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Research Article

Restoration ability of newly developed restorer gene pool inbreds on two different CMS sources in sunflower (*Helianthus annuus* L.)

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Abstract
Classical cytoplasmic male sterility (PET-1) discovered by Leclercq and a few restorer lines are being utilized for commercial hybrid seed production in sunflowers over the world since 1972. The utilisation of a single source of male sterility results in a high level of vulnerability to biotic and abiotic stresses. The goal of this study was to identify new fertility restorers for two different cytoplasmic male sterile sources, i.e., PET-1 (COSF-6A, COSF-7A, CMS-38A, ARM-248A) and CMS PEF (FMS-852A). Five CMS lines were crossed with newly developed drought tolerant 10 inbred lines in Line x Tester fashion and an evaluation of test cross progenies was conducted. All the inbreds were categorized into complete, partial restorers and maintainers through cytological observation. Six inbreds, *viz.,* RGP-157, RGP-184, RGP-190, RGP-223, RGP-225 and RGP-233 behaved as restorers for all the CMS lines and restored fertility, while RGP-222 and 298R inbreds maintained sterility. The CMS line, FMS-852A was maintained by four inbreds indicating the involvement of different gene(s), while six inbreds, RGP-157, RGP-184, RGP-190, RGP-223, RGP-225 and RGP-233 were restored its fertility. None of the inbreds behaved as partial restorers for any of the CMS lines. The fertility restoration ranged from 91.4 to 97.4 per cent in different cross combinations. In the present study, pollen fertility of up to 80 per cent was observed for the PET-1 source. However, only 60 per cent of the pollen fertility was observed for CMS *fallax*. From the study, it was evident that among the two CMS sources, restorers for PET-1 and PEF were available. Efforts should be made to utilize CMS PEF for the production of more productive sunflower hybrids resistant to biotic and abiotic stresses.

Keywords: CMS sources, Inbreds, Maintainer, Restorer, Sunflower

INTRODUCTION

The availability of cytoplasmic male sterile (CMS) and fertility restoring sources and the highly cross-pollinating nature of sunflower has made the exploitation of heterosis possible on a commercial scale (Rajanna *et al.,* 2001). The development and commercialization of sunflower hybrids came into existence due to the discovery of the first PET-1 cytoplasmic male sterility by Leclercq (1969) through an interspecific cross between diploid annual wild species *Helianthus petiolaris* x *H. annuus* and the identification of pollen fertility restorer sources by Kinman (1970), Leclercq (1971) and Vranceanu and Stoenescu (1971), which shifted interest from population breeding to heterosis breeding. Sunflower hybrids are more preferred by farmers compared to varieties due to hybrids offer various advantages in terms of uniformity, early maturity,

more seed setting due to high autogamy and more resistance to important diseases and insect pests (Hernández *et al.,* 2017). In India, the first ever sunflower hybrid, BSH-1 (CMS-234A x RHA-274), was released in Bangalore (Seetharam, 1980). Afterwards, a total of twenty-nine hybrids were released from the public sector suitable for different agroclimatic situations in India (Sujatha *et al.,* 2019). All hybrids in cultivation around the world get their cytoplasm from *H. petiolaris*. This has resulted in genetic uniformity in the sunflower's cytoplasmic background. If the cytoplasm becomes vulnerable to a new strain of disease or pest, as happened in maize when Texas cytoplasm became susceptible to *Helminthosporium maydis* in the United States, the prevalence of genetic uniformity of this kind over a vast area could result in the genetic vulnerability of hybrids (Tatum, 1971; Anonymous, 1972). There is an urgent need to find new cytoplasmic sources to identify and utilize new restorers to increase genetic diversity. Diversification of CMS and restorer sources are likely the simplest and most effective ways available to tackle this challenge. Although several new CMS sources are available, one of the most significant challenges in employing the diverse sterile cytoplasm for commercial hybrid seed production has been the exceptionally low frequency of pollen fertility restoration genes in the currently cultivated sunflower (Anaschenko, 1974). To reduce the genetic vulnerability of commercial sunflower hybrids caused by the current usage of a single source of cytoplasm and a few fertility restoration genes, alternative sources of cytoplasmic male sterility and fertility restorers are required (Ardilla *et al.,* 2010; Meena and Sujatha, 2013; Meena and Prabakaran 2016). Increasing the diversity of hybrid parents, especially in restorer lines, is always the main breeding goal to solve this problem (Dudhe *et al.,* 2021). The purpose of the present study was to explore the possibility of identifying high fertility restorers based on sterility and fertility reactions in two diverse CMS sources. The simple method (Chaudhary *et al.,* 1981) was used to determine the pollen fertility of crosses, which leads to the identification of superior inbreds as maintainers and restorers of two different CMS sources, with the goal of using these inbreds in future sunflower breeding programmes to increase the genetic diversity of sunflower hybrids. In view of this constraint, an attempt was made at the ICAR-Indian Institute of Oilseeds Research, Hyderabad, to find efficient restorers for the various CMS sources indicated above. Before adopting the best maintainer inbreds in the hybrid development programme, they must first be converted to CMS lines through the backcross method. In the hybrid programme, inbreds from the restorer gene pool can be utilized as male lines.

MATERIALS AND METHODS

The sunflower breeding materials included two different cytoplasmic male sterile sources, *viz., H. petiolaris* and *H. petiolaris* sp. *fallax*. In this study, four CMS lines from the PET-1 background *viz.,* COSF-6A, COSF-7A, CMS-38A and ARM-248A and one CMS from the CMS PEF (*H. petiolaris* sp. *fallax*) background *viz.,* FMS-852A maintained by ICAR-Indian Institute of Oilseeds Research, Hyderabad, and newly developed ten drought tolerant inbred lines (testers) *viz.,* RGP-118, RGP-157, RGP-173, RGP-184, RGP-190, RGP-222, RGP-223, RGP-225, RGP-233, 298R were used. The five cytoplasmic male sterile lines, five rows each from both cytoplasmic backgrounds, and two rows each of the ten testers (inbreds), were planted during the *kharif* 2019-20, with a spacing of 60 cm x 30 cm in a row length of 4.5 m. Staggered sowing of male and female parents, at a weekly interval, was done to synchronize the flowering. To obtain healthy plants, the recommended packages and practices were followed. At the ray floret stage, just before the start of flower opening, the heads of male sterile (female) lines and inbreds (male lines) were covered with cloth bags to avoid outcrossing. The five CMS lines from two diverse CMS sources were crossed with 10 inbred lines in Line x Tester fashion. Between 8:00 and 11:00 a.m., pollen from the inbreds was collected in a petri plate and applied to five flower heads of each of the corresponding CMS lines with a small brush, and the procedure was repeated until all disc florets had opened. To avoid contamination, precautions were taken. To determine the fertility restoration of the 10 male lines on the five female lines, F_1 seeds from each of the 50 crosses were collected separately at the maturity stage. The hybrids were raised during *rabi* 2020-21 at Rajendranagar Research Farm ICAR-IIOR, Hyderabad to identify inbred behaviour, with respect to maintenance and restoration of the two diverse CMS sources of sunflower. F_1 seeds from the 50 crosses were planted in a randomized block design with three replications. Two rows of 3.5 m for each F_1 entry were planted, maintaining a row-to-row distance of 60 cm and a plant-to-plant distance of 30 cm. The hybrids were classified as male fertile/male sterile based on the dehiscence and pollen shedding at anthesis. Further, pollen fertility was confirmed in the laboratory by using 1% acetocarmine. The pollen parents that resulted in sterile crosses were classified as maintainers, while those that resulted in fertile crosses were designated as restorers of the corresponding CMS lines, based on visual observation. The pollen fertility percentage was calculated using a method suggested by Chaudhary *et al.* (1981). Anthers were obtained from all the fertile F_1 hybrids for pollen study. Pollen grains were tweezed out of the anther on glass slide. The stained (fertile) and unstained (sterile) pollen grains were counted under the light microscope. The pollen fertility was calculated as the ratio between the number of fertile and sterile pollen grains in the microscopic field using the formula suggested by (Meena and Prabakaran, 2017).

RESULTS AND DISCUSSION

Based on cytological observation, inbreds that produced sterile F_1s were designated as maintainers, while those

that produced fertile F_1s were classified as restorers of the respective CMS sources (per cent pollen fertility). It can be seen from **Table 1** that two inbreds, namely RGP-222 and 298R produced sterile F_1s on the PET-1 as well as the PEF CMS background. RGP-118 and RGP-173 inbreds produced sterile F_1s on PEF as well, while only six inbreds *viz.,* RGP-157, RGP-184, RGP-190, RGP-222, RGP-223 and RGP-233 behaved as restorers on PEF cytoplasm. Though a minute fraction of aborted pollen (sterile pollen) was also observed, it can be seen from **Table 1** that eight (RGP-118, RGP-157, RGP-173, RGP-184, RGP-190, RGP-222, RGP-223, and RGP-233) out of ten inbreds produced fertile F_1s with PET-1 (COSF-6A, COSF-7A, CMS-38A and ARM-248A). Two inbreds, namely, RGP-118 and RGP-173 acted as restorers on PET-1 CMS source but maintainers on PEF CMS lines. It is suggested that the cytoplasm of FMS-852A is distinct from those of other PET-1 CMS lines. Similar conclusions were drawn regarding the differences between three new CMS sources and the French CMS source PET 1 (Petrov and Nenov, 1992). In terms of the frequency of fertility restoration, the current findings support Spirova (1990) conclusion. This is clear in the case of FMS-852A, whose fertility was restored by only six inbreds evaluated in the present study (**Table 2**). The data clearly indicates that at least 40% of the inbreds tested behaved as maintainers for the new CMS source. For diverse CMS backgrounds, similar results of differences in fertility restoring genes have been observed (Whelan, 1981; Virupakshappa *et al.,* 1991). Many other authors also reported a lack of fertility restorers other than PET-1 (Rukminidevi *et al.,* 2006; Sujatha and Reddy, 2008; Venkanna *et al.,* 2008; Channamma, 2009; Satish Chandra *et al.,* 2011; Zhao Liul *et al.,* 2013). In general, only 60% of the inbreds tested behaved as restorers for the new CMS sources. Even the identified effective restorers (RGP-118 and RGP-173) of the traditional PET-1 cytoplasm behaved mostly as maintainers understudy on PEF. Similar observations were also made by Serieys and Vincourt (1987) and Bijral *et al.* (1987). In the material under research, the same pollen parent demonstrated varied types of fertility restoration behaviour in different

Table 1. Evaluation of hybrids for pollen fertility

Inbred	COSF-6A				COSF-7A				CMS-38A				ARM-248A				FMS-852A			
	SP	USP	TP	% PF	SP	USP	TP	%PF	SP	USP	TP	%PF	SP	USP	TP	%PF	SP	USP	TP	%PF
RGP-118	313	22	335	93.4	333	17	350	95.1	341	32	373	91.4	326	12	338	96.4	0	0	0	0
RGP-157	317	18	335	94.6	320	18	338	94.6	323	18	341	94.7	330	15	345	95.6	318	25	343	92.7
RGP-173	332	11	343	96.8	336	13	349	96.2	332	16	348	95.4	321	19	340	94.4	0	0	0	0
RGP-184	326	10	336	97.0	329	12	341	96.4	336	10	346	97.1	332	16	348	95.4	334	12	346	96.5
RGP-190	321	10	331	97.0	338	18	356	95.0	332	12	344	96.5	333	11	344	96.8	319	21	340	93.8
RGP-222	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
RGP-223	317	8	325	97.5	337	19	356	94.6	334	14	348	96.0	320	25	345	92.7	310	21	331	93.6
RGP-225	322	10	332	97.0	322	20	342	94.1	336	14	350	96.0	321	23	344	93.3	317	20	337	94.0
RGP-233	328	9	337	97.3	328	25	353	93.0	342	9	351	97.4	325	17	342	95.0	326	20	346	94.2
298R	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

SP = Stained pollen grains USP = Unstained pollen grains TP = Total pollen grains %PF = Per cent pollen fertility

Table 2. Maintainer and restorer behaviour of 10 inbreds in two diverse CMS sources of sunflower

Inbred	Cytoplasmic male sterile line				
	PET-1 CMS (*H. annuus* L.)				FMS (*H. fallax*)
	COSF-6A	COSF-7A	CMS-38A	ARM-248A	FMS-852A
RGP-118	R	R	R	R	M
RGP-157	R	R	R	R	R
RGP-173	R	R	R	R	M
RGP-184	R	R	R	R	R
RGP-190	R	R	R	R	R
RGP-222	M	M	M	M	M
RGP-223	R	R	R	R	R
RGP-225	R	R	R	R	R
RGP-233	R	R	R	R	R
298R	M	M	M	M	M

CMS line combinations. The restoration ability of restorer lines for the same cytoplasm varied, suggesting that the cytoplasm of CMS interacts differently with different restorer lines. According to Divya Ambati (2010), the varied pollen parent behaviour on different CMS sources indicated significant diversity among cytoplasmic sources and fertility restoration inbreds. Different types of fertility restoration behaviour were observed among the crosses, with some inbreds restoring fertility on one source while failing to restore fertility on another, indicating that different genes are involved in fertility restoration (Venkanna *et al.,* 2008). Similar differences in fertility restoration in different CMS backgrounds have been reported (Hu, 1983). The distinct behaviour of the inbreds for fertility as well as sterility reaction may be due to the genetic architecture, particularly the number of genes controlling and their interactions with cytoplasm in restoring fertility.

Although the appearance of male sterile or fertile plants in a few hybrids is a routine phenomenon in sunflower, in the present investigation none of the crosses displayed segregation with one or two fertile/sterile plants in their progeny. It indicates that the inbred lines used for crossing were stable in terms of maintainer or restorer genes. Virupakshappa *et al.* (1991) reported segregation in F_1 sunflower hybrids and pointed out that it is because of contamination of foreign pollen or the heterozygosity of the lines to restorer genes or perhaps because of the modifying effects of genes (Dominguez-Gimenez and Fick, 1975). In the absence of pollinators, partial restoration of male fertility causes a reduction in the amount of viable pollen, which reduces seed setting and ultimately reduces the seed yield. The degree of fertility restoration mediated by such partial restorer lines is heavily influenced by environmental factors as well as seed parent-pollinator line interactions. In the present investigation, all the inbreds studied displayed a complete restorer (> 90% pollen fertility) reaction, while maintainer lines showed no pollen in F_1 hybrids. To determine plant fertility, the proportion of stained pollen and/or the percentage of aborted pollen should be employed. Pollen fertility may be a key factor in determining fertility, according to the majority of studies. The percentage of fertile pollen was the ultimate criterion for fertility in rice (Ahirwar *et al.,* 2013) and wheat (Gouri Shankar *et al.,* 2007). The pollen study is very important because it provides a clear picture of the restorer or maintainer behaviour of any useful breeding material. A complete restorer male parent (> 90% pollen fertility) is very essential for the development of good hybrids in sunflowers. (**Table 3**).

In the current investigation, 80 per cent of pollen fertility was reported for PET-1. The maximum per cent of pollen fertility (80%) was observed for all PET-1 CMS lines (COSF-6A, COSF-7A, CMS-38A and ARM-248A) (**Table 4**). However, only a 60 per cent frequency of restorer was observed for CMS PEF. The maximum frequency (40%) of tested inbreds as maintainers was recorded for CMS PEF. This finding is in line with Hu (1983), who reported that most of the lines reported fertility restorers for classical cytoplasm PET-1. Hardly a small number of inbreds could act as fertility restorers for new CMS sources. Only two inbreds out of 40 were successful in restoring CMS PF fertility, while three inbreds were successful in restoring *H. lenticularis* fertility (Satish Chandra *et al.,* 2011). For different CMS sources, many more researchers from India and elsewhere reported a very low frequency of fertility restoration genes (Gouri Shankar *et al.,* 2007). Because effective restorers for these novel CMS sources were not available, they concluded that hybrids could not be generated. The findings of this study revealed that the fertility restoration

Table 3. Classification of F_1 hybrid based on pollen fertility

Class	Pollen fertility per cent	Number of hybrids
Complete restorer	>90	38
Partial restorer	10-80	-
Complete maintainer	<1 or 0	12

Table 4. Per cent pollen fertility frequency in tested sunflower inbred lines

CMS line	Number of inbred lines tested	Maintainer (M)	Percent	Restorer (R)	Percent	Segregating types (SG)	Percent	Partial restorer (PR)	Percent
COSF-6A	10	2	20	8	80	-	-	-	-
COSF-7A	10	2	20	8	80	-	-	-	-
CMS-38A	10	2	20	8	80	-	-	-	-
ARM-248A	10	2	20	8	80	-	-	-	-
FMS-852A	10	4	40	6	60	-	-	-	-

reaction of genotypes differs depending on the genetic background of CMS lines. Success in identifying restorers for PET-1 from the limited number of genotypes tested indicates the availability of restorer gene(s) in the cultivated populations of sunflower. Because only six restorers for CMS PEF could be identified, it was concluded that PEF was different from the commercially utilized PET 1 cytoplasm. As a result, it may be concluded that at least PEF can be exploited for the development of commercial sunflower hybrids. The newly identified maintainers will also be more useful in developing new and superior agronomic backgrounds as well as new CMS lines with good general combining ability, for further utilization in hybrid breeding programmes to generate diverse hybrids with better heterosis and resistance to biotic stress. The new restorers identified for the two different CMS sources will help in generating hybrids with a broad cytoplasmic base, which could lead to increased production and productivity of sunflowers by breaking the yield stagnation and enhancing heterosis and intensifying the future hybrid sunflower breeding programme.

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