AN EMS MUTATION ALTERING OIL QUALITY IN SUNFLOWER INBRED LINE

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ABSTRACT

The main objective of this research was to increase genetic variability of sunflower in terms of oil quality and productivity using induced mutations. A preliminary sensitivity test was performed to establish optimal ethyl-methane-sulphonate (EMS) doses for seed treatement. The results showed that high EMS concentrations (0.5-2.5%) caused low survival rates, therefore lower EMS doses were used. Thousand seeds of the sunflower high-oleic inbred line L31 were treated with 0.1% solution of EMS to induce mutations. In the M_2 generation, seeds were screened for fatty acid composition and alterations occured in individual plants. In the next generation a putative mutant line, ML31-1, was isolated with significantly lower oleic acid content compared to the wild type L31 grown in the same year. We assumed that heterozygous mutation occurred, manifested by changing a dominant allele *Ol* to the recessive *ol*. After self-polination in the next generation the segeregation of oleic acid was from 346.6 to 949.1 g/kg and of linoleic acid from 39.9 to 339.3 g/kg. In subsequent generations, individual selection and evaluation of progenies continued in several directions depending on the content of oleic acid: low, increased or high. The stable progenies were evaluated in micro-plot tests for seed yield and other agronomic traits in comparison with their respective wild type.

Key words: sunflower, induced mutation, ethyl-methane-sulphonate, fatty acids, oleic acid

INTRODUCTION

Sunflower oil has been traditionally appreciated as a high-quality commodity in the world oil market (Fernandez-Martinez et al., 2009). Standard sunflower oil is liquid at room temperature due to high content of unsaturated fatty acids. The most abundant is polyunsaturated linoleic acid (C 18:2), about 550-700 g/kg, followed by monounsaturated oleic acid (C 18:1) with 200-250 g/kg. Keeping up with the trends of the food and other industries, sunflower breeders have been able to significantly change the quality of the oil (Cvejić et al., 2014). The high-oleic sunflower hibrids have increased content of oleic acid 800 g/kg and more, compared to a standard type of sunflower. Oil of the high-oleic hybrids has excellent nutritional properties, is a suitable raw material for many derivatives of the chemical industry and for the production of high quality biodiesel, is more favorable because of higher oxidative stability, more resistance to heating and heart-healthy properties (Haddadi et al., 2011). Sunflower breeders have developed a large number of high-oleic hybrids because of the rapidly increasing interest of oil industry (Škorić et al., 2008). However, selection pressure to one particular trait can influence variability of other traits.

Sunflower genetic variability is often limited, as its genetic base of available inbred lines is narrowed. Genetic variability can be broadened by interspecies hybridization with wild species and mutation breeding (Cvejić et al., 2015). The great variability arising after mutagen treatment offers breeders unique challenge for the development of new genetic combinations (Velasco et al. 1999). Induced mutations have been applied for the past 40 years to produce mutant cultivars in sunflower by changing plant characteristics that significantly increase seed yield and quality (Cvejić and Bado, 2009). The first high-oleic sunflower variety Pervenec was obtained by induced mutagenesis by

seed treatment of the variety VNIIMK 8931 with the solution of dimethyl sulfate (DMS) and selection for increased content of oleic acid over 840g/kg (Soldatov 1976). Worldwide, Pervenec is used as a high-oleic trait donor in breeding programs. There are publications about other sources of high-oleic mutants with 800g/kg of oleic acid (Ivanov et al. 1992) and with 900g/kg of oleic acid (Andrich et al., 1992). Recently, the new high oleic sunflower mutant was obtained which ultra-high oleic content was not affected by temperature during grain filling, representing an advantage over the high oleic Pervenets and traditional genotypes (Leone et al. 2013, Alberio et al. 2016). The mode of inheritance of oleic acid content proved to be complex and has been studied by numerous authors, but there is no unanimity among scientists over the number of genes which control this trait (Fick, 1984; Urie, 1984; 1985; Miller et al., 1987; Fernandez-Martinez, 1989; Fernandez et al., 1999; Demurin and Škorić, 1996; Velasco et al., 2000; Lacombe and Berville, 2001; Lacombe et al., 2002; Perez-Vich et al., 2002; Vares et al., 2002; Schuppert et al., 2006). The common conclusion of all studies is that the presence of gene *Ol* is crucial for creating high oleic sunflower genotypes, while number and function of genes controlling this inheritance of this trait remain to be determined.

The main objective of this research was to increase genetic variability of sunflower inbred line in terms of oil quality and productivity. The first step was to assess the efficiency of ethyl-methane-sulphonate (EMS) mutagenic treatments, while the second is to detect mutant lines with different (changed) oil quality; this would provide new genetic variability and better crop productivity and stability.

MATERIAL AND METHODS

Plant material: Sunflower inbred line L31 (wild type) was used for mutagenesis. Line was developed in Institute of Field and Vegetable Crops in Novi Sad, Serbia. This line has over 800 g/kg oleic acid and has potential for further improvement of productivity and stability.

Mutagenic treatment: Ethyl-methane-sulphonate (EMS) mutagenesis of seeds from line L31 was performed in the Joint FAO/IAEA Laboratories in Seibersdorf, Austria. In order to determine the survival rate, fifty seeds were treated with 5 concentrations of EMS solution, 0.5, 1.0, 1.5, 2.0 and 2.5% (v/v), respectively; treatment concentrations were based on studies of other species (Kodym and Afza, 2003). Before the treatment, seeds were transferred to nylon meshes and pre-soaked in distilled water for 24 hours at room temperature. Seeds were then incubated in 200 ml of sodium phosphate buffer (0.1 M, pH 7.4) with gentle shaking (100 rpm) and different EMS concentrations were added. Incubation lasted 4 h. After the EMS treatment, the seeds were washed in distillated water several times. The control, non-mutagenized seeds were treated similarly, except for exposure to the mutagen. All treated seeds and the controls were sown in boxes using the flat method (Gaul, 1963) in a glasshouse under controlled environmental conditions (22-35ºC, lighting of 12h photoperiod). The parameter used to assess the dose response was the survival rate. The number of viable seedlings were calculated after a week of sowing and survival rate was determined by calculating number or survived seedlings per total number of planted seeds. Based on these results, batches of seeds were treated with two concentration of EMS, 0.1% (v/v) and 0.25% (v/v), respectively, and planted in the field.

Selection method: After the mutagenesis, M_1 seeds were planted in the nursery field of the Institute of Field and Vegetable Crops in Rimski Šancevi, Novi Sad, Serbia and after self-pollination of M_1 surviving plants, M_2 seeds were harvested. Seeds from each head were screened for fatty acid composition. Seeds of the wild type were grown and screened at the same time/. Mutants with altered fatty acid content were selected by screening. Seed from selected plants were planted next year in the field and after self-pollination, the M_3 seeds were collected. In next

generations plants were selected by pedigree method and seeds were screened for fatty acid composition. Fatty acid composition was measured by gas chromatography.

Agronomic evaluation: Selected mutants (M_6) and wild type were planted in comparative trial. The trials were organized in randomized block design with three replicates. Following traits were analyzed: days to flowering (from plant emergence to full flowering - UPOV - stage F3.2), plant height (10 plants per replication), seed yield per plant, thousand seed weight, oil content (NMR) and fatty acid composition by gas chromatography (AOCS Official Method Ce 1-62, 1993).

Statistical analysis: The statistical data analysis of mutant generation was performed using Statistica 12 (StatSoft, DEL, USA). The selection progress in successive generations is illustrated in table and figures. Statistically significant differences between examined traits was determined by of t-test? In order to compare distributions of oleic and linoleic acid among mutant generations it was necessary to make corrections for their fluctuation over the years (Spasibionek, 2006).

RESULTS AND DISCUSSION

In order to obtain optimal concentration of EMS solution, seeds were treated with five different doses. The effect of treatment was evaluated by calculating the survival rate. The survival rate varied from 25% (2.5% EMS solution) to 32% (0.5% EMS solution) in the glasshouse (Table 1). This drastical reduction of survival rate showed that all five doses were too high for mutagenic treatment. For that reason further bulk treatments were adjusted with 0.1 and 0.25% of EMS. Depending on the concentration of EMS treatment, the survival rate was 86% (by use of 0.1% EMS solution) and 31% (by use of 0.25% EMS solution) of the M_1 seedlings growing in the field (Table 1). Since the plants treated with 0.25% EMS solution had poor seed set (19.2%), further analysis were based on plants treated with 0.1% EMS solution. In general, results of the sensitivity test showed high frequency of lethality leading to the conclusion that less drastic EMS concentrations should be used for sunflower inbred line L31 seed mutation induction. Generally, optimal EMS concentration for mutation induction differs not only between plant species, but also between different genotype of the same crop. Osorio et al. (1995) reported that EMS concentration of 70m*M* (0.87% EMS) was used to obtain mutagenic sunflower line CAS-3. In *Arabidopsis thaliana*, the LD50 rate determined for Ler and Cor-0 seeds was 0.2% EMS for 16h and 0.13-0.25% for 12.5h, respectively (Jander et al., 2003). The LD50 rates for sugar beet seed balls were 1% EMS for 12h (Hohmann et al., 2005).

Table 1. Results from EMS treatment.

EMS Treatments	No of treated seeds	M_1 seedlings - Survival (%)	Sterility (%)	Seed set (%)
Glasshouse				
0.5%	50	32		
1%	50	30		
1.5%	50	28		
2%	50	27		
2.5%	50	25		
Total	250			
Field				
0.1%	500	86.0	0.0	75.6
0.25%	500	31.0	0.4	19.2
Total	1000	58.5	0.2	47.4

Since no mutant selection is recommended in M_1, as mutation may remain masked or undetectable due to chimerism presence (Bado et al. 2015), M_2 generation of 0,1% EMS-mutagenized population was developed. To isolate the mutants, 378 individually harvested M_2 seed stocks were screened for fatty acid composition and alterations occured in individual plants. These individual plants were planted in the next generation and the mean content of oleic acid in the seed oil decreased from 867 to 603 g/kg while mean linoleic content increased from 40 to 305 g/kg. Mutant line, designated ML31-1, was identified (Table 2). Mutant line had significantly changed oleic acid content compared to the wild type, L31, grown in the same year. We assumed recessive mutation occurred, manifested by changing a dominant allele *Ol* to the recessive *ol*, especially due to the fact that the effect of recessive gene is manifested in the later generations (Knowles, 1989). For that reason, seeds were collected from each ML31-1 plant and used as a source of segregating mutant plants.

Table 2. Oleic and linoleic acid concentrations (g/kg) in the seed oils of mutants (ML31-1, ML-31-11, ML31-12, ML31-13) and the wild type (L31) of sunflower in five M generations.

Gen eration	No of plants	Fatty acid (g/kg)	Mutants				Check	CV			
			ML31-1	ML31-11	ML31-12	ML31-13	L31	ML31-1	ML31-11	ML31-12	ML31-13
M_2	378	Oleic	790.0** (590.0[a])				821.0	22.32			
		Linoleic	144.0** (325.0[a])				94.0	25.01			
M_3	45	Oleic	603.0**				867.0	40.82			
		Linoleic	305.0**				40.0	45.84			
M_4	163	Oleic		519.9**	649.9**	863.6	850.8		12.25	22.55	8.24
		Linoleic		339.3**	231.5**	39.9	40.1		19.88	20.01	12.32
M_5	275	Oleic		514.7**	624.0**	855.0	832.5		12.26	11.22	4.86
		Linoleic		359.3**	269.0**	57.0	50.9		15.48	12.83	8.38
M_6	308	Oleic		482.0**	613.0**	887.0	867.0		9.62	10.21	8.66
		Linoleic		391.3**	270.0**	13.0	40.0		8.29	10.11	8.73

*,**significant at P=0.05 and P=0.01, respectively

[a]meaen value of individual plants

In the next generation (M_3), it was convenient to maintain mutant selection as in segregating population. After harvesting seeds were screened for fatty acid composition. The content of oleic and linoleic acid was significantly changed comparing to the wild type (Table 2). The segregation of oleic acid ranged from 346.6 to 949.1 g/kg and of linoleic acid from 39.9 to 339.3 g/kg (Fig. 1 and 2). In subsequent generation (M_4), individual selection and evaluation of progenies continued in several directions depending on the content of oleic acid: low, increased or high. We identified three subsequent mutants, designated ML31-11, ML31-12 and ML31-13.

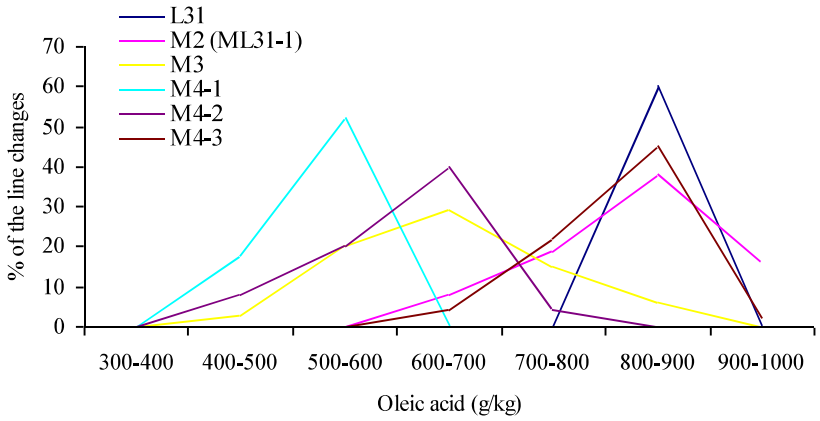

Fig.1. Distribution of oleic acid content (g/kg) in four M generations of sunflower mutant ML31-1 and subsequent mutants ML31-11 (M4-1), ML31-12 (M4-2) and ML31-13 (M4-3) compare to wild type L31

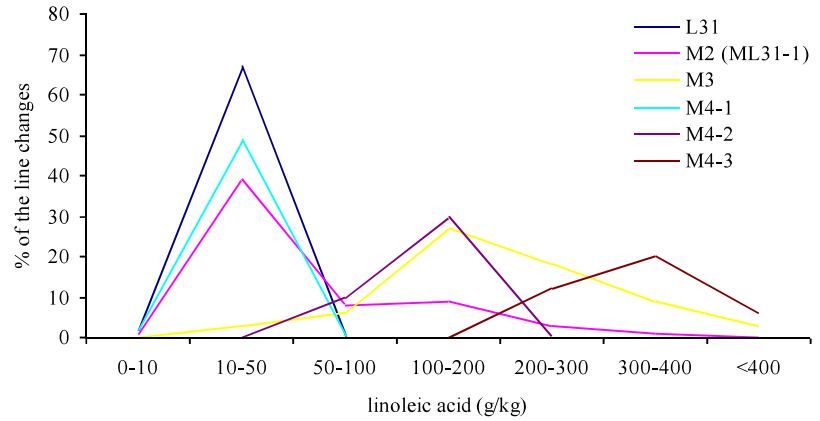

Fig.2. Distribution of linoleic acid content (g/kg) in four M generations of sunflower mutant ML31-1 and subsequent mutants ML31-11 (M4-1), ML31-12 (M4-2) and ML31-13 (M4-3) compare to wild type L31

In M_6 generation subsequent mutants ML31-11, ML31-12 and ML31-13 were evaluated and showed significant differences in one or more characteristics in regards to wild type (Table 3). Due to fatty acid content, mutant lines ML31-11 and ML31-12 had significantly lower concentration of oleic acid and significantly higher concentration of linoleic acid compared to the wild type. The content of oleic acid was higher in ML31-12 mutant line than in ML31-11. The thousand-seed-weight of these mutant lines was significantly higher than of the wild type. With respect to oleic acid content, values obtained were similar between the wild type and the mutant ML31-13, however, other examined traits such as oil content, thousand-seed-weight and seed yield were significantly higher in mutant line than the wild type (Table 3). This improvement represents the progress of wild type (line L31) thought mutation breeding since the seed yield and its components are the most important traits in sunflower production. Two mutant lines (ML31-12, ML31-12) exhibited highly significant increase in seed yield compared to the wild type. The oil content in the seed is closely linked to seed yield, which is the main purpose of sunflower growing (Škorić, 2012). Significant increase in oil content was observed in the mutant line ML31-13. This obtained increase is a very notable result, since no drastic mutation has been reported for seed oil content in sunflower (Vranceanu and Iuoras, 1991, Cvejić et al., 2015).

Table 3. Comparison between mutants ML31-11, ML31-12, ML31-13 and wild type L31 for some agronomic traits and fatty acid composition investigated in the field trials.

Traits	Mutants			Wild type
	ML31-11	ML31-12	ML31-13	L31
Full flowering (days)	58.0(±0.33)	57.0(±0.33)	58.0(±0.67)	57.0(±0.01)
Plant height (cm)	134.8(±2.10)	126.6(±0.62)	133.2(±1.21)	133.6(±0.15)
Seed yield (g/plant)	25.9(±0.13)	29.5**(±0.08)	30.1**(±0.32)	24.7(±0.05)
Thousand-seed-weight (g)	63.61**(±0.13)	63.13**(±0.11)	64.79**(±0.15)	59.5(±0.12)
Oil content (%)	50.56(±0.13)	50.09(±0.14)	54.1**(±0.13)	50.4(±0.10)
Palmitic acid (g/kg)	54.3(±0.20)	48.2(±0.08)	34.8(±0.01)	39.2(±0.01)
Stearic acid (g/kg)	59.5(±0.41)	57.6(±0.02)	50.8(±0.10)	49.6(±0.08)
Oleic acid (g/kg)	482.0**(±1.13)	613.0**(±2.23)	887.0(±2.40)	867.0(±3.08)
Linoleic acid (g/kg)	391.3**(±2.14)	270.0**(±0.21)	13.0**(±0.01)	40.0(±0.70)
Linolenic acid (g/kg)	1.1(±0.00)	1.0(±0.00)	1.2(±0.01)	1.0(±0.00)

*,**significant at P=0.05 and P=0.01, respectively

Induced mutagenesis lead to genetically inherited variability of sunflower inbred lines in terms of oleic and linoleic acid content, which will be more suitable for use in breeding programmes. Further studies will include identification of molecular changes that led to changes in oleic acid content in new mutant lines.

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