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Identification of Cotton Haploids by Stomatal Chloroplast-count Technique[1]

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ABSTRACT

Chloroplast counts were made from the stomatal guard cells in haploid (n = 2x = 26) and in natural tetraploid (2n = 4x = 52) plants of *Gossypium hirsutum* L. and *G. barbadense* L. The mean chloroplast number was 10.46 ± 2.18 for haploids and 21.00 ± 3.31 for tetraploids. The means from haploid plants did not overlap with the means from tetraploid plants in 280 plants evaluated. Cotton plants can be classified much easier and quicker from the cotyledon stage to maturity as haploids or tetraploids and as accurately as by chromosome counts.

Additional index words: **Androgenesis, Chromosome number count, Guard cells, Plastids, Ploidy, Semigamy, *Gossypium hirsutum* L., *G. barbadense* L.**

HAPLOIDS have aroused great interest among plant scientists and have received increased attention from genticists and plant breeders due to recent developments that have enabled their production at will in some species. Haploids have extensive use in genetic, cytogenetic, physiological, and breeding research (1).

The chloroplast-count technique has been used as a rapid method of determining ploidy levels in some plants at any stage of plant growth. It has been applied to alfalfa, *Medicago sativa* L., (3); mulberry, *Morus* spp., (8); potato, *Solanum tuberosum* L., (7); red clover, *Trifolium pratense* L., (5); white clover, *T. repens* L., (11); sugarbeet, *Beta vulgaris* L., (10); and turnip, *Brassica rapa* L., (13). It was found efficient and accurate in the screening of large populations of seedlings for different ploidy levels.

Hamada and Baba (8) in 1930 investigated the number of stomatal chloroplasts in several cultivars of mulberry and were the first to point out that the guard cells of cultivars with higher chromosome numbers tended to contain more plastids. In sugarbeet, a relationship was found between the number of plastids in the guard cells and the number of chromosomes (4, 5, 6, 10). Mochizuki and Sueoka (10) observed a significant increase in number of chloroplasts in guard cells with the increase in ploidy levels and reported 14, 20, and 26 plastids/stoma in 2x, 3x, and 4x sugarbeets, respectively. Dudley (6) counted 6.6 and 11.8

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as average numbers of chloroplasts/stoma for diploid and tetraploid beets, respectively. This average was a 79% increase in number of plastids with doubling of chromosome numbers.

For *Trifolium* species, differences in plastid numbers were reported by Butterfass in 1960 (5) and later investigated by others (11, 12). Butterfass (5) showed that the number of plastids increased by 76% in tetraploid clover and by 54% in *T. hybridum* L. by doubling the chromosome number. Nuesch (12) reported in 1966 that the guard cells and other cells in the lower epidermis of tetraploid red clover leaflets contained 80% more chloroplasts than those in diploids, at all stages of growth. He recommended using chloroplast counts for rapid screening of large seedling populations for ploidy levels. In 1968, Najcevska and Speckman (11) studied chloroplasts of different levels of ploidy in red clover, white clover, and berseem (*T. alexandrinum* L.). They observed an increase in number of stomatal plastids with doubling of chromosome numbers. The magnitude of increase was 61% in white clover and berseem and 73% in red clover.

Frandsen (7) observed 12, 16, and 22 plastids, respectively, in the stomata of haploid, triploid, and tetraploid potatoes. He concluded that preliminary screening of haploids and triploids could be done effectively by chloroplast counts. In studies on chloroplasts of guard cells of *S. bulbocastanum* Dun. and *S. acaule* Bitt., Hermsen and De Boer (9) noticed a significant increase of 67% in chloroplast numbers from 2x to 4x. Speckmann, Dikstra, and Ten Kate (13) identified diploid and tetraploid plants of turnip by chloroplast counts. They found 11.4 and 19.3 chloroplasts/stoma in 2n and 4n plants, respectively. Bingham (3) reported respectively 8, 9.2, 9.4, 12.8, and 15.2 chloroplast/stoma in haploid, diploid, triploid, tetraploid, and hexaploid alfalfa. He suggested that this technique could be used as early as the seedling stage to screen alfalfa populations for desired ploidy levels and restrict chromosome counting to verification of selected plants.

In cotton (*Gossypium* spp.), haploids can be produced in most strains by semigamy (2, 15, 17). The semigametic phenomenon was described by Turcotte and Feaster (15, 16, 17) as an abnormal fertilization process in which the male and female gametes fail to fuse. They subsequently divide, independently forming a monoembryonic seed, chimeral for haploid maternal and paternal tissue. In practice, the semigametic parent is used as female and contains the virescent foliage marker v_7v_7. Haploids can be synthsized from most normal strains of cotton, usually of the green foliage color V_7V_7, when the green plants are crossed as male with the semigametic strains. Most of the progeny of such a cross are green hybrid seedlings, V_7v_7. Chimeral seedlings resulting from semigamy are observed and selected for green haploid tissue V_7. These male tissue haploid chimerals are grown and treated with colchicine to produce homozygous strains for breeding and genetic research.

The objectives of this study were i) to estimate the number of stomatal chloroplasts in cotyledons and leaves of haploid and natural tetraploid cotton plants and ii) to determine if the chloroplast-count technique could be used to identify these two levels of ploidy so that the efficiency of haploid production by semigamy could be increased.

MATERIALS AND METHODS

Three strains of cotton, VSg, DH 57-4, and VSg × (DP 16 × 9450), each with two levels of polyploidy, haploid (n = 2x = 26) and tetraploid (2n = 4x = 52), were studied. VSg and DH 57-4 belong to the species *G. barbadense* L. and produced more than 50% haploids along with tetraploids by semigamy in their selfed progenies (14, 15, 16, 17). These two strains provided the respective haploid and tetraploid plants used in this work. The DP 16 × 9450 cross provided an intraspecific hybrid of *G. hirsutum* L. which when crossed with VSg provided tetraploid hybrids of *G. barbadense* and *G. hirsutum* and chimeral haploid seedlings of *G. hirsutum* in the F_1 generation. Some of the chimeras ultimately gave rise to green haploids. The hybrids and green haploids used for observations came from this cross.

All the plants were grown and observed in the greenhouse or under a light bench to accelerate germination and growth. The seeds of all three strains were germinated in peat pellets in trays in the greenhouse. Some of the VSg plants were grown on a lighted table. The cotyledons were collected after 20 days for chloroplast counts. The seedlings were then transplanted to 20.3-cm pots with a mixture of 1/3 peat moss, 1/3 sand, and 1/3 soil. After two months, two mature leaves were collected for additional chloroplast counts.

Chloroplasts were counted from guard cells in the stomata on the abaxial surface of leaves. The lower epidermis was stripped from the middle of lamina. We avoided the midrib and large veins by twisting and tearing the leaf blade by hand. Then the strips of epidermis were placed on a glass slide, removed from the attached leaf with a razor blade, mounted in a drop of 1:2 iodine-potassium iodide solution (1 g iodine and 2 g potassium iodide dissolved in 100 ml distilled water), covered, and examined immediately.

Data for analysis of chloroplast numbers were collected from four groups of plants, namely, VSg under light bench (VSg*), VSg, DH 57-4, and the cross VSg × (DP 16 × 9450). Each group had 10 haploid and 10 tetraploid plants. Two cotyledons and two mature leaves were taken from each plant, except from the green haploids, on which cotyledons were not available. The guard cells of five stomata were examined from each leaf and cotyledon for the number of chloroplasts. Thus, there were 15 entries from all groups on the basis of cotyledons, leaves, haploids, and tetraploids. In all, 1,500 stomata and 40 haploid and 40 tetraploid plants were examined. The average number of chloroplasts per stoma for haploid and tetraploid was determined on the respective basis of 700 and 800 stomatal observations.

The data were analyzed statistically by one-way analysis of variance to determine the variation in chloroplast numbers between and within the entries as well as groups.

In addition, 200 seedlings from a semigametic parent were identified as haploids and tetraploids by the chloroplast-count technique. They were vertified at maturity by morphological features that readily identify the haploids from the normal tetraploids, such as size and appearance of vegetative and floral parts and male sterility indicated by indehiscence of anthers.

RESULTS AND DISCUSSION

The mean number of chloroplasts in the guard cells of haploid plants was 10.46 and of tetraploid plants, 21.00 (Fig. 1). This difference was highly significant (Tables 1 and 2).

The ratios of chloroplasts in haploids to tetraploids was 1:2, the same ratios as their chromosome numbers 26:52. This difference was comparable to ratios reported for sugarbeets (5, 6, 10) and slightly greater than the ratios reported for *Trifolium, Solanum,* and *Brassica* species. These results indicate that the chloroplast technique compares with the root-tip technique in accuracy. The root-tip method requires several hours and numerous techniques for tissue prepara-

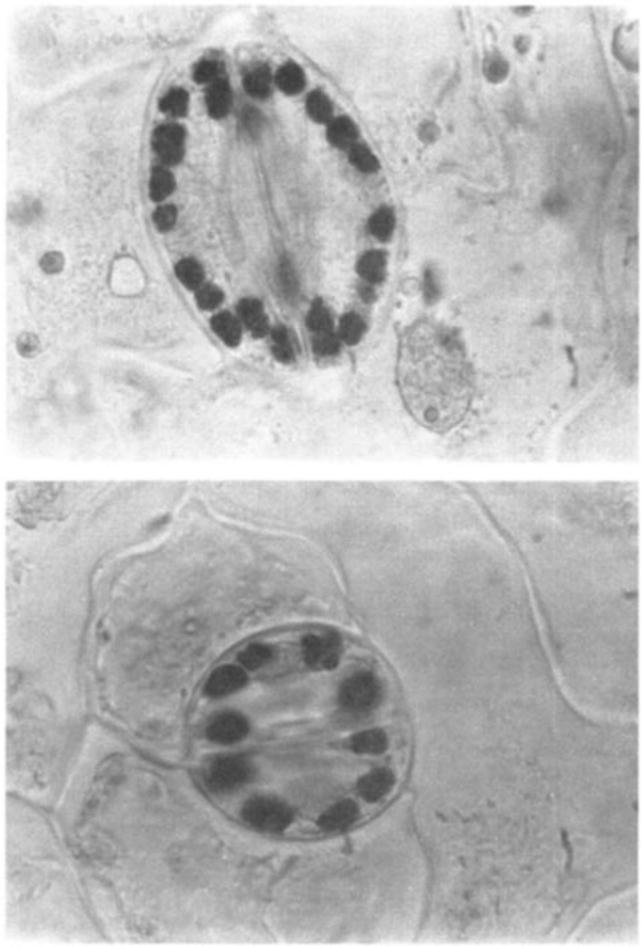

Fig. 1. Contrast in chloroplast number of haploid (lower) and diploid (upper) guard cells × 1,350.

tion for chromosome counts. In cotton, it is difficult with known techniques to get good spreads of the mitotic chromosomes in root-tips because of stickiness and clumping. The chloroplast technique requires less skill and takes about 5 minutes for the preparation and count.

Other significant differences in chloroplast counts in this study were observed between cotyledons and leaves, within haploids and tetraploid plants, and among 2n strains. These differences, however, were subtle and did not affect the 1:2 ratio of chloroplasts in any comparison (Table 1).

The stomatal-chloroplast technique was used to screen 200 progeny from a semigametic parent. The semigametic strain normally produces 50% or more haploids with its progeny. Of the 200 seedlings, 109 were classed as tetraploid, 69 haploid, and 22 chimeral, with some sectors being tetraploid and others haploid. All seedlings were marked, grown to maturity, and classified by morphological differences and sterility. All haploid and tetraploid plants were classified correctly. The chimeral plants for haploid and tetraploid tissue rarely remained chimeral; they became either haploid or tetraploid at an early stage, and the mature plant parts were either haploid or tetraploid. This behavior was consistent with our observations among all semigametic progenies.

Table 1. The means, standard deviations, and plant mean ranges of stomatal chloroplasts in cotyledons and leaves of haploid and tetraploid plants.

Plant group	No. of plants	Cotyledons or leaves	Ploidy level	No. of stomata	Mean of plastids	Plant mean ranges
VSg†	10	2 Cotyl-LT	n	100	11.09 ±2.38	10.2-12.5
		2 Leaves	n	100	10.36 ±2.08	9.4-11.8
	10	2 Cotyl	n	100	10.77 ±2.09	10.2-11.5
		2 Leaves	n	100	10.44 ±2.21	9.8-10.9
DH 57-4	10	2 Cotyl	n	100	10.64 ±2.47	9.9-11.5
		2 Leaves	n	100	10.10 ±2.06	9.4-10.8
GH	10	2 Leaves	n	100	9.80 ±1.77	9.3-10.4
Total	40	6 Cotyl	n	300	10.83 ±2.30	9.9-12.5
		8 Leaves	n	400	10.17 ±2.04	9.3-11.8
VSg*	10	2 Cotyl-LT	2n	100	22.72 ±4.28	20.0-24.6
		2 Leaves	2n	100	20.66 ±3.27	19.3-22.2
VSg	10	2 Cotyl	2n	100	20.67 ±2.92	19.0-21.9
		2 Leaves	2n	100	20.05 ±2.49	18.6-21.0
DH 57-4	10	2 Cotyl	2n	100	22.27 ±3.31	20.4-24.1
		2 Leaves	2n	100	20.36 ±2.71	19.2-21.8
Hybrids	10	2 Cotyl	2n	100	21.29 ±3.74	19.7-22.7
		2 Leaves	2n	100	20.01 ±2.34	19.3-20.9
Total	40	8 Cotyl	2n	400	21.74 ±3.67	19.0-24.6
		8 Leaves	2n	400	20.27 ±2.73	18.6-22.2
Grand total	40	6 C + 8 L	n	700	10.46 ±2.18	9.3-12.5
	40	8 C + 8 L	2n	800	21.00 ±3.31	18.6-24.6

* VSg = Plants grown under a light bench for about 20 days. Cotyl-LT = Light-treated cotyledons.

Table 2. Pertinent mean squares for chloroplast numbers.

Source of variation	df	Mean square
Among entries	14	3,025.25**
Haploid vs. tetraploid	1	41,526.20**
Among haploids	6	18.35*
Cotyledons vs. leaves	1	74.30**
Among cotyledons	2	5.37ns
Among leaves	3	8.36ns
Among tetraploids	7	102.46**
Cotyledons vs. leaves	1	430.71**
Among cotyledons	3	86.29**
Among leaves	3	9.21ns
Within entries	1,485	7.59ns
Among plants	135	7.46ns
Among stomata (error)	1,350	7.61

*, ** Significant at the 0.05 and 0.01 probability levels, respectively. ns = Non-significant.

Most chimerals are predominantly virescent, haploid, female tissue at flowering. Of the remaining, predominantly green chimeral plants, two out of three are hybrid, 2n V_7v_7 tissue. There is evidence that a portion of these hybrid green sectors V_7v_7 are also chimeral for haploid V_7 tissue (18). These chimerals are indistinguishable as seedlings. The chloroplast-count technique could detect these haploid sectors and increase of haploid plants of paternal origin.

We conclude from these results that the stomatal-chloroplast technique is rapid and accurate for classifying haploid and tetraploid plants at any stage of growth, as long as the leaves are living at the time of examination. It should have considerable value for detection of haploid plants at an early stage of development from F_1 chimeral plants, which have resulted from the crossing of selected normal green cotton strains with semigametic female parents, to produce specified haploid plants.

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