A GENETIC ANALYSIS OF THE ORIGIN OF MATERNAL HAPLOIDS IN MAIZE[1]

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Received March 14, 1966

THE first case of haploidy in higher plants was reported in *Datura stramonium* (BLAKESLEE *et al.* 1922). Subsequently, haploid sporophytes have been discovered in many plant species; STADLER and RANDOLPH first reported haploidy in maize (1929 unpublished, cited by RANDOLPH 1932). Numerous investigations have been conducted on the occurrence of spontaneous and induced haploids in a number of species, but the mechanism of haploid origin remains elusive. Several comprehensive reviews and discussions on haploidy have appeared (GATES and GOODWIN 1930; IVANOV 1938; KOSTOFF 1942; KIMBER and RILEY 1963; MAGOON and KHANNA 1963).

A haploid embryo can arise through sporophytic development of a male gamete or unfertilized egg or other cell of the embryo sac. Androgenesis, development of the embryo from a male nucleus, can be detected easily by the use of suitable cytological or genetic markers, and its occurrence has been demonstrated in several species (MAGOON and KHANNA 1963). Androgenesis occurs very rarely in maize; a frequency of 1 per 80,000 fertilizations has been estimated (GOODSELL 1961; CHASE 1963).

Exact modes of origin of maternal haploids are difficult to establish by cytological and genetic analysis, but several postulates have been put forward (see IVANOV 1938; KOSTOFF 1942; MAGOON and KHANNA 1963). These include origination from the egg by incomplete fertilization, from other nuclei of the embryo sac by degeneration of the fertilized or unfertilized egg, and from other nuclei or other embryo sacs in connection with polyembryony. Little is known of the fate of male nuclei following entry and the initiation of maternal haploid development. Low frequency of occurrence (0.1% in maize, for example—CHASE 1949) makes critical cytological analyses of the entire process difficult to conduct.

Genotypic control of the frequency of maternal haploidy has been observed (see MAGOON and KHANNA 1963 for discussion). In maize, both female (CHASE 1952) and male parents (CHASE 1952; COE 1959) are known to influence haploid frequency. Identification of a high haploidy line in maize, designated "stock 6"

[1] Contribution from Crops Research Division, Agricultural Research Service, United States Department of Agriculture, and Field Crops Department, Missouri Agricultural Experiment Station, Journal Series No. 3054. Aided by Grant No. GB-1079 from the National Science Foundation to EHC. Adapted from portions of a Ph.D. thesis in Genetics presented to the Graduate School of the University of Missouri by KRS.

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(COE 1959), which produced 3.23% haploids in selfed progeny, has provided material for investigations into the genotypic control of haploidy, and a useful device for obtaining haploids for experimental purposes. An efficient screening technique for identification of haploid embryos in dry seed (COE and SARKAR 1964) has allowed us to undertake a genetically marked study of some of the mechanisms involved in the origin of maternal haploids.

MATERIALS AND METHODS

The female parent in all crosses carried *C C* (colored aleurone). The strains used were stock 6 (the high haploidy line), hybrid 6 × 3 (stock 3 is a line with low haploid frequency), and a backcross of the hybrid to stock 6 (designated 6/6/3). The 6/6/3 parent was homozygous *gl* (glossy seedling) and heterozygous for markers in each chromosome, a strain obtained by crossing a 6/3 hybrid of genotype *bm*$_2$, *lg*, +/*a*, *su*, *pr*, *y*, *gl*, *j*, *wx*, +/*g* with *gl* stock 6. Male parents were all *C*^{I} *C*^{I} (color inhibitor), derived from first generation backcrosses of a *C*^{I} stock to stocks 3 and 6, and the F_1 between the two derived stocks. The genotypes and descriptions of the stocks used are presented in Table 1.

The method used for identifying haploids in the dry seed stage has been described elsewhere (COE and SARKAR 1964). Kernels with colored-scutellum embryos (maternal embryos) recognized in dry seed examination of *C C* × *C*^{I} *C*^{I} crosses were planted in the field for observation. Most of these produced haploid seedlings, rare exceptions being diploid. The haploids were identified by their distinctive morphology (COE 1959; CHASE 1964b), by the *gl* marker (when 6/6/3 was used as female parent), and by stomatal guard cell measurements. Average guard cell length for haploid plants was about 30 microns as compared with 40 microns for diploids.

Since some of the factors controlling pigmentation of the scutellum (SPRAGUE 1932) were not homozygous in the material used, the criterion of colored scutellum alone could not be used to determine over-all haploid frequencies. For this purpose stock 6/6/3, carrying the seedling marker *gl* in homozygous condition, was used. All the seeds, after screening for scutellum color, were germinated in the sand bench, and the glossy seedlings were sampled for epidermal guard cell measurements before transplanting to the field.

Genomic constitution of the endosperms was determined by observing the number and distribution of colored spots due to loss of *C*^{I} on the kernels (COE 1962). The number of spots was counted at 25× magnification for a portion of the cases and for control populations from *C C* × *C*^{I} *C*^{I} and *C*^{I} *C*^{I} × *C C* crosses.

For one set of crosses, frequencies of haploids were determined separately in top and bottom halves of the ear to test for possible effects of attenuation during pollen tube growth.

Twin embryos were sought to examine the relation between haploidy and polyembryony. Genotypes of diploids among the twins, for markers derived from the heterozygous 6/6/3 maternal parent, were determined by selfing and crossing to the multiple tester line.

TABLE 1

Description and genotypes of the stocks used

Brief symbol	Stock background	Genotype
6, 6/3	6, 6 × 3	*C C Pr Pr*
6/6/3	(6 × 3) × 6	+/*bm*$_2$, +/*lg*, +/*su*, +/*pr*, +/*y*, *gl*/*gl*, +/*j*, +/*wx*, *A*/*A* or *A*/*a*, *G*/*G* or *G*/*g*; *C*/*C*
C^I, 3, 6, 6/3	3, 6, 6 × 3	*C*^{I} *C*^{I} *wx wx y y*
Testers	6 × 3 or (6 × 3) × 6	*bm*$_2$ *lg a su pr y gl j wx g* (MANGELSDORF's tester)

TABLE 2

Frequency of colored scutellum cases in different C C × C^I C^I *crosses (in percent; number of seeds examined is indicated in parentheses)*

Female parent	Male parent		
	C^I, 3	C^I, 6/3	C^I, 6
6/3	0.04 (4,603)	0.40 (20,336)	0.46 (3,056)
6/6/3*	.....	0.87 (20,728)	0.99 (29,175)
6	0.47 (2,737)	3.05 (5,217)	2.94 (34)

* Corrections have been made for plants segregating *A a* in the population.

RESULTS

In crosses involving stock 6 and stock 3 and derivatives, the yields of kernels with colored scutellum showed a definite trend (Table 2). The data were not subjected to statistical analysis because the maternal parents were not homozygous for all the factors responsible for the production of color in the scutellum. Thus, not all the haploids were recognized. Since no other maternal marker was universally present for further screening of haploids in this experiment, the results have been expressed in terms of yields of kernels with colored scutellum, rather than as true haploid frequencies. Influences of both parents on the rate of occurrence of maternal haploids as detected by scutellum color were clearly involved. In all crosses, as the proportion of stock 6 increased in the female, haploid frequency increased sharply. This suggests maternal genotypic control of high haploid-yielding ability in stock 6. Although the male gamete does not contribute directly to the maternal haploid embryo, when pollen of stocks 6/3 and 6 was used the frequency of haploids was greater than when stock 3 was used.

Stock 6 has no comparable influence on the production of androgenetic haploids. In a study of material genetically marked for scutellum color and glossy seedling, 57,953 kernels were examined for androgenetic haploids; none was found. Selfed progeny of stock 6 show about a 30-fold increase in maternal haploidy over the standard rate in corn inbreds; on the basis of a comparable increase in paternal haploidy, 22 cases would have been expected.

Using the seedling character, *gl*, in the 6/6/3 female parent, actual haploid frequencies were determined for individual males of C^I, 6 and C^I, 6/3 sources (Table 3). Heterogeneity of the group involving stock 6 males was highly significant, indicating variability in the haploid-inducing potential of the individual pollen parents. In Figure 1, the distribution of the ears according to percent haploidy is presented graphically; there was a much greater spread for stock 6 than for 6/3 males. Haploid frequencies as high as 9.14% were registered, although the mean was only 2.67%. Variability was not so pronounced with stock 6/3 males; the heterogeneity chi-square was significant only at the 5% level. This difference between the two types of males might be interpreted to indicate that stock 6, by virtue of its higher haploid-inducing potential, will reveal more of the variability on the female side. Stock 6/3 may be more stabilized because

TABLE 3

Effects of individual male plants on maternal haploid frequencies in gl, C C, *6/6/3* × C^{I} C^{I} *crosses*

Cross	Genotypes	No. of ears	No. of kernels	No. of haploids	Percentages of haploids		χ^2§
					Range	Mean	
70 × 68.2–1	6/6/3 × *C^{I}*, 6	6	1,362	55	2.37–7.41	4.04	9.77**
× 68.2–2		16	3,861	104	0.00–7.02	2.69	0.01
× 68.2–3		4	1,185	28	1.24–3.52	2.36	0.42
× 68.2–5		14	3,361	95	0.41–7.89	2.83	0.32
× 68.2–6		15	3,456	60	0.56–3.42	1.74	11.61**
× 68.3–1		6	1,470	23	0.77–2.70	1.56	6.88**
× 68.3–2		11	2,229	70	0.79–4.62	3.14	1.86
× 68.3–3		10	2,318	61	0.99–4.26	2.63	0.01
× 68.3–5		10	2,552	72	0.00–5.52	2.82	0.23
× 68.3–7		13	2,750	112	2.38–9.14	4.07	20.86**
× 68.3–8		14	3,822	81	0.70–4.45	2.12	4.44*
× 68.3–9		4	631	14	0.00–6.45	2.22	0.48
Total:		123	28,997	775	0.00–9.14	2.67	$\chi^2_h = 56.89$**†
70 × 69.2–2	6/6/3 × *C^{I}*, 6/3	13	3,788	102	0.59–4.61	2.69	5.33*
× 69.2–3		17	6,579	129	0.94–3.42	1.96	1.11
× 69.2–4		6	1,854	44	1.57–3.40	2.37	0.43
× 69.2–7		18	5,659	121	0.00–4.46	2.14	0.00
× 69.2–8		10	2,774	49	0.97–3.33	1.77	1.89
Total:		64	20,654	445	0.00–4.61	2.15	$\chi^2_h = 8.76$*‡

* Significant at 5% level. ** Significant at 1% level.
† df = 10.
‡ df = 3.
§ Expected number was calculated from mean frequency of haploids from all the crosses involving the same male family.

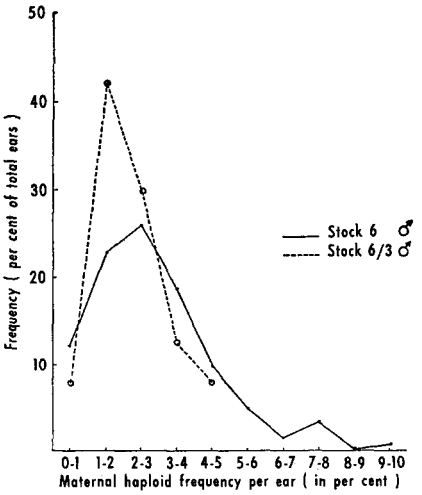

FIGURE 1.—Distribution of ears from stock 6/6/3 under different classes of maternal haploid yields. Twelve plants from stock 6 and five from 6/3 were used as pollen parents.

TABLE 4

Frequency distribution of kernels according to number of colored spots due to loss of C^{I} *in the endosperm*

	Number of colored spots															No. of seeds examined
	0	1	2	3	4	0–4	5–9	10–14	15–19	20–24	25–29	30–34	35–39	40–44	45–49	50–54	
Kernels from $C^I C^I \times C C$	84	9	5	1	1	100	0	0	0	0	0	0	0	0	0	0	100
Colorless scutellum kernels from $C C \times C^I C^I$	1	0	0	3	5	9	24	41	49	31	22	16	6	2	0	0	200
Colored scutellum cases from $C C \times C^I C^I$	2	2	3	2	3	12	36	50	41	39	36	14	3	8	2	4	241

it derived only half its genetic material from stock 6. Since the inducing character is subject to quantitative modification, there may be further scope for selection of higher haploidy lines from stock 6.

Numbers of colored spots due to C^I loss followed different patterns in $C\,C\,C^I$ and $C\,C^I\,C^I$ endosperms (Table 4). With two doses of C^I, colored spots appeared in only 16% of the seeds and the number of spots did not exceed four in any kernel. On the other hand, only 4.5% of the kernels with one dose of C^I in the endosperm showed 4 or fewer spots. Among 241 kernels with colored scutellum from $C\,C \times$

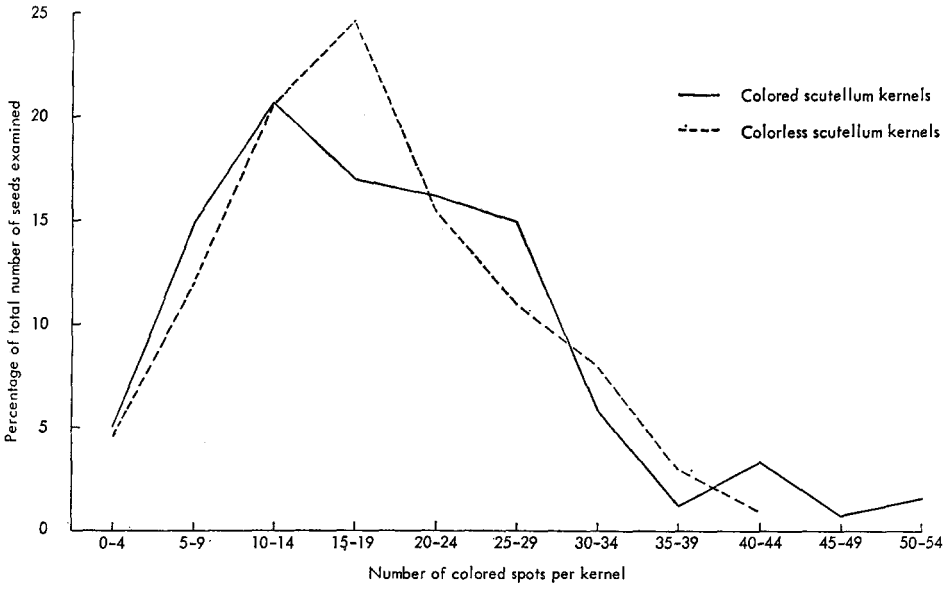

FIGURE 2.—Frequency distribution of colored spots due to loss of C^I on colorless endopserm from $C\,C \times C^I\,C^I$ cross, for kernels with colorless scutellum (diploid embryo) and with colored scutellum (haploid embryo).

C^I C^I crosses, only 12 showed four or fewer spots per kernel. The frequency distribution of spots due to C^I loss in those kernels bearing haploid embryos followed a pattern strikingly similar to that obtained from a population of C C C^I kernels (Figure 2). Since the appearance of colored spots on kernels bearing two doses of C^I depends on simultaneous loss of both C^I genes, kernels bearing haploid embryos from the C C × C^I C^I cross must have only one C^I in the endosperm, and hence only one complement from the male.

Standard procedure for hand pollination in corn includes clipping off the silks 1 to 2 cm above the ear tip and allowing a short brush of silks to grow out overnight, for convenience in pollination. Silks from the ovules near the base of the ear will be considerably longer than those from the tip, and pollen tubes will require more time and growth to reach the base than to reach the tip. RANDOLPH (1936) observed that the time interval from pollination to fertilization was approximately 16 hours for silk lengths of 3 to 5 cm, and 23 hours for silk lengths of 15 to 18 cm. If attenuation (reduced capacity to function) of one of the sperms during pollen tube growth contributes to haploid frequency, the bottom half of the ear should contain more haploids than the top half. Data on frequencies of haploids in top and bottom halves of the ear are presented in Table 5 for 6/3 maternal parent with 3, 6, and 6/3 males, and for stock 6 with 3 and 6/3 males. On 6/3, which produces larger ears having a greater distance from tip to base than stock 6, there were more haploids in the top half than in the bottom half with all males. On stock 6, little difference was found between top and bottom halves. In the over-all analysis, no significant difference was observed between top and bottom, although more haploids were obtained from top halves of the ears.

Stimulation of one of the synergids into development following normal fertilization of the egg may be a source of maternal haploids (KOSTOFF 1942; COOPER 1943). This situation would primarily result in a haploid-diploid twin pair in which the diploid member would be a hybrid of the two parents and the haploid member would be maternal. Death or arrest in growth of the hybrid embryo

TABLE 5

Distribution of haploids identified by colored scutellum method on the ears from C C × C^I C^I *crosses*

Cross	Top half of the ear			Bottom half of the ear			Over-all frequency (Percent)	χ^2-test P =
	Kernels examined (No.)	Colored scutellum cases (No.)	Haploids (Percent)	Kernels examined (No.)	Colored scutellum cases (No.)	Haploids (Percent)		
6/3 × C^I, 3	2,283	2	0.09	2,320	0	0.00	0.04	.10–.25
× C^I, 6	1,501	14	0.93	1,531	0	0.00	0.46	< .005
× C^I, 6/3	10,097	50	0.50	10,239	32	0.31	0.40	.025–.05
Subtotal	13,881	66	0.48	14,090	32	0.23	0.35	< .005
6 × C^I, 3	1,329	3	0.23	1,297	10	0.77	0.50	.025–.05
× C^I, 6/3	2,405	73	3.04	2,530	78	3.08	3.06	.75
Subtotal	3,734	76	2.04	3,827	88	2.30	2.17	.25–.50
Total	17,615	142	0.81	17,917	120	0.67	0.74	.10–.25

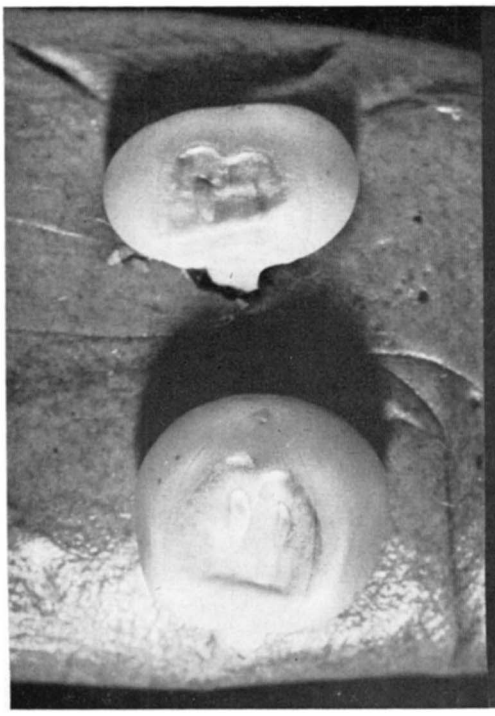

FIGURE 3.—Kernels with twin maternal haploid embryos.

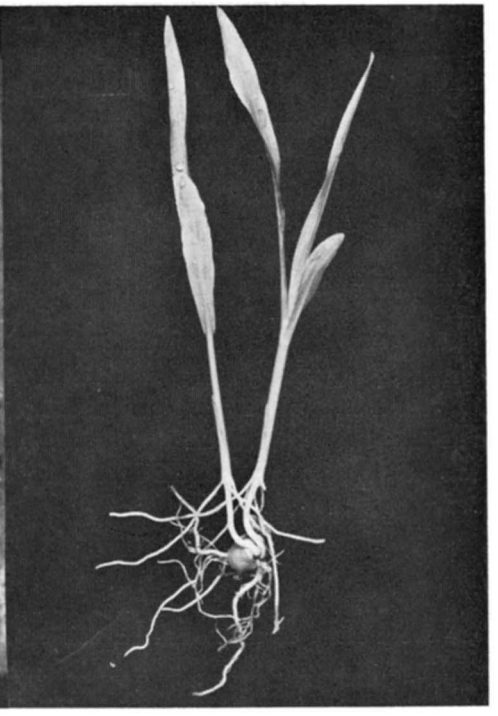

FIGURE 4.—A maternal (glossy) haploid-haploid twin seedling selected from sandbench plantings.

could, however, result in a maternal haploid embryo only. From the crosses of *gl*, multiple marked, *C C* females with C^I C^I males, 49,903 kernels were carefully examined for the presence of twin embryos. Kernels with two embryos showed either both colored (Figure 3) or both colorless scutellums. The remaining seeds were planted thinly in the sand bench to allow detection of twin seedlings (Figure 4). All of these were then grown to maturity. In all, 49 twin pairs were obtained, and could be classified as follows: diploid-diploid, 32; haploid-haploid, 15; haploid-diploid, 2.

The haploid member of both haploid-diploid pairs did not survive to maturity. Their haploid nature and maternal origin were confirmed by stomatal guard cell measurements and presence of glossy seeding (*gl*) character, respectively. The diploid members of the two pairs could be progeny tested by selfing and crossing to a multiple tester line. One proved to be a *C* C^I hybrid, segregating markers from both parents. The other was found to be of entirely maternal origin from the heterozygous female, and showed homozygosity at all loci for the markers used. This plant might have originated from doubling of the egg or union of two reduced members of the egg apparatus.

Both members of those haploid-haploid twin pairs that survived were observed for phenotypic expression of the genes bm_2, *lg*, *j*, and *g*, and in all the cases the

two members of a pair completely corresponded with each other. It can be concluded that each pair originated from a single embryo sac, but whether they resulted from an early cleavage of a haploid embryo or by simultaneous development of two cells of the egg apparatus could not be ascertained. Cleavage is suggested by the fact that many pairs were developmentally identical and mutually oriented.

The diploid-diploid pairs were of hybrid origin as evidenced by nonglossy phenotypes of the seedlings. Selfed as well as testcrossed progenies from both members of a pair could be obtained from 11 diploid-diploid pairs. The two members of each twin pair showed complete genotypic correspondence. They must each have originated from a single embryo sac, most likely by early embryonic cleavage, or alternatively, as a result of fertilization of two reduced female cells in the embryo sac by two sperms. These observations on twinning indicate that stimulation of one of the synergids following fertilization is not likely to contribute substantially to the rather high over-all maternal haploid frequency of 2.45% in the crosses.

DISCUSSION

Previously reported influences of both pollen and seed parents in controlling the frequency of maternal haploidy have been substantiated in these studies. Male parent effects on the induction of haploidy have been observed previously (FORTUNO 1948; CHASE 1949; GERRISH 1956; COE 1959); variation in haploid frequency due to different seed parents was demonstrated by CHASE (1949, 1952) with a uniform pollen parent. STADLER (1940) and CHASE (1949) observed that the differential haploid frequencies of inbreds tend to be transmitted to their crosses. Transmission of the high haploidy potential of stock 6 is clearly established here by the results of tests involving stock 6 and its derivatives with a low haploidy line, stock 3, in various combinations. When a comparison is made between seed parents of 6/3, 6/6/3 and 6 with a common pollen parent, haploid frequency increases as the stock 6 component of the female line increases. A similar gradient due to pollen parent is found. The variability in stock 6, C^I male itself suggests quantitative variation in the high haploidy potential, and indicates the possibility of further increase in haploidy frequency by selection in stock 6.

Elucidation of the mechanism of haploid induction is of importance for practical purposes, and for understanding the fundamental processes of fertilization. A key to the problem presumably lies in determination of the fate of the sperm destined to fertilize the egg cell. One possibility has been that both the sperm participate in fertilizing the polar nucleus, producing a tetraploid endosperm and a haploid embryo. GAINES and AASE (1926), for example, obtained a haploid from a large, plump seed from the cross *Triticum compactum* × *Aegilops cylindrica*, which usually produces shriveled small seeds, and concluded that possibly both the male gametes fused with the polar nuclei. Cytological data compatible with this interpretation have been given by WANGENHEIM *et al.* (1960) in 4n × 2n potato crosses. However, the present studies clearly establish that maize kernels bearing a haploid embryo have triploid endosperms (one set of chromosomes

from the male), ruling out union of both sperms with the polar nuclei. A similar observation has been made by CHASE (1964a), using dosage effects of *Y* and R^{st} in the endosperm. Restitution in the division of the generative nucleus resulting in a single diploid sperm, which might fertilize the polar nuclei and cause the egg to develop alone, could also be ruled out conclusively on the same grounds. Recently, cytological examination by J. VENKATESWARLU (personal communication; conducted at the University of Wisconsin) of 3,830 pollen grains of stock 6 showed the normal 3-nucleate situation in all of the grains. These observations eliminate monospermy (n or 2n) as a cause of haploid induction in this strain.

Destruction or attenuation of one of the sperms in the pollen grain or tube might be considered to contribute to haploid frequency in the experimental production of haploids by physical treatments (for review see MAGOON and KHANNA 1963). However, this is not the situation in the spontaneously arising haploids in maize, since the bottom half of the ear (attenuated sperms) failed to show any increase in haploid frequency over the top half. To explain increased haploidy in delayed pollination, SMITH (1946) suggested that haploid embryos begin to develop during the period of delay, so that when pollination provides an endosperm, a seed with a haploid embryo develops. This view does not receive support in maize from our observation that the receptive ovules in the bottom half of the ear, which are mostly more advanced in maturity than receptive ovules in the top half, actually produced fewer haploids.

KOSTOFF's postulate (KOSTOFF 1942) suggests that a great many haploids originate from haploid-diploid twins, where normal fertilization produces the diploid member and a haploid cell of the embryo sac produces the haploid member of the pair. This postulate is supported by a cytological demonstration of stimulation of a synergid following normal fertilization (COOPER 1943), and by the preponderance of haploid-diploid twins in polyembryony in a number of plant species (KIMBER and RILEY 1963; MAGOON and KHANNA 1963; OWINGS *et al.* 1964). In maize, however, this explanation is untenable, as no relation between haploidy and occurrence of haploid-diploid twins could be observed. Only two haploid-diploid twins were observed in our studies as compared to 1,220 haploids from the same population, and only one of these two contained a hybrid diploid member. Similar observations on low occurrence of haploid-diploid twins in maize have been noted by others (MORGAN and RAPPLEYE 1951; JONES 1953). If stimuation of synergids along with normal fertilization, followed by subsequent death or slowing down in development of the diploid zygote, contributed heavily to spontaneous haploids, more haploid-diploid twin pairs would be expected in the population. Moreover, it seems reasonable to assume that the chances of survival of the diploid member of a haploid-diploid pair are greater than those of the haploid member, except perhaps when the source is an interspecific cross. The possibility of zygotic incompatibility leading to death of the hybrid embryo and stimulation of one of the other components of the embryo sac (most likely a synergid) cannot be completely ruled out on the basis of present evidences, however. Whether syngamy is absolutely necessary for initiation of any kind of embryo development is not known definitely. There is some indication that fusion

of the male gamete with the egg cell is not essential for stimulation of a reduced female cell to develop parthenogenetically (JORGENSEN 1928; SMITH 1946). Occurrence of androgenetic haploids in maize also indicates that embryo development may initiate without stimulation from syngamy (assuming that only two sperms enter and are available, one being involved in endosperm formation and the other in the embryo).

From the foregoing results and discussion, maternal haploidy in maize is concluded to result primarily from failure of fertilization caused by an abnormal condition, either inherent or induced, in a male or female gamete, and subsequent development of the reduced egg into the embryo. The reciprocal event, failure of fusion of the male gamete with the polar nuclei, is not expressed because fertilization of the endosperm nuclei is a prerequisite for proper seed development. That the fertilization abnormality is not simply an accident but is under genetic control is shown by inherited differences in haploid yielding potential for both male and female parents. Cytological studies might well reveal the exact mechanism, but material with higher haploid frequency would be necessary for such studies.

We appreciate the stimulating interest of J. B. BECKETT in this study; suggestions on the manuscript by J. R. LAUGHNAN; and permission of J. VENKATESWARLU to cite his unpublished observations on nuclear contents of pollen of stock 6.

SUMMARY

Genotypic influences of both male and female parent on the frequency of maternal haploidy in maize have been confirmed. Transmission of the high potential for haploid induction of an inbred line, stock 6, was demonstrated in crosses involving parents containing varying genomic proportions of stock 6 and stock 3, a low haploidy line. The variation in haploid frequency obtained among individual males of stock 6 background suggests that sublines with even higher haploid-inducing potential could be derived.—Employing the criterion of loss of the C^I allele (dominant color inhibitor) in the endosperm tissue from crosses involving $C\ C$ and $C^I\ C^I$ parents, it was clearly demonstrated that kernels bearing haploid embryos have endosperms bearing only one male genomic complement. The fate of the second sperm has not been determined, but it is clear that maternal haploids do not arise from fusion of both sperms with the polar nuclei, nor from monospermy.—Sperms must travel a longer distance and time to reach the ovules at the base of the ear than at the top. However, haploid frequency was equal, or lower in the bottom half, indicating that destruction or attenuation of one of the sperms during pollen tube growth is not a major contributing factor in the production of haploids. Similarly, it was concluded that the more advanced ovules in the bottom half are not disposed to production of more haploids through delay causing the egg to start developing into an embryo.—Stimulation of a synergid following regular fertilization of the egg would result in a haploid maternal embryo; a haploid-diploid twin pair or a single maternal haploid embryo could result depending on the viability of the zygotic embryo. Only one twin pair (out

of 49 twins found in a marked cross from which 1,220 haploids arose) was a haploid-diploid hybrid, lending no support to a stimulation hypothesis for the origin of haploids in maize.—Normal fertilization of the polar nuclei to give a triploid endosperm, concurrent with stimulation of the unfertilized egg to divide, is considered to give rise to most maternal haploids in maize; the fate of the remaining sperm nucleus is undetermined. Genotypes of both the male and female parents influence the occurrence of the incomplete fertilization. Embryological studies are required to clarify some of the problems further.

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