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Assessment of Cytomorphological Differences in Sorghum Fertility Restoration

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Abstract: Sorghum (*Sorghum bicolor* L. Moench) is ranked fifth as a cereal crop after maize, rice, wheat, and barley based on global cultivation area. However, heat and drought stresses cause improper fertility restoration and inefficient pollination, severely affecting sorghum productivity. The discovery of cytoplasmic male sterility (CMS) is a milestone for commercializing hybrids. This study assessed the pollen fertility and in vitro pollen germination percentage of male and female lines and F_1 hybrids of sorghum using two years of pooled data with multivariate analysis. The principal component analysis (PCA) of female and male lines showed that PC1 represented 82.8% of the variation, whereas PCA of hybrids revealed a significant genetic divergence of 97.1%. Agglomerative hierarchical clustering marked that cluster II genotypes have a high pollen fertility contribution, which can generate superior and high-yielding hybrids. Three male-sterile lines exhibited 100% pollen sterility, with morphological attributes, viz., pinpointed, flattened, low anther extrusion, and starch-digested pollens. Pollen fertility restoration behavior revealed that nine hybrids were fully fertile, eighteen were partially fertile, and three were completely sterile amongst thirty hybrids. The findings of this study will facilitate the identification of potential restorers for the exploitation of high-yielding hybrids in sorghum breeding programs.

Keywords: AHC; CMS; in vitro pollen germination; PCA; pollen fertility; programmed cell death

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1. Introduction

Sorghum (*Sorghum bicolor* L. Moench) is ranked as the fifth most widely cultivated cereal crop in the world, after maize, rice, wheat, and barley, according to the Food and Agriculture Organization of the United Nations (FAO) [1,2]. The cytoplasmic male sterility (CMS) was exploited in sorghum for commercial hybrid seed production [3,4]. CMS is nuclear–mitochondrial interaction inherited maternally, restricting the plant from producing functional pollens [5]. CMS is a crucial breeding platform to harness heterosis in hybrid crops and for the assessment of cytomorphological investigation of floral organs and cytoplasmic–nuclear interaction studies [6].

Failure to produce pollen is associated with anther development defects [7]. CMS is a three-line breeding system for hybrid seed production [3,8]. The CMS (A line) is completely male-sterile. The maintainer (B line) has a fertile cytoplasm responsible for its fertility

owing to the absence of *Rf* genes and is required to pollinate the A line to maintain sterility. The restorer line (R line) is responsible for fertility restoration. R line restores the fertility in the CMS line to develop a hybrid by complementing the cytoplasm defect via restoring dominant nuclear genes [9,10]. Amongst several cytoplasmic sources available in sorghum, only the A_1 (*milo*) CMS system has been used predominantly for the commercial production of hybrids [10]. Principal component analysis (PCA) suggests the genetic divergence in the sorghum lines and hybrids. Agglomerative hierarchical clustering assesses the genetic divergence among the genotypes and cross combinations.

Cytological investigation in sorghum stated that meiosis in CMS (A line) appeared normal. Still, partially fertile pollen grains get shriveled with significantly reduced size and the absence of viable pollens before dehiscence. The meiosis was normal in the CMS parent, but pollen grains deteriorated after formation. It also found that the non-viable character of pollen grains in male-sterile plants was associated with the nutritional role of tapetum [11–13]. Investigating pollination potential relies on pollen quantity, viability, and germination [14], which are crucial for crop improvement [15,16].

Temperatures above 37 °C and below 10 °C alter pollen morphology, resulting in an abnormal exine wall, degeneration of tapetum cells, and membrane damage. It also leads to poor anther dehiscence, impairs pollen tube growth, and hampers fertilization, resulting in partial or complete sterility and a lower seed set [16]. Starch was reportedly the energy source for pollen germination and a checkpoint for pollen maturity [17]. The starch deposition is controlled gametophytically. The fertile pollens are starch-positive engorged with more starch deposition, whereas sterile pollens are starch-deficient [18,19]. The starch deficiency in pollens hampers pollen morphology and pollen tube growth, leading to exine structure degeneration. The pollen grain degeneration in the CMS line causes tapetum degeneration, leading to apoptosis or programmed cell death. This was studied in several crops, viz., sugar beets [20], maize [21], wheat [22–24], tomato [25], etc. Therefore, the present investigation assessed the cytological and morphological behavior of anthers and pollens, including pollen viability and in vitro pollen germination percentage in female and male lines and their hybrids, to identify potential restorers. Most landraces show segregation of fertility restoration or sterility maintenance ability in the post-rainy season. The cytological investigation, such as in vitro germination and pollen fertility percentage, is key for putative restorers. Very limited research has been undertaken to study sorghum's cytology. This study may form the foundation to identify the putative restorers that could be exploited commercially to develop high-yielding post-rainy season sorghum hybrids.

2. Materials and Methods

2.1. Experimental Site and Soil Topography

Female and male lines and their F_1 hybrids were grown in two *post-rainy* seasons (September 2018 and September 2019) in randomized block design (RBD) with three replications at the Sorghum Research Unit (SRU), Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola, Maharashtra, India (latitude 20°42′10.59′′ N and longitude 76°59′57.97′′ E), located at an altitude of 285 m above mean sea level. The soil was medium black with clay, properly leveled with uniform topography and a precise drainage system. The electrical conductivity of the soil was 0.38 dsm^{-1}, and the soil pH was slightly alkaline (pH 7.2). The experiment was conducted to improve major fertility constraints in the post-rainy season. Due to improper restoration, there is no acceptable seed setting and high-yielding post-rainy season hybrids.

2.2. Genotypes

Forty-three sorghum genotypes were involved in the study. These were three maintainers, ten restorers, and thirty hybrids. Restorers used for the present investigation are the collection from the different sorghum research stations. These putative restorers performed best over the CMS lines used in the previous study. Therefore, these restorers were considered for this study to evaluate the performance based on fertility restoration traits in

CMS lines. The sorghum parental genotypes and hybrids used in the present investigation are listed in Table 1. All thirty hybrids were generated using ten restorers and three CMS lines following the Line × Tester mating design.

Table 1. Sorghum genotypes and hybrids used in the present investigation.

S. No.	Genotypes	Status	S. No.	Genotypes	Status
	Parents		10	AKMS 30A × AKRB-431	Hybrids
1	AKMS 30B	Maintainer	11	AKRMS 45A × RS 585	Hybrids
2	AKRMS 45B	Maintainer	12	AKRMS 45A × AKR-354	Hybrids
3	AKRMS 66-2B	Maintainer	13	AKRMS 45A × AKRB-335-3	Hybrids
4	RS 585	Restorer	14	AKRMS 45A × SLR 24	Hybrids
5	AKR 354	Restorer	15	AKRMS 45A × AKRB-428	Hybrids
6	AKRB-335-3	Restorer	16	AKRMS 45A × RB-324	Hybrids
7	SLR 24	Restorer	17	AKRMS 45A × Rb-413-1	Hybrids
8	AKRB 428	Restorer	18	AKRMS 45A × AKRB-429	Hybrids
9	RB 324	Restorer	19	AKRMS 45A × AKRB-430	Hybrids
10	Rb-413-1	Restorer	20	AKRMS 45A × AKRB-431	Hybrids
11	AKRB 429	Restorer	21	AKRMS 66-2A × RS 585	Hybrids
12	AKRB 430	Restorer	22	AKRMS 66-2A × AKR-354	Hybrids
13	AKRB 431	Restorer	23	AKRMS 66-2A × AKRB-335-3	Hybrids
	Hybrids		24	AKRMS 66-2A × SLR 24	Hybrids
1	AKMS 30A × RS 585	Hybrids	25	AKRMS 66-2A × AKRB-428	Hybrids
2	AKMS 30A × AKR-354	Hybrids	26	AKRMS 66-2A × RB-324	Hybrids
3	AKMS 30A × AKRB-335-3	Hybrids	27	AKRMS 66-2A × Rb-413-1	Hybrids
4	AKMS 30A × SLR 24	Hybrids	28	AKRMS 66-2A × AKRB-429	Hybrids
5	AKMS 30A × AKRB-428	Hybrids	29	AKRMS 66-2A × AKRB-430	Hybrids
6	AKMS 30A × RB-324	Hybrids	30	AKRMS 66-2A × AKRB-431	Hybrids
7	AKMS 30A × Rb-413-1	Hybrids			
8	AKMS 30A × AKRB-429	Hybrids			
9	AKMS 30A × AKRB-430	Hybrids			

2.3. Morphological Investigation of Anthers

Restorer and male-sterile lines were assessed for phenotypic traits, viz., plant height, panicle size (compactness), anther extrusion, morphology, and stigma receptivity. Fresh anthers were dissected from parental materials (restorer and sterile lines) just before dehiscence and fixed in a fixative solution (70% ethanol). Single anthers were then examined under the light microscope at 40× magnification, and morphological attributes were studied. The experiment was carried out in five individuals from each replication.

2.4. Stigma Receptivity

Stigma receptivity lasted for about 3 h after anthesis [26]. Spikelets from five different fertile and sterile plants were excised between 3:00 p.m. and 5:00 p.m. (as the pollination rate was higher during this period), placed on a glass slide, and observed for stigma receptivity for anthers and pollens. The photograph was taken at 40× magnification with a digital camera (Nikon COOLPIX S6300, Tokyo, Japan) mounted on a phase-contrast microscopy.

2.5. Cytological Behavior of Pollens: Pollen Viability and Pollen Fertility

In the F_1 generation, the flower of each genotype was bagged 5–10 days before flowering. Five individual florets from five different plants were collected from each genotype for the pollen viability assessment during anthesis. Anthers were collected just before dehiscence early in the morning (08:00 to 09:00 A.M.) and fixed for 1 h in fixative solution (70% ethanol). For the pollen quantification, the third or fourth spikelets from branches were used. Pollen fertility was assessed using a 1% KI (Potassium Iodide) stain. Anthers were then squashed with a needle to disperse pollen grains on the slide and covered with a drop of KI solution. After 5 min of incubation, slides containing pollens were observed,

viability/fertility of pollen was counted under the light microscope at 10× and 40× magnification, and photographs were taken with a camera (Nikon COOLPIX S6300, Japan). Dark-brown-stained pollen grains indicated viable pollen grains (fully fertile). In contrast, faintly stained indicated partially fertile, and unstained or yellow pollen grains indicated sterile ones that appeared empty and deflated. The experiment was carried out in five replicates. Pollen fertility percentage was estimated as the ratio of stained pollens to the total number of pollen grains [25,27–29]. Based on pollen viability/fertility percentage, F_1 plants were classified into four categories: fully fertile (FF) (>80–100% pollen fertility), partially fertile (PF) (50–80% pollen fertility), partially sterile (10–50%), and fully sterile (FS) (0–10% pollen fertility) [29,30].

2.6. Preparation of Iodine Stain

Freshly prepared 1% KI stain was used for the staining purpose. Iodine (0.1 g) was dissolved in 80 mL of 70 percent ethanol, and 0.9 g of potassium iodide (KI) crystal was added. The solution was then warmed gently to dissolve all iodine crystals, and the final volume was made up to 100 mL using 70 percent ethanol [31]. The dissolved solution was cooled and filtered using Whatman® filter paper No. 1.

The percent pollen fertility was calculated using the following formulae,

$$Pollen\ fertility\ (\%) = \frac{(Number\ of\ dark\ stained\ pollen\ grains\ in\ the\ microscope\ field)}{(Total\ number\ of\ pollen\ grains\ in\ the\ microscope\ field)} \times 100$$

2.7. In Vitro Pollen Germination

Pollens were incubated under dark conditions at 28 °C. Germination was assessed on a culture medium containing 100 mg potassium nitrate (KNO_3), 200 mg magnesium sulfate heptahydrate ($MgSO_4.7H_2O$), 150 mg boric acid (H_3BO_3), 500 mg calcium nitrate tetrahydrate Ca $(NO_3)_2.4H_2O$, and 300 g sucrose dissolved in 1 L of Millipore water to which 12 g of agar was added. The culture medium was heated gently till the agar gets completely dissolved [25,28]. The culture medium was then poured into petri dishes and allowed to solidify. These plates were kept in the dark at 28 °C. During anthesis, pollen grains were dusted onto a petri dish, incubated in the dark for 72 hrs at 28 °C, and photographed at 10× and 40× magnification with a digital camera mounted on a phase-contrast microscopy, and pollen germination percentage was estimated. The pollen grain is said to be germinated when a pollen tube emerges from the pollen grains [28,29]. The experiment was carried out in five replicates.

2.8. Statistical Analysis

XLSTAT software (Addinsoft Corporation, Long Island, NY, USA) [32] was used to perform principal component analysis (PCA) and agglomerative hierarchical clustering for the pooled data. XLSTAT software [32] was used to perform PCA to estimate the total variance among the genotypes. PCA involves a mathematical procedure that transforms several correlated variables into several uncorrelated variables called principal components.

The objective of the analysis is to take p variables X1, X2, ... Xp and find combinations of these to produce uncorrelated indices Z1, Z2, ... Zp. The absence of correlation indicates the indices measure from different dimensions in the data. The Z1, Z2, ... Zp means Zi displays the variations among the Xi variables. The Zi are called principal components. The PCA depends only on the covariance matrix $\sum$ or the correlation matrix of the variable under study. The best results are obtained when the original variables correlate positively or negatively. Agglomerative hierarchical clustering (AHC) assesses the genetic divergence and genetic similarities in different clusters.

The present investigation was subjected to PCA and AHC involving female and male lines and their cross combinations, revealing the genetic divergence and variation among the genotypes. This genetic divergence furthers the uses for the exploitation of good post-rainy season hybrids.

3. Results

3.1. Morphological Observation of Anthers

Male fertile (Restorer) and male sterile (CMS) were assessed for phenotypic traits, viz., plant height, panicle size (compactness), anther morphology, and stigma receptivity. The differences between CMS (AKMS 30A) and restorer (AKRB 431) are depicted in Figure 1A–N. The sterile plants were taller (75–78 cm) (Figure 1A) compared to the fertile plant (140 cm) (Figure 1H). A sparse panicle was observed in the sterile plant (Figure 1B), whereas a dense and compact panicle was observed in the fertile plant (Figure 1I). Anther extrusion is less in the sterile line (Figure 1C) compared to fertile plants with a copious amount of dark yellow anthers with extrusion (Figure 1J). Pointed, flattened, and slender anthers were observed in the sterile plant (Figure 1D), whereas fertile anthers were plumpy (Figure 1K). In the restorer (Figure 1L), round red pollen grains were distributed evenly in another lobe, whereas none of the pollens were found inside CMS anther lobes (Figure 1E). In the CMS line (Figure 1F), there are two extruding pale yellow, pinpointed anthers, and they were not present on the stigmatic surface with none of the pollen grains. In the restorer line, two plumpy and dark yellow anthers were present on the stigmatic surface, having a good amount of pollen load (Figure 1M). Figure 1G represents a hairy stigma with no pollen grains, whereas the restorer stigma has a copious amount of pollen load released from fertile anthers (Figure 1N).

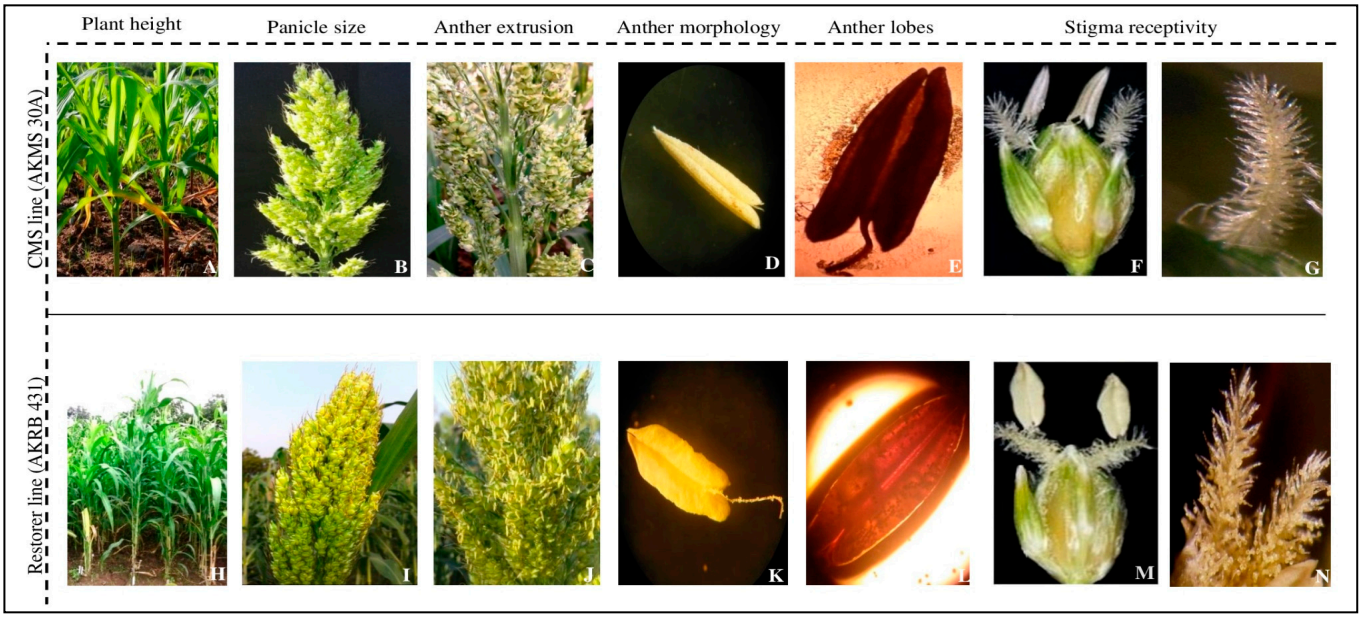

Figure 1. Morphological variations of male-sterile (AKMS 30A) and male-fertile (AKRB 431) plants. (**A**): Whole sterile plant, (**B**): sparse panicle, (**C**): less anther extrusion, (**D**): flatted sterile anthers, (**E**): absence of pollen grains inside anther lobes, (**F**): sterile light yellow, flatted anther, not on the stigma, (**G**): no pollen grains on the stigma, (**H**): Whole fertile plant, (**I**): compact panicle, (**J**): dark yellow anthers with extrusion, (**K**): plumpy fertile anther, (**L**): pollen grains load inside anther lobes, (**M**): mature anthers on hairy stigma, (**N**): pollen grains stacked on hairy stigma.

3.2. Cytological Assessment for Fertility and In Vitro Germination of Pollen

The ten restorers were initially crossed with three CMS lines in a Line × Tester mating design, and their resultant thirty hybrids were evaluated for pollen fertility and in vitro germination (Figure 2). The maximum pollen fertility and in vitro germination response were observed in AKRB 431, and the least was recorded in maintainer AKRMS 66-2B in both the years' pooled data (Supplementary Table S3). Hybrid, AKMS 30A × AKRB 431, exhibited the highest pollen fertility and in vitro pollen germination and, therefore, can be considered fully fertile hybrids. In contrast, hybrid AKRMS 66-2A × SLR-24 exhibited

the least pollen fertility and in vitro germination (Supplementary Table S6). Three hybrids, AKMS 30A × RB 324, AKRMS 45A × RB 324, and AKRMS 66-2A × RB 324, were sterile and showed no pollen fertility and in vitro germination, indicating that their restorer (RB 324) was a perfect maintainer (Supplementary Table S6).

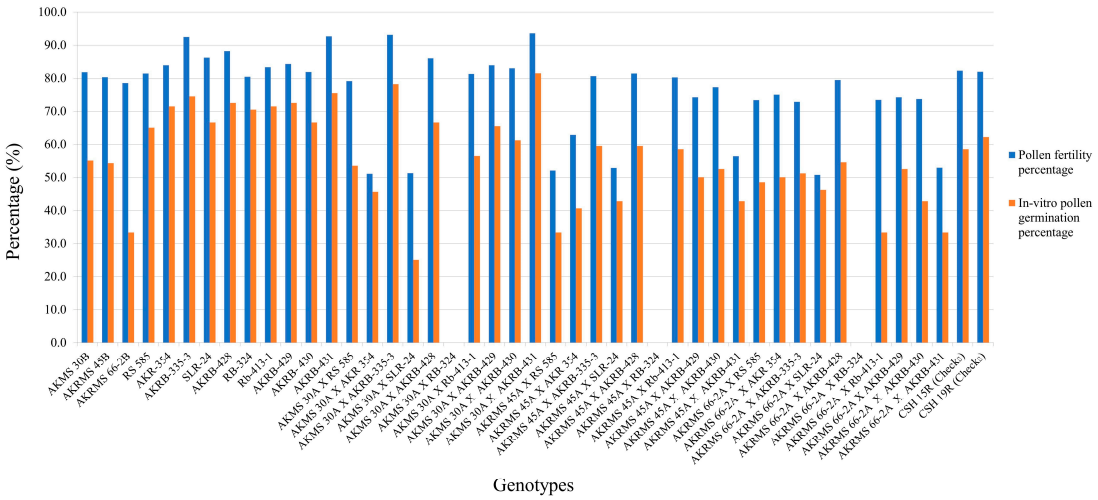

Figure 2. Graphical expression of pollen fertility and percent in vitro pollen germination of parental lines and hybrid used in the present study. Note: Pollen fertility: Fully fertile (>80–100%), partially fertile (50–80%), partially sterile (10–50%), fully sterile (0–10%).

3.3. Cytomorphological Observations of Fully Fertile, Partially Fertile, and Fully Sterile Hybrids

A cytological study for pollen viability was conducted using KI staining. Pollen morphology and in vitro germination assays were performed. Figure 3 indicates that pollen grains with dark brown stain were viable (fertile pollen, Figure 3A). Fertile pollens are considered starch-positive, engorged without any distorted structure, and exhibited remarkably no morphological abnormality (Figure 3B). The partially fertile line having few fertile pollen grains with light stains indicates the low viability of pollen grains (Figure 3C).

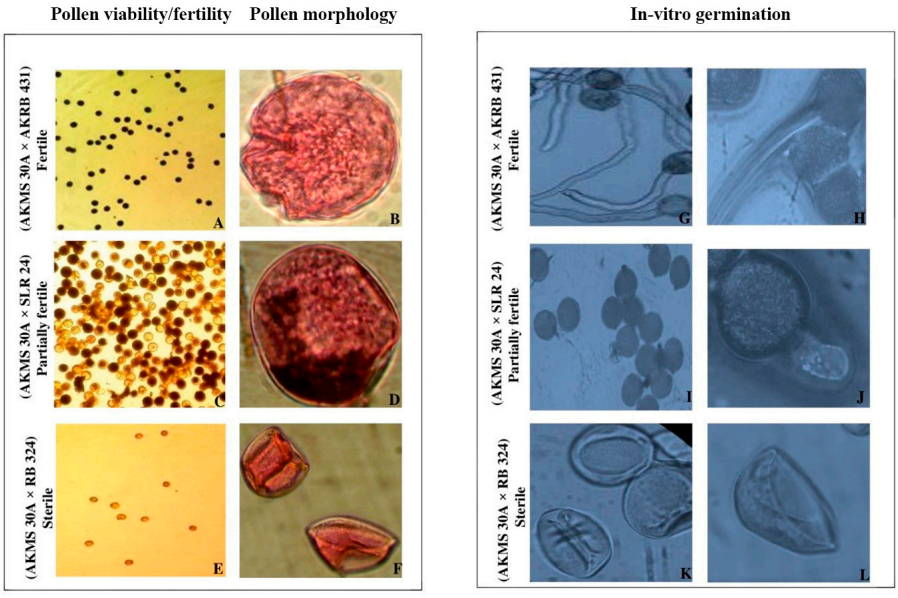

Figure 3. Cytological differences of pollens of fertile hybrid (AKMS 30A × AKRB 431), partial fertile hybrid (AKMS 30A × SLR 24), and sterile hybrid (AKMS 30A × RB324), (**A**–**F**): Pollen Viability and morphology, (**G**–**L**): in vitro germination of pollen grains.

Partially fertile pollens appear to have digested starch with irregular pollen grains (Figure 3D). The unstained, pale, or completely yellow starch digested indicates sterile pollen grains (Figure 3F). Similarly, pollen germination can be confirmed as pollen tube initiated. In fertile lines (Figure 3G), many pollen grains germinated with long pollen tubes (Figure 3H). Partially fertile lines showed the least germination (Figure 3I) with shorter pollen tubes (Figure 3J). In contrast, abortive pollens (Figure 3K) with no germination response were observed in sterile hybrid lines (Figure 3L).

In the present investigation, all the male lines in both the years' pooled data showed 80–93% seed set (Supplementary Tables S1–S3), and nine hybrids were considered fully fertile due to showing >80% seed set (Supplementary Tables S4–S6). Eighteen hybrids are partially fertile due to showing 2–80% seed set, and three hybrids are considered maintainers due to showing 0–20% seed set.

3.4. PCA for Pollen Fertility and In Vitro Pollen Germination

3.4.1. Female and Male Lines

One of the two components investigated in this study had an eigenvalue of more than one. For selecting various female and male lines, the first component with more than one eigenvalue showed greater variability among the parental sorghum genotypes. According to PCA, the PC1 explained 82.87% of the variation among genotypes AKRB-431, AKRB-335-3, AKRB-428, and SLR-24, contributing more favorably. The PC2, which AKRB-429, Rb-413-1, and AKR-354 predominantly contribute, revealed 17.13% of the total variation (Figure 4).

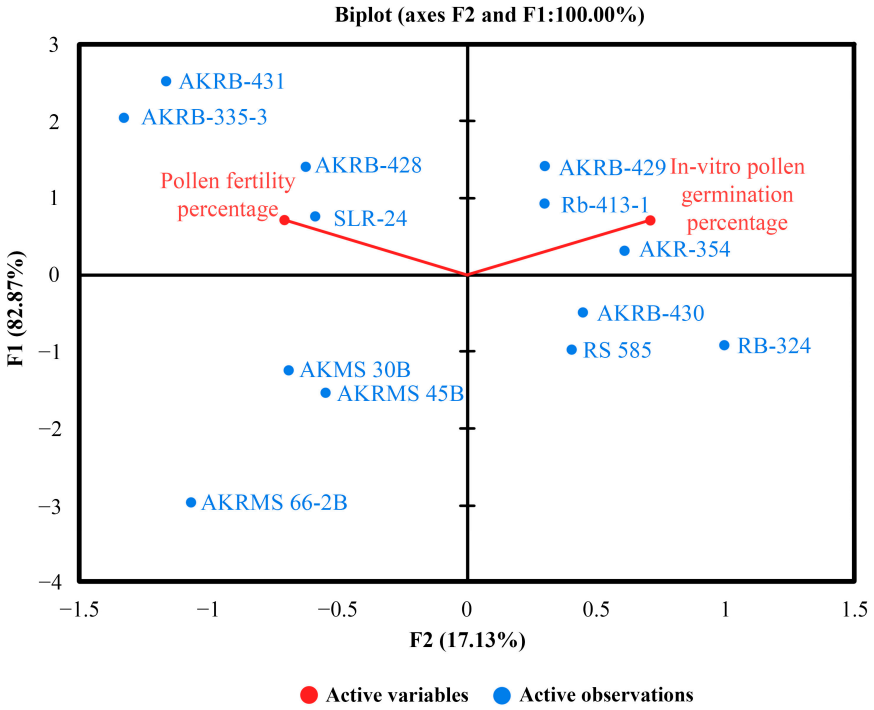

Figure 4. Pooled PCA of female and male lines for pollen fertility and in vitro pollen germination. The F1 and F2 are the biplot axes, representing the total variance.

3.4.2. Hybrids

One of the two components investigated in this hybrid had an eigenvalue of more than one. The first component with more than one eigenvalue showed greater variability among the hybrid genotypes. The PC1 accounted for 97.10% of the total variation with AKMS 30A × AKRB-431, AKMS 30A × AKRB-335-3, AKMS 30A × AKRB-428, AKMS 30A × AKRB-429, AKMS 30A × AKRB-430, and CSH 19R. The PC2, predominantly contributed by RB-324, accounted for an additional 2.90% of the total variation (Figure 5).

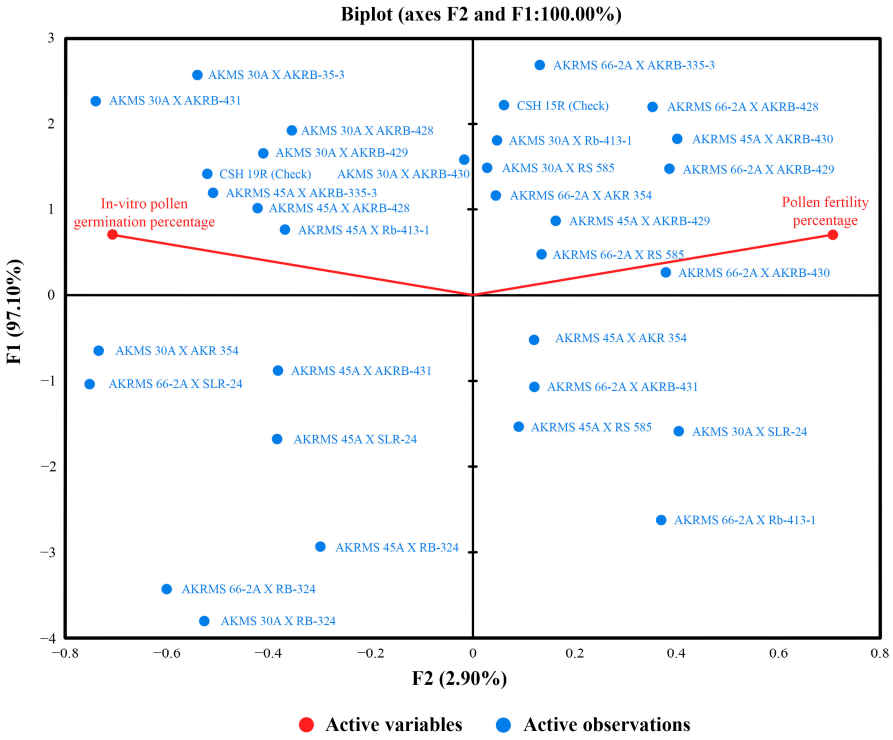

Figure 5. Pooled PCA of hybrids for pollen fertility and in vitro pollen germination assays. The F1 and F2 are the biplot axes, representing the total variance.

3.5. AHC

3.5.1. Female and Male Lines

Thirteen parents (3 female, 10 male lines) were categorized into four groups using a similarity level (Figure 6). A key conclusion drawn from AHC is that, based on composition, the variations between the accessions analyzed are still significant enough to identify them appropriately using the criteria used in this study.

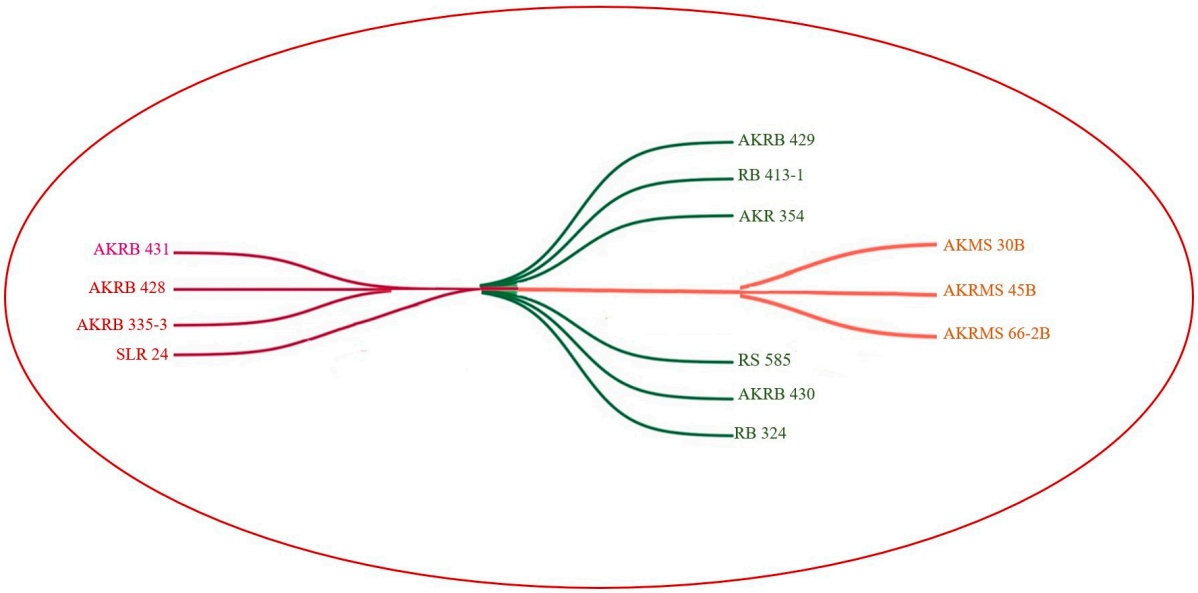

Figure 6. Dendrogram of female and male lines based on pollen fertility percentage and in vitro pollen germination assays.

3.5.2. Hybrids

Thirty hybrids and checks were categorized into five groups using a similarity level (Figure 7). A major conclusion from AHC is that, based on composition, the differences between the hybrids analyzed are still significant enough to identify them accurately using the criteria used in this study.

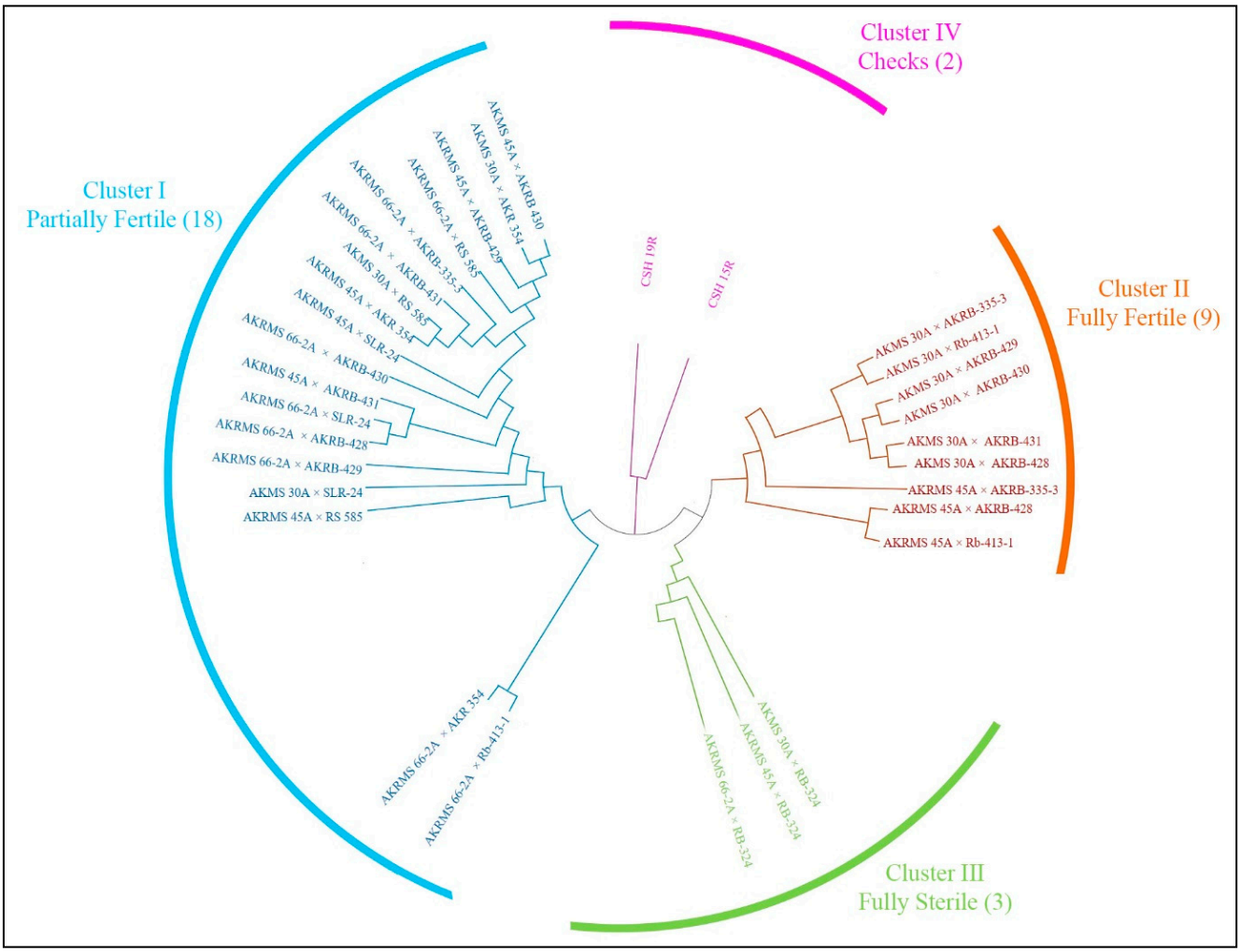

Figure 7. Dendrogram of hybrids and checks based on pollen fertility percentage and in vitro pollen germination assays.

4. Discussion

4.1. Morphological Observations of Male-Sterile and Fertile Anthers

Pollen abundance and dispersal in the restorer line affect seed setting in hybrid seed production. The fertile plant was taller than the sterile plant (Figure 1H). Anther extrusion, anther color, and pollen staining with KI are other traits that differentiate a male-sterile plant from a fertile plant. Less anther extrusion was observed in sterile plants, pointed, flattened, slender sterile anthers without pollen grains on the stigmatic surface. In contrast, higher anther extrusion with two plumpy and dark-yellow-colored anthers was present on the stigmatic surface, having a good amount of pollen load observed in the restorer line (Figure 1L). Similar findings were observed in the Brassica anthers of MF7-2, which developed normally and possessed many plump pollen grains. The filaments of MS7-2 were short, and the anthers were abnormally degraded, with no obvious pollen grains on the surface [33]. The BTx623's (wild-type sorghum) mature spikelet (sessile spikelet) contains three extruding yellow anthers and two stigmas with pollen grains. However,

in the ms8 mutant spikelet (sessile spikelet), three extruding anthers are pale-colored and flattened [34]. The individual and pooled morphology data of two seasons, such as plant height, panicle length, panicle width, anther extrusion, and anther morphology for male lines, female lines, and hybrids, are described in Supplementary Tables S7 and S8.

4.2. Cytological Assessment of Pollen Fertility and In Vitro Germination

Pollen fertility or spikelet fertility is used as an index for the fertility and sterility assessment of lines [16,34]. Almost all restorers exhibited higher pollen fertility and in vitro germination response, showing that these restorers have more viable pollen grains than their corresponding maintainer lines. These can be incorporated into the breeding program without any fertilization barriers. Restorers RS 585, AKR 354, and SLR 24 showed good pollen fertility and in vitro germination, but their cross combinations were partially fertile on three CMS lines.

The graphical representation of pollen fertility and in vitro pollen germination of female and male lines and their F_1 hybrids (Figure 2) revealed that nine were fully fertile based on the above data. Therefore, their corresponding restorers AKRB 335-3, AKRB 428, Rb-413-1, AKRB 429, AKRB 430, and AKRB 431 were classified as putative restorers and restore good fertility in some hybrids on CMS line AKMS 30A and AKRMS 45A, whereas, none of the hybrids were found better on AKRMS 66-2A. Almost all hybrids were partially fertile, which concludes that they restored fertility in their corresponding CMS lines partially and hence were assigned as partially fertile. The sterile hybrids supported the findings that if restorer lines (*Rf* gene) do not restore fertility in CMS, then hybrids become sterile after crossing, and their corresponding restorers maintain the sterility of the CMS lines. Therefore, it is assumed to be a maintainer line in this condition. The partially fertile segregants defined the potential role of modifier genes [35,36]. The present investigation found that RB-324 did not restore fertility in the corresponding CMS and is a maintainer. Overall, the results indicated that pollen fertility is a potential trait for identifying maintainers and spikelet fertility for identifying restorers in the early flowering stage and can be effectively applied for three-line heterosis breeding [35,36].

4.3. Cytomorphological Observations of Fertile, Partially Fertile, and Fully Sterile Hybrid

Pollen fertility percentage and pollen germination criteria were used to select fertile, partially fertile, and sterile hybrids. Three selected cross combinations, AKMS 30A × AKRB 431 (fertile), AKMS 30A × SLR 24 (partially fertile), and AKMS 30A × RB 324 (sterile), were used for cytomorphological investigation of pollens.

The cytological investigation (Figure 3) revealed that the sterile line with the immature pollen was devoid of starch and remained metabolically active at the late vacuolated stage. Reduced hexose sugar levels in starch-deficient male-sterile were due to impaired sugar uptake/transport to the microspore/pollen grains [35]. Abnormalities in the pollen grains (degeneration changed coloration) could be caused by disturbances in the development of tapetum that hampers the nutrition function of the tissue, which leads, in turn, to the sterilization of pollen grains [12,13,37]. The waxy loci encode granule-bound starch synthase (GBSS), which regulates the synthesis of amylase, and mutation in the waxy locus leads to pale/light yellow pollen grains. Mutation in this locus leads to the replacement of amylose with amylopectin [38]. The starch deposition is controlled gametophytically, and hence fertility restoration in CMS plants is gametophytic in nature [18,19,33]. The findings in sorghum suggested that the deterioration of pollen grains was associated with an increase in reactive oxygen species (ROS) with a significant reduction in phospholipid levels. The lower pollen germination could result from hampered carbohydrate metabolism and have a possibility for tapetum degeneration; hence, it could be predicted that premature or delayed tapetal PCD leads to male sterility. This leads to the abnormal development of anthers in sterile lines [39,40]. Temperatures above 37 °C and below 10 °C alter pollen fertility and pollen tube morphology, disturbing the metabolic state of carbohydrates. This leads to lower pollen germination, associated with tapetum degeneration, and hence premature

or delayed tapetal programmed cell death could be predicted, which is the cause of male sterility, resulting in abnormal development of anthers in sterile lines.

In the case of the morphological investigation (Figure 1A–N), similar results in the sorghum have been reported using cross BT×623 and observed the same morphological attributes that we found in sterile anthers and normal fertile anthers [41]. Nine hybrids are fully fertile, and their corresponding restorers AKR 354, AKRB 335-3, AKRB 428, Rb-413-1, AKRB 430, AKRB 429, and AKRB 431 are considered putative restorers, which restore good fertility in CMS lines. These lines could be incorporated into different breeding programs to develop high-yielding post-rainy season sorghum hybrids.

4.4. PCA and AHC of Female, Male Lines and Hybrids

PCA transforms large data sets into smaller principal components without losing details, considering the characters' interdependence. One of the two components investigated in this study had an eigenvalue of more than one. For the selection of various female and male lines, the first component with more than one eigenvalue showed greater variability among the sorghum male and female genotypes. The first principal component of Biplot axes (pc1, 65% of variance) distinguishes the fertile genotypes, whereas the second principal component (pc2, 14% of variance) differentiates between fertile and sterile genotypes (41). In the case of hybrids, one of the two components investigated in this study had an eigenvalue of more than one, which showed greater variability among the sorghum hybrids. AHC analysis discovered clustering patterns between the female and male sorghum genotypes. Female and male lines were categorized into four groups using a similarity level. Cluster I had six genotypes (AKRB 429, Rb 413-1, AKR 354, RS 585, AKRB 430, and RB 324), Cluster II had four genotypes in it (AKRB-335-3, AKRB-428, AKRB-431, and SLR-24), whereas Cluster III had three genotypes (AKMS 30A, AKRMS 45A, and AKRMS-66-2A) (Figure 6). In this context, it may be desirable to cross female lines or germplasm lines with Cluster II genotypes, which have a high pollen fertility contribution and genetic divergence, which can generate superior high-yielding hybrids (Figure 6). Thirty hybrids and checks were categorized into four groups using a similarity level (Figure 7). A major conclusion from AHC is that, based on composition, the differences between the hybrids analyzed are still significant enough to identify them accurately using the criteria used in this study. Cluster I had 18 partially fertile hybrids, Cluster II had two checks completely fertile, Cluster III had nine hybrids completely fertile, and Cluster IV had three genotypes completely sterile (Figure 7). It has been reported that the hybrids grouped in the same Cluster have a narrow genetic base with more genotypic similarity [41] (Figure 7). These diverse populations can be used as base populations to derive new breeding lines with desired traits to broaden the genetic base of current pearl millet breeding programs [42].

5. Conclusions

The present investigation indicated that the genotypes' fertility restoration reaction varies with the genetic background of CMS lines in both post-rainy seasons (September 2018 and September 2019). The PCA of male, female, and hybrids pooled data revealed significant genetic variance and agglomerative hierarchical clustering grouped into four clusters. It interprets that most diverse parents (male and female lines) and their crosses (hybrids) are confined to separate clusters, indicating their dissimilarity from other parents. The parents with a broad genetic base and high genetic divergence could be incorporated into the breeding program to generate superior high-yielding post-rainy sorghum hybrids. In this study, three cross combinations were found to be sterile; therefore, restorer RB 324 could be a maintainer line that maintained the sterility of CMS (A line). The putative restorers identified in the present investigation having high pollen fertility and in vitro germination percentage were AKRB 335-3, AKRB 428, Rb-413-1, AKRB 429, AKRB 430, and AKRB 431.

Pollen fertility restoration behavior revealed that nine hybrids were fully fertile with high pollen fertility and in vitro germination response, eighteen hybrids were partially

fertile, and three hybrids were to be sterile. Programmed cell death (PCD) may cause abnormal development of anthers in sterile lines. The findings of this study will facilitate the identification of potential restorers for the exploitation of high-yielding hybrids in sorghum breeding programs. Nine hybrids are fully fertile, and their corresponding restorers AKR 354, AKRB 335-3, AKRB 428, Rb-413-1, AKRB 430, AKRB 429, and AKRB 431 are considered putative restorers, which restore good fertility in CMS lines. These lines could be incorporated into different breeding programs to develop high-yielding post-rainy season sorghum hybrids. The present investigation found that RB-324 did not restore fertility in the corresponding CMS and is a maintainer. Restorers RS 585, AKR 354, and SLR 24 showed good pollen fertility and in vitro germination, but their cross combinations were partially fertile on three CMS lines.

PCA is a multivariate technique for examining the relationships among several quantitative variables and was conducted to identify the patterns of variation and to estimate the relative contribution of various characters for total variability. In the present investigation, PCA of female and male lines showed that PC1 represented 82.8% of the variation, whereas PCA of hybrids revealed a significant genetic divergence of 97.1%. PCA provides information that could facilitate a better selection of parental genotypes with specific traits and formulation of breeding strategies for trait improvement.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture13050985/s1, Table S1: Sorghum parental genotypes pollen fertility percentage and in vitro pollen germination percentage in 2018; Table S2: Sorghum parental genotypes pollen fertility percentage and in vitro pollen germination percentage in 2019; Table S3: Pooled data of Sorghum parental genotypes pollen fertility percentage and in vitro pollen germination percentage; Table S4: Sorghum F1 hybrids pollen fertility percentage and in vitro pollen germination percentage in 2018; Table S5: Sorghum F1 hybrids pollen fertility percentage and in vitro pollen germination percentage in 2019; Table S6: Pooled data of Sorghum F1 hybrids pollen fertility percentage and in vitro pollen germination percentage; Table S7: Morphological pooled data of Sorghum parental lines; Table S8: Morphological pooled data of Sorghum hybrids and checks.

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