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Staining techniques to ascertain CMS/FR system in maize *(Zea mays L.)* for hybrid development

ISHFAQ ABIDI*, G ALI[1], ZA DAR[2], SHABIR H WANI[3], ASIF M IQBAL[3] AND ASIMA GAZAL[1]

Directorate of Research SKUAST-Kashmir Shalimar Srinagar J&K, 190 025, India

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ABSTRACT

The major hurdle impeding the wide adoption of hybrid maize, in developing countries particularly in high altitudes of Indian Himalayas has been the cost consideration. Hybrid maize seed is generally 4-10 times more expensive than the seed of OPVs and often beyond the purchasing power of poor farmers who have limited resources and little access to the credit facilities. The seed production costs, therefore, need to be reduced drastically to make it available to the farmers at affordable prices. Currently, the hybrid maize seed is produced by detasseling of seed parent is labour intensive and costs 280-300 US dollars per hectare. Cytoplasmic male sterility (genetic emasculation) therefore, has been looked since long back as a sound and sustainable alternative to bring down the hybrid seed production costs besides adding purity to the end product. CMS plants are characterized by their inability to produce viable pollen while having little or no effect on female fertility. The unique pattern of inheritance (CMS is transmitted only through female parent) has enabled the CMS trait to be of great utility to plant breeders and the commercial seed industry. Staining techniques used in present investigation helped in the objective classification of maize pollen into fertile and sterile phenotypes in maize.

Keywords: Staining, Maize, Emasculation, Pollen, Hybrid, CMS

INTRODUCTION

A major shift in global cereal demand has been predicted by 2020, demand for maize will surpass the demand for both wheat and rice (Pingali, 2001). The underlying reason of shift to maize includes demand as livestock feed following higher poultry, meat and dairy product consumption due to an improved standard of living in most of the countries of the developing world. This unprecedented demand for maize presents a daunting challenge for breeders, seed producers, and policymakers.

As a monoecious plant, maize develops unisexual male and female flowers in physically separate portions of the plant. Pollination occurs by transfer of viable pollen from staminate flowers in the tassel to silk. Wind is the principal agent in this uncontrolled/open pollination of maize plant. About 95 per cent of the ovules on the shoot are usually cross-pollinated and 5 per cent self-pollinated (Poehlman and Sleper, 1995).The fact that the male and female flowers of maize are located on different parts of the plant enables large-scale hybridization of maize.

Cytoplasmic male sterility (genetic emasculation) therefore, has been looked since long back as a sound and sustainable alternative to bring down the hybrid seed production costs (Duvick, 1965), besides adding purity to the end product. CMS plants are characterized by their inability to produce viable pollen while having little or no effect on female fertility. The unique pattern of inheritance (CMS is transmitted only through female parent) has enabled the CMS trait to be of great utility to plant breeders and the commercial seed industry, eliminating the need for mechanical or hand emasculation in the production of hybrid seed. A precise understanding of physiology, biochemistry, genetics and molecular bases of male sterility/fertility restoration systems in crop plants would significantly help to extend these systems for hybrid breeding of a wide range of crops for the production of superior and stable hybrids. The present work was undertaken with the aim of identifying sterility/fertility genes in the available germplasm through genetic and cytogenetic analysis & staining techniques.

Studies have revealed that CMS is a tool for enabling efficient coexistence between genetically modified (GM) and non- GM cultivation by biocontainment of GM maize pollen. Cytoplasmic male sterility is a reliable solution to the problem of coexistence between genetically modified (GM) and non-GM maize because producers of non –GM maize are usually concerned about the potential risks of outcrossing by pollen from GM maize. Indeed, suitable transgenic CMS plants would not release pollen to the environment and, therefore, would not fertilize non–GM adjacent plants. It is particularly important to avoid the release of pollen by GM plants that are bred for the production of pharmaceuticals (Weider *et al.*, 2016).

Expanding the array of stable CMS lines with desirable agronomic attributes constitutes a key step in a hybrid breeding scheme. In this regard, placing a cytoplasm known to condition male sterility in different genetic backgrounds is a widely accepted strategy for the development of new CMS

[1]Division of Plant Breeding and Genetics SKUAST-Kashmir, 190025, India
[2]Dryland Karewa Agricultural Research Station SKUAST-Kashmir, Budgam India
[3]Mountain Research Centre for Field Crops, Khudwani SKUAST-Kashmir (J&K), India
*Corresponding Author Email : ishfaqabidi@gmail.com

lines. Equally important is the establishment of an effective restoration system for the available CMS lines, which paves the way for their subsequent deployment in hybrid breeding (Bohra *et al*., 2017).

Harnessing heterosis is the best approach to handle the current challenge of the sustainable enhanced yield of plant crops. The heritable nature of Cytoplasmic male sterility (CMS) allows manifesting non-functional male gametophyte and hence is a cost-effective system for promotion of efficient hybrid seed production. The phenomenon of CMS stems from a complex interplay between maternally-inherited (mitochondrion) and bi-parental (nucleus) genomic elements. Molecular tools including DNA markers have been implicated in crop hybrid breeding in order to greatly expedite the progress (Bohra *et al*., 2016). The relevance of CMS-based hybrid breeding is evident across a wide range of crop species including rice (Pranathi *et al*., 2016), Brassica (Heng *et al*., 2014), cotton (Zhang *et al*., 2012) and soybean (Lin *et al*., 2014).

MATERIALS AND METHODS

Inbred Lines

This study included genetically diverse converted male sterile line namely I-15C(A),their F_1sI-15C(A) x I-318(R) and maintainer line I-15C(B) , which were produced and maintained at High Altitude Maize Research Substation, Sagam, Anantnag but have not been used for commercial hybrid seed production as attempts were on to fix the sterility/ Fr genes in an indigenous and stable genetic background of maize.

Experimental material

The experimental material consisted of the cross I-15C(A) x I-318(R) and involving parents I-15C(A), and I-318(R), genetically diverse converted male sterile line namely I-15C(A), their F_1s with restorer line I-318(R) and maintainer line I-15C(B).

The study was carried out as follows:

1. The crosses I-15C(A) x I-318(R) was attempted at High Altitude Rice Research Sub-station, Larnoo (Anantnag) during the *First year* to generate F_1-generation.
2. The F_2 and backcrosses generations (BC_1 and BC_2) were developed at Offseason Winter Nursery Centre (ICAR) Hyderabad, during 2nd year.
3. The six generations of the cross thus obtained P_1, P_2 (parental generation), F_1, F_2, BC_1 and BC_2 and I-15C(B) were raised and evaluated at the Research/ Experimental Farm of Division of Plant Breeding & Genetics, SKUAST-Kashmir, Shalimar during 3rd*year*.

Experimental Design

All the six basic set of generations of the cross under study developed in the year 2016 were evaluated in 2017 in a randomized complete block design with three replications at the Research/Experimental Farm of Division of Plant Breeding & Genetics, SKUAST-K, Shalimar. In The non-segregating (P_1, P_2 and F_1) and segregating generations (F_2, BC_1 and BC_2) were raised in four and six rows, respectively. An inter and intra-row spacing of 60 cm and 25 cm respectively were adopted. Recommended package of practices was followed to raise a good crop. The details of plant population (Table 1) raised and selected for observations is as:

Table 1: The details of plant population selected for observations

Generation	No. of plant raised	Plants chosen/ replication
Parent (P_1)	40	10
Parent (P_2)	40	10
Hybrid (F_1)	40	10
Backcross (BC_1)	80	60
Backcross (BC_2)	80	60
Second generation hybrid (F_2)	120	100

Pollen stainability

Before crossing, CMS-line was tested for pollen sterility/ fertility. This was determined by staining pollen grains in 1% potassium iodide solution. About 2-4 tassels from each treatment were collected prior to pollen shed and fixed in 70 per cent alcohol. All anthers were excised with the help of forceps and placed in the stain. The pollen grains were released with a needle and gently crushed. After the debris was removed a cover slip was placed over the pollen material and it was observed under a microscope at 10x resolution (Virmani *et al*., 1997 and Chaudhary *et al*., 1981).

Pollen Viability

To evaluate pollen viability of parents and F_{1s} by staining, anthers were fixed in Carnoy's fluid (3 parts ethyl alcohol: 1 part propionic acid) and pollen grains were stained in 2 per cent acetocarmine. Five glass slides of each genotype were evaluated for pollen viability. The pollen grains were classified based on their shape, size and extent of staining (Virmani *et al*., 1997, Tang *et al*., 1998) as follows (Table 2):

Table 2: Categories of maize pollen and their features

Category of pollen	Shape and staining behaviour	Classification
Unstained withered sterile (UWS)	Withered, undeveloped and unstained	Sterile
Unstained spherical sterile (USS)	Spherical and smaller unstained	Sterile
Stained round sterile	Round and small, lightly /incompletely stained	Sterile
Stained round fertile (SRF)	Round and large, darkly stained	Fertile

Additional staining according to Alexander (1969) was conducted. Alexander's stain contains malachite green which stains cellulose in pollen walls and acid furchin which stains pollen protoplasm. It differentially colours viable (fertile) and unviable (sterile) pollen grains.

RESULTS AND DISCUSSION

Staining procedures as per (Alexender, 1969; Virmani *et al*., 1997 and Tang *et al*., 1998) was adopted to assess the sterility/fertility of pollen grains of CMS lines, restorer, and F_1 hybrids. Fertility/ sterility status of genotypes was recorded as per scoring from field tests and cross-checked by staining with 2 per cent acetocarmine and 1% potassium iodide solution respectively. Five pollen glass slides of each genotype were collected prior to anthesis (based on visual scoring), anthers were excised and disrupted, pollen of appropriate genotype placed on to the slide, stained under a cover slip and scored by microscopic examination. The first 100 pollen grains were scored on left to right transect starting on the left center of the cover slip (Figs.1 to 8). Pollen grains in five randomly selected microscopic fields were counted. The pollen grains were classified based on their shape, size, and extent of staining. The results are summarized in Table 3. Different categories of pollen which have been observed have been visualized and are shown in (Figs. 6 to 8).

Table 3: Pollen categories and types of male sterility in male sterile lines, restorer and hybrids of maize

CMS/hybrid/ test line	Total pollen examined	Frequency (%)				
		UWS	USS	SRS	SRF	Pollen sterility (%)
I-15C(A)	270	33.98	62.59	1.86	1.48	97.63
I-318(R)	340	0.30	2.20	8.68	88.36	11.24
I-15C(A) x I -318(R)	425	0.34	2.77	9.10	87.79	12.21
Range	(270-425)	(0.30 -33.98)	(2.20 62.59)	(1.86 9.10)	(1.48 88.36)	(11.24 97.63)
Mean	345	11.54	22.52	6.54	59.21	40.36
S.E	±44.8	±11.21	±18.34	±2.35	±28.86	±30.55
S.D	77.62	19.43	31.76	4.06	49.99	52.92

Additionally staining according to Alexander (1969) also substantiated the results obtained. Pollen samples of parents and their crosses were stained with Alexanders stain and it was observed that fertile pollen was fully stained and showed dark purple colour but sterile pollen was either unstained or partially stained and had either a pale green or splotchy light dark purple colour (Table 4 and Figs. 6 to 8).

Table 4: Categories of maize pollen and their features

Category of pollen	Staining behaviour	Classification
Unstained withered sterile (UWS)	Withered, undeveloped and unstained	Sterile/ non-viable
Unstained spherical sterile (USS)	Smaller and unstained	Sterile/ non-viable
Stained round sterile (SRS)	Small, round and lightly/ incompletely stained	Sterile/ non-viable
Stained round fertile (SRF)	Round, darkly stained	Fertile/ viable

Because of problems associated with detasseling female plants to produce hybrid seed, male sterile cytoplasms resistant to diseases, available in an agronomically acceptable form would be especially attractive for commercial hybrid seed production. CMS sources resistant to *H. maydis* race T. have been widely reported but these CMS sources generally lack stability across space and time. Therefore the success of using CMS source in hybrid seed production would largely depend on its stability coupled with resistance to the common maize diseases.

Comprehensive evaluation of potentially useful sources of CMS was undertaken with an aim of ascertaining the fertility restoring ability of R-line in an indigenously developed and highly stable cytosterile source of maize, study inheritance of fertility restoring gene(s) so as to pave way for development and commercialization of CMS based maize hybrids.

The present results were further validated by ascertaining the degree of fertility/sterility using Beckett's modification of Ducviks scale-1 which also indicated the dominance nature of fertility restoring genes as was observed in F_1-generation of the cross whereas genes for sterility exhibited recessive nature. Cytological studies were carried out to cross-check field test results via staining techniques. Fertile and sterile pollen grains can be readily distinguished by staining with 2 per cent acetocarmine or 1 per cent KI solution (Neuffer *et al*., 1997). So fertile and sterile parents were identified in the present study through staining techniques. It was observed that fertile pollen is stained dark brown and sterile pollen was either partially stained or unstained (Figs. 1 to 6).

Fertile pollen stained with 2 per cent acetocarmine/1 per cent potassium iodide showed dark brown colour because there was normal starch deposition in this pollen and probable viability and evident male fertility, whereas sterile pollen produces little or no starch so they were either unstained or partially stained. Pollen grains of CMS line I-15C (A) were unstained indicating abnormal starch deposition and probable sterility. Additional staining according to Alexander (1969) and procedures used by Virmani *et al*. (1997) and Tang *et al*. (1998) helped to partition pollen grains into different categories (Figs. 6 to 8) and thus simplified their identification. The pollen grains were classified based on their shape and extent of staining into four classes as unstained withered sterile (UWS), unstained spherical sterile (USS), stained round sterile (SRS), and stained round fertile (SRF). Pollen sterility above 97% was noticed in CMS-line I-401 (A)

and highest pollen fertility (88.5%) in restorer line I-318 (R). The frequency of SRF pollen was high in R-line (I-318 R) followed by F_1 hybrid I-15C (A) x I-318 (R) whereas the frequency of USS pollen was more in CMS-line 15C (A). As reported by Virmani *et al*. (1997) genotypes showing greater than 80 per cent pollen fertility are used as a restorer and those showing 0-2 per cent pollen fertility are used as maintainers. So the CMS and R-lines used in the present study can successfully be used as restorers and maintainers. Alexanders stain contains malachite green which stains cellulose in pollen walls and acid fuschin which stains the pollen protoplasm and thus differentially colours the viable and non-viable pollen grains, with viable pollen staining dark purple and non-viable pollen staining either green or splotchy dark, purple or pink.

Cytological investigations of pollen biogenesis in CMS-maize lines in the present study indicate that degeneration of pollen in CMS maize lines occurs abruptly following intine deposition and before the disappearance of the central vacuole. Examination of pollen stainability in the F_1 generation of cross revealed gradation of pollen stainability. Also, sterile pollen grains exhibited variation in staining from light green to blotchy pink and many fertile pollen grains stained dark brown/black. Visually intermediate classes were also evident wherein starch synthesis may have been delayed or slow so that part of these pollen grains stained brown/pink. An indication that non-stained/partially stained pollen of CMS line had lost integrity was reflected in observations that these pollen grains desiccate and collapse within a few hours following anther exertion.

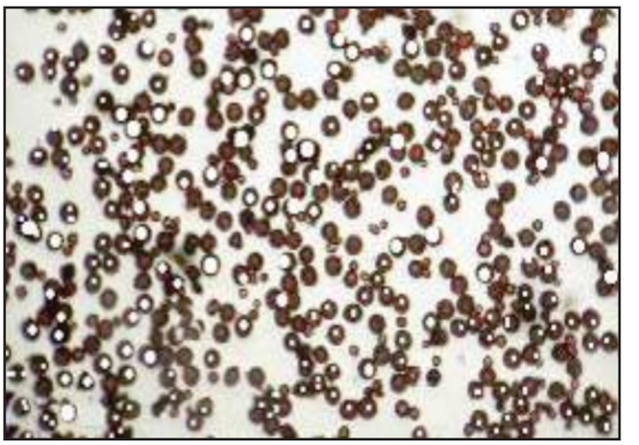

Fig 3 : Mixture of fertile/sterile pollen

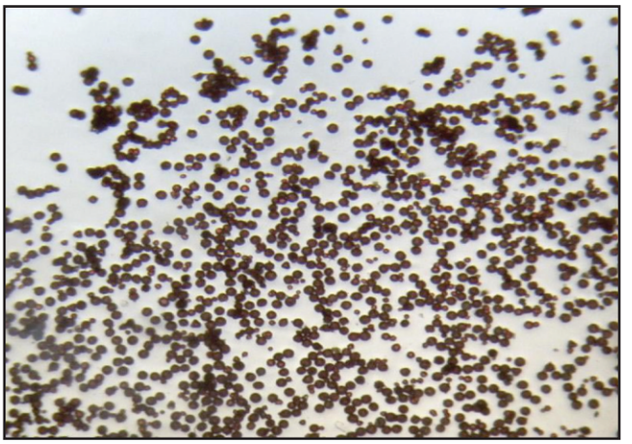

Fig 1 : Fertile pollen stained

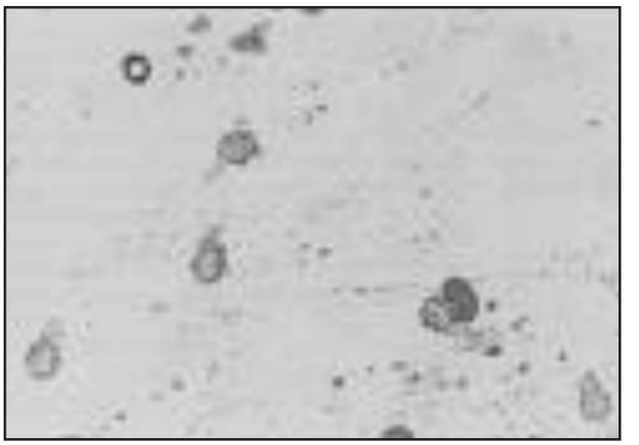

Fig 4 : Sterile pollen of CMS parent [I-15C (A)]

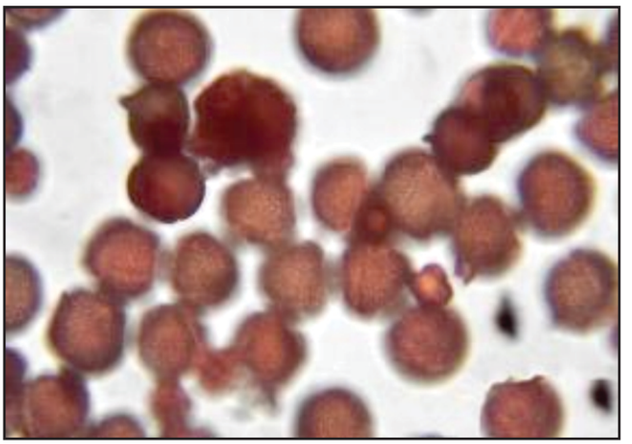

Fig 2 : Magnified view of fertile pollen

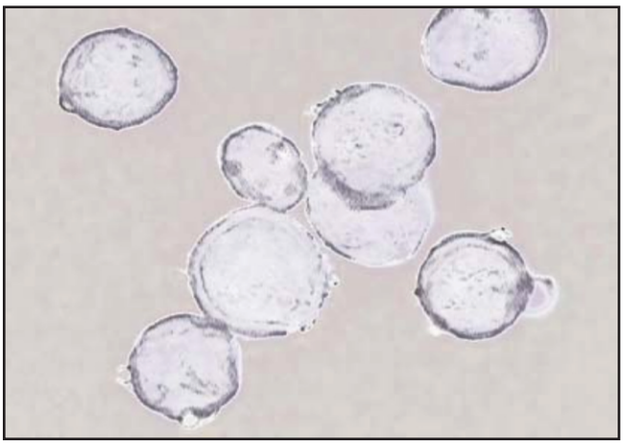

Fig 5 : Magnified view of sterile pollen of CMS-lines

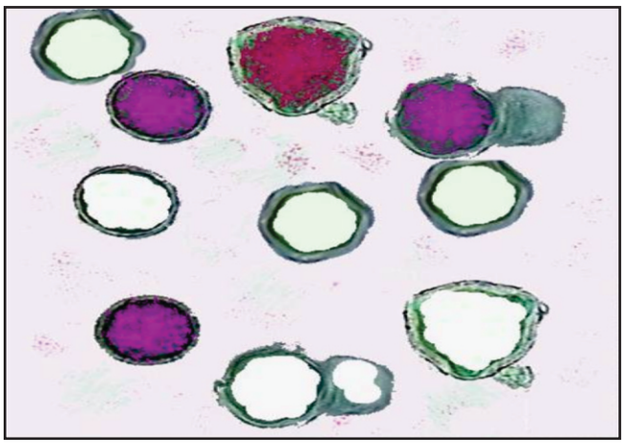

Fig 6 : Categories of pollen after staining with Alexanders stain

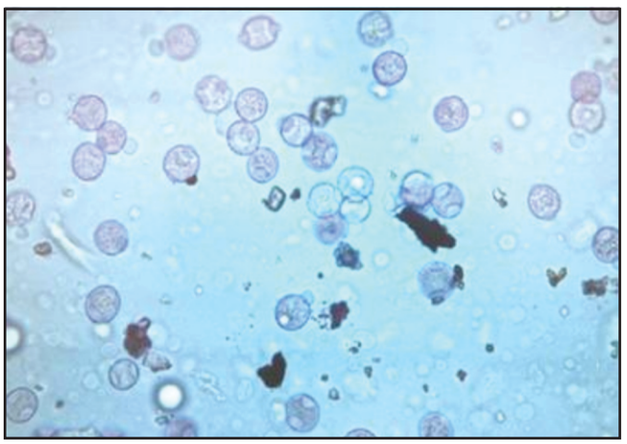

Fig 7 : Differential staining of fertile / sterile pollen as per Alexanders (1969)

Low self-compatibility in the progeny makes pollen stainability a better indicator of fertility restoration than visual scoring or seed set. The heterozygous male fertile F_1-progenies for fertility restoration gene(s) of I-318 (R) restored pollen stainability to 85-88 per cent indicating nearly complete dominance. To be useful for hybrid seed production a CMS-line needs complete male sterility and female fertility. So the cytological studies of fertility restoration in present

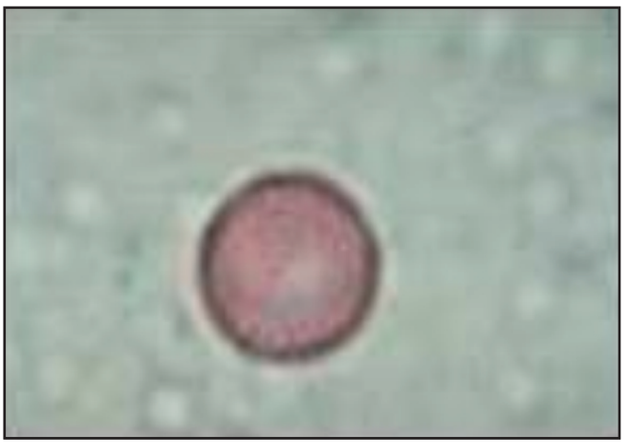

Fig 8 : Fertile F1 pollen of cross I-15C(A) x I-318 (R) (Magnified view)

investigation substantiated the results obtained from field reports.

CONCLUSION

The staining techniques used in this study helped in developing a rapid, repeatable and objective classification of maize pollen into fertile and sterile phenotypes allowing rapid identification of genotypes at anthesis and thus will be highly useful to make further genetic studies and germplasm improvement more efficient in maize crop. Cytoplasmic substitutions were made in the nuclear backgrounds of early-maturing pigeon pea varieties or lines. Three new CMS lines (ICPL 88039A, Pusa 992A, and DPP 3-2A) resulted from genetic crosses involving cytoplasmic donors from A2 (GT 288A) and A4 (ICPA 2089) categories. In addition to visual inspection of anthers, pollen-staining techniques and scanning electron microscopy (SEM) analysis were used to confirm pollen sterility (Bohra et al., 2017).

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