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POLLEN FORMATION IN SUNFLOWER HYBRIDS ON THE BASIS OF CYTOPLASMIC MALE STERILITY

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ABSTRACT

In externally fertile plants, the quality of the pollen may be different, and it is possible to distinguish the so-called "semi-fertile" forms. This is typical for sunflower F_2 hybrid populations from crossing CMS PET1 line and restorer lines. We have characterized the fertility/sterility manifestation in a sample set of 17 F_2 and 7 F_3 genotypes derived from a cross between the CMS line VIR 116A and a restorer line VIR195. The segregation pattern for fertility/sterility in the original F_2 population of 262 genotypes fitted three (fertile): one (sterile) model that supports the hypothesis on controlling the fertility restoration by a single locus (putatively *Rf1*). Each plant was phenotyped under field conditions and genotyped using SSR marker ORS511 linked the to the *Rf1* locus. After flowering, the fertile or sterile plants were registered. The pollen of fertile and chimeric plants was stained with acetocarmine. F_1 plants produced appr. 90% of fertile pollen. Among F_2 plants examined, five plants were classified as purely fertile, seven as sterile, three as semi sterile, and two as chimeric ones. Among the F_3 plants three fertile, three sterile, and one chimeric plant have been noted. The chimeras possessed fertile flowers in the 1st to 3rd cycles of flowering, and further to the center the flowers were sterile. In most cases, fertile and chimeric plants possessed the ORS511 marker whereas sterile plants lacked the marker. The occurrence of the marker in sterile genotypes, and the absence of it in fertile ones can be a consequence of recombination events.

Keywords: *pollen fertility, CMS, sunflower, SSR marker ORS511, Rf1* gene.

INTRODUCTION

Cultivated sunflower (*Helianthus annuus* L.) is one of the main oilseed crops. The industrial production of high seed yields is based on heterotic interlinear hybrids obtained using the phenomenon of cytoplasmic male sterility. The CMS trait manifests itself in certain combinations of mutant (usually chimeric) mitochondrial genes resulting from rearrangements of the mitochondrial genome and nuclear genes called *Rf* (Restoration of fertility) pollen restoration genes (Anisimova, Gavrilenko, 2017; Anisimova, 2020). Fertility restoration genes are present in

some varieties and lines that can participate in crossing for heterosis to obtain high-yielding hybrids. In sunflower, lines with PET1-type of cytoplasmic male sterility (CMS) are currently used worldwide for producing F_1 hybrid seeds. The effects of CMS are suppressed when functional alleles of *Rf* genes are included in the genotype. Based on this hypothesis, fertility should be restored in the first generation of hybrids, and the second generation should segregate for this trait. It is known that the F_2 plants can differ in the pollen characteristics including pollen fertility indices and morphometric parameters. According to recent studies, some externally fertile plants from F_2 segregating populations may not be completely fertile. As a result, an F_2 population can be highly heterogeneous for pollen characteristics (Karabitsina *et al.,* 2019; Voronova and Gavrilova, 2019). Therefore, in the present study we checked the fertility distribution of pollen grains by cytological methods.

Externally fertile plants may not be fully fertile, resulting in heterogeneous pollen. Based on this, the purpose of our study was to carry out phenotyping, genotyping and analyze the fertility of hybrid plants by cytological methods.

MATERIAL AND METHODS

Field experiments were conducted in 2021 at Pushkin and Pavlovsk Laboratories of the VIR (St. Petersburg, Pushkin). F_2 and F_3 hybrid seeds from crossing a CMS PET1 inbred line VIR 116A with fertility restorer line-VIR 195 were sown at the end of May.

During the growing season, phenological observations were carried out, visual assessment of-fertility / sterility trait was performed, and the damage of plants by pests and diseases was monitored. Moreover, leaf material for DNA isolation was collected at the 2-3 pairs of true leaves. For cytological examination, part of head was collected and fixed in FAA solution. To assess pollen fertility, a modified acetocarmine staining method was used (Voronova and Gavrilova, 2019).

DNA was isolated from frozen leaves. Genomic DNA was isolated by the modified CTAB method (Li *et al.*, 2007).

The obtained DNA fractions were stored at a temperature of +4 to -20 ° C. The concentration and quality of DNA preparations were determined by electrophoresis in 1% agarose gel. In order to identify the genotypes of hybrid plants for alleles of Rf1 locus, the microsatellite diagnostic marker ORS511 was used.

For PCR, a standard reaction mixture was used (volume – 25 µl, but could vary slightly), which included 1.5 µl of DNA template, 15.95 µl of H2O, 2.5 µl of 10X reaction buffer, 2.4 µl of 2.5 mM dNTP, 1.25 µl of 50 mM MgCl2, for 0.5 µl of forward and reverse primers at a concentration of 10 pM, 0.4 µl of Taq polymerase (5U/µl).

The amplification products were separated by electrophoresis on 1.5% agarose gel and stained with 0.05% ethidium bromide solution. The gels were visualized using the Gel Doc XR+ Bio Rad gel documentation system in ultraviolet light.

Pollen fertility was evaluated in the field (phenotypically) by the presence of normally developed anthers containing pollen and on cytological preparations stained with acetocarmine.

Cytological analysis was performed using a Zeiss Axio Imager.Z1 microscope with an AxioVision digital camera and Zen 2.1 software. The percentage of fertile pollen grains was calculated based on the analysis of at least 12 visual fields at a 20-fold magnification.

RESULTS AND DISCUSSION

We have characterized the fertility/sterility manifestation in a sample set of 17 F_2 and 7 F_3 genotypes derived from a cross between the CMS line VIR 116A and a restorer line VIR195.

As previously defined, F_1 hybrid plants produced appr. 90% of fertile pollen. The segregation pattern for fertility/sterility in the original F_2 population of 262 genotypes fitted three (fertile): one (sterile) model that supports the hypothesis on controlling the fertility restoration by a single locus (putatively *Rf1*) (Anisimova *et al.*, 2021).

Each plant was phenotyped under field conditions. After flowering, the fertile or sterile plants were registered (Fig. 1, *1-3*).

Among F_2 plants examined, five plants were classified as highly fertile, seven as sterile, three plants had intermediate phenotype and marked as sterile/fertile. They had visible anthers, but the anther tube was not as well developed as in truly fertile plant. Moreover, the two plants were classified as chimeric ones. The chimeras possessed fertile flowers in the 1st to 3rd cycles of flowering, and further to the center the flowers were sterile (Fig. 1, 2). Among the F_3 plants three fertile, three sterile, and one chimeric plant have been noted.

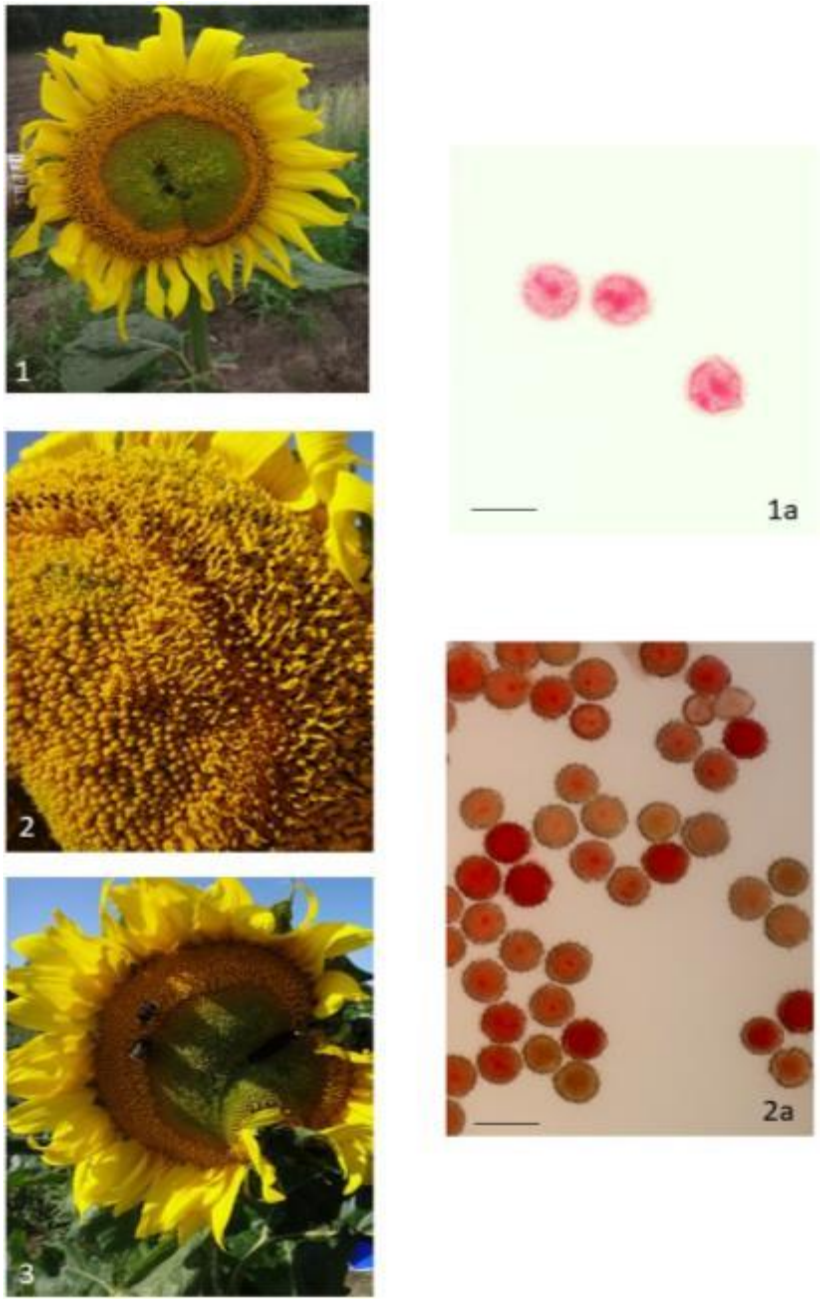

Figure 1. F_2 plants from cross combination VIR 116A × VIR 195: 1) head of a fertile plant, 1a) fertile pollen, 2) head of a chimeric plant, 2a) pollen of a chimeric plant, 3) head of a sterile plant (St. Petersburg, Pushkin, 2021)

The presence of chimeric plants seems especially interesting to us, because in the early stages (flowering 1-3 circles) they look like truly fertile ones. If we did not carry out monitoring until the end of flowering, then these plants would be counted as ordinary fertile ones.

The pollen of fertile and chimeric plants was stained with acetocarmine (Fig. 1, *1a*, *2a*). When pollen was evaluated by staining with acetocarmine, it is customary to say not about stained or not stained, but about fertile and sterile pollen grains. We considered the pollen grains of normal and enlarged size with a uniformly stained cytoplasm as a fertile one; unstained and heterogeneously stained pollen grains of all sizes, as well as stained grains of a much smaller size (micropollen), were classified as sterile ones.

In general, the analysis of pollen by the acetocarmine method revealed a significant diversity among hybrid plants by coloration and the diameter of pollen grains. The range of variations of these parameters was from highly fertile plants with a fertility rate of 85-97%, through a group of plants with medium fertility of 72-74%, to low-fertile plants with a pollen fertility rate of 10-56% (Fig. 2).

Highly fertile plants have the pollen that is aligned with diameter. In the pollen of plants with reduced fertility, there is a significantly greater heterogeneity in diameter compared to highly fertile forms. Also, in general, a decrease in the total amount of pollen was noted in plants with reduced fertility.

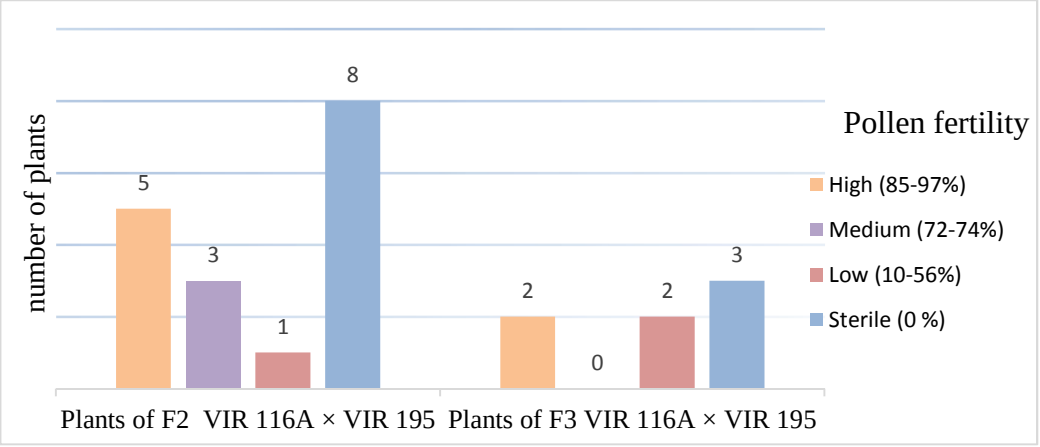

Figure 2. Distribution of plants by pollen fertility

In total there were only 7 highly fertile (HF) plants (44%), medium fertile (MF) - 3 (23%), low fertile (LF) - 3 (23%) and 11 sterile plants. The distribution according to the level of fertility among the F_2 plants was as follows: 5 HF, 3 MF and 1 LF. Plants from F_3 generation had 2 HF, 0 MF, and 2 LF. In general, there is a tendency that highly fertile plants (HF) belong to plants with reduced fertility (MF + LF) in a ratio of 1 : 1 (Fig. 2).

Also, the ratio of sterile and fertile plants is close to 1:1. Of course, we do not yet have enough material for convincing statistics, but we plan to continue research to

clarify this issue. Similar cases with partially fertile pollen have already been found earlier in lines with CMS. For example, in some plant species, along with classes of fertile/sterile plants, semi-fertile or semi-sterile ones were also observed. Such plants were characterized by reduced pollen fertility. The so-called "pollen deficiency" effect has been repeatedly noted by authors on different plant species (Elkonin, 2005; Sinha *et al.*, 2013; Karabitsina *et al.*, 2019), but there is still no consensus on the reason for this phenomenon. For example, variants of microsporogenesis with partial degeneration of tapetum cells and young microspores were described in cultivated sunflower. It leads to the appearance, in addition to aborted pollen grains, and a certain amount of normal pollen (Kovacik and Sykorova, 1979).

Most plant was genotyped using SSR marker ORS511 linked the to the *Rf1* locus (Table 1). Unfortunately, clear data could not be obtained for several plants. In most cases, fertile and chimeric plants possessed the ORS511 marker whereas sterile plants lacked the marker. The occurrence of the marker in sterile genotypes, and the absence of it in fertile ones can be a consequence of recombination events.

Table 1. Distribution of SSR marker ORS 511 and pollen fertility index among F_2 and F_3 hybrid plants from cross combination VIR 116A x VIR 195

№	Plant number	Amount of fertile pollen grains, in %	Plant phenotype	Presence of the ORS 511 marker
	F_2 VIR 116A × VIR 195			
1	1	0	Sterile	no data
2	5	0	Sterile	–
3	9	0	Sterile	–
4	10	0	Sterile	–
5	16	0	Sterile	–
6	3	0	Sterile	+ (?)
7	8	0	Sterile	+ (?)
8	7	0	Sterile/fertile	–
9	11	55	Fertile	no data
10	2	72	Sterile/fertile	+
11	14	73	Fertile	+
12	15	74	Chimeric	+
13	12	85	Fertile	no data
14	17	86	Fertile	+
15	4	87	Sterile/fertile	– (?)
16	6	92	Fertile	no data
17	13	93	Chimeric	no data
	F_3 VIR 116A × VIR 195			
1	20	0	Sterile	–
2	21	0	Sterile	–
3	24	0	Sterile	–

4	22	10	Fertile	no data
5	18	56	Fertile	no data
6	19	82	Fertile	+
7	23	97	Chimeric	+

CONCLUSIONS

We found that long-term monitoring (until the flowering of the head is completed) and cytological analysis of pollen (determination of fertility by the acetocarmine method) makes it possible to reveal interesting features of the F_2 and F_3 progeny from crossings of the CMS line VIR 116 with the pollen fertility restorer line VIR 195. Hybrid plants not only differentiated for fertile and sterile in appearance, but chimeric plants were found among the fertile ones. Also, among the fertile plants, heterogeneity was found in the quality of pollen, which made it possible to distinguish highly fertile, medium fertile, and low fertile forms.

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