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# The sporophytic type of fertility restoration in the $A_3$ CMS-inducing cytoplasm of sorghum and its modification by plant water availability conditions

L.A. Elkonin ✉, V.V. Kozhemyakin, M.I. Tsvetova

Agricultural Research Institute for South-East Region, Saratov, Russia
✉ e-mail: lelkonin@gmail.com

The $A_3$ type of CMS in sorghum is one of the most difficult to restore fertility because of the low frequency of fertility-restoring genes among sorghum accessions, the complex mechanism of fertility restoration that occurs with the complementary interaction of two gametophytic genes *Rf3* and *Rf4*, and the sensitivity of their expression to air and soil drought. In order to test the hypothesis of the sporophytic type of fertility restoration in CMS lines with $A_3$ type cytoplasm developed in our laboratory, we analyzed segregation in the self-pollinated progeny of fertile $F_1$ hybrids grown under different water availability conditions (in a dryland plot, in plots with additional irrigation, in a growth chamber, and in an experimental field with a natural precipitation regime) and in their backcrosses to the maternal CMS-line. The presence of sterile plants in the $F_2$ and $BC_1$ families with the maternal CMS line grown in all tested water availability conditions argues for the sporophytic mechanism of fertility restoration. Cytological analysis of fertile $F_1$ hybrids revealed a significant amount of degenerating pollen grains (PGs) with impaired starch accumulation and detachment of the PG contents from the cell wall. It is assumed that the expression of the fertility-restoring genes *Rf3* and *Rf4* in the hybrids with studied CMS lines starts already in the sporophyte tissues, normalizing the development of a certain part of the PGs carrying the recessive alleles of these genes (*rf3* and *rf4*), which are involved in fertilization and give rise to sterile genotypes found in $F_2$ and $BC_1$ families. For the first time, the transgenerational effect of water availability conditions of growing a fertility-restoring line on male fertility of the $F_2$ generation was detected: a pollinator grown in a plot with additional irrigation produced more fertile and less sterile individuals compared to the same pollinator grown under a rainfall shelter ($p < 0.01$), and the segregation pattern changed from digenic to monogenic, indicating heritable inhibition of the expression of one of the fertility-restoring genes (kind of "grandfather effect"). The possibility of selection for the stability of the fertility restoration system of the $A_3$ cytoplasm to functioning under conditions of high vapor pressure deficit during the flowering period was shown. These data may contribute to the creation of effective fertility restoring lines for this type of CMS in sorghum.

Key words: *Sorghum bicolor* (L.) Moench; cytoplasmic male sterility; $A_3$ cytoplasm; fertility-restoring genes; epigenetics; transgenerational inheritance; drought; vapor pressure deficit.

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# Спорофитный тип восстановления фертильности в ЦМС-индуцирующей цитоплазме сорго типа $A_3$ и его модификация условиями влагообеспеченности растений

Л.А. Эльконин ✉, В.В. Кожемякин, М.И. Цветова

Научно-исследовательский институт сельского хозяйства Юго-Востока, Саратов, Россия
✉ e-mail: lelkonin@gmail.com

$A_3$-тип ЦМС сорго – один из самых трудных для восстановления фертильности вследствие низкой частоты встречаемости генов-восстановителей, сложного механизма восстановления фертильности, происходящего при комплементарном взаимодействии двух гаметофитных генов, *Rf3* и *Rf4*, чувствительности их экспрессии к воздушной и почвенной засухе. С целью проверки гипотезы о спорофитном типе восстановления фертильности у созданных нами ЦМС-линий на основе цитоплазмы типа $A_3$ анализировали расщепление в самоопыленном потомстве фертильных гибридов $F_1$, выращенных при разных режимах влагообеспеченности (на делянках с засушливым фоном, с влагообеспеченным фоном, в климатической камере и на опытном поле при естественном режиме влагообеспеченности). Присутствие стерильных растений в семьях $F_2$ и $BC_1$ с материнской ЦМС-линией, выращенных при всех испытанных режимах влагообеспеченности, свидетельствует в пользу спорофитного механизма восстановления фертильности. Цитологический анализ фертильных ги-

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бридов $F_1$ выявил значительное число дегенерирующих пыльцевых зерен (ПЗ) с нарушением накопления крахмала, отрывом содержимого ПЗ от клеточной стенки. Предполагается, что у гибридов с изученными ЦМС-линиями гены-восстановители *Rf3* и *Rf4* начинают функционировать уже в тканях спорофита, нормализуя развитие некоторой части ПЗ, несущих рецессивные аллели генов *rf3* и *rf4*, которые участвуют в оплодотворении и дают начало стерильным генотипам в семьях $F_2$ и в $BC_1$. Впервые обнаружено трансгенерационное влияние условий влагообеспеченности растений линии-восстановителя на характер расщепления по мужской фертильности в поколении $F_2$: опылитель, выращенный в грядке с дополнительным поливом, давал больше фертильных и меньше стерильных индивидуумов по сравнению с опылителем, выращенным в «засушнике» ($p < 0.01$). При этом характер расщепления изменялся с дигенного на моногенный, свидетельствуя о наследуемом ингибировании экспрессии одного из генов-восстановителей (своеобразный «эффект дедушки»). Показана возможность отбора на устойчивость системы восстановления фертильности в цитоплазме $A_3$ к функционированию в условиях дефицита влажности воздуха в период цветения, что может способствовать созданию новых восстановителей фертильности этого типа ЦМС.
Ключевые слова: *Sorghum bicolor* (L.) Moench; цитоплазматическая мужская стерильность; цитоплазма $A_3$; гены-восстановители фертильности; эпигенетика; трансгенерационное наследование; засуха; дефицит влажности воздуха.

## Introduction

The development of the reproductive structures is the stage of plant ontogeny most sensitive to environmental stresses. One of the causes for this sensitivity is the high energy intensity in microspore- and gametogenesis (Dolferus et al., 2013). It is known that the energy demand of plant is covered by the mitochondriome of the plant cell. Besides, mitochondria are the primary targets of environmental stresses, which disrupt their functioning and information exchange between the mitochondrial and nuclear genomes (Atkin, Macherel, 2009; Jacoby et al., 2012; Ng et al., 2014; Liberatore et al., 2016). This fact is of particular importance for hybrids with cytoplasmic male sterility (CMS), resulting from remote hybridization, in which the information exchange between the nuclear and mitochondrial genome, established during coevolution, is disturbed (Touzet, Meyer, 2014) and the resistance to environmental stresses is debilitated (Li et al., 2012).

Among the different types of sterile cytoplasm found in sorghum (Reddy et al., 2005), $A_3$ cytoplasm, the source of which is IS1112C accession, is one of the most difficult to restore fertility. This difficulty arises from the low frequency of fertility-restoring genes among sorghum accessions (Worstell et al., 1984; Torres-Cardona et al., 1990; Dahlberg, Madera-Torres, 1997) and the sensitivity of their expression to air and soil drought (Kozhemyakin et al., 2017). Genetic analysis of fertility restoration in hybrid combinations between CMS lines with $A_3$ cytoplasm of the Texas Agricultural Experimental Station (USA) and IS1112C indicated the functioning of a digenic gametophytic fertility restoration system, in which the complementary interaction of two genes (*Rf3* and *Rf4*) is necessary for the formation of viable pollen (Tang et al., 1998; Pring et al., 1999). $F_1$ hybrids with restored male fertility, heterozygous for the *Rf3* and *Rf4* genes, yield 25 % of the fertile pollen that reduces the seed set and limits the practical use of $A_3$ cytoplasm in sorghum breeding, compared to other types of sterile cytoplasms ($A_1$ and $A_2$), providing the 100 % seed set in $F_1$ hybrids.

Later, a sporophytic fertility restoration system for the $A_3$ CMS type was found in Sudan grass accessions (Tang et al., 2007). It is known that gametophytic and sporophytic fertility restoration systems have fundamental differences (Chase, Gabay-Laughnan, 2004). In a sporophytic fertility restoration system, a fertility-restoring gene, functioning in the anther tissues (sporophyte), prevents degeneration of pollen grains (PGs) after meiosis, which is typical of most types of CMS (Kaul, 1988). As a result, hybrids that are heterozygous for fertility-restoring genes (*Rf*/*rf*) produce PGs carrying the fertility-restoring allele (*Rf*) and PGs carrying the recessive allele (*rf*), unable to restore fertility. Therefore, sterile plants are present in the self-pollinated progeny of such hybrids ($F_2$), as well as in backcrosses with the maternal CMS line (*rf*/*rf*). With the gametophytic fertility restoration system, the fertility-restoring gene functions in male gametophytes (developing PGs). As a result, hybrids that are heterozygous for fertility-restoring genes (*Rf*/*rf*) contain both fertile PGs, carrying the *Rf* allele, and sterile PGs, which degenerate because of the lack of the restoring allele. Therefore, sterile plants are absent from the self-pollinated offspring of such hybrids, as well as from their backcrosses with the maternal CMS line, and only plants with full restoration of male fertility (*Rf*/*Rf*) and semi-sterile individuals (*Rf*/*rf*), arising from the transmission of the *rf* allele through egg cells, are present.

We showed in our previous study that severe vapor pressure deficit during flowering inversely correlated to the level of fertility of $F_1$ hybrids in the $A_3$ cytoplasm; also, the cultivation of hybrid populations with artificial irrigation favored the selection of lines restoring fertility for this type of CMS (Kozhemyakin et al., 2017). The presence of a significant number of sterile plants in $F_2$ families formally supported the sporophytic type of male fertility restoration in $F_1$ hybrids, but careful analysis showed that sterile plants flowered on dates with severe vapor pressure deficit, which could be the cause of their sterile phenotype.

The purpose of the experiments presented in this paper was to test the hypotheses of the sporophytic type of fertility restoration of CMS $A_3$ by the *Rf3* and *Rf4* genes, known as gametophytic fertility-restoring genes, and of the transgeneration effect of plant water availability conditions on the expression of male fertility in the offspring of $F_1$ hybrids. In addition, this paper presents the first data on the efficiency of selection for the sustainable functioning of the male fertility restoration system in the $A_3$ cytoplasm under conditions of air drought during the flowering phase.

## Materials and methods

Experiments were conducted with $F_1$–$F_3$ hybrid populations obtained by crossing the CMS lines $A_3$ KP-70 and $A_3$ Topaz, created earlier by us, to the $A_3$-type CMS fertility restorer KVV-96. These CMS lines were developed using a nonrecurrent backcrossing program with $A_3$ Tx398 as the nonrecurrent parent. The genotype of $A_3$ Tx398 is *rf3rf3rf4rf4* (Tang et al., 1998). We created the KVV-96 line by selection of fertile plants from the hybrid combination $A_3$ Karlikovoe beloe × IS1112C (Kozhemyakin et al., 2017). The IS1112C line has the $A_3$ type of cytoplasm and is a donor of the *Rf3* and *Rf4* genes (Pring et al., 1999; Kulhman et al., 2006).

The CMS lines, fertility restorers, $F_1$, $F_2$, and testcross hybrids were grown in experimental fields of the Agricultural Research Institute for the South-East Region (Saratov, Russia) (51° 32' N, 46° 02' E) in 2016–2018. Sowing was made in the last third of May. Plants were grown in 4-meter rows with row spacing 70 cm and a distance between plants 12–15 cm. Standard agricultural techniques for grain sorghum growing were applied.

Agricultural climatic conditions of vegetation periods varied from year to year (Table 1). Year 2016 was characterized by dry conditions: the average daily temperatures in each calendar month during the growing season (June-August) were 1.6–4.8 °C higher than the average annual value, and the amount of precipitation was three times less (hydrothermal coefficient 0.02). Year 2017 was characterized by a significant excess of precipitation in the first half of the growing season (from germination to flowering), while in June, during the booting phase, the average daily temperature was lower than June temperatures averaged over years. In 2018, the first half of the vegetation, before flowering, proceeded under dry conditions, whereas in the second half of the growing season, from mid-July (during the flowering period), a significant amount of precipitation was recorded.

To study the effect of plant water availability on the expression of male fertility and the inheritance of ability for fertility restoration, plots with different irrigation regimes were used: with additional watering (two times per week, 7–8 L/m$^2$, starting from the booting phase to the end of flowering) and without watering (dryland environment). To obtain a dryland environment, the plot was covered with a translucent polycarbonate rainfall shelter before the beginning of the booting phase.

The $F_1$ hybrids used in this study were obtained by individual crosses of plants of CMS line $A_3$ KP-70 grown at natural water availability to KVV-96 plants grown in dryland and irrigated plots (two pollinators from each environment). The $F_1$ hybrids obtained from individual crosses were grown in 2016–2018 in dryland and irrigated plots (four hybrid combinations in each environment). The offspring of four paternal plants were also grown in such plots as a control.

In 2017–2018, $F_2$ families obtained from individual self-pollinated $F_1$ hybrids from different selective environments and $BC_1$ obtained from crossing these $F_1$ hybrids to the CMS line $A_3$ KP-70 were grown in the experimental field with the natural plant water availability regime. Some $F_2$ families were also grown in irrigated plots. One of the hybrid populations of $F_2$ ($A_3$ Topaz/KVV-96) was grown in an LGC-4203 growth chamber (Daihan, Korea) at the 14L : 10D light/dark schedule, 70 % relative humidity, and temperatures 28 ºC in the daytime/24 ºC at night.

To determine the level of male fertility, all plants were isolated with parchment bags before flowering. Depending on the level of seed set, plants were classified as sterile (s) (0 % seed set), partially sterile (ps) (< 40 %; most often, 10–20 %), fertile (f) (> 40 %; most often, 80–100 %).

For the cytological analysis of pollen, branches from different parts of panicles of the $F_1$ hybrids and the offspring of paternal plants were fixed in acetoalcohol (1 : 3), washed twice, and stored in 75 % alcohol at 4–6 °C. Slides were prepared from a mixture of anthers isolated from 10–15 branches of individual plants. Pollen was stained with 1 % iodine solution in potassium iodide. In each plant, pollen from hermaphrodite and male flowers was analyzed separately. In each slide, 100 PGs were counted in four replicates. In each selective environment, 15–17 $F_1$ plants and the same numbers of individuals from the offspring of paternal plants were studied.

Indicators of vapor pressure deficit (VPD) during the flowering phase were provided by the Laboratory of Hydrometeorology of the Agricultural Research Institute for the South-East Region. For each plant an individual indicator was calculated, which was the average of indicators within five days before the flowering of the top of the panicle and five days after the beginning of flowering.

Statistical analysis of segregations in the $F_2$ and $BC_1$ populations was performed using the $\chi^2$ test with Yates' correction and the exact binomial test (McDonald, 2014), checking the

**Table 1.** Agroclimatic conditions during the experiment[1]

| Month | Indices | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Average daily air temperature, °C | | | | Average daily relative humidity, % | | | | Precipitation, mm | | | |
| | Perennial | 2016 | 2017 | 2018 | Perennial | 2016 | 2017 | 2018 | Average perennial | 2016 | 2017 | 2018 |
| May | 15.0 | 16.1 | 14.0 | 18.3 | 52 | 62.9 | 57.5 | 50.0 | 43 | 77.2 | 99.3 | 28.1 |
| June | 19.4 | 21.0 | 18.0 | 19.9 | 54 | 52.8 | 60.7 | 46.9 | 45 | 9.0 | 66.7 | 14.1 |
| July | 21.4 | 23.6 | 21.8 | 22.8 | 56 | 53.9 | 62.1 | 60.2 | 51 | 28.8 | 51.3 | 88.7 |
| August | 19.9 | 24.7 | 22.8 | 23.7 | 58 | 48.6 | 58.0 | 51.2 | 44 | 8.3 | 9.1 | 4.4 |

[1] According to the Laboratory of Hydrometeorology of the Agricultural Research Institute for South-East Region (Saratov, Russia).

hypotheses of mono- and digenic control. The significance of differences in the level of fertility in different experimental variants was assessed by variance analysis using the AGROS software package, version 2.09 (S.P. Martynov, Institute of General Genetics, RAS). The proportions of fertile and sterile plants in different progenies were also compared by Fisher's tests (Zaitsev, 1984).

## Results

**Effect of drought stress on restoration of fertility in the $F_1$ hybrids.** Analysis of seed sets in $F_1$ ♀ $A_3$ KP-70 × ♂ KVV-96 hybrids grown under different water availability conditions shows that the KVV-96 line can restore the fertility of $A_3$ type CMS (Table 2). However, the proportion of fertile plants among $F_1$ hybrids decreases significantly in dryland plots, and the proportion of partially sterile plants increases as compared to irrigated plots. These data indicate that plant water availability conditions regulate the expression of the fertility-restoring genes of the KVV-96 line, and this fact should be taken into consideration when analyzing segregation in the $F_2$ and $BC_1$ families.

**Table 2.** Characterization of male fertility of $F_1$ sorghum hybrids ♀$A_3$ KP-70 × ♂KVV-96 grown under different plant water availability regimes (2016)

| Water availability conditions | Percent of plants with fertility level (%)[a] | | |
|---|---|---|---|
| | f | ps | s |
| Irrigated plots | 98.0 a | 1.3 | 0.7 |
| Dryland plots | 76.9 b | 16.9 | 6.2 |
| F | 36.05** | 8.80 | 4.18 |

Note: [a] – average over four replications; f – fertile (seed set > 40 %, usually 80–100 %); ps – partially sterile (< 40 %, usually, 10–20 %); s – sterile (0 % seed set); Data within the columns marked with different letters differ significantly at $p < 0.05$; **$p < 0.01$.

**Sporophytic type of male fertility restoration in CMS $A_3$.** In accordance with the hypothesis of the gametophytic nature of male fertility restoration in the CMS-inducing cytoplasm $A_3$ by two fertility-restoring genes (Tang et al., 1998; Pring et al., 1999), $F_1$ hybrids obtained by crossing CMS lines (*rf3rf3rf4rf4*) with the line IS1112c (*Rf3Rf3Rf4Rf4*), have the genotype *Rf3rf3Rf4rf4*. As a result of meiosis, four classes of gametes are produced in such hybrids (*Rf3Rf4*, *Rf3rf4*, *rf3Rf4*, *rf3rf4*), while only PGs with the genotype *Rf3Rf4* turn out to be fertile and PGs with other genotypes degenerate. In this regard, only plants with restored male fertility should be present in the $F_2$ generation, since only pollen grains carrying both fertility-restoring genes are involved in fertilization. Similarly, only plants with restored male fertility (*Rf3rf3Rf4rf4*) should be expected in the backcross of $F_1$ hybrids to the maternal CMS line.

However, in our experiments in the $F_2$ families obtained by crossings $A_3$-CMS lines to the KVV-96 line, created on the basis of the IS1112C line, grown in different environmental conditions (in irrigated plots, in field plots with the natural water availability regime, and in the growth chamber), male-sterile plants were regularly observed. In this regard, data from the irrigated plots and the growth chamber with high relative humidity (70 %) are the most impressive, because in such conditions the drought effects, which prevent the restoration of fertility in $A_3$ type CMS (Kozhemyakin et al., 2017), were absent.

In 2016 (see Table 3), in the irrigated plot, the ratio of plants with restored fertility (f+ps) and sterile plants (s) corresponded to digenic segregation 15 : 1, or 12f : 3ps : 1s, when considering partly sterile plants a separate class. Such segregation testified that the restoration of fertility in the conditions of water abundance is controlled by two fertility-restoring genes. When growing the same $F_2$ family in a dryland plot, the ratio of plants with fully or partially restored fertility and sterile plants differed from the digenic segregation, but corresponded to the monogenic segregation 3 : 1 (Table 3), which indicated inhibition of the expression of one of the fertility-restoring genes in drought conditions. The causes of the segregation 3 : 1 in the growth chamber are uncertain. Perhaps, with a larger sample size, the segregation pattern in the growth chamber would have been different.

In the 2017 season, the segregation in the progeny of $F_1$ hybrids ♀$A_3$ KP-70 × ♀KVV-96 was studied, while the size of the $F_2$ populations grown in the irrigated plot was increased. Again, as in the progeny of $F_1$ ♂$A_3$ Topaz × ♂KVV-96 hybrids, sterile plants were present in the $F_2$ families, which favored the sporophytic type of fertility restoration. The ratio of fertile, partially sterile, and sterile individuals corresponded to the digenic segregation 12 : 3 : 1 (Table 4). It is noteworthy that sterile plants from the $F_2$ families, transferred from the plots with additional irrigation to the growth chamber with 70 % relative humidity, formed male-sterile panicles. This fact testified that the male sterility of such plants was caused by their genotype rather than environmental factors (severe vapor pressure deficit) at flowering. However, sterile plants were absent from the $BC_1F_1$ families obtained by pollinating the maternal CMS-line with the pollen of $F_1$ hybrids (see Table 4).

The causes of the discrepancy between the segregations in $F_2$ and $BC_1$ are not clear. They may be related to differences in the functioning of fertility restoration systems in $BC_1$ and $F_2$ or with gamete selection in $F_1$ plants, since the progeny of panicles from the main stem was used to obtain $F_2$, whereas the pollen from the second and third panicles was used to obtain $BC_1$. Also, the average daily vapor pressure deficit during the flowering of the main panicles was 24.9 hPa, whereas during the flowering of the second and third panicles, 15.8 hPa. It is possible that the lack of air humidity was a factor affecting the functioning of gametophytic fertility-restoring genes, and thus contributing to the selection of certain classes of pollen grains.

In 2018, $F_2$ progeny obtained from $F_1$ hybrids grown in the dryland and high water availability environments was analyzed. The studied progenies were grown concomitantly under conditions of the experimental field and irrigated plots. All the analyzed families included significant numbers of sterile and semi-sterile plants (Table 5); however, when analyzing the observed segregations, only those sterile plants that bloomed on the same dates as the fertile plants were taken into account, because their sterility was caused by the genotype but not by the severe vapor pressure deficit.

**Table 3.** Segregation of the hybrid ♀$A_3$Topaz × ♂KVV-96 in the $F_2$ generation under different plant water availability conditions (2016)

| Water availability conditions | Number of plants | | | Ratio | $\chi^2$ | *p* |
|---|---|---|---|---|---|---|
| | f | ps | s | | | |
| Irrigated plot | 35 | 9 | 4 | 15(f+ps):1s | 0.154 | 0.694 |
| | | | | 12f:3ps:1s | 0.157 | 0.924 |
| Dryland plot | 27 | 18 | 18 | 3(f+ps):1s | 0.161 | 0.688 |
| Growth chamber | 11 | 9 | 4 | 3(f+ps):1s | 0.505 | 0.477 |

**Table 4.** Segregation for male fertility in the $F_2$ generation of the ♀$A_3$KP-70 × ♂KVV-96 hybrids and in $BC_1F_1$ with the maternal CMS line grown in irrigated plots (2017)

| Hybrid combination | Number of plants | | | Ratio | $\chi^2$ | *p* |
|---|---|---|---|---|---|---|
| | f | ps | s | | | |
| $F_2$ (♀$A_3$ KP-70 × ♂KVV-96-1) | 71 | 10 | 4 | 12f:3ps:1s | 1.859 | 0.395 |
| $F_2$ (♀$A_3$ KP-70 × ♂KVV-96-5) | 74 | 21 | 3 | 12f:3ps:1s | 1.231 | 0.540 |
| $BC_1$ [♀$A_3$ KP-70 × (♀$A_3$ KP-70 × ♂KVV-96-1)] | 7 | 23 | 1[b] | | | |
| $BC_1$ [♀$A_3$ KP-70 × (♀$A_3$ KP-70 × ♂KVV-96-5)] | 6 | 26 | 3[c] | | | |

Note: [a] – average indicators of vapor pressure deficit (VPD) at flowering: average daily VPD = 15.1 hPa; maximum VPD = 31.2 hPa; [b] – when transferred to the growth chamber, panicles with 30 % seed set developed; [c] – 1 % seed set.

In the $F_2$ families grown in the irrigated plots, the ratio of fertile, partially sterile, and sterile plants corresponded to the digenic segregation 9f : 6ps : 1s. It is plausible that under conditions of 2018, only those plants whose genotype contained both fertility-restoring genes (*Rf3–Rf4–*) were fertile, whereas plants whose genotype contained only one dominant gene (*Rf3* or *Rf4*), had the partially sterile phenotype. In $BC_1$, the ratio of plants with partial restoration of fertility and sterile plants corresponded to the segregation 3ps : 1s (see Table 5), which should have been observed when there are four classes of male gametes (*Rf3Rf4*; *rf3Rf4*; *Rf3rf4*; *rf3rf4*) in diheterozygous paternal plants ($F_1$ hybrids) crossed to plants of the CMS line (*rf3rf3rf4rf4*). These data confirm the sporophytic type of fertility restoration in $F_1$ hybrids.

It is noteworthy that in the $F_2$ families derived from the $F_1$ hybrids obtained with the participation of the paternal parents grown in the dryland environment (KVV-96-♂1dr), the segregation pattern in the field conditions deviated from the digenic segregation (9 : 6 : 1) and corresponded to the monogenic segregation 3 : 1, indicating that one of the fertility-restoring genes of the paternal parent did not function in these families (see Table 5).

A detailed analysis of the influence of growing conditions of fertility-restoring lines and $F_1$ hybrids on the expression of male fertility in the $F_2$ generation showed that water availability conditions of $F_1$ hybrids did not affect the segregation pattern in $F_2$. However, the conditions of growing of the fertility-restoring line (donor of the *Rf* genes) influenced the segregation pattern in the progeny of $F_1$ hybrids (see Table 5). The proportion of sterile plants in the $F_2$ families derived from the donor of the *Rf*-genes from a dryland environment (KVV-96-♂1dr) was significantly higher ($p < 0.01$), and the proportion of fertile plants was lower ($p < 0.01$) than in the $F_2$ families derived from the $F_1$ hybrids obtained by using the *Rf* donor grown under high water availability conditions (KVV-96-♂2ir). As a result, the $F_2$ families derived from the donor of the *Rf* genes from the high water availability conditions showed the digenic segregation 9 : 6 : 1, whereas in $F_2$ families derived from the *Rf* donor from the dryland environment (KVV-96-♂1dr), the proportion of sterile plants was significantly higher ($p < 0.01$), and of fertile plants, lower ($p < 0.01$) than with sterile individuals, and the segregation was monogenic (3 : 1). This result presumably indicates that the inhibition of one of the *Rf* genes in the fertility restorer grown under a rainfall shelter had a transgenerational effect. This effect was inherited over two generations. However, when growing the $F_2$ family derived from the $F_1$ hybrid obtained with the *Rf* donor from the dryland plot (KVV-96-1dr) in the irrigated plot (see Table 5, first row), the segregation corresponded to the ratio 9 : 6 : 1. This fact testifies to the reversibility of drought-induced inhibition of the fertility-restoring gene under a high level of plant water availability. In addition, this fact provides an example of the transgenerational effect of growing conditions of the donor of *Rf* genes on the expression pattern of these genes in the $F_2$ generation.

**Cytological analysis of pollen of $F_1$ hybrids.** Assuming that with the sporophytic type of fertility restoration, pollen in $F_1$ hybrids contains mostly fertile PGs, whereas under gametophytic fertility restoration, segregation for different types of PGs (fertile:sterile) takes place in the anthers of $F_1$ hybrids, we performed a cytological analysis of the pollen of $F_1$ ♀A3 KP-70 × ♂KVV-96. We noted a significant number of sterile PGs in anthers of fertile hybrids; the proportion of fertile PGs with no signs of degeneration was low (3–7 %),

**Table 5**. Characterization of the male fertility of hybrid combinations in the $A_3$ cytoplasm (2018)[a]

| Hybrid combination | Gene-ration | Water availability conditions[b] | | | Number of plants | | | Ratio | $\chi^2$ | *p* |
|---|---|---|---|---|---|---|---|---|---|---|
| | | ♂ | $F_1$ | $F_2$ ($BC_1$) | f | ps | s | | | |
| ♀$A_3$ KP-70 × ♂ KVV-96-1 dr | $F_2$ | dr | dr | ir | 42 | 25 | 7 | 9f:6ps:1s | 0.943 | 0.624 |
| ♀$A_3$ KP-70 × ♂ KVV-96-2 ir | $F_2$ | ir | ir | ir | 44 | 27 | 2 | 9f:6ps:1s | – | 0.707 |
| ♀$A_3$ KP-70 × ♂ ($A_3$ KP-70/KVV-96-2 ir) | $BC_1$ | ir | ir | ir | – | 23 | 8 | 3ps:1s | 0.011 | 0.916 |
| ♀$A_3$ KP-70 × ♂ KVV-96-1 dr | $F_2$ | dr | ir | Field | 29 (42.0 %) | 23 | 17 (24.6 %) | 9f:6ps:1s | 40.13 | 0.0 |
| | | | | | | | | 3(f+ps):1s | 0.005 | 0.944 |
| ♀$A_3$ KP-70 × ♂ KVV-96-2 ir | $F_2$ | ir | ir | Field | 51 (54.3 %) | 34 | 9 (9.6 %)* | 9ϕ:6ps:1s | 1.225 | 0.542 |
| ♀$A_3$ KP-70 × ♂ KVV-96-1 dr | $F_2$ | dr | dr | Field | 18 (26.5 %) | 35 | 15 (22.1 %) | 9f:6ps:1s | 41.451 | 0.0 |
| | | | | | | | | 3(f+ps):1s | 0.176 | 0.675 |
| ♀$A_3$ KP-70 × ♂ KVV-96-2 ir | $F_2$ | ir | dr | Field | 49 (51.6 %)** | 35 | 11 (11.6 %) | 9f:6ps:1s | 3.796 | 0.150 |
| Total for (♀$A_3$ KP-70 × ♂ KVV-96-1 dr) | $F_2$ | dr | | Field | 47 (34.3 %) | 58 | 32 (23.4 %) | | | |
| Total for (♀$A_3$ KP-70 × ♂ KVV-96-2 ir) | $F_2$ | ir | | Field | 93 (53.1 %)** | 62 | 20 (11.4 %)** | | | |

Note: [a] – the average indicators of the vapor pressure deficit during the flowering period: the average daily = 10.1 hPa; the maximum = 19.5 hPa; [b] – dr – dryland plot; ir – irrigated plot; *, ** – differs from the proportion of plants of a similar fertility class in the offspring obtained using a pollinator grown in a dryland plot at $p < 0.05$ and $p < 0.01$, respectively.

and it did not match the 25 % expected from the literature data (Pring et al., 1999) (Table 6). A significant part of the PGs that looked fertile at low magnification showed disorders of starch accumulation and detachment of the PG contents from the cell wall under a detailed study at high magnifications (Figure). In addition, starch color in some PGs was changed, apparently due to amylose replacement by amylopectin. However, if these violations did not affect the ability of the PGs to fertilize, then the ratio of all the "fertile" PGs and "sterile" PGs did correspond to the 1:3 segregation in some plants (No. 161-1 ($\chi^2 = 1.08$, $p = 0.299$) and No. 165-2 ($\chi^2 = 0.013$, $p = 0.909$) from the irrigated plots), while one of the plants from the dryland plot had a higher proportion of fertile pollen, and the segregation of fertile and sterile PGs did not fit the 1 : 3 ratio (No. 165-9: $\chi^2 = 9.72$, $p < 0.01$).

These data indicate that PGs containing both dominant genes *Rf3* and *Rf4* and the recessive alleles of these genes could develop in the $F_1$ hybrids. It is noteworthy that PGs with the aberrations described above were also observed in plants of paternal *Rf* donor lines; however, the proportion of fertile pollen was significantly higher than in the hybrids.

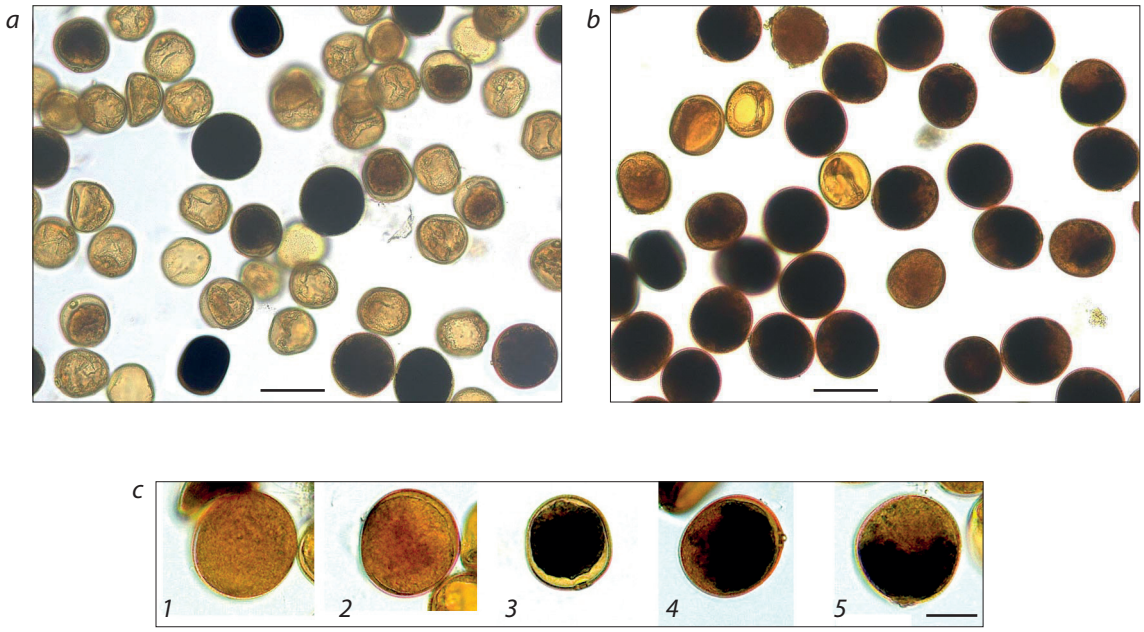
Thus, the results of cytological analysis indicate that pollen grains bearing recessive alleles of these genes could develop in the $F_1$ hybrids heterozygous for the fertility-restoring genes *Rf3* and *Rf4*. It is possible that in the CMS-lines created by us the fertility-restoring genes *Rf3* and *Rf4* begin to function in the tissues of the sporophyte, normalizing the development of part of the PGs carrying the recessive alleles of these genes (*rf3* and *rf4*), which participate in the fertilization and give rise to sterile genotypes detected in $F_2$ or $BC_1$. However, such "precocious" functioning of these genes contributes to the development of only part of the PGs, while a significant portion of pollen grains without restoring alleles still degenerates. The presence of numerous "intermediate" PGs phenotypes with signs of partial degeneration (see Figure), which were not described in earlier studies on $A_3$ type CMS of sorghum, argues for this hypothesis.

**Selection for severe VPD tolerance.** Water and its movement inside the anther play important roles in pollen development and anther dehiscence (Wilson et al., 2011). An important factor regulating anther dehiscence is air humidity. As is known, an indicator characterizing air humidity is vapor pressure deficit: the difference between the maximum possible and actual water vapor tension at specific temperature and pressure.

Assuming that vapor pressure deficit at the flowering phase suppresses fertility restoration in $A_3$ CMS of sorghum (Kozhemyakin et al., 2017), we set up an experiment to study the possibility of selection for tolerance for a high level of this factor (Table 7). In the $F_2$ ♀A3 Topaz × ♂ KVV-96 population, two groups of fully fertile plants were selected: flowering at VPD = 13.8 hPa and at VPD = 23.0 hPa, respectively. In the $F_3$ generation, under conditions of 16.4–16.6 hPa VPD at flowering, the levels of fertility in these groups differed significantly: 61.0 and 80.7 %, respectively ($p < 0.01$) (see Table 7). These differences were due to the presence of plants with low seed sets (< 50 %) in the first group, whereas such plants were absent at all from the second group. These data indicate the presence of a genetic factor contributing to tolerance for severe VPD, which acts at the flowering stage and affects the expression of the fertility-restoring genes. Apparently, under conditions of high VPD level (23.0 hPa), fertility restoration was possible only under homozygosity for this genetic factor; as a result, there was no segregation in the progeny of these plants, whereas during selection under the conditions of low VPD level (13.8 hPa) there could be heterozygotes, which segregated in the $F_3$ generation (see Table 7). These data indicate the efficiency of the selection for tolerance of the fertility restoration system of $A_3$-type CMS for severe VPD.

**Table 6.** Results of the cytological analysis of pollen formed in hermaphrodite flowers of the $F_1$ sorghum hybrids with $A_3$-type CMS and the paternal line with the same type of cytoplasm grown in dryland and irrigated plots (2017)

| Plant No., seed set | Percentages of pollen grains | | |
|---|---|---|---|
| | Fertile | | Sterile |
| | Total | Without signs of degeneration | |
| Irrigated plots | | | |
| KVV-96 (100 %) | 75.2 ± 2.4 | 42.3 ± 7.5 | 24.5 ± 4.0 |
| 161-1 ($F_1$ $A_3$ KP-70/KVV-96), 70 % | 30.2 ± 3.6 | 2.7 ± 1.1 | 69.8 ± 3.6 |
| 165-2 ($F_1$ $A_3$ KP-70/KVV-96), 100 % | 26.0 ± 1.1 | 5.0 ± 0.5 | 74.0 ± 1.1 |
| Dryland plots | | | |
| KVV-96 (60 %) | 80.7 ± 3.7 | 31.3 ± 3.3 | 20.2 ± 4.0 |
| KVV-96 (40 %) | 75.4 ± 2.4 | 18.5 ± 2.6 | 24.5 ± 4.0 |
| 161-9 ($F_1$ $A_3$ KP-70/KVV-96), 100 % | 29.0 ± 3.4 | 10.0 ± 2.1 | 71.0 ± 3.4 |
| 165-9 ($F_1$ $A_3$ KP-70/KVV-96), 90 % | 39.2 ± 2.3 | 4.0 ± 0.4 | 60.8 ± 2.3 |
| 167-7 ($F_1$ $A_3$ KP-70/KVV-96), 100 % | 32.2 ± 1.4 | 3.2 ± 1.2 | 67.8 ± 1.3 |




Pollen of fertile $F_1$ hybrid ♀$A_3$ KP-70 × ♂KVV-96 (*a*); pollen of fertility-restoring line KVV-96 (*b*), scale bar 50 μm; *c* – types of pollen grains (PGs): *1*, *2* – change of starch coloration, *2–4* – detachment of PG content from the pollen cell wall, *4*, *5* – abnormal starch accumulation; scale bar 20 μm.

## Discussion

The current notion of the genetic control of CMS is that the sterilization of the male reproductive system results from the operation of mitochondrial CMS-inducing genes, whereas the nuclear genes restoring fertility generally arrest the expression of the former at the transcriptional or posttranscriptional level (Chase, Gabay-Laughnan, 2004; Horn et al., 2014; Bohra et al., 2016). If the expression of the restorer gene starts in sporophyte (anther) tissue, the fertility restoration follows the sporophytic mode. Both fertile PGs, carrying the dominant fertility-restoring gene, and PGs carrying its recessive allele, unable to restore fertility, develop in the anthers. If the restorer expression starts in gametogeny, only PGs carrying the restorer gene are fertile, whereas those not carrying it degenerate and fail to participate in fertilization (gametophytic type of fertility restoration) (Kaul, 1988).

Our experimental results indicate that this classification is somewhat conventional. The appearance of sterile plants in $F_2$ and $BC_1$ families with the maternal CMS line grown at different water availability conditions (in an irrigated plot, in

**Table 7.** Effect of selection under contrasting air humidity conditions during the flowering period on the manifestation of male fertility in the self-pollinated progeny ($F_3$) of the $F_2$ hybrids ♀$A_3$Topaz × ♂KVV-96

| Family | Number of plants with seed set levels | | | Mean seed set level, % | Vapor pressure deficit during the flowering period, hPa |
|---|---|---|---|---|---|
| | 100–71 % | 70–51 % | < 50 % | | |
| $F_3$ families from the $F_2$-plants that bloom at the vapor pressure deficit 13.8 hPa | | | | | |
| 8/2 | 4 | 5 | 6 | 57.7 | 16.4 |
| 9/2 | 7 | 3 | 5 | 63.0 | 16.4 |
| 10/1 | 5 | 3 | 7 | 60.7 | 16.2 |
| 10/2 | 6 | 3 | 6 | 62.7 | 16.5 |
| Total | 22 | 14 | 24 | 61.0 a | 16.4 |
| $F_3$ families from $F_2$ plants that bloom at the vapor pressure deficit 23.0 hPa | | | | | |
| 11/1 | 9 | 5 | 0 | 76.0 | 16.9 |
| 11/2 | 10 | 4 | 0 | 81.0 | 16.6 |
| 13/1 | 11 | 4 | 0 | 83.7 | 16.7 |
| 13/2 | 10 | 4 | 0 | 81.9 | 16.3 |
| Total | 40 | 17 | 0 | 80.7 b | 16.6 |
| *F* | | | | $300.3^{**}$ | |
| $LSD_{05}$ | | | | 3.6 | |

Note: Data denoted by different letters differ at $p < 0.05$, in accordance with the Duncan Multiple Range test; $^{**}p < 0.01$.

a growth chamber at 70 % humidity, under a rainfall shelter, and in an experimental field at natural water supply) argues for the sporophytic control of fertility restoration by two restorer genes in hybrid combinations studied. Taking into account that the source of the fertility-restoring genes was IS1112C, carrying the fertility-restoring genes *Rf3* and *Rf4*, which, according to literature data (Tang et al., 1998; Pring et al., 1999), are gametophytic fertility-restorers, we consider this fact exceptionally interesting and unusual, since the gametophytic and sporophytic types of fertility restoration are controlled by different genetic systems (Kaul, 1988).

However, the cytological analysis of pollen from fertile $F_1$ hybrids revealed a significant percentage of degenerating pollen grains to argue for the gametophytic type of fertility restoration. It is conceivable that in the genotype of the CMS-lines created by us, these fertility-restoring genes *Rf3* and *Rf4* act differently than in the genotype of CMS lines created in the USA at the Texas Experimental Station. Indian researchers also showed the sporophytic type of inheritance of fertility restoration in CMS $A_3$, controlled by three complementary fertility-restoring genes (Reddy et al., 2010).

Probably, the expression of the fertility-restoring genes *Rf3* and *Rf4* in the hybrids with studied CMS lines starts already in the sporophyte tissues, normalizing the development of a certain part of the PGs carrying the recessive alleles of these genes (*rf3* and *rf4*), which are involved in fertilization and give rise to sterile genotypes found in $F_2$ and $BC_1$ families. A similar hypothesis was put forward to explain the appearance of sterile plants in the progeny of some maize hybrids with the S type of CMS, having the gametophytic type of fertility restoration (Duvick, 1965).

This is the first finding of the transgenerational effect of water availability conditions of growing a fertility-restoring line on male fertility in the $F_2$ generation: a pollinator grown in a plot with additional irrigation formed much more fertile and less sterile individuals compared to the same pollinator line grown under dryland conditions. The segregation pattern changed from digenic to monogenic, indicating a heritable inhibition of the expression of one fertility-restoring gene under drought conditions (kind of "grandfather effect"). It is known that such transgenerational effects arise from a diversity of epigenetic changes (Hauser et al., 2011; Paszkowski, Grossniklaus, 2011; Kumar et al., 2015; Tricker, 2015; Alsdurf et al., 2016). They are based on changes in the methylation pattern, induced, among all, by environmental factors, in particular, drought stress (Lukens, Zhan, 2007; Wang et al., 2011; Tricker et al., 2012; Zheng et al., 2013).

Perhaps, just the methylation changes cause the loss of the function by one of the $A_3$ CMS restorer genes under drought conditions. By the MSAP analysis of $F_1$ hybrids $A_3$ KP-70/KVV-96, we found DNA fragments whose methylation patters under contrasting water availability conditions correlated with the manifestation of male fertility (Elkonin et al., 2019). Apparently, methylation pattern changes are an important mechanism controlling fertility restoration in the $A_3$ sorghum cytoplasm.

The possibility of selection for the stability of functioning of the fertility restoration system for the $A_3$ cytoplasm in

conditions of severe VPD during the flowering period was demonstrated in our work (see Table 7) also deserves attention. Such selection may contribute to the creation of new fertility-restoring lines for this CMS type.

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**ORCID ID**

L.A. Elkonin orcid.org/0000-0003-3806-5697
V.V. Kozhemyakin orcid.org/0000-0002-8758-5819
M.I. Tsvetova orcid.org/0000-0002-5239-5097

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