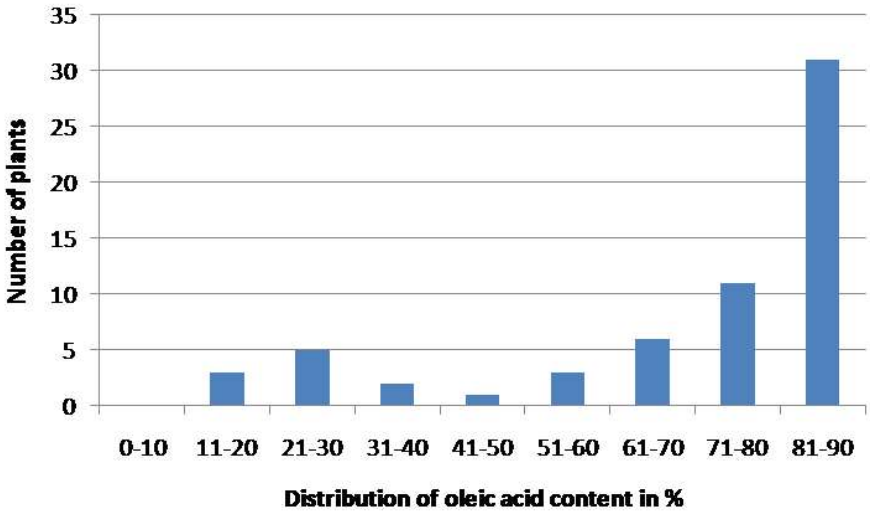


**Figure 1.** Distribution of oleic acid content (%) in examined sunflower genotypes

In order to examine the presence of *Ol* mutation in tested lines F4-R1 primer was used (Schuppert et al., 2006). Out of 62 chosen genotypes, seeds of three lines did not germinate; consequently they were excluded from the molecular analysis. Molecular marker used amplified a band of expected length (approximately 650 bp) in all sunflower lines, except in lines L50-L58 that had low OAC ranging from 14.24 to 34.46% (Figure 2). Presence of an amplified band in all tested mutant lines showed that there is an *Ol* mutation present in examined lines, consequently

some other changes on a molecular level must have happened and caused significant decrease in OAC. Since EMS was used for treatment of wild-type line, small nuclear changes could have occurred in *FAD2-1D* sequence, as EMS most frequently induces SNPs (G to A and C to T point mutations) (McCallum et al., 2000), as was the case with high oleic mutant lines developed by León et al. (2013). Consequently, there is a possibility that some small changes occurred in amplified sequence which could not be detected by electrophoresis. Alternatively, some changes might have occurred in other parts of *FAD2-1D* sequence or somewhere else in sunflower genome. However, this is unlikely since most of the reports on molecular changes in fatty acid composition occurred in the sequence of encoding enzymes (León et al., 2013).

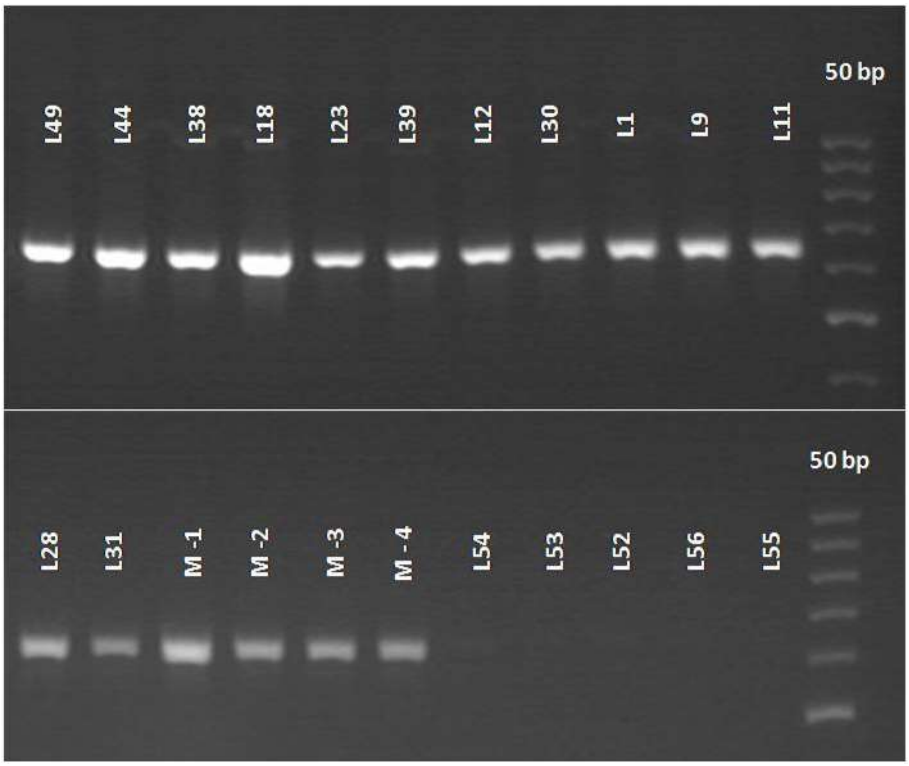


**Figure 2.** Molecular profiles of high oleic, low oleic and mutant sunflower lines obtained by amplification with F4-R1 (Schuppert et al., 2006) (DNA ladder 50 bp, Thermo Scientific)

In this study, we examined OAC in a set of sunflower lines and established that there is a great variation in OAC in comparison to studies performed by Lacombe et al. (2004), since in this research high oleic lines with OAC ranging from 83 to 91%. and low oleic lines with OAC ranging from 23 to 39% were used for molecular studies. The great variation in OAC could be explained by the fact that OAC is influenced not only by genetic background (Lancombe et al., 2001; Schuppert et al., 2006), but also by the environmental conditions, primarily temperature, but also by sowing date etc. (Triboï-Blondel et al., 2000; Flagella et al., 2002; Izquierdo et al., 2002; Del Gatto et al., 2015).

Molecular marker used in this study successfully identified high oleic genotypes and could therefore be used in marker assisted selection in IFVCNS. However, *Ol* mutation was detected in mutant lines that had lower OAC, as well. This means that molecular breeders should always be aware of the genetic background used in breeding and verify results with GC.

**Acknowledgment:** This work was supported by Ministry of Education, Science and Technological Development, Republic of Serbia, project TR 31025.

## LITERATURE

Dehmer, K.J., Friedt, W. (1998). Development of molecular markers for high oleic acid content in sunflower (*Helianthus annuus* L.). Industrial Crops and Products 7(2): 311-315.

Del Gatto, A., Mengarelli, C., Pedretti, E.F., Duca, D., Pieri, S., Mangoni, L., Signor, M., Raccuia, S.A., Melilli, M.G., 2015. Adaptability of sunflower (Helianthus annuus L.) high oleic hybrids to different Italian areas for biodiesel production. Industrial Crops and Products, 75: 108-117.

Dimitrijevic, A., Imerovski, I., Miladinovic, D., Tancic, S., Dusanic, N., Jocic, S., Miklic, V. (2010). Use of SSR markers in identification of sunflower isogenic lines in late generations of backcrossing. Helia 33(53): 191-198.

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

Fernández-Martínez, J.M., Jiménez, A., Domínguez, J., García, J.M., Garcés, R., Mancha, M. (1989). Genetic control of the high oleic acid content in cultivated sunflower. Euphytica 41: 39-51.

Fick, G.N. (1984). Inheritance of high oleic acid in the seed oil of sunflower. Proc. 6th Sunflower Res. Workshop. Natl. Sunflower Assoc., Bismarck, USA. 9.

Flagella, Z., Rotunno, T., Tarantino, E., Di Caterina, R., De Caro, A. (2002). Changes in seed yield and oil fatty acid composition of high oleic sunflower (*Helianthus annuus* L.) hybrids in relation to the sowing date and the water regime. European journal of agronomy 17(3): 221-230.

Hongtrakul V., Slabaugh M.B., Knapp S.J. (1998). A seed specifc D12 oleate desaturase gene is duplicated, rearranged, and weakly expressed in high oleic acid sunflower lines. Crop Science 38: 1245-1249.

Izquierdo, N., Aguirrezábal, L., Andrade, F., Pereyra, V. (2002). Night temperature affects fatty acid composition in sunflower oil depending on the hybrid and the phenological stage. Field Crops Research 77(2): 115-126.

Kabbaj, A., Vervoort, V., Abbott, A.G., Tersac, M., Bervillé, A. (1996). Polymorphism in *Hetianthus* and expression of stearate, oleate and linoleate desaturase genes in sunflower with normal and high oleic contents. Helia 19(25): 1-18.

Lacombe, S. Bervillé, 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(2): 129-137.

Lacombe, S., Guillot, H., Kaan, F., Millet, C. Bervillé, A. (2000). Genetic and molecular characterization of the high oleic content of sunflower oil in Pervenets. Proc. 15th Int. Sunflower Conf, Toulouse, France. 12-15.

Lacombe, S., Kaan, F., Griveau, Y. Bervillé, A. (2004). The pervenets high oleic mutation: methodological studies. Helia 27(40): 41-54.

Lacombe, S., Leger, S., Kaan, F., Berville, A. 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(1): 17-23.

Lacombe, S., Souyris, I. and Bervillé, A.J. (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(1): 43-54.

León, A.J., Zambelli, A.D., Reid, R.J, Morata, M.M., Kaspar, M., Martínez-Force, E., Garcés, R., Salas, J.J., Venegas-Caleron, M. (2013). Isolated Mutated Nucleotide Sequences That Encode a Modified Oleate Destaurase Sunflower Protein, Modified Protein, Methods and Uses. WIPO Patent WO/2013/004280, Jan 10, 2013.

Martínez-Rivas, J.M., Sperling, P., Luehs, W., 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 (2): 159-168.

McCallum, C.M., Comai, L., Greene, E.A., Henikoff, S. (2000). Targeted screening for induced mutations. Nature biotechnology 18(4): 455-457.

Miller, J.F., D.C. Zimmerman, Vick, B.A. (1987). Genetic control of high oleic acid content in sunflower oil. Crop Science 27(5): 923-926.

Okuley, J., Lightner, J., Feldmann, K., Yadav, N., Lark, E., Browse, J. (1994). Arabidopsis FAD2 gene encodes the enzyme that is essential for polyunsaturated lipid synthesis. Plant Cell 6(1): 147–158.

Pérez-Vich, B., R. Garcés, Fernández-Martínez, J.M. (2002). Inheritance of high palmitic acid and its relationship with high oleic acid content in the sunflower mutant CAS 12. Plant Breeding 121: 49-56.

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

Schuppert, G.F., Tang, S., Slabaugh, M.B., Knapp, S.J. (2006). The sunflower high-oleic mutant Ol carries variable tandem repeats of FAD2-1, a seed-specific oleoyl-phosphatidyl choline desaturase. Molecular Breeding 17(3): 241-256.

Soldatov, K.I. (1976). Chemical mutagenesis in sunflower breeding. Proc 7th Intl Sunflower Conf Krasnodar, USSR, Vlaardingen, the Netherlands: Intl. Sunflower Assoc., pp. 352–357.

Triboï-Blondel, A., Bonnemoy, B., Falcimagne, R., Martignac, M., Messaoud, J., Philippon, J. Vear, F. (2000). The effect of temperature from flowering to maturity on seed composition of high oleic sunflower inbreeds and mid oleic hybrids. In Proc of the 15th Intern. Sunf. Conf. Toulouse, France. A67-A72.

Urie, A.L. (1985). Inheritance of high oleic acid in sunflower. Crop Science 25(6): 986-989.

Urie, A.L. (1984). Inheritance of very high oleic acid content in sunflower. Proc 6th Sunflower Res. Workshop. Natl. Sunflower Assoc., Bismarck, USA. 9-10.

Velasco, L., Pérez-Vich, B., Fernández-Martínez, J.M. (2000). Inheritance of oleic acid content under controlled environment. Proc. 15th Int. Sunflower Conf, Toulouse, France. A31-A36.