Short Communication Open Access

# Anomalous fertilization in haploidy inducer lines in maize (*Zea mays* L)

**M Swapna[1,2]*, Kumud R Sarkar[1]**

[1]Division of Genetics, Indian Agricultural Research Institute, New Delhi-110012, India
[2]present address: Division of Crop Improvement, Indian Institute of Sugarcane Research, Lucknow, Uttar Pradesh-226002, India
*Corresponding author: E-mail: sarikkath@yahoo.com

## Abstract

A detailed investigation was carried out to study the exact cause and the sequence of events leading to the origin of high frequency of haploids in the high haploidy inducer lines (Stock 6 lines) in maize (*Zea mays* L). Microtome sections and isolated embryo sacs were observed to understand the fertilization process in maize. These studies helped in understanding the normal fertilization process and also in tracing out a few anomalous fertilization events in these ovules. The study indicated that, haploid embryos in these lines arise due to a failure in the fusion of the sperm nucleus and the egg cell, after the release of sperm nuclei from synergid into the embryo sac, with the egg cell being induced to develop parthenogenetically into a haploid embryo.

**Keywords**: anomalous fertilization, haploids, maize, parthenogenesis, stock 6

## Introduction

The potential that the haploids have in developmental, genetical and evolutionary studies make them valuable tools for basic as well as applied research. The low incidence of haploids, the lack of effective screening techniques and the difficulties encountered in chromosome doubling have however limited an extensive exploitation of haploids. In maize, the "genetic selection technique" has been the technique of choice for the production of haploids (Sarkar, 1974) since other techniques like anther culture, chromosome elimination following wide crosses etc do not yield desired results. This technique has two components - use of haploidy inducer lines (HILs) to increase the frequency of maternal haploids and application of genetic markers for the early, easy and efficient screening. This technique involves the use of dominant inhibitor gene CI, which inhibits anthocyanin pigmentation in both endosperm and scutellum (Coe and Sarkar, 1964). Female plants with coloured scutellum (CC) are crossed with pollen from male parents with the dominant inhibitor C-I C-I. In normal fertilization, both endosperm and embryo are colourless whereas the haploid kernels can be easily identified by the presence of colored embryo against a sharply contrasting colourless endosperm (Figure 1A, B).

In 1959, Coe discovered a genetic strain `Stock 6', which on selfing gave a haploid frequency of 3.23%. The high heritability of this trait allowed its transfer to other stocks of maize (Sarkar et al, 1972). Rigorous selection for this trait over generations led to the development of haploidy inducer lines (HILs), in which, the haploid frequency was greater than 5% (Sarkar et al, 1994). Use of these lines along with C, C-I or R-nj markers facilitated detailed investigations on the origin of maternal haploids. Though several studies have been carried out, the exact sequence of events which leads to the induction of haploids in these lines has not been traced out yet. The normal fertilization process itself raises a few questions for which the answers remain elusive. The pollen-stigma interaction, pollen tube guidance, stimuli that helps in differentiating the various components of the embryo sac, the sperm dimorphism observed in some plants, the exact mechanism which determines the fate of egg cell and polar nuclei are some of the grey areas in the entire fertilization process. Keeping these in mind, an attempt was made to study the fertilization process in the high haploidy inducer lines.

## Materials and Methods

The materials used in the study were the high haploidy inducer lines derived from a haploidy inducer stock, Stock 6 (Coe, 1959; Coe and Sarkar, 1964; Sarkar and Coe, 1966). The female parent had the anthocyanin pigmentation genes A1, C1 and R1 (stock designated as ACR) and the male parent had the genes A1, C-I and R1 (stock designated as C-I) in the homozygous condition. Thus the genetic constitution of the female and male parents was AA CC RR and AA C-IC-I RR respectively. Nine haploidy inducer lines and two testers were initially tried in the crosses (Table 1). Out of these, a set of three high haploidy inducer lines (HIL1-HIl3) were crossed with the testers. Cobs from these crosses were used for microtome studies and embryo sac isolation. The silks of the testers were covered with butter paper bags to prevent contamination with any foreign pollen. After pollination, the ears were fixed at 22, 24, 26, 30 and

 RECEIVED 03/06/2011

Table 1 - Plant material used for crossing

| Stock designation | Genetic constitution | Haploidy induction potential |
|---|---|---|
| HIL 1* | AA C-IC-I RR | High |
| HIL 2* | AA C-IC-I RR | High |
| HIL 3* | AA C-IC-I RR | High |
| HIL 4 | AA C-IC-I RR | Medium |
| HIL 5 | AA C-IC-I RR | Medium |
| HIL 6 | AA C-IC-I RR | Medium |
| HIL 7 | AA C-IC-I RR | Low |
| HIL 8 | AA C-IC-I RR | Low |
| HIL 9 | AA C-IC-I RR | Low |
| Tester1 (7851) | AA CC RR | High |
| Tester 2 (7852) | AA CC RR | Low |
| CM 105 | | Low haploidy indred line |

*high haploidy inducer lines used as male parents in crosses for microtome studies and embryo sac isolation

36 hours after pollination (HAP). Along with the HILs, a low haploidy breeding inbred line CM 105 also was used for comparison.

For microtome studies, the cobs were detached from the plant and the outer bracts were removed. The ears were cut into longitudinal pieces and fixed in formaldehyde: acetic acid: alcohol (1:1: 18) at the stipulated intervals, i.e., 22, 24, 26, 30 and 36 HAP. The processing of the sections and the subsequent staining were done based on the technique proposed by Johannsen (1940). The slides with the sections were passed through xylene, xylene- alcohol series (3:1, 1:1, and 1:3), alcohol series (from 90-35%) and washed with distilled water. The sections were stained with 2% iron-heamatoxylin for 10-15 minutes, washed in running water thoroughly and then again with distilled water. The sections were differentiated in 1% iron-heamatoxylin and again washed in running water. The slides were passed through alcohol series (35%-90%) and xylene-alcohol series (1:3, 1:1, 3:1) and then placed in 100% xylene. The sections were finally mounted in DPX mountant.

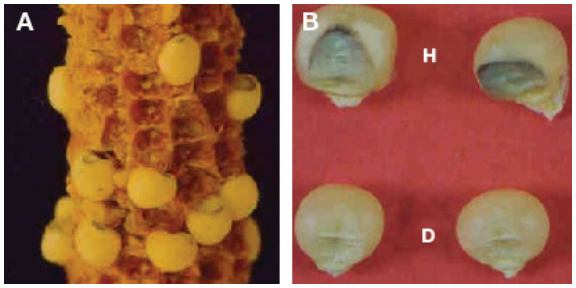


Figure 1 - Haploid and diploid kernels from an ACR x C-I cross. A) Cob with haploid kernels retained. B) Haploid and diploid kernals with coloured embryo and colourless endosperm (haploid H) and both the components colourless (diploid D).

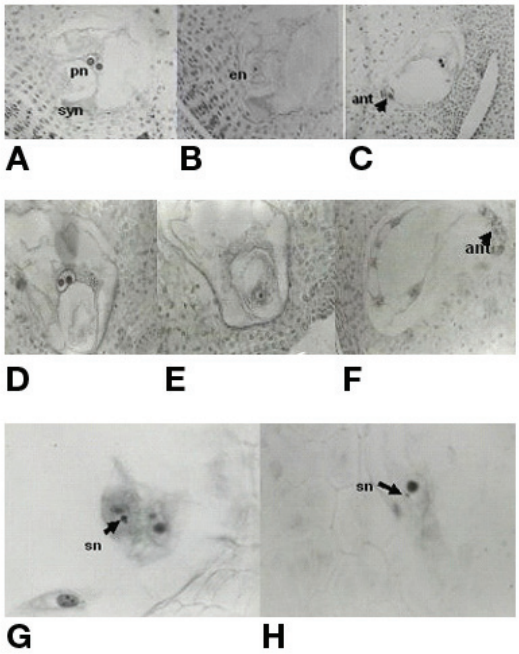


Figure 2 - A, B, C) sections of an unfertilized embryo sac showing the polar nuclei (pn), egg cell (en), synergids (sn) and dividing antipodals (ant); D, E, F) sections of a fertilized ACR ovary pollinated by C-I pollen 25HAP with fertilized polar nuclei, egg nucleus and profusely dividing antipodals (ant); G, H) sections of two pollinated ovaries, 31 and 30 HAP respectively, showing failure of fusion of egg nucleus and sperm nucleus. The arrows show the sperm nucleus (sn) lying unfused near the egg nucleus.

From a pilot study it was confirmed that under normal conditions, fertilization will be effected within 22-24 HAP. Hence the ovules were fixed at different hours after pollination starting from 22 to 36 HAP. For observing the embryo sacs after isolation, the procedure proposed by Wagner et al (1989) was followed, with some modifications. Ovules were separated from the outer bracts and the nucellar portion along with the embryo sac was incubated in cavity slides in 2% cellulose + 1% pectolyase in equal proportions. The incubation was carried out at room temperature overnight or alternatively, at 35°C for 5-6 h.The embryo sacs were teased out under the microscope, put in clearing solution for 24h and observed after staining with haematoxylin-erythrosin B stain.

## Results

Examination of ovule sections revealed that the ovules were deeply embedded inside the integuments and the nucellar tissue. Inside the embryo sac, the chalazal end pointed towards the silk attachment region and the micropylar end towards the base where the ovary was attached to the cob. In the true sense, the components inside the embryo sac could be visualized by the darkly staining nucleolus (Figures

2A, B).

*Unpollinated embryo sacs*

A typical unpollinated embryo sac (Figures 2A-C) depicted the two polar nuclei at the centre, with round and darkly stained nucleoli. They remained partly fused with their membranes just touching or partially overlapping each other. The egg apparatus was situated towards the micropylar end of the embryo sac. The synergids were seen flanking the egg cell. Once pollination was effected one of the synergids was found to display darker staining, showing signs of degeneration, into which the sperm nuclei was released from the pollen tube.

The group of cells situated at the chalazal end is the antipodals. A large number of antipodals both before (Figure 2C) and after pollination (Figure 2F) were observed. This is the characteristic feature of the maize embryo sac (Vollbrecht and Hake, 1995). This proliferation in maize, in contrast to the degeneration observed in Arabidopsis, which also has a Polygonum type of embryo sac, may have important implications with respect to the post-fertilization development of the embryo sac (Vollbrecht and Hake, 1998) and endosperm.

*Embryo sacs after pollination*

Out of a total of approximately 600 ovules processed, about 100 ovules could not be observed due to losses at different steps. Since the time taken by the pollen tube to reach the embryo sac depends on the distance traveled inside the silk, at a given time, different ovules within the same cob were found to be at different stages of fertilization process. Figures 2D and 2E show the sections of an ovule at 25 HAP, with the endosperm at the bi-nucleate stage. The egg nucleus is still to start division. Of the two synergids, one is darkly stained. The antipodals are found to divide profusely. Figure 2G shows one of the serial sections of an ovule fixed 31 HAP. The endosperm has started division. The unfused sperm nucleus is seen near the egg cell. This is a clear case of failure of fertilization of the egg nucleus, while the endosperm nucleus has started division. Figure 2H also shows the section of another ovule at 30 HAP displaying failure of fertilization of egg cell. Three more sections could be traced with a similar situation.

*Isolated embryo sacs*

Figure 3A shows the isolated embryo sac of an HIL 21 HAP. The synergids, polar nuclei and egg nucleus are seen at their respective positions. Here also, profuse division of antipodals could be observed.

The whole mount of an embryo sac at 30 HAP is seen in Figure 3B. The endosperm nuclei have started dividing, it being at the eight nucleate stage. The egg nucleus has not started dividing. But a small nuclear structure is seen in the vicinity of the egg cell. From its size and shape it appears similar to a sperm nucleus. Under normal conditions it is observed that the fertilization of the polar nuclei and the egg cell is completed 21-24 HAP. Within 3-5 HAP, the primary endosperm nucleus starts dividing. Normally the fertilization of the egg cell would have been completed by this time. Thus there is every chance that there is a failure of fusion of the egg cell and the sperm nucleus, while the endosperm formation and its subsequent divisions are normal. Three more embryo sac showing such an anomaly could be traced out.

Thus, even though a failure of fertilization of the egg cell could not be traced out in a large number of cases, the observations point towards the possibility of occurrence of this phenomenon, followed by the parthenogenetic development of the egg cell into a haploid embryo.

## Discussion

*Haploids in Stock 6 - a case of sperm attenuation?*

The high incidence of haploids in the Stock 6 lines of maize has previously been investigated to some extent (Sarkar and Coe, 1966; Aman et al, 1978; Aman and Sarkar, 1981). Though the process by which they are induced have been speculated, the exact cause has not been pin pointed. From the present study and also from the inferences of the previous workers, some conclusions may be drawn regarding the origin of haploid embryos in 'Stock 6' lines of maize.

It is possible that there may be an arrest of the meiotic/mitotic division at the time of pollen grain formation resulting in a single diploid sperm nucleus. This diploid sperm nucleus can fuse with the polar nuclei alone. This can give rise to a haploid embryo along with a tetraploid endosperm. Such a possibility could be ruled out from the present investigation, where binucleate pollen could be detected only in 0.17% of the high HIL pollen grains, 0.03% of low haploid pollen grains and also in 0.39% of the inbred pollen grains. These frequencies are too low to account for the relatively high haploid frequencies observed in these lines. Mahendru (2000) has also come to similar conclusions in her studies using the pollen grains of haploidy inducer lines. In a detailed investigation by Sarkar and Coe (1966) using crosses involving Stock 6, the genomic constitution was studied in

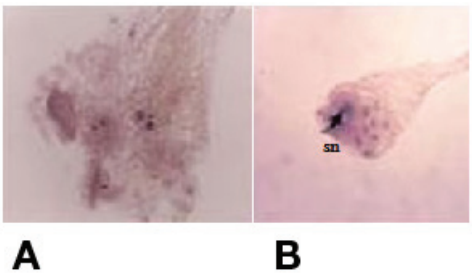


Figure 3 - A) an isolated embryo sac of an unfertilized ovary showing the polar nuclei (pn), egg cell (en), synergids (sn); B) a fertilized ovary 30 HAP showing unfused sperm nucleus (sn) near the egg nucleus.

crosses involving CC and C-IC-I, to look into the possibility of a 4n endosperm formation, making use of the number and constitution of the colored spots in the aleurone layer in these crosses. They concluded that the haploid embryo formation might be due to a failure in the fertilization of the egg cell due to some abnormality in the sperm nucleus, either inherent or induced, with the haploid egg cell being induced to develop parthenogenetically into a haploid embryo.

Abnormalities in the pollen tube growth can lead to slowing down of the pollen tube and a subsequent delay in the fertilization process. In 99% of the cases pollen tube entry into the micropyl could be detected by 21-23 HAP, with no abnormalities like branching being observed. Similar observations were made by Mahendru (1994). Delayed pollination has also been found to produce haploids in *Triticum monococcum*, Einkorn wheat etc (Kihara, 1940; Smith, 1946). This view also does not find support in this particular case, since all the endosperms showed a triploid condition, indicating the occurrence of fertilization.

Normal fertilization of the egg cell and the polar nucleus can occur, with the subsequent abortion of the zygote, as suggested by Kostoff (1942) and Cooper (1943). This would presumably give rise to n-2n twin pairs. Death or arrest of the 2n embryo will result in the development of a maternal haploid embryo. However in the present investigation, we failed to detect any degenerating zygote or dividing synergid. A preferential elimination of the paternal chromosomes during the course of development of the embryo can be suggested, but no such reports exists on the development of a haploid embryo due to wide crosses involving maize as the female parent (reviewed in Chase, 1966). Thus the observations so far point to a failure of fertilization, leading to haploid production in 'Stock 6' lines, rather than a normal zygote formation and subsequent anomalies.

Thus, having ruled out all these possibilities, observations from the present investigation point towards a possible attenuation of the sperm nucleus inside the embryo sac. The present study enabled us to trace out seven cases where the endosperm was at 2-16 nucleate stage after 24 HAP, with a small nuclear structure in the vicinity of the egg cell. It appears that it is the failure of fertilization of the egg cell with the sperm nucleus, which gives rise to a haploid embryo in these lines. There is an attenuation of one of the sperm nuclei inside the degenerated synergid, which fails to fuse with the egg cell, after being released from the synergid. Due to the stimulus received from the single fertilization which takes place normally, the unfertilized egg develops parthenogenetically into a haploid embryo.

Since the two sperm nuclei arise from the same generative nucleus, it is difficult to explain this differential behaviour of the two nuclei with respect to their functional potential. This prompts us to recall the possibility of sperm cell dimorphism which has been reported in some crops like *Plumbago zeylanica* (Russell, 1985; 1986) and *Nicotiana tabacum* (Tian et al, 2001). It is possible that in maize also, there may be some sort of sperm nuclei dimorphism operating, as reported in Plumbago (Russell, 1985, 1986), resulting in a well programmed fertilization process.

An anomaly with respect to the functional capacity of the female gametophyte is another possibility. During the female gametogenesis, the megaspore mother cell gives rise to a linear tetrad, out of which, the lower most one alone survives to give rise to an embryo sac. Recent studies have also indicated the role of calcium dynamics in megaspore degeneration (Qiu et al, 2008). An alteration in the nutritional status (Haig, 1990) and genetic variation exhibited among the tetrads may lead to functional changes. Detailed studies are needed to prove or disprove these speculations. It has been reported by Vollbrecht and Hake (1998) that maize mutants with morphologically intact embryo sacs can show failure of fertilization. Characterization of the male gamete repertoire, thereby aiding in understanding male-female molecule interaction during fertilization process is also underway (reviewed in Singh et al, 2008). The role of sperm fertilizin and egg integrin in the adhesion, and probably in the gametic fusion in mammals has been speculated (Snell and White, 1996). Similar compounds may be present in the case of plants also, which facilitate the gametic attraction and fusion. These may also have a role to play in the anomalous fertilization observed in the Stock 6 maize.

## Acknowledgements

The Senior Research Fellowship provided to the first author by Indian Council of Agricultural Research, New Delhi, India is gratefully acknowledged.

## References

Aman MA, Mathur DS, Sarkar KR, 1981. Effect of pollen and silk age on the maternal haploid frequencies on maize. Indian J Genet 41: 362-365

Aman MA, Sarkar KR, 1978. Selection for haploidy inducing potential in maize. Indian J Genet 38: 452-457

Cass D, 1973. An ultrastructural and Nomarski interference study of the sperms of barley. Can J Bot 51: 601-605

Coe EH, 1959. A line of maize with high haploid frequency. Am Nat 93: 381-382

Coe EH, Sarkar KR, 1964. The detection of haploids in maize. J Hered 55: 231-233.

Cooper DC, 1943. Haploid-diploid twin embryos in Lilium and Nicotiana. Am J Bot 30: 408-413.

Dupius I, Roeckel P, Matthys-Rochon E., Dumas C, 1987. Procedure to isolate viable sperm cells from corn (*Zea mays* L) pollen grains. Plant Physiol 85: 876-878

Haig D, 1990. New perspectives on the angiosperm female gametophyte. Bot Rev 56: 236-274

Johansen DA, 1940. Plant Microtechnique. Mc Graw Hill, New York

Kiesselbach TA, 1949. Structure and Reproduction of Corn. University of Nebraska Agric Exp Stn Bull 161: 96

Kihara H, 1940. Formation of haploids by means of delayed pollination in *Triticum monococcum*. Bot Mag Tokyo 54: 178-185

Kostoff D, 1942. The problem of haploidy: cytogenetic studies in Nicotiana haploids and their bearing to some cytogenetic problems. Bibl Genet 13: 1-148

Kranz E, Bautor J, Lorz H, 1991. In vitro fertilization by single isolated gametes by electrofusion. Sex Plant Reprod 4: 12-16

Kranz E, Lorz H, 1993. In vitro fertilization with isolated and single gametes results in zygotic embryogenesis and fertile maize plants. Plant Cell 5: 739-746

Mahendru A, 1994. Studies on the origin of maternal haploids in maize (*Zea mays* L) PhD Thesis, Indian Agricultural Research Institute, New Delhi

Mahendru A, 2000. Cytological analysis of the pollen of haploidy inducer lines in maize. Indian J Genet 60: 37-43

Mathur DS, Aman MA, Sarkar KR, 1980. Induction of haploids in maize through heat treatment of pollen. Curr Sci 49: 744-746

Mathur DS, Sachan JKS, Sarkar KR. 1976 Radiation induced haploidy and heterofertilization in maize. J Nuclear Agric Biol 5: 76-77

Qiu LY, Liu RX, Xie CT, Russell SD, Tian HQ, 2008. Calcium changes during megasporogenesis and megaspore degeneration in lettuce (*Lactuca sativa* L.) Sex Plant Reprod 21: 197-204

Russell SD, 1985. Preferential fertilization in Plumbago: Ultrastructural evidence for gamete-level recognition in an angiosperm. Proc Nat Acad Sci USA 82: 6129-6132

Sarkar KR, Coe EH, 1966. A genetic analysis of the origin of maternal haploids in maize. Genetics 54: 453-464

Sarkar KR, 1974. Genetic selection technique for the production of haploids in higher plants, pp. 33-41. In: Haploids in Higher Plants: Advances and Potential. Kasha KJ ed. Guelph University Press, Canada

Sarkar KR, Panke S, Sachan JKS, 1972. Development of maternal haploidy inducer lines in maize (*Zea mays* L). Indian J Agric Sci 142: 781-786

Sarkar KR, Pandey A, Gayen P, Madan JK, Kumar R, Sachan JKS, 1994. Stabilization of haploidy inducer lines. Maize Genet Coop Newsl 68: 64-65

Smith L, 1946. Haploidy in einkorn wheat. J Agric Res 73: 291-301

Singh MB, Bhalla, PL, Russell SD, 2008. Molecular repertoire of flowering plant male germ cells. Sex Plant Reprod 21: 27-36

Snell WJ, White JM, 1996. The molecules of mammalian fertilization. Cell 85: 629-637

Tian HQ, Zhang Z, Russell SD, 2001. Sperm dimorphism in *Nicotiana tabacum* L. Sex Plant Reprod 14: 123-125

Vollbrecht E, Hake S, 1995. Deficiency analysis of female gametogenesis in maize. Dev Genet 16: 44-63

Vollbrecht E, Hake S, 1998. Developmental genetics of female gametophyte function in maize. Proc 40th Annual Maize Genet Conference, Lake Geneva, Wisconsin, USA

Wagner VT, Song YC, Matthys-Rochon E, Dumas C, 1989. Observations on the isolated embryo sacs of *Zea mays* L. Plant Sci 59: 127-132