CO-EXTRA

GM and non-GM supply chains: their CO-EXistence and TRAceability

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1 Summary

Sunflower is a crop of major economic importance, being second among oilseed crops in the world. Presently no genetically modified sunflowers are commercially grown in Europe. However, this could be changed even in a not so distant future. If transgenic sunflower will ever be grown in Europe, methods for preventing unwanted transgene escape has to be developed in order to facilitate the co-existence of GM and non-GM plants on the farms. Sunflower is an outcrossing crop that is pollinated by insects, mainly honey bees. This places it in a high risk category in terms of a likelihood of transgene escape. The methods for preventing gene flow, such as physical distances, barriers and hand emasculation of anthers, are not trustworthy in sunflower.

The proposed study aimed to evaluate the reliability of CMS in sunflower as a possible way for preventing the flow of transgenic pollen. Results from the field trials conducted in 2005 and 2006 in Bulgaria dealing with the assessment of pollen flow at different distances and stability of sterility of 18 CMS lines under the different climatic conditions are represented. Microsatellite analysis was applied for determination of genetic authenticity and homogeneity of the investigated lines and the source of pollen contributed to formation of seeds.

The flow of pollen of male fertile line at distance 10m, 1.0 km and 1.5 km was proven by microsatellite analysis.

Individual plants from the four CMS lines assessed in 2005 showed reversal to fertility under the climatic condition of excessive rainfalls and low temperatures. Three of them were stable in relation to the CMS trait in the climatic condition of 2006, while the fourth line was unstable during the two year of assessment. The eighteen CMS lines evaluated in 2006 were stable in the climatic conditions of north-west Bulgaria, while two of them were found to reverse to fertility in the conditions of mid-west Bulgaria after the sudden change in the weather,15 days after beginning of flowering.

The obtained data suggest that the climatic condition (temperature, humidity and daily light intensity) may have an effect on the stability of sterility of the investigated CMS lines and that this effect is genotype dependent.

2 Introduction

With the development and exploitation of transgenic crops in agricultural practice more attention is paid on the issues concerning the possible escape of transgene and the development of techniques for preventing the gene flow from transgenic crops to the related cultivated varieties, weeds and wild relatives. Such a concern is necessary in order to predict and to restrict the potential agricultural and ecological consequences of releasing the transgenes in the environment.

There are several routes of transfer of transgenes during the conventional seed production: adventitious presence of GM seeds in non-GM seeds, crosspollination with nearby non GM and/or GM crops, crosspollination with a population of GM volunteers or a wild population, crosspollination with F1 crop-wild hybrids, human movement of crop seeds. The transfer of transgene to the wild relatives as well as to other domesticated cultivars via hybridization has been considered a major environmental risk. Gene flow is a naturally occurring process and for many taxa often is sufficiently high to homogenize even neutral alleles (Morjan and Reizenberg, 2004). A number of studies report gene flow from crops to wild relatives. If a transgenic trait (disease, insect resistance, tolerance to herbicide, or to stress environmental conditions like drought or salinity) provides fitness advantages to cultivated and wild relatives, the corresponding favorable allele may spread quickly and maintain for a long term period in a given population, even more in the presence of selective pressure. This may lead to the creation of invasive weeds and hampering of weed control. The spread of engineered traits depends on the cross-compatibility of a given transgenic crop with its wild relatives, the nature of trait and the ability of the trait to persist in wild populations.

Sunflower is now considered a crop of major economic importance, being second among oilseed crops in the world. Presently no genetically modified sunflowers are commercially grown in Europe. However, this could change, even in the not so distant future. Transgenic sunflower with resistance to important fungal diseases has already been developed. Introduction of new traits in sunflower like herbicide and insecticide tolerance, pathogen resistance and production of high-value compounds in oil will ensure the better place for sunflower in the competition with other oil crops.

If GM sunflower is ever to be grown commercially on a large scale, the corresponding methods for preventing transgene escape will have to be developed and applied in the commercial production of sunflower. This will enable co-existence of GM and non GM sunflower, which will help farmers what to grow.

Sunflower is an outcrossing crop that is pollinated by insects, mainly honey bees, so the risk of unwanted long distance spread of pollen that carries transgene is much greater than in crops pollinated by wind. This places a sunflower in a high risk category in terms of a likelihood of transgene escape.

Most studies of gene flow in sunflower are focused on the dispersal of pollen between *H. annuus* species and between cultivated sunflower and its wild relatives. Wild sunflowers are native to North America, where they occur as weeds in the fields where cultivated sunflowers are grown. The inter species and intraspecies crossing in *H. annuus* and its closed relatives have been determined (Dorado et al., 1992, Rieseberg et al.1988, Rieseberg et al.1992). Chromosomal structure differences between species were suggested as significant barriers to gene flow between sunflower species (Reizenberg et al., 1995, 1999).

A number of studies documented the introgression and persistence of cultivar alleles in wild relatives (Arias and Reisenberg,1994, Ellstrad et al., 1999, Ellstrad, 2003, Massigna et al., 2003, Reagon et al. 2006). Several factors have been shown to assist exchange of genes

between wild and cultivated sunflowers: overlapping of flowering time, shared pollinators, self-incompatibility of wild sunflower, self compatibility of domesticated sunflower, and high rate of outcrossing.

Arias and Reisenberg, 1994, studied the gene flow between cultivated and wild sunflower over long distance. They obtained that 42% of the progeny of wild plants near domesticated fields was F1 hybrids. The rate of pollination was found to decrease with the distance, but the gene flow in sunflower occurred up to a distance of 1000m from the source of pollination. Arias and Reisenberg 1994, suggested the role of climatic condition on vitality of pollen, and correspondingly on the rate of pollination over long distances. Both biotic and abiotic factors were pointed to influence pollen dispersal and hybridization (humidity, temperature, cultivar genotype, bees).

The alleles introgressed in wild populations can be farther spread by both pollen and seed dispersal, providing an additional source for secondary gene flow. The F1 hybrids between cultivated and wild sunflower are fertile and may hybridize more easily with wild relatives than cultivated sunflower (Snow et al 1998).

Whitton et all, 1997 showed that cultivar genes persisted in wild population up to five generations. Similarly, Linder et al., 1998 reported the effect of up to 40 years of persistence of cultivar alleles in the genetic structure of three wild *H. annuus* populations that are adjacent to cultivated fields. They revealed the persistence of at least one marker per individual by analyzing 115 individuals with 18 cultivar specific RAPD markers

The methods, such as physical distances and barriers, used in wind pollinated crops to prevent gene flow between crops and its compatible relatives, are not applicable in sunflower. The distance required for isolation of sunflower must be greater than 1000m and this barrier cannot be considered impermeable (Arias and Reisenberg, 1994). For ccommercial sunflower seed production the isolation distances between hybrid seed production fields and wild sunflower and/or other cultivated sunflower are required to be 1.6-2.4 km. Methods that include genetic isolation by male sterility or transformation of protoplast seem to be more reliable for preventing transgene escape.

The conventional production of seeds in most modern crops, including sunflower, is based on the F1 hybrid seeds that were derived by the cross between inbred lines. The resulted progeny is characterized with higher yield, enhanced quality, tolerance to stress, etc. (heterosis effect). To avoid the self-pollination of mother line the cytoplasmic male sterile (CMS) lines are used. CMS lines are male sterile mutants that are disrupted in pollen development. Cytoplasmic male sterility is a maternally inherited trait resulting in pollen abortion, while the female fertility and the normal vegetative development are not interfered. In most cases the CMS phenotype is associated with mutation in mitochondrial genome that is controlled by dominant nuclear restorer (Rf) genes. The inbred lines that harbor the Rf alleles, restorer (R) lines, are used for pollination of CMS lines in hybrid seed production. The cross between CMS line and restorer line results in a progeny that is heterozygous for the Rf gene. As a result the male sterility is suppressed; the plant produces pollen and is fertile. Isonuclear fertile lines - maintainer (B) lines - that lack Rf alleles are used for reproduction of CMS lines and maintenance of their CMS trait. The restorer and maintainer lines are reproduced by self-pollination. The process of development of a new CMS line is a substitution of the genome of a given CMS with the genome of another line by a number of backcrosses between the initial CMS line and the new line.

Currently CMS inbred lines, used for the production of hybrid seeds in sunflower, are developed on the basis of a single CMS source, CMS-PET1 cytoplasm. This CMS was developed by P.Leclercq (1969) in an interspecific cross between the wild species *H.*

petiolaris Nutt and *H. annuus*. From this time till 2005 seventy two CMS new sources were identified in sunflower and described worldwide, but still are not used in agricultural practice. (X Consultation meeting, European Cooperative Research network on Sunflower, Novi Sad, Serbia and Montenegro, July 17-20, 2005). Most of them were derived from interspecies crosses between wild annual species and cultivated sunflower or were induced by mutagenesis. CMS may also occur in wild *H. annuus* populations as spontaneous mutations or naturally occurring interspecies cross.
The proposed study aims to evaluate the reliability of CMS in sunflower as a possible way for preventing the flow of transgenic pollen.

3 Development of a model for pollen/gene flow mitigation of cultivated sunflower based on CMS

During the two-year period under review investigations were aimed at assessment of: i) the impact of distance on pollen flow of sunflower and ii) the stability of sterility of different CMS lines.
A total of 18 CMS lines, which differ in respect to the origin of cytoplasm (*H. petiolaris*, *H. argophylis*, *H. rigidus* and *H. praoxes*) and putative CMS stability, were included in a field trials. The CMS inbred lines were obtained from the sunflower collection of Dobrudja Agricultural Institute (Table 1). Fourteen of them were developed in the same institute by Doctor Michail Hristov.

Table 1. List of CMS lines/origin, origin of cytoplasm, supposed stability of the sterility

CMS lines/origin	Origin of cytoplasm	Supposed stability of the sterility
1. 2607A (Bulgaria)	*petiolaris*	unstable
2. 6116A (Bulgaria)	*petiolaris*	unstable
3.197A (Bulgaria)	*petiolaris*	stable
4. 879A (Bulgaria)	*petiolaris*	unstable*
5. 6134A (Bulgaria)	*petiolaris*	unstable*
6. 6148A (Bulgaria)	*petiolaris*	unstable*
7. 6149A (Bulgaria)	*petiolaris*	unstable*
8. 6164A (Bulgaria)	*petiolaris*	stable
9. 6075A (Bulgaria)	*petiolaris*	stable
10. U14A (Ukraine)	*petiolaris*	unknown
11. U66A (Ukraine)	*petiolaris*	unknown
12. HA89 (USA)	*petiolaris*	stable
13. HA402 (USA)	*petiolaris*	stable
14. 6004A (Bulgaria)	*hirsutus*	stable
15. 6068A (Bulgaria)	*argophillus*	stable
16. 6048A (Bulgaria)	*argophillus*	stable
17. 2607A (Bulgaria)	*rigidus*	stable
18. 6023 (Bulgaria)**	*petiolaris*	stable

*- CMS lines included in the field trial accomplished during the first 12 month of Co-extra project, which showed some degree of instability in respect to CMS.
**-this line was sown only in General Toshevo

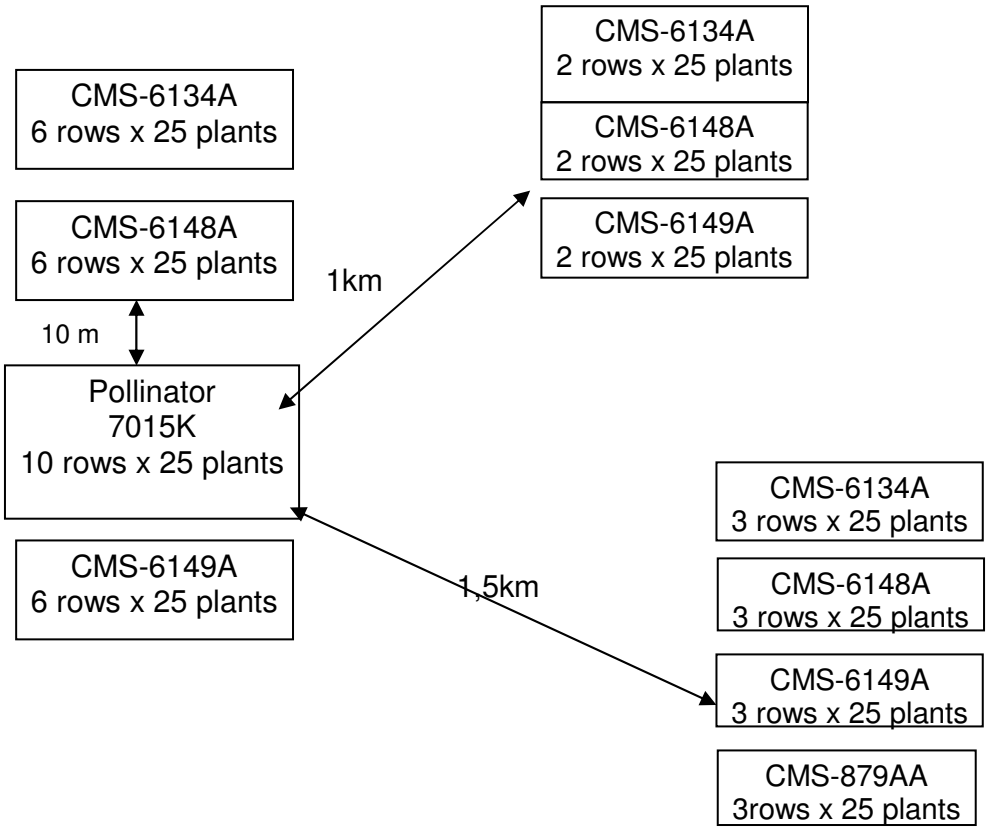
3.1 Impact of distance on the pollen flow of sunflower

The goal of the first year of the project was to assess the rate of pollen flow of sunflower at different distances.

3.1.1 Field trial

According to this aim the design of the field trial was based on the parallel cultivation male fertile and distantly situated male sterile lines. The field trial in summer 2005 was situated in village Opicvet, Sofia district, Bulgaria in collaboration with Dobrudja Agricultural Institute.
The plant material included male-fertile restorer line 7015 R and 4 CMS lines 6134A, 6148A, 6149A and 879A. CMS plants were sown in three lots located at different distances- 10m, 1km and 1, 5 km- from the pollinator, according to the scheme (Fig 1).
All plants heads were left without bags for free pollination and after seed formation were covered with bags in order to protect them from the birds. The covered heads were collected and stored for air drying.
Assessment of the pollen flow at the pointed distances (10m, 1km and 1,5 km) was carried out by: i) determination of the rate of pollination of CMS lines in the three lots and ii) determination of the source of pollen by microsatellite markers.

Fig.1. Design of field trial in 2005



3.1.2. Determination of the rate of pollination of CMS lines sown at different distances from the male-fertile line.

Totally 229 sunflower heads from three lots shown on the scheme were collected. The rate of pollination at different distances is estimated by counting the number of fertile seeds per sunflower head for each combination- CMS line/distance. Table 2 presents the number of full seeds calculated as a percentage of all seeds found in the head. The analysis of the obtained data pointed at the effect of the distance on the rate of pollination. At distance 10 m the percentage of full seeds varied between 50 and 80, depending on the CMS line. The pollination decreased in different degree in lot 2 and 3. While in lot 2 (1km) the percentage of full seeds varied between 4 and 11, in lot 3 (1,5 km) these values were between 20% and 44%.

Table 2. Percentage of fertile seeds found at distance 10m, 1 km and 1.5 km

Percentage of fertile seeds CMS line	lot 1	lot 2	lot 3
6134A	80,5	11,0	20,4
6148A	59,1	8,9	44,2
6149A	50,3	4,4	34,7
879A		10,3	

3.1.3. Determination of source of pollen by microsatellite markers.

Microsatellite analysis was used for characterization of the parent lines and the hybrid seeds. The four CMS lines and line 7015 R were genotyped with 15 highly polymorphic microsatellite markers, randomly distributed trough the genome of sunflower, in order to select markers which discriminate between them (Tang et al,. 2002, Tang et al,. 2003).
The microsatellite markers were scored for polymorphism between the CMS lines and the pollinator line 7015R. Two of the tested microsatellite markers (ORS316 and ORS 10088) were obtained to discriminate male-fertile line on the one hand and CMS lines on the other hand. They were selected for further examination of hybrid seeds obtained on CMS plants.
Microsatellite profiles of CMS lines and the male fertile line at locus ORS316 and locus ORS1088 are shown at Fig.2 A and B. The size of alleles of the corresponding microsatellite loci is shown in Table 3.

Table 3. Allele sizes (in bp) of 4 CMS lines and male-fertile line at loci ORS 316 and ORS 1088.

CMS line/locus	ORS 316	ORS 1088
879A	128	246
6134A	138	250
6148A	136	250
6149A	128	246
7015R	130	254

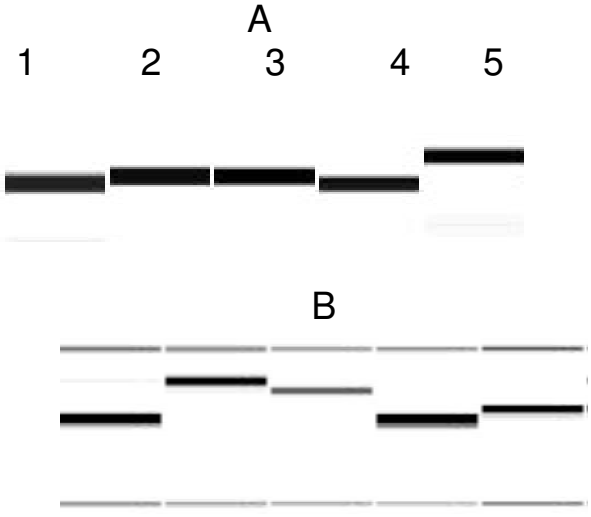


Fig. 2. Microsatellite profiles of CMS lines, male-fertile line 7015R and hybrid Albena at loci ORS 1088 (A) and ORS 316 (B). 1-line879A, 2-line 6134A, 3-line 6148A, 4-CMS line 6149A, 5- male-fertile line 7015R

Microsatellite marker ORS1088 discriminated the four CMS lines from the pollinator line. Lines 6149A and 879A had identical profiles. Lines 6134A and 6148A also had the same

microsatellite profile. Microsatellite marker ORS 316 distinguished all lines with the exception of lines 879A and 6149A, which had identical profiles at the corresponding locus.
Both selected markers were used for discrimination of the pollen source of the hybrid seeds. Hybrid seeds obtained after pollination of the four CMS lines were genotyped by using markers selected in the previous item. The bulk samples, containing 10 seeds of each combination (CMS line/distance), were initially used for microsatellite analysis. The results of the bulk analysis indicated the presence of additional alleles, which were not characteristic for the supposed parent, line 7015R. For this reason analysis was performed with individual seeds. 15 seeds of each combination (CMS line/distance) were amplified with both selected microsatellite markers.

In total, 11 genotypes were obtained for all variants (Fig. 3). Most of the tested seeds from CMS lines 6134A,6148A, 6149A and 879A in lots 1,2 and 3 were found to contain two alleles originating from male fertile line 7015R and the corresponding CMS line. For all four CMS lines in lots 1, 2 and 3 individual seeds were observed, which were homozygous with alleles identical to the alleles of the mother CMS line. Another group of seeds were heterozygous and possessed the combination of alleles characteristic for two of the mother CMS lines. Based on these results it could be concluded that the obtained seed genotypes were originated by: (i) cross between CMS line and the male fertile line 7015R, (ii)cross between the CMS lines and (iii) self pollination of the CMS lines. These results suggested a certain rate of instability of the investigated CMS lines

The analysis of 15 seeds per variant showed that the male fertile line 7015R contributed to the formation of most of the seeds in all three lots, but the rate of this contribution was not possible to assess due to the presence of seeds produced by self pollination of the CMS lines and cross between them. Probably the excessive rate of rainfalls and the low temperature during the summer of 2005 year might have influenced both the stability of sterility of the investigated CMS lines and the flow and germination of the pollen of the male fertile line 7015R.

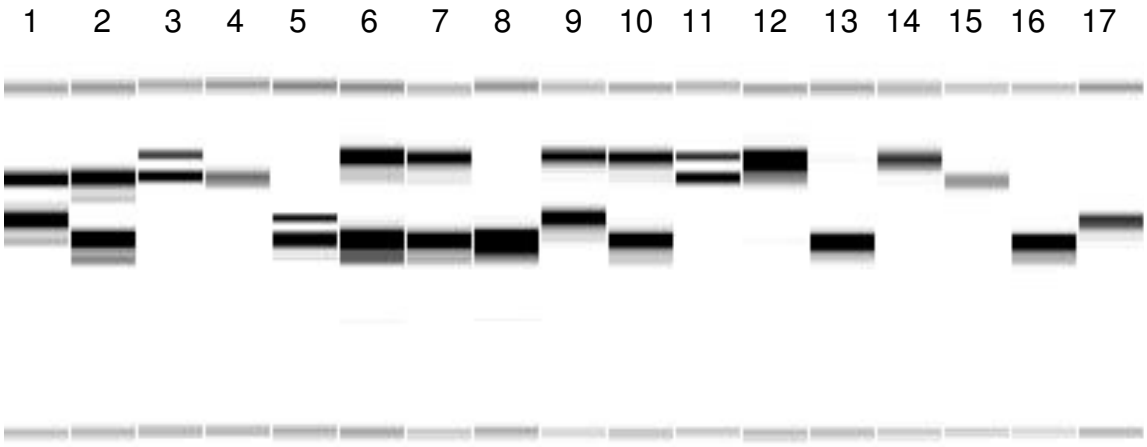


Fig. 3. Microsatellite profile of seeds collected from CMS lines in lots 1, 2, and 3, obtained at locus ORS 316, Lines 1-4-genotypes of seeds from CMS line 6148A, lines 5-8- genotypes of seeds from CMS line 6149A, lines 8-12 genotypes of seeds from CMS line 6134A.

3.1.4. Conclusions:

1. The pollen flow of the male fertile line 7015R at distance 10m, 1 km and 1, 5 km was proven by microsatellite analysis.
2. The investigated CMS lines showed a certain rate of reversal to fertility under the climatic condition of the summer of 2005-unusual excessive amount of rain and low temperature.
3. The fertile line 7015R contributed to the formation of seeds in all three lots, but the assessment of the rate of this contribution was hampered by the presence of seeds produced by selfpollination and by cross between the CMS lines.
4. Microsatellite markers could be successfully applied to:
   - analysis of hybrid seeds
   - assessment rate of pollination
   - determination of pollen source
   - assessment of stability of CMS lines
5. The results obtained in this investigation showed the necessity of evaluation of stability of the CMS lines and the influence of different climatic conditions on this stability.

3.2 Stability of sterility of different sunflower CMS lines

The experiments performed in 2006 aimed at the assessment of stability of sterility of sunflower CMS lines under two different climatic conditions in Bulgaria.

3.2.1. Field trial

Eighteen sunflower CMS lines, which differ in respect to the origin of cytoplasm (*H. Petiolaris*, *H. Argophylis*, *H. Rigidus* and *H. Praoxes*) and putative CMS stability were tested for stability of sterility

Two identical field trials were performed in two districts in Bulgaria with correspondingly different climatic conditions: General Toshevo in north-east Bulgaria and Research Station Kubratovo in mid-west Bulgaria, near Sofia. Two CMS lines, 6023 and 6027, were sown only in General Toshevo. For each line 30 plants were sown in each area. At the beginning of florescence 10 heads of each line were covered with bags to prevent the pollination with pollen from the nearby plants.

3.2.2. The assessment of formation of pollen and seeds in CMS lines

During the flowering period the assessment of formation and release of pollen for each line in Kubratovo was carried out by: i) determination of the presence/absence of pollen. ii) observation of the release of pollen in the plants covered with bags. The vitality of pollen was determined by the stain of pollen with acetocarmin. Table 4 presents the results from the field trial in Kubratovo.

Table4. Results from field trial performed in 2006

*plants that released the pollen from the beginning of flowering

CMS lines/origin	Origin of cytoplasm	Supposed stability	Number of plants observed for pollen formation	Number of observed flowers	Number of stamens with pollen	Number of plants, that release a pollen in the bag	Number of scored heads	Number of heads with fertile seeds
1. HA402 (USA)	Petiolaris	stable	6	54	36	0	10	0
2.197A (Bulgaria)	Petiolaris	stable	3	20	16	0	8	0
3.879A (Bulgaria)	Petiolaris	Unstable[1]	2	15	33	0	9	0
4.6134A (Bulgaria)	Petiolaris	unstable[1]	4	26	0	0	9	0
5.6148A (Bulgaria)	Petiolaris	unstable[1]	4	35	37	2*+ 1**	11	3
6.6149A (Bulgaria)	Petiolaris	unstable[1]	1	9	9	0	11	0
7.6164A (Bulgaria)	Petiolaris	stable	1	12	6	1*	10	1
8.2607A (Bulgaria)	Petiolaris	unstable	4	27	41	2* + 1**	10	3
9.6116A (Bulgaria)	Petiolaris	unstable	9	42	33	0	10	0
10.6004A (Bulgaria)	Praecox	stable	2	14	0	1**	10	1
11.6068A (Bulgaria)	Argophillus	stable	1	8	0	0	10	0
12.6048A (Bulgaria)	Argophillus	stable	2	21	20	0	4	0
13.2607 (Bulgaria)	Rigidus	stable	3	27	15	1**	12	1
14.U14A (Ukraine)	Petiolaris	unknown	3	22	32	2*	10	2
15.U66A (Ukraine)	Petiolaris	unknown	2	14	12	1*+ 1**	10	2
16. HA89 (USA)	Petiolaris	stable	2	9	11	0	10	0

**plants that began to release pollen 15 days after the beginning of flowering (after the changing the temperature and humidity)

It was observed that 14 out of 18 lines generated pollen in a low frequency. Twelve plants belong to 7 CMS lines, 6148A, 6164A, 2607A (in cytoplasm *H. petiolaris*), 6004A, 2607 (in cytoplasm *H. rigidus*), U14 and U66 released pollen in the bags. Seven plants released pollen from the beginning of the flowering, and four – 15 days after the beginning of flowering, after July 27th. In the period 25 – 27 of July the temperature, humidity and daily light intensity in Sofia district were changed (Fig 4 A,B,C).
The formation of seeds, respectively the stability of the sterility for each line, was evaluated (10 plants per line) by counting the number of plants with full seeds. A total of 153 sunflower heads were collected from Kubratovo and 180 – from General Toshevo. Twelve plants grown in Kubratovo that belong to seven CMS lines were found to have fertile seeds. These plants corresponded to the plants that released the pollen. In contrast, lack of plants with fertile seeds was found in General Toshevo, where the temperature and humidity during the flowering period were relatively stable in comparison to those in Sofia district.

3.2.3. Genotyping of CMS lines and determination of genetic authenticity of fertile plants and their progeny

The investigated CMS lines were characterized through 18 microsatellite markers uniformly distributed in the genome of sunflower: ORS 16, ORS 316, ORS 857, ORS613, ORS 230, ORS 1079, ORS 309, ORS 307, ORS 925, ORS 894, ORS 885, ORS 949, ORS 610, ORS 878, ORS 7, ORS 1065, ORS 761, ORS 561 (Tang et al., 2002,2003)

For each line a unique microsatellite profile was determined. The observed level of polymorphism among investigated lines was found to be quite low (0.51). This is probably due to the use of genetically similar initial lines for development of the studied CMS lines. The set of 5 markers (ORS 16, ORS613, ORS 230, ORS 307, ORS 925) was composed, which allowed distinguishing all lines. Evaluation of homogeneity of the studied CMS lines was performed by analysis of 10 seeds per line with 18 SSRs (Fig 3 A, B). Most of the lines were found to be heterogeneous consisting of 2 or 3 different genotypes. Based on the microsatellite data the additional genotypes were considered as a contamination from hybrid seeds presented a given CMS line, which probably occurred during the development of a given CMS line or/and the process of the production of the seeds. Two types of contaminations were observed: i) mechanical, which included seeds from another CMS line or/and variety (Fig 5 B, wells 10, 30, 34) and ii) heterozygous seeds that share one allele with the given CMS line (Fig 5 B, wells 11, 12, 16). The latter can originate from cross pollination with nearby sunflowers during the production of seeds, or could be remainders of heterozygous genotypes, containing the alleles from CMS and maintainer B inbred lines that have been used for the development of a given new CMS line.

A

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35

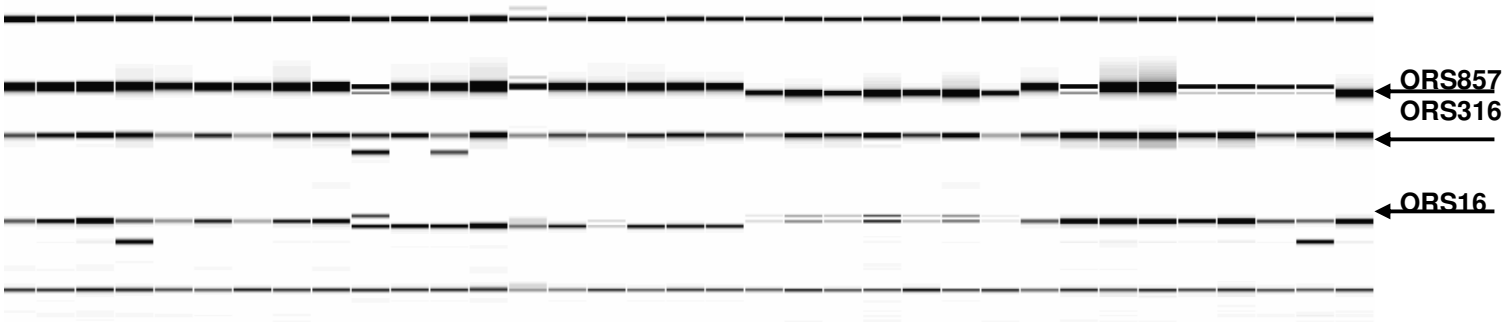


Fig. 5 A Analysis of homogeneity of investigated CMS lines. Microsatellite profiles of individual seeds from CMS line 6134A (1-9), CMS line 6148A (10-19), CMS line 6023A (20-26), CMS line 6075A (27-35) at microsatellite loci ORS 16 and ORS 316 and ORS 857.

B

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35

Fig. 5. B Analysis of homogeneity of investigated CMS lines. Microsatellite profiles of individual seeds from CMS line 6134A (1-9), CMS line 6148A (10-19), CMS line 6023A (20-26), CMS line 6027A (27-36) at microsatellite loci ORS610 and ORS 878.

Taking into consideration the heterogeneity of the studied CMS lines, leaf samples from each covered plant were collected. The samples were analyzed with the previously selected 5 microsatellite markers and 12 chloroplast SSR markers, ccmp1, ccmp 3, ccmp 4, ccmp 6, ccmp7, ccmp 8, ccmp 9, ccmp 10, NTCP 7, NTCP 9, NTCP18, NTCP 30, NTCP 39, (Weising et al., 1999, Wills et al., 2005, Wills et al., 2006) in order to determine genetic authenticity and type of cytoplasm of the covered plants and thus to avoid the mistakes due to the heterogeneity of the CMS lines. The seeds, obtained from the 12 plants that released pollen in the bags and formed seeds, were also analyzed with the same 5 nuclear SSR markers with the aim to prove the source of pollen contributed to the formation of seeds.

The analysis of the investigated CMS lines with 12 chloroplast SSR, markers did not reveal polymorphism between the different types of cytoplasm (*H. annuus*, *H. petiolaris*, *H. argophylis*, *H. rigidus* and *H. praoxes*). Additional chloroplast SSR markers should be applied in order to distinguished different types of cytoplasm and correspondingly the contaminations, referred to the presence of seeds from (with *H. annuus* cytoplasm) used for development of a given CMS line. The maintainer B line seeds should have a genotype identical to the genotype of the seeds of the CMS line, but have to be fertile.

The results showed that of 10 out of 12 fertile plants, belonging to 5 CMS lines-6148A, 6164A, 6004A, U14 and U66 had a genotype that was not identical to the genotype of the corresponding line. All 10 plants were heterozygous that shared one allele with the corresponding CMS line. These plants were considered as a contamination of fertile hybrids, presented in the corresponding lines. The obtained segregation of alleles in the seed progeny of these plants suggests that the seeds are result of self-pollination (Fig. 6).

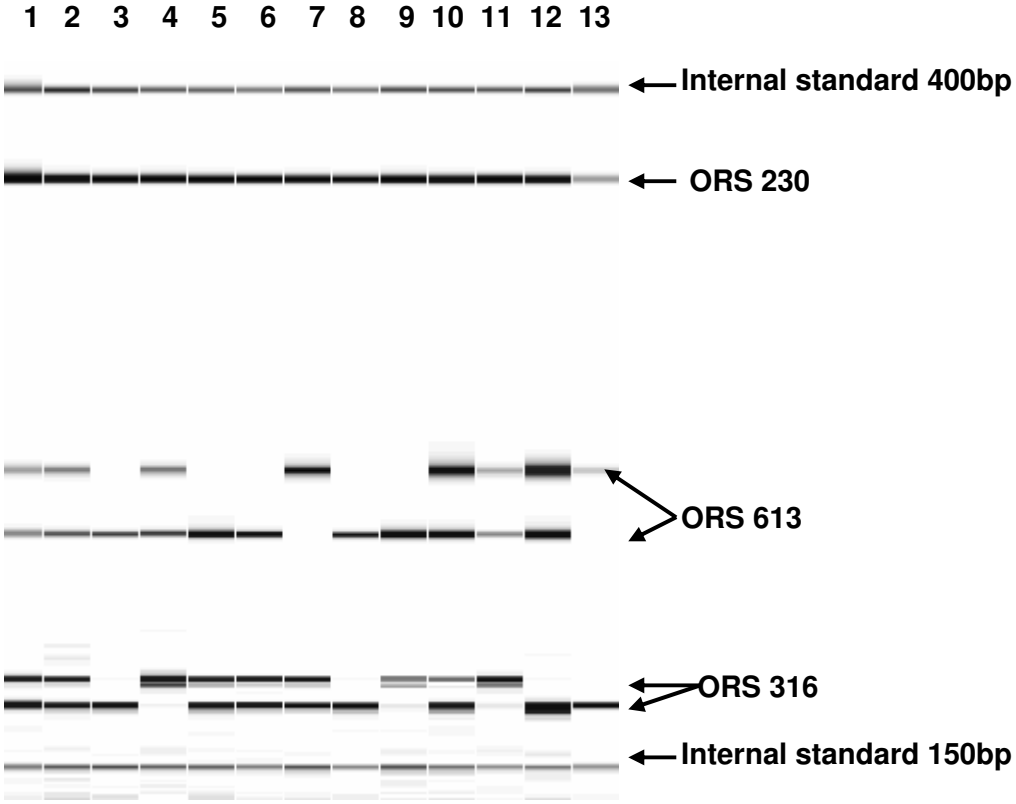


Fig. 6. Analysis of seeds obtained after self-pollination of heterozygous plant from line U66. Line1-microsatellite profile of leaf sample, lines 2-13 microsatellite profiles of individual seeds obtained on this plant.

The remaining two fertile plants had a microsatellite profile, identical to the corresponding CMS lines- 2607A and 6148A. Microsatellite analysis of the seeds obtained from these plants proved that they originated from self-pollination. It is interesting to note that the latter did not flower at the beginning of flowering period and the flowers appeared in the middle of the sunflower heads after the changing of weather. This finding is an indication that the stability of sterility of these lines is influenced by climatic condition. The diagrams in Fig 4 A, B, C show the sudden change in the weather in Sofia district during the period 25-29 of July. Taking into consideration that during this period the change of humidity and daily light intensity were stronger, than the decrease of temperature, it could be supposed that the first two climatic factors affected the stability of sterility of the two plants from line 2607A and 6148A. However, most likely is that the combination of the changes in the three climatic factors could affect the stability trait.

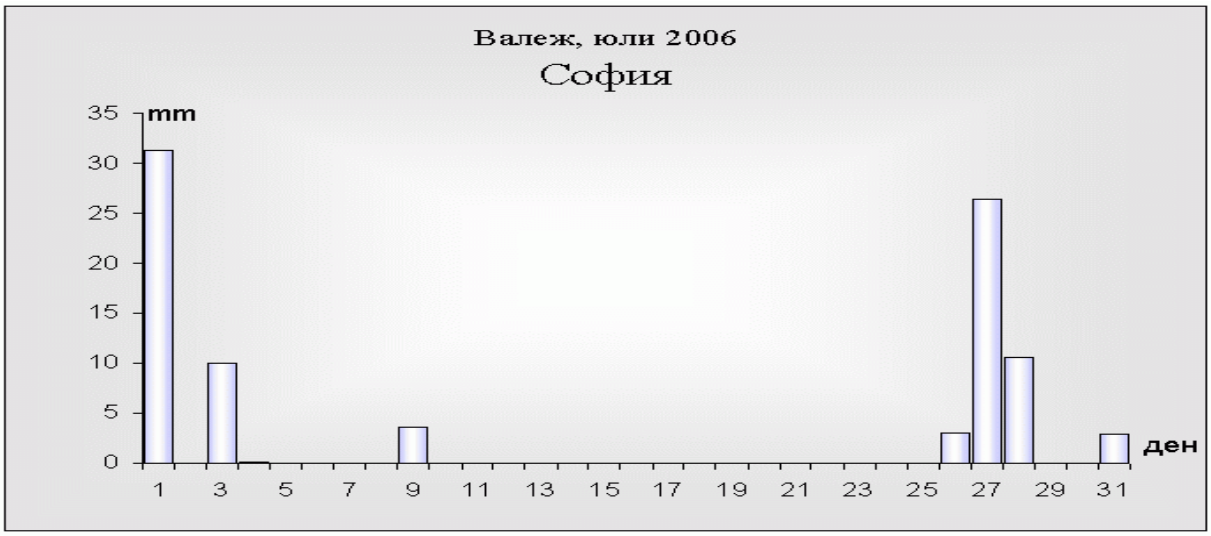


Fig.4a: Diagram of rainfalls during July

Fig.4b: Diagram of temperature during July

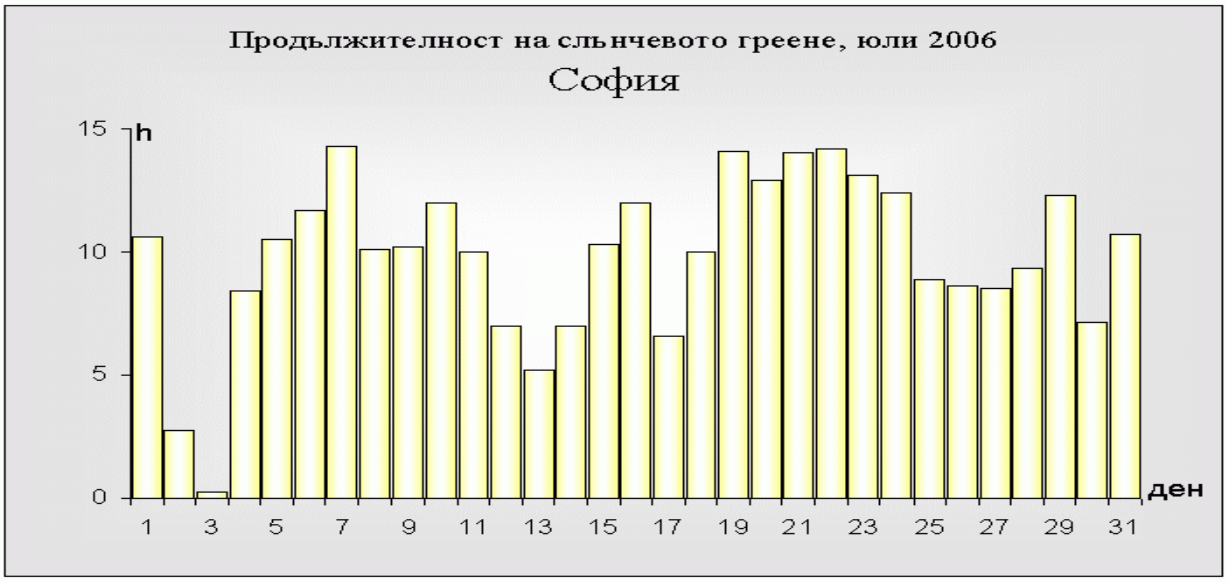


Fig.4c: Diagram of daily light duration during July

3.2.4. Conclusions:

1. Fourteen out of 16 CMS lines, sown in 2006 year in mid-west Bulgaria, showed stable sterile trait and two CMS lines – reversal to fertility 15 days after the beginning of flowering, when the humidity, daily light intensity and temperature changed.
2. All 18 CMS lines were stable in 2006 in respect to male sterility in the climatic condition in north-east Bulgaria, i.e. relatively stable temperature, humidity and daily light intensity.
3. Four CMS lines (6134A, 6148A, 6149A and 879A) assessed in 2005 showed reversal to fertility under the climatic condition of the same year, which is characterized by excessive rate of rainfalls and low temperatures. Three of them (6134A, 6149A, 879A) were stable in 2006, while line 6148A was unstable during the two years of assessment.
4. The data obtained from the two years of experiments suggest that the climatic condition (temperature, humidity and daily light duration) may have an effect on the stability of sterility of the investigated CMS lines and that this effect is genotype dependent. Consequently stability of sterility of CMS lines has to be assessed during several vegetation periods. This will enable the evaluation of a greater number of variations in climatic factors and their effect on stability of sterility of CMS lines.
5. Due to the heterogeneity of investigated CMS lines the field trial for the next year, has to include less CMS lines, but more plants per line. Fifty plants/line (5 lines) have to be sown and their genetic authenticity has to be proved before the covering with bags for assessment of self-pollination/ stability of sterility.

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