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CYTOLOGICAL ANALYSIS OF THE POLLEN OF HAPLOIDY INDUCER LINES IN MAIZE (*ZEA MAYS* L.)

ANJU MAHENDRU* AND K. R. SARKAR

Division of Genetics,
Indian Agricultural Research Institute, New Delhi 110 012

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ABSTRACT

Cytological analysis was attempted to study the mechanism of origin of maternal haploids involving Haploidy Inducer Lines (HILs) developed from a high haploid inducer strain 'Stock 6' of maize. The possibility of degeneration or loss of a sperm nucleus in the pollen grain and tubes was studied using flourescent stains in the HILs and compared with that of the control inbreds. Binucleate pollen grains appeared in HILs albeit with frequencies too low to account for anomalies leading to haploid induction. Pollen tubes with two nuclei were observed with high frequency in both the HIL and the control. In such tubes, two gamete nuclei were regularly present and the vegetative nucleus was not visible, thus arguing against any anomaly in the number of male gametes in the HIL pollen tubes. Other anomalies like presence of more than one pollen tube per pollen or branched pollen tubes were also not detected. It is concluded that nuclear anomaly does not occur in the pollen and tubes of HILs and is, therefore, not the cause of haploidy induction in these lines.

Key Words : Maize, haploids, pollen grain, cytology, fluorescence

Haploids are of special interest from an embryological standpoint besides being a valuable tool in plant breeding and cytogenetical investigations. However, cytological analyses of events leading to the origin of haploids are limited, since larger number of ovules must be sectioned because of the rather low frequency of haploids in natural populations.

For enhancement of haploid frequencies, several techniques have been developed in different crop species [1-4]. Of these, use of strains with a tendency to produce haploid individuals at greater frequencies than normal has been found to be quite effective in crops like maize, potato and cotton. In maize, Coe [5] discovered a genetic strain, 'Stock 6' that on selfing produced a haploid frequency of 3.23 per cent. The high heritability trait of 'Stock 6' was transmitted into another background by crossing it to a locally adapted line 'Stock 2' [6]. By backcrossing to 'Stock 6'

*Corresponding author, E-mail : ama_gene@iari.ernet.in

and continued selection, high haploid inducer lines (HILs) with up to 3 per cent haploidy induction were developed [7]. Further selections have increased the yields from particular lines reaching a mark of 5 per cent [8]. Genetic selection technique in maize is an easy, efficient and low cost method more so because the haploids can be detected in the dry seed stage itself by the embryo marker technique [9-10]. Besides being of applied value, this technique is a valuable tool for basic research on haploidy. Sarkar and Coe [11] attempted a genetic analysis to study the mechanism of origin of maternal haploids in crosses involving Stock 6. They were able to exclude some of the possible ways in which haploids could have originated and suggested that haploid induction may be resulting from the failure of fertilization due to an abnormal male or female gamete and the reduced egg may then develop to form a haploid. Cytological studies were, however, needed to reveal the exact mechanism and fate of the male nucleus destined to fuse with the egg. An attempt, therefore, was made to find the possible anomalies in the male gametes of HILs of maize.

MATERIALS AND METHODS

Plants of *Zea mays* L. from Haploidy Inducer Lines (HILs) and low haploidy inbred (3085) as control were employed in this study (Table 1). Pollen grains from dehiscing anthers were fixed in 1:3 acetoalcohol solution overnight, transferred to 70% alcohol and stored at 4°C for use. In another experiment, pollen grains were germinated in semi-solid media containing 0.35M sucrose, 0.01% borate, 0.03% calcium chloride and 0.06% agar- agar. After six hours of germination at 28 ± 2°C and high relative humidity, the pollen were scraped from the surface of media and suspended in 1:3 acetoalcohol solution overnight, transferred to 70% alcohol in eppendorfs and stored at 4°C for use.

Table 1. Average haploid frequencies obtained from HILs on selfing.

Family no.	Average haploid frequency (%)
3518	5.88
3529A.3	5.99
3530C	2.55
3085 (control)	-

For cytological studies, DNA binding fluorescent compound 4, 6- diamidino, 2-phenylindole dihydrochloride (DAPI, Sigma lot #1388) was used @ 0.5ug/ml solution prepared in citrate phosphate buffer, pH 4, following the procedure described by

Coleman and Goff [12] for nuclei in pollen grains and tubes. Fluorescence was observed using Nikon Microphot diafluorescence with a 200 W mercury lamp and a combination of excitation filter U (UV) and barrier filter BA 420. Photographs were taken using Nikon Microphot system. High speed (Ilford HP 400) film for fluorescence was used.

RESULTS AND DISCUSSION

The pollen grain is shed in the trinucleate state in maize as the generative nucleus divides to form two male gametes while the vegetative nucleus remains undivided. Disintegration or abortion of one of the gametes may result in a binucleate grain. The availability of a single gamete (monospermy) may lead to a single fertilization. Then, if a normal triple fusion (i.e. of the two polar nuclei with one male gamete forming the primary endosperm nucleus) occurs, the unfertilized haploid egg may be induced to develop into a haploid sporophyte. Therefore, the number of nuclei in the mature pollen grains of HILs were studied. In the control, all the 3000 pollen grains screened showed to contain 3 nuclei each (Fig. 1). Binucleate pollen grains were found with a frequency of 0.41% (17 binucleate grains out of 4178) and 0.09% (2 binucleate grains out of 2320) in the two HILs viz. 3529A.3 and 3518 respectively (Fig. 2). The haploidy frequency of these lines is 5.99 and 5.88% respectively. If binucleate condition were the cause of haploidy induction, binucleate pollen grains with frequencies higher than 5.88% would be expected, since a binucleate pollen grain may not always effect fertilization. In all possibility, the few binucleate pollen grains observed have resulted due to a chance asynchrony of the division of generative nuclei of a few tetrads in relation to others. Thus, absence or degeneration of a male gamete is not the cause of haploidy in HILs of maize. On the same basis, the possibility of restitution of the generative nucleus giving a '2n' nucleus was excluded since this situation would also lead to a binucleate condition.

Pollen grains having two tubes (bisiphonous grains) in 'Stock 6' derivative were reported to occur with higher frequency (4.2%) than those in the inbred by Pogna and Marzetti [13]. They also reported an asymmetrical distribution of nuclei in these tubes. Pogna and Marzetti believed that such tubes may result in incomplete fertilization, and subsequently a haploid may develop from the egg. In our experiment, presence of more than one pollen tube or branching in pollen tube was studied by growing *in vitro* the pollen of control inbred and HIL (haploid induction frequency 2.55%). None of the 3233 pollen grains of control line and 879 pollen grains of HIL had a branched pollen tube or two tubes coming out simultaneously from a pollen. This observation excludes the possibility of bisiphonous pollen grains or asymmetric distribution of gamete nuclei in such tubes being the cause of anomalous fertilization.

The two male gametes and the vegetative nucleus of the pollen move into the tube upon pollination. Mathur *et al* [14] reported that X-ray treatment of HIL pollen before pollination substantially increases the incidence of haploidy and suggested that attenuation or slowing down of one of the sperms may be the cause of haploidy induction. In our studies, the possibility of degeneration or loss of nuclei in the pollen tubes of HIL was studied. Both binucleate and trinucleate pollen tubes were seen in the HIL as well as the control inbred. Three fluorescent nuclei appeared in 54 out of 89 tubes in the control and in 317 out of 514 tubes studied in the HIL (Table 2). In 52 out of 54 tubes in the control and 263 out of 317 tubes in the HIL, the three nuclei could be distinguished into vegetative and sperm nuclei on the basis of their shape and staining pattern. The sperm nuclei were small, thicker, spherical to rod shaped in appearance and brightly fluorescing (Fig. 3). The vegetative nucleus was less intense in fluorescence. It was longer than broad and many times appressed close to pollen tube wall. The wall also showed some autofluorescence. In a few cases, the three nuclei could not be distinguished as vegetative and sperm nuclei on the basis of shape and/or staining intensity (Fig. 4). This was seen in both HIL and control. A careful analysis and comparison of binucleate with the trinucleate pollen tubes indicated that the nucleus missing in the pollen tubes is the vegetative nucleus. This interpretation was made on the basis of two observations:

Table 2. Analysis of nuclei in the pollen tubes of high haploidy inducer line

Lines	Number of pollen tubes studied	Number of pollen tubes with	
		Three nuclei	Two nuclei
3085 (control)	89	52(2)	34(1)
3530C(HIL)	514	263(54)	191(6)

Note. Figures in parentheses denote cases where nuclei are not distinguishable as vegetative and sperm nuclei

1. The vegetative nucleus in the pollen tubes with three nuclei in both the low haploidy inbred and HIL did not stain brightly and uniformly along its length. It was seen appressed to the tube wall in many cases. The wall itself showed some fluorescence in both the pollen grain as well as tubes. The weak fluorescence of vegetative nucleus might, therefore, have been suppressed by wall fluorescence in cases where the vegetative nucleus may be lined too close to the wall. Moreover, neither of the nuclei observed in the binucleate pollen tubes of either the inbred or the HIL (Fig. 5) were similar in shape to the vegetative nucleus, rather they resembled the male gametes in the trinucleate pollen tubes. The behaviour of the vegetative

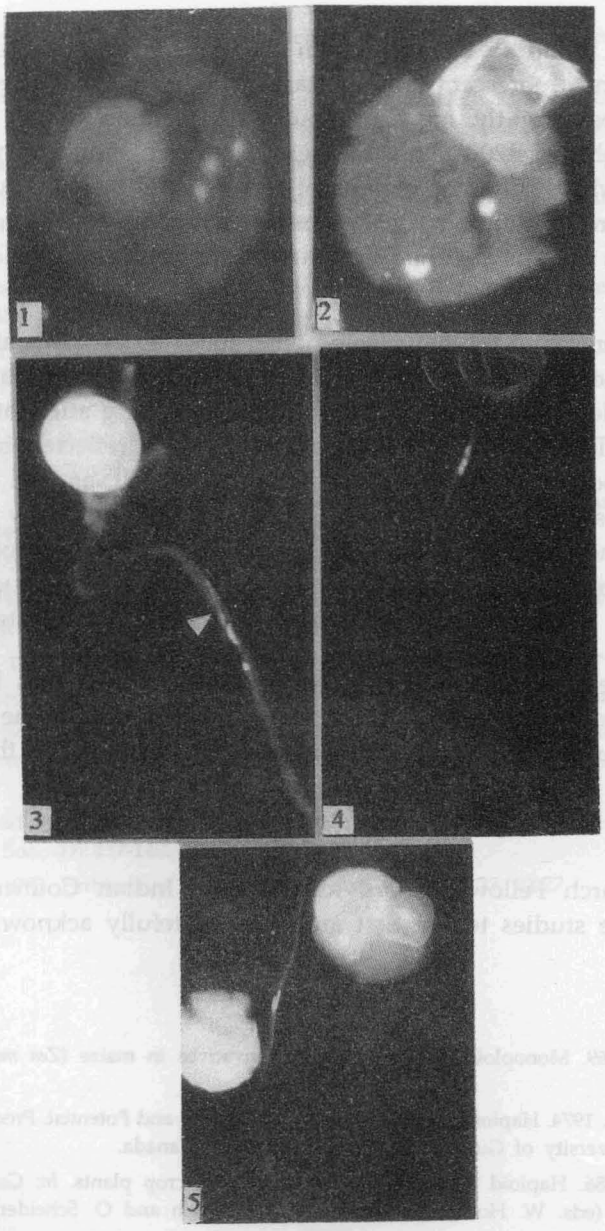

Fig. 1. Pollen grain of HIL showing three fluorescent nuclei. X150; Fig. 2. Pollen grain of HIL showing two fluorescent nuclei. X150; Fig. 3. Elongated, faintly stained vegetative nucleus (shown by arrow head) behind two male gametes in pollen tube of HIL. X150; Fig. 4. Three nuclei similar in shape and fluorescence intensity in pollen tube of control. X120; Fig. 5. Two fluorescent nuclei in the pollen tube of HIL. X200.

nucleus has been found to be peculiar in the pollen tubes stained with both the non- fluorescent and fluorescent nuclear stains in other plant species also [12, 15-19]. Mostly, it stains less brightly, has a pronounced elongation and an irregular outline. Wulff and Maheshwari [20] felt that these features, combined with the lack of uniformity with which different regions of this nucleus stain, should be taken into consideration before forming any conclusions. Even in the phase contrast, it has been difficult to see this nucleus as it has an unusual shape and lacks condensed chromatin [21].

2. The frequencies of the binucleate pollen tubes in the HILs and control inbred are high and almost equal (38.32% in the HIL and 39.32% in control line). Thus, even if one considered the tubes with two nuclei reflecting attenuation of the sperm nucleus, one would expect this serious anomaly to be reflected in ovule abortion, gap setting or production of haploids etc. in both the HIL as well as in the control. Since none of these effects are seen in the control but seen only in the HIL, it is interpreted that the detection of only two nuclei in many tubes of control and the HIL is due to weak stainability of the vegetative nucleus. It is much less likely due to the loss of the vegetative or the male nucleus in the binucleate tubes.

Based on these observations, it is concluded that anomalies like restitution in the division of generative nucleus, absence or disintegration of the male gametes in pollen grains or tubes are not the cause of haploidy induction in the HILs of maize.

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REFERENCES

1. S. S. Chase. 1969. Monoploids and monoploid derivatives in maize (*Zea mays* L.). Bot. Rev., **35**: 117-167.
2. K. J. Kasha (ed.). 1974. Haploids in Higher Plants : Advances and Potential. Proc. I Intern. Symp.,10-14 June, 1974, University of Guelph. University of Guelph, Canada.
3. C. J. Jensen. 1986. Haploid induction and production in crop plants. *In*: Genetic manipulation in Plant Breeding (eds. W. Horn, C. J. Jensen, W. Odenbach and O. Scheider). Walter de Gruyter, Berlin., 231-256.
4. K. J. Kasha and K. N. Kao. 1970. High frequency haploid production in barley. Nature., **255**: 874-875.
5. E. H. Coe Jr. 1959. A line of maize with high haploid frequency. Amer. Nat., **93**: 381-382.
6. K. R. Sarkar, S. Panke and J. K. S. Sachan. 1972. Development of maternal haploidy inducer lines in maize. Indian J. Agric. Sci., **42**: 781-786.

7. A. M. Aman and K. R. Sarkar. 1978. Selection for haploidy inducing potential in maize. Indian J. Genet., **38**: 452-457.
8. K. R. Sarkar, A. Pandey, P. Gayen, Jasbir Kaur Madan, Rajesh Kumar and J. K. S. Sachan. 1994. Stabilization of high haploidy inducer lines. Maize Genet. Coop. Newsl., **68**: 64-65.
9. E. H. Coe Jr. and K. R. Sarkar. 1964. The detection of haploids in maize. J. Hered., **55**: 231-233.
10. K. R. Sarkar. 1974. Genetic selection techniques for production of haploids in higher plants. *In*: Haploids in Higher Plants : Advances and Potential. Proc. I Intern. Symp., 10-14 June, 1974, University of Guelph (ed. K. J. Kasha). University of Guelph, Canada, pp 33-41.
11. K. R. Sarkar and E. H. Coe Jr. 1966. A genetic analysis of the origin of maternal haploids in maize. Genetics., **54**: 453-464.
12. A. W. Coleman and L. J. Goff. 1985. Application of fluorochromes to pollen biology. I. Mithramycin and 4', 6-diamidino 2-phenyl indole (DAPI) as vital stains and for quantification of nuclear DNA. Stain Technol., **60**: 145-154.
13. N. Pogna and A. Marzetti. 1978. Frequrency of two tubes in *in vitro* germinated pollen grains. Maize Genet. Coop. Newsl., **57**: 44.
14. D. S. Mathur, J. K. S. Sachan and K. R. Sarkar. 1976. Radiation induced haploidy and heterofertilization in maize. J. Nuclear Agric. Bio., **15**: 76-77.
15. R. B. Wylie. 1923. Sperms of *Vallisnaria spiralis*. Bot. Gaz., **75**: 191-202.
16. M. Magde. 1929. Spermatogenesis and fertilization in the cleistogamous flowers of *Viola odorata var. praecox. Gregory*. Ann. Bot. **43**: 545-577.
17. G. O. Cooper. 1936. Development of male gametes in *Lilium*. Bot. Gaz., **97**: 169-177.
18. T. H. Goodspeed. 1947. Maturation of gametes and fertilization in *Nicotiana*. Madrono., **9**: 110-120.
19. J. Heslop-Harrison and Y. Heslop-Harrison. 1984. The disposition of gamete and vegetative cell nuclei in the extending pollen tube of a grass species, *Alopecurus pratensis* L. Acta Bot. Neerl., **33**: 131-134.
20. H. D. Wulff and P. Maheshwari. 1938. The male gametophyte of angiosperms (a critical review). J. Indian Bot., Soc., **17**: 117-140.
21. W. A. Jensen. 1973. Fertilization in flowering plants. Science., **23**: 21-27.