Experimental Indriction of Haploid Parthenogenesis in Black Cottonwood

(Populus trichocarpa T. & G. ex Hook.)

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Introduction

Haploids are rare among forest trees. The reasons for this are that male or female gametes rarely undergo embryogeny without prior fertilization and, if they do, the haploid sporophytes thus generated typically grow slowly and will eventually be eliminated by competition from diploids. If not physically eliminated, haploids encounter serious problems at meiosis that reduce their fitness. Thus, it is not surprising that the first haploid forest tree was reported only recently (TRALAU, 1957), and in a species with effective vegetative propagation (Populus tremula L.).

Yet, to the forest geneticist, haploid sporophytes are of great intrinsic interest because all their recessive genes are expressed and because they can be turned into homozygotes by chromosome doubling. These two phenomena are central to the various uses for which haploid tree material is well suited (discussed by ROHMEDER and SCHÖNBACH, 1959; GUSTAFSSON, 1960; KOPECKY, 1960; SIMAK, 1965; STETTLER, 1966). In fact, for some purposes haploid tree material may offer greater experimental efficiency than diploid material, even at low frequencies of occurrence (NEI, 1963; STETTLER, BAWA, and LIVINGSTON, 1969).

The abundant literature on the spontaneous or experimentally induced occurrence of haploid in higher plants was ably reviewed by KIMBER and RILEY (1963) and by MAGOON and KHANNA (1963). In contrast, few cases of haploids have been reported in forest tree species. In gymnosperms, ILLIES (1964) found ten haploid seedlings in the course of an extensive cytological study covering 435 progenies in Picea abies KARST. The haploids showed various abnormalities. Five were members of a pair of twins, the other five germinated radicle-first and all were unusually small. No attempt was madr to culture the abnormal seedlings for subsequent study. In the same species SIMAK, GUSTAFSSON and CHING (1968) screened 220 polyembryonal seeds and found one mosaic aneuploid with a chromosome number ranging from 12 to 14. Of much interest is POHLHEIM's 1968 report of a haploid form of Thuja plicata called Thuja gigantea '*gracilis*' BEISSN. which was first described in 1896 (BEISSNER, L., cited by POHLHEIM, 1968) although not recognized as a haploid at that time. POHLHEIM propagated it vegetatively and found a high tendency to sporting in the ramets. Already BEISSNER had observed several branches of the gracilis form to revert to the normal form; POHLHEIM verified this observation and found the reverted tissue to be diploid.

In angiosperm forest trees, haploids have only been described in the genus Populus. KOPECKY (1960) obtained six maternal haploids from treating P. alba L. catkins with P. tremula L. pollen that had been kept moist in a stoppered test tube for several days. The pollen showed weak germination and apparently was capable of stimulating, but not fertilizing, the egg cells. Also, five maternal haploids were obtained from a P. alba X P. nigra L. cross. The haploids were slower growing, had smaller leaves and a more variable form than diploids but were still alive at two years of age. WINTON and EINSPAHR (1968) produced four haploid/diploid chimaeras in a P. tremuloides MICHX. X P. tremuloides cross involving heat-treated pollen. They attributed the chimaeral condition to spontaneous chromosome doubling and suspected the same phenomenon to have occurred in four seedlings from a P. tremuloides X P. alba cross, which were of maternal phenotype but diploid. A putative aspen haploid was also found by VALENTINE, LA BUMBARD, and FOWLER (1968) in experiments involving the same parental species.

Altogether, these accounts indicate that in some forest-tree species haploids occur spontaneously, that they can be experimentally induced, and that some are viable but have a tendency to undergo chromosome doubling. The question unanswered by these accounts is whether haploid forest trees can be produced at frequencies of practical consequence.

In 1964, we began a series of systematic studies aimed at answering this question. Black cottonwood (Populus trichocarpa T. & G. ex HOOK.) was chosen as experimental material, for the reasons that (1) it is a dioecious species, thus not susceptible to contamination from selfing; (2) it can be bred under controllable ccnditions in the greenhouse and the branch-culture technique allows large numbers of ovules to be kept in a small space; (3) it has a short seed-maturation period (4—6 meeks), the seed germinates readily and seedlings grow rapidly to allow early scoring for haploids; (4) it is ideal for cloning and once haploids are found they can be propagated vegetatively; (5) it has an extensive distribution range harboring much variation and this genetic diversity increases the probability of finding females that are responsive to haploid induction. We adopted a three-step strategy, namely

Step 1: Screening of randomly-picked females trees for individuals responsive to the generally successful haploid-induction technique of remote hybridization.

Step 2: Perfecting the induction technique on the responsive females.

Step 3: Testing the perfected induction technique on a larger number of females randomly picked from a wide array of natural populations.

This paper presents the results of two studies conducted over three years. In the first study, performed in 1966—67, the emphasis was placed on producing haploid seedlings. In the second study, performed in 1968, the emphasis was placed on producing haploid embryos. The results show that we found responsive females, and gained experience for further improving the haploid induction technique.

1966—67 STUDY: EMPHASIS ON PRODUCTION OF HAPLOID SEEDLINGS

Design

Induction technique

Remote hybridization was chosen as the induction technique. Four species were selected that had given few or no

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hybrid progenies in previous crosses with black cottonwood (*P. alba* L., *P. canescens*, *P. grandidentata* MICHX., and *P. tremuloides* MICHX. all of section *Leuce*). A fifth species *P. deltoides* BARTR. (Section *Aigeiros*), known to hybridize freely with black cottonwood, was added as a contrast to detect potential correlation between haploid induction and hybridization success.

To prevent catkin abortion, a problem commonly encountered in remote hybridization of poplars, we added irradiated pollen of the female species ("mentor" pollen) to that of the foreign species. Exploratory experiments had shown promise for the effectiveness of such a scheme (STETTLER and HOWE, 1966).

Selection of female trees

It was reasonable to expect differences between females, regarding both their sensitivity to induction stimuli and their ability to produce viable haploids. Yet, short of an induction test there was no way of recognizing a desirable female tree. Thus, females had to be chosen at random. To heighten their differences we selected some from large, continuous, and other from small, isolated populations. Because of the higher probability for past inbreeding we expected greater haploid success in females from the small, isolated populations.

Numbers of ovules required

We arbitrarily set the lower performance limit for our screening test at 0.1 percent, that is if less than one per thousand potentially fertile ovules gave rise to a haploid seedling we considered the phenomenon too rare to have practical merit. This figure seemed reasonable in light of other findings with angiosperms (KIMBER and RILEY, 1963). We further assumed that in the absence of known marker genes we would only detect half the haploid seedlings. Finally, we assumed that the frequency of ovules giving rise to detectable haploid seedlings followed a Poisson distribution. Based on these assumptions we calculated that when the true frequency of haploidy was 0.1 percent it would take 6,000 potentially fertile ovules to detect one or more haploids with 95 percent probability (BURINGTON and MAY, 1953). This figure served to determine the numbers of catkins required for conducting the experiments.

Materials and Methods

In 1966, nine female trees were selected, one from each of nine geographically separated natural populations in the State of Washington (*Fig. 1*). Trees had to have a well developed crown with abundant floral buds to qualify as sample trees. Five trees were located in large, continuous populations on five major river drainages in western Washington. Four were located in small, isolated stands along creek bottoms in the arid portion of the state and each of these stands was at least one mile apart from the closest neighboring stand, usually located in another drainage.

In February of 1966 and 1967 dormant branches bearing floral buds were collected and wrapped in moist plastic bags. Some were taken directly to the greenhouse; others were stored up to three months in plastic bags at 5° C before being used for breeding. In the greenhouse, the branches were placed in troughs with running tap water and trimmed weekly to facilitate their water uptake. Greenhouse temperatures were under moderate control and ranged from 15° C to 25° C. In 1966, an unusual heat wave sent temperatures up to 32° C, causing much mortality of branches. To minimize pollen contamination all branches to be treated with live black cottonwood pollen (control) were kept in a separate greenhouse from the others. Protection against outside pollen was attempted by timing critical pollinations prior to natural pollen release in the

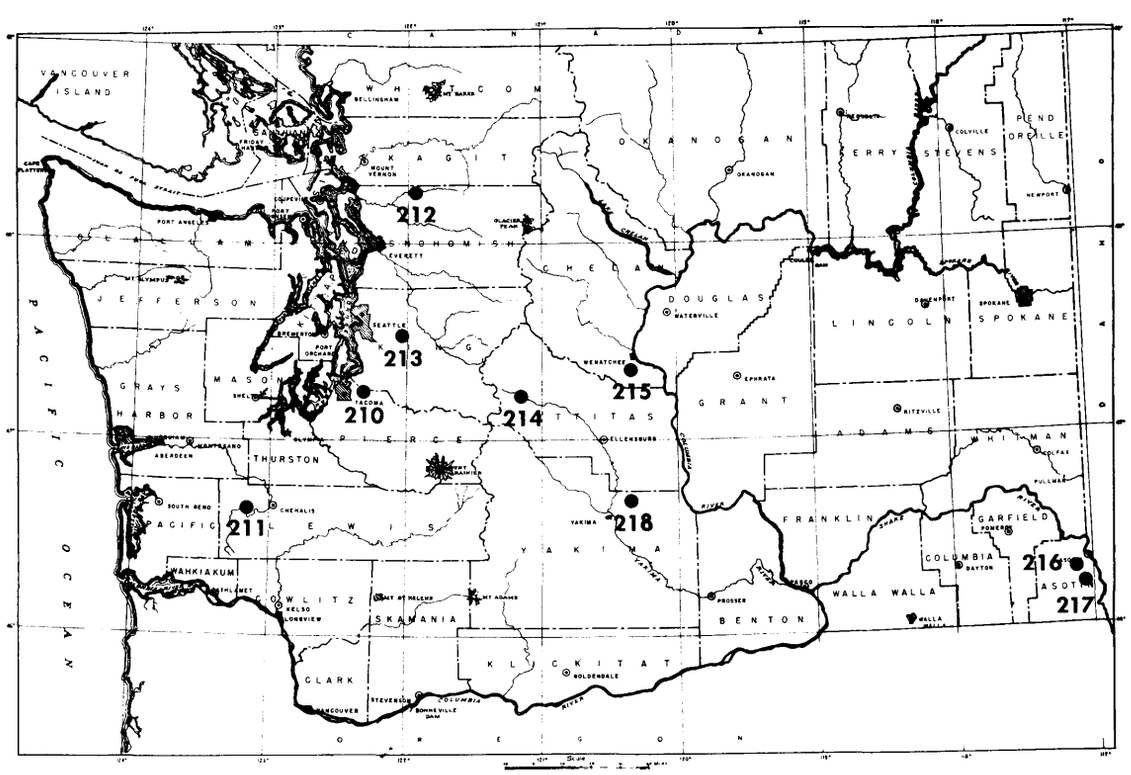

Fig. 1. — Map of the State of Washington showing the location of the nine trees used as female parents.

area. Experiments with isolation bags gave negative results. All in all, isolation was sufficient to prevent mass contamination but insufficient to exclude contamination altogether.

Pollen was extracted during January and February by "forcing" dormant male branches in a moderately cool room (10—12° C) at a 24-hour photoperiod (HEIMBURGER, personal communication). Pollen from species not available to us was supplied by cooperating agencies[2]). In all but one species *(P. alba)* pollen was obtained from more than one tree per species. All pollen was air-dried and stored in vials with rubber stoppers at 3—5° C until used. "Mentor" pollen of black cottonwood was exposed in air-dried condition to a cobalt-60 source for a dose of 100 kR. Pollen mixes were prepared by thoroughly mixing pollen of a given species with mentor pollen in a ratio of 2 : 1 by weight.

At pollination, the pollen was dusted evenly over each receptive catkin. Most catkins were pollinated at least twice on consecutive days, but those with asynchronous flower development were pollinated several times. Twenty-four hours after pollination each batch of pollen was tested for germination. For this purpose, two stigmas from each of the nine female trees were squashed in acetocarmine and examined under phase contrast. In every treatment, even in those involving irradiated "mentor" pollen alone, we found good pollen germination.

Mature seeds were obtained 24—41 days after pollination, the period varying from one female tree to another. Mature catkins were collected, air dried in the laboratory, and the cotton containing the seeds was removed and stored in vials at 3—5° C until germination. For germination, the seeds were blown out of the cotton, soaked for 10 minutes in a sterilizing solution (0.7 ml Pan-0-drench in 1000 cc of water), and germinated on moist filter paper in sterile petri dishes at room temperature. Most seeds germinated within 24 hours. Within 48 hours after germination, the seedlings were transferred to sterilized soil in sub-irrigated containers and kept in a growth chamber at 20—28° C with a 14-hour photoperiod of 250 f—c. Pan-0-drench treatments at weekly intervals during the first four weeks kept damping-off at a minimum. At three to four weeks old the seedlings were moved to a greenhouse. At one year old the seedlings were planted in the University of Washington Arboretum where they will be kept for further study and breeding tests.

Seedling phenotypes were carefully examined at regular intervals. Leaf shape, undersurface and margin, as well as the cross section and relative length of petioles were most diagnostic in distinguishing hybrids from black cottonwood phenotypes. These traits could be reliably assessed on the third and later leaves. Chromosome counts were made from root or shoot squashes stained with Feulgen reagent (SHARMA and SHARMA, 1965).

Results and Interpretation

The 1966—67 experiments involved the treatment of nine females with five different pollen mixes. The major object was to detect differences in the quality and quantity of resulting seedlings as a function of (a) the female parent, and (b) the treatment. Due to unequal numbers and low scores the data did not lend themselves to conventional

Table 1. — Summary of 1966/67 breeding results for the nine female trees[1])

	Female parent																			
	210		211		212		213		214		215		216		217		218			
	1966	1967	1966	1967	1966	1967	1966	1967	1966	1967	1966	1967	1966	1967	1966	1967	1966	1967		
C_1=Number of catkins pollinated	307	234	181	175	203	198	204	204	269	310	649	706	228	225	444	582	190	661		
O_1=Mean number of functional ovules per catkin[2])	267.78		321.55		253.92		398.85		180.18		164.14		256.45		471.36		131.04			
O_2=Calculated number of functional ovules pollinated [3])	82,208	62,661	58,201	56,271	51,546	50,276	81,365	81,365	48,468	55,856	106,527	115,883	58,471	57,188	209,284	274,332	24,898	86,617		
C_2=Number of mature catkins	25	85	14	18	6	140	53	119	1	103	91	224	23	17	43	25	3	9		
O_3=Calculated number of mature functional ovules [4])	6,695	22,761	4,502	5,788	1,524	35,549	21,139	47,463	180	18,559	14,937	36,767	5,898	4,360	20,268	11,784	393	1,179		
Number of filled seed	336	112	178	168	20	504	70	823	4	909	879	2,013	11	7	20	764	5	15		
Number of germinating seed	90	43	25	10	5	89	27	258	3	45	69	794		2		239		1		
Number of seedlings																				
Hybrid phenotypes [5])	31	11	12	1	1	7	12	49		12	11	123				11				
Maternal phenotypes																				
Haploid												1[6])								
Diploid or uncountable	13					6	1	1	1		2	5								
Unclassified phenotypes																				
Haploid							3	1	1											
Diploid or uncountable	46	32	13	9	4	76	11	207	1	28	56	665		2		228		1		

[1]) Numbers are totals from all five treatments
[2]) determined from the number of filled seed per mature catkin in control
[3]) $O_2 = C_1 \cdot O_1$
[4]) $O_3 = C_2 \cdot O_1$
[5]) includes both positively identified hybrids and probable hybrids
[6]) Mixoploid.

[2]) We thank Dr. D. W. EINSPAHR, Institute of Paper Chemistry, Appleton, Wisconsin; Dr. R. E. FARMER, U. S. Forest Service, Stoneville, Mississippi; Dr. C. HEIMBURGER and Dr. L. ZUFA, Ontario Department of Lands and Forests, Maple, Ontario, Canada, for their pollen shipments.

Table 2. — Summary of 1966/67 breeding results for the five treatments.[1])

	Treatment									
	P. alba mix		*P.canescens* mix		*P. grandid.* mix		*P. tremuloides* mix		*P. deltoides* mix	
	1966	1967	1966	1967	1966	1967	1966	1967	1966	1967
Number of catkins pollinated	327	642	776	649	389	657	750	746	433	599
Number of mature catkins	51	158	96	139	19	143	56	171	37	129
Per cent mature catkins	15.6	24.6	12.4	21.4	4.9	21.8	7.5	22.9	8.5	21.5
Number of filled seed	27	131	280	698	321	1331	120	826	775	2329
Number of filled seed per mature catkin	0.53	0.83	2.92	5.02	16.89	9.31	2.14	4.83	20.95	18.05
Number of germinating seed	9	34	69	243	17	573	97	462	27	164
Number of seedlings[2])										
Hybrid phenotypes	3	7	9	35	3	73	40	72	12	27
Haploid						1[3])				
Maternal phenotypes										
Diploid or uncountable		2	3			8	14	1		1
Unclassified phenotypes										
Haploid			1	1			3			
Diploid or uncountable	6	25	56	207	14	491	40	389	15	136

[1]) Numbers are totals from all nine female parents.
[2]) Same categories as in Table 1.
[3]) Mixoploid.

statistical treatment. However, major trends can be detected from *Tables 1—3.* Since consistent differences were found between 1966 and 1967 in all parameters studied, data for the two years are listed separately. Althogether, the 1967 experiments were more successful than those in 1966, which suffered mortality from drought and pesticide.

Catkin maturation. Catkin maturation, or the proportion of catkins reaching the mature-seed stage, was critical to the succes of the study. As already noted irradiated "mentor" pollen of the female species was added to the foreign pollen, to prevent the early abortion common in remote hybridization of poplars. Catkin maturation for the five treatments averaged 9.7 percent in 1966, and 22.5 percent in 1967. The corresponding figures for the control were 14.0 percent and 35.8 percent respectively. Thus, the performance in response to the pollen mixes was about two thirds as high as that of control — a fairly high value that probably can be attributed to the beneficial effect of the "mentor" pollen. This interpretation is supported by the remarkable uniformity in catkin maturation among the five different pollen mixes, particularly in 1967 *(Table 2).* In other words, the different phyletic relationships between the female species and the five pollen species were not reflected by differential catkin maturation, but rather were masked by the "mentor"-pollen effect, common to all. We were able to verify this in 1968 by showing that catkins treated with irradiated "mentor" pollen alone were capable of reaching maturity.

Catkin maturation seemed to be unaffected by the number of embryos contained in a catkin. This can be seen in *Table 2* where, for example, catkins treated by *P. alba* mix had a similar survival rate to those treated by *P. deltoides* mix, yet contained much fewer embryos (filled seed) than the latter. In fact, we harvested many mature catkins that did not contain any embryos, and we also observed several mature catkins where all but 1—3 pistil had abscissed prematurely. Although no statistical comparison was made between abscissed and mature catkins relative to the number of embryos, occasional checks indicated the presence of embryos in abscissed catkins. Altogether, this evidence suggests that the irradiated "mentor" pollen provided the critical stimulus for catkin maturation, although it probably did not participate in fertilization.

In contrast to the uniformity among pollen treatments, catkin maturation varied markedly among the nine females. In some females it was high in both years (e. g. Nos. 213, 215), in some it was low both years (e.g. Nos. 217, 218), in others it differed between the two years *(Table 1).* Part of this variation can be attributed to differential sensitivity of the different females to the branch-culture technique, as manifested by the varied performance of control branches. Another part of the variation has to be ascribed to the differential response by different females to a given pollen mix.

Seedlings obtained

Three categories of seedlings were obtained: (1) putative hybrids whose phenotypes were intermediate between the maternal and paternal species; (2) black cottonwood (maternal) phenotypes, and (3) "unclassified" seedlings that remained small or died prior to a reliable diagnosis. The numbers of seedlings in these three categories are listed separately by female parent in *Table 1* and by treatment in *Table 2.*

Hybrid phenotypes. In both years we obtained all five possible hybrids, each from several female parents. The broader significance of this result has been discussed in an earlier paper (Stettler, 1968). Here, it must suffice to say that the two aspen hybrids may have some practical promise since they could act as a bridge for the transfer of rooting genes from the easily-rooting black cottonwood to the difficult-rooting aspen. A more detailed description of the hybrids and their performance will be given at a later date.

The relative scarcity of *P. deltoides* hybrids was not expected since the two species of cottonwoods hybridize quite freely (Brayshaw, 1965). However, this can be explained by the high mortality during germination. Many more full seeds were recovered from the *P. deltoides* than from any other mix, but during early germination the majority was lost due to a bacterial agent that we were unable to isolate.

Periodic chromosome counts performed on randomly sampled hybrids always gave diploid numbers of 2n = 38.

Maternal phenotypes. Since none of the nine females carried any known marker gene we had to treat all seed-

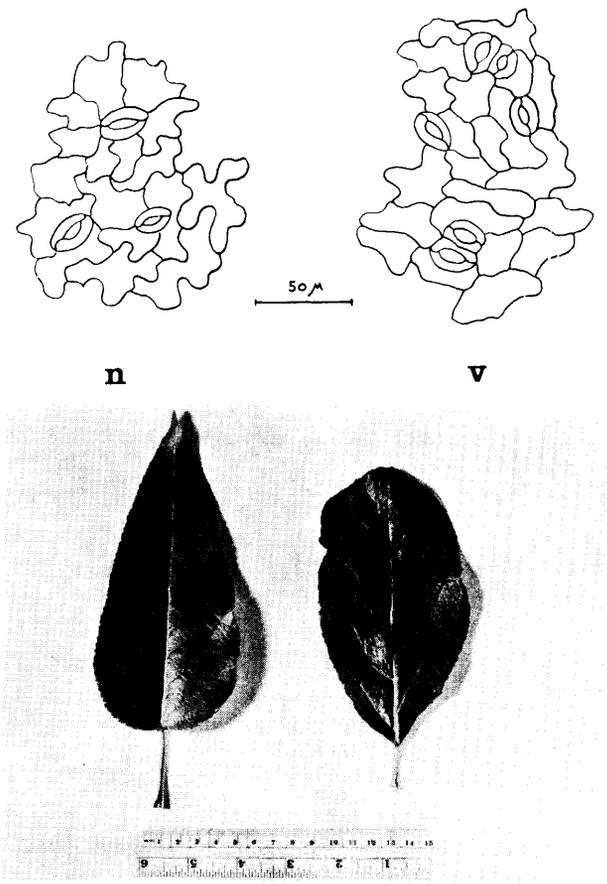

Fig. 2. — Normal (n) and forma *verrucosa* (v) leaves and their corresponding stomatal variation.

lings with black cottonwood phenotypes as putative haploids. Only one such seedling from tree No. 215 had haploid chromosome counts in one root tip squash. The same tissue also had diploid counts, indicating that the chimaeral nature of this root originated from the spontaneous diploidization of haploid cells during early root growth. Unfortunately, this seedling remained stunted and died after two months.

Twenty-nine seedlings gave either diploid chromosome counts or died before reliable counts were made. Two of them remained stunted until the time of this writing and had unusually narrow leaves. But most phenotypes fell within the variation range of control seedlings, thus making it impossible to trace their mode of origin to any one of three possible routes: (1) haploid parthenogenesis followed by spontaneous diploidization during embryogeny; (2) fertilization by irradiated "mentor" pollen; or (3) fertilization by stray pollen of the maternal species. However, many of these seedlings are permanently planted, and eventually will be amenable to analyses such as isozyme analysis, progeny testing and other methods capable of resolving among the three alternatives.

One exceptional seedling was recovered among the 1966 progeny of tree No. 210. It was diploid and had all the diagnostic characteristics of a black cottonwood but its leaves were elliptical-oblanceolate rather than lanceolate-ovate and had small wart-like excrescences and epidermal ridges on the upper surface *(Fig. 2)*. Unofficially, this forma was named *"verrucosa"*. Close examination of its leaves also showed many stomata occurring in pairs rather than singly as in normal cottonwood seedlings. The described syndrome persisted throughout all the leaves produced by the plant to date but did not seem to affect its growth. Until a genetic analysis will be possible it is suggested that the seedling is homozygous at one, possibly at all loci, having arisen from a haploid through chromosome doubling.

Unclassified phenotypes Most seedlings obtained from the 1966—67 study remained stunted and died within 3—4 weeks, before a diagnosis was made of their phenotype. Furthermore, cytological examination was often hindered by poor staining and the lack of dividing cells. We found

Table 3. — 1967 Embryo Sampling[1]) listed by female parent and treatment.

Category of Embryos	Female parent								
	210	211	212	213	214	215	216	217	218
Haploid				2					
Diploid	14	23	11	21	23	36		18	2
Uncountable	18	8	13	7	10	13		6	6
Total	32	31	24	30	33	49		24	8
Number of Ovules sampled	(200)	(200)	(200)	(200)	(200)	(200)	(120)	(180)	(160)

Category of Embryos	Treatment					
	P. tricho. (control)	*P. alba* mix	*P. canesc.* mix	*P. grandid.* mix	*P. tremul.* mix	*P. deltoides* mix
Haploid			1	1[3])		
Diploid	27[2])	2	9	19	1	119
Uncountable	294	2	3	10	2	63
Total	321	4	13	30	3	182
Number of Ovules sampled	(347)	(360)	(360)	(320)	(320)	(320)
Percent Filled Ovules	92.5	1.1	3.6	9.4	0.9	56.9

[1]) Four weeks after pollination, four of the largest pistils per female parent and treatment were collected. From each pistil, the ten largest ovules were examined for embryos. Occasionally, the required number was not available.

[2]) Not all control embryos were cytologically examined; these 27 were a random sample picked from the 32 available.

[3]) Contained both haploid and diploid cells.

five haploids and many more diploids among these unclassified phenotypes. Morphologically, the haploids could not be distingusihed from the other small seedlings. This means that additional haploids may have remained undetected among this category of seedlings.

Embryos. A small-scale sampling *(Table 3)* was conducted in 1967 to detect possible differences between female parents and/or treatments in the numbers of embryos. The non-random sampling was done four weeks after pollination, concentrating on those pistils that, according to their size, were likely to contain embryos. Tree No. 213 was the only female parent in which haploid embryos were found, resulting from pollen mixes of *P. canescens* and *P. grandidentata.* The *P. deltoides* mix produced by far the highest number of diploid embryos (presumably hybrids), consistent with the high number of full seeds produced *(Table 2).*

Evaluation of success

The most direct parameter of success in our 1966—67 study is the number of haploids detected as a function of the female parent and the treatment. This parameter contains non-random errors, introduced by sampling bias and inadequacies in our haploid-detection scheme, all tending to underestimate the actual success of haploid induction. An additional parameter is the number of maternal phenotypes found (diploid or uncountable). It is based on the assumption that all maternal phenotypes were of haploid origin, rather than contaminants. Although we have no independent estimate of the amount of contamination it is safe to say that this assumption is not entirely valid and, therefore, this parameter is an overestimate of actual success.

It seems reasonable to evaluate success in relation to the numerical requirements of the experimental design. Under the assumptions made, 6000 functional, i. e. potentially fertile, ovules were required in any female parent/treatment combination to allow detection of at least one haploid with 95 percent probability if haploidy occurs at a frequency of 0.1 percent. To compare actual with required numbers, we determined for each tree the number of functional ovules per catkin, as measured by the number of filled seed per catkin produced on the control branches. These numbers (0_1 in Table 1), which varied greatly among the nine trees, were then multiplied by the number of catkins pollinated (0_2) and by the number of mature catkins (0_3).

Table 4 shows that from 90 combinations only 17 had enough mature, functional ovules to meet the requirement (framed boxes); the majority had enough functional ovules pollinated but not enough survived to maturity; whereas 10 had not enough functional ovules pollinated (dotted boxes). The deficiencies apparently resulted from the higher-than-expected catkin mortality due to factors inherent in the trees themselves, and/or to experimental conditions. Certain trees, such as Nos. 211, 216, 217, 218, were *a priori* unsuitable for our experiments, as shown by their control performance, thus reducing the total possibility for haploid parthenogenesis. If catkin mortality was independent of haploid parthenogenesis, as our information on catkins maturation indicates, and if the assumptions underlying our experimental design are correct, we would expect success only in those cases where the number of mature functional ovules exceeded the requirement (framed boxes). Our results agree reasonably well with this expectation in that most haploids and maternal phenotypes

Table 4. — Evaluation of the success of haploid induction.

Female parent	Treatment									
	P. alba mix		P. canescens mix		P. grandidentata mix		P. tremuloides mix		P. deltoides mix	
	1966	1967	1966	1967	1966	1967	1966	1967	1966	1967
210							(13)			
211										
212		(1)				(3)		(1)		(1)
213		(1)		2		1	3 (1)			
214			1 (1)							
215			(2)			1 (5)				
216										
217										
218										

3 — Number of haploid embryos and seedlings.
(1) — Number of maternal phenotypes.
Number of mature functional ovules greater than 6000.
Number of pollinated functional ovules, but not of mature functional ovules, greater than 6000.
Number of functional ovules pollinated less than 6000.

were found where expected. But they were not found in all cases where expected.

Of the five responsive trees, three (Nos. 213, 214, 215) produced both haploids and maternal phenotypes, two (Nos. 210, 212) only maternal phenotypes. The most responsive female was tree No. 213 which produced three haploids in each of the two years. Only one responsive tree, No. 215, came from a small, isolated population, the others from large continuous populations. However, in view of the small number of individuals and because of confounding factors, no inference can be drawn on how population size affected success.

Among the five treatments, *P. canescens, P. grandidentata,* and *P. tremuloides* mixes were more successful than the two other mixes. The low success of the *P. deltoides* mix is partly attributed to the high frequency of diploid hybrid embryos produced in this combination *(Tables 2 and 3);* in competition for available metabolites hybrid embryos presumably outgrew haploids in the same pistil.

The 1966—67 studies showed that haploids and putative homozygotes could be produced at detectable frequencies in some trees but not in others. It seemed reasonable to continue the studies by attempting to improve the haploid-induction treatments on the responsive females. Since haploid detection was inadequate at the seedling stage it also seemed reasonable to shift our attention to the embryo stage.

1968 STUDY: EMPHASIS ON PRODUCTION OF HAPLOID EMBRYOS

Design

The four responsive females chosen for the embryo study were Nos. 210, 213, 214 and 215. The same haploid-induction technique was used as in the 1966—67 study, using a

mixture of foreign species pollen and irradiated "mentor" pollen. However, the mix ratios and irradiation levels were varied. We also were interested in the effects of irradiated pollen alone. Was haploid success dependent on the presence of a functional endosperm produced by double fertilization and, thus, dependent on the foreign-species pollen? Or, was haploid success lowered in the presence of hybrid embryos and, thus, hindered by the foreign-species pollen?

Three different mixes of 4 : 1, 1 : 4, 0 : 1, foreign to "mentor" pollen were combined with three different irradiation levels (20, 40, 80 kR) to give a total of nine treatments. Pollinations with two different mixes of foreign and non-irradiated "mentor" pollen (4 : 1, 1 : 4) served as control. The two species that rated the highest success in 1966—67 (*P. canescens* and *P. tremuloides*) were chosen as sources for the foreign pollen. Because the number of branches was limited, the four female trees were split into two groups. Trees Nos. 213 and 214 received *P. canescens* treatments, Nos. 210 and 215 received *P. tremuloides* treatments. Embryos were examined 15—30 days after pollination.

Materials and Methods

The breeding operations were the same as in the previous study. Foreign-species pollen was again supplied by the same cooperating agencies. In *P. canescens*, it came from one, in *P. tremuloides* from seven, trees. "Mentor" pollen came from three trees of black cottonwood. Equal weights of pollen from each tree were mixed together before the mixes of foreign and mentor pollen were prepared. Mentor pollen was irradiated in air dried condition with cobalt-60 at doses of 20, 40, and 80 kR.

For embryological examinations, about one third of the available catkins were sampled, at 12, 24, and 28 days after pollination, and fixed in 1 : 3 acetic-alcohol. Each pistil was split open at the carpel seams to allow better penetration of the fixing fluid. After 24—48 hours, the catkins were transferred to 70 percent alcohol and stored at 5° C until examined.

For cytological study, the full ovules were separated from the empty ovules under a dissecting microscope. Usually, the embryos were visible through the integument, thus facilitating the distinction. Occasionally, however, we had to tease the integuments apart. In several such cases we lost the small embryos during staining. Since haploid embryos were generally smaller than diploid embryos, the former were more likely to be affected by this error than the latter. Full ovules were stored in 70 percent alcohol separately for each pistil. They were then hydrolyzed for 10—12 minutes in 1*N* HCl in a water bath at 60° C, rinsed twice in tap water, then placed in Feulgen reagent (Sharma and Sharma, 1965). The tightly closed vials were kept for 2 hours in the dark at 4° C. The Feulgen reagent was then poured off, and the ovules placed in tap water until the embryos were dissected out of the ovules on a slide and squashed in 45 percent acetic acid. This was generally done within 1—4 hours after transfer to tap water although we found that staining was not affected even if the material remained in the tap water up to 2 days. All observations, chromosome counts, and photomicrographs were made from temporary slide preparations observed under phase contrast.

Preliminary screening showed that 15 and 24-day old embryos were too small to permit reliable detection of haploids on a large scale. Thus, we based our analysis entirely on 28-day-old embryos. Since seed maturation in our females occurred about five weeks after pollination, these 28-day-old embryos had completed about four fifths of the maturation period.

Results and Interpretation

Types of ovules found

Black cottonwood pistils contain about 50—70 ovules, of which 25—40 are potentially functional. Under optimal conditions all potentially functional ovules contain embryos.

In our 28-day material, the ovules within the same pistil fell into two quite distinct size classes. Small ovules were more or less globular and comparable in size to ovules from non-pollinated pistils. They were almost all empty, and only in one treatment of tree No. 213 did we recover some haploid embryos from small ovules.

Large ovules were elongate and several times the size of ovules from non-pollinated pistils, but they did not always contain embryos. Nor was variation in size among these large ovules indicative of the presence or absence of embryos. Some ovules with embryos were often smaller than those without, even within the same pistil. Among the large ovules, those containing haploid embryos were usually smaller than those containing diploid embryos. All large ovules were considered as potentially functional.

Types of embryos found

The major objective of the 1968 study was to determine the frequency of haploid embryos as a function of both the female parent and treatment. Its fulfillment depended on the cytological discrimination between haploid and diploid embryos.

Although embryos are not normally used for chromosome counts, we found the 28-day-old material well suited for this purpose. Diploid embryos from control branches had many cells in division, and we generally found a dozen or so cells in late prophase or early metaphase to make counts. The same was true for diploid embryos from experimental treatments. Typically, such embryos had counts of 34—38 chromosomes, but occasional cells with poorly separated chromosomes gave counts as low as 28. In contrast, haploid embryos had fewer cells in division and gave counts of 18—20. Differences between diploid and haploid metaphases are illustrated in *Fig. 3*. In agreement with Smith's (1943) observations on meiotic chromosomes in the genus *Populus* we found the chromosome size to follow a continuous variation pattern. The smallest chromosomes were dot-like, the largest 3—4 times the length of the smallest. Since chromo-

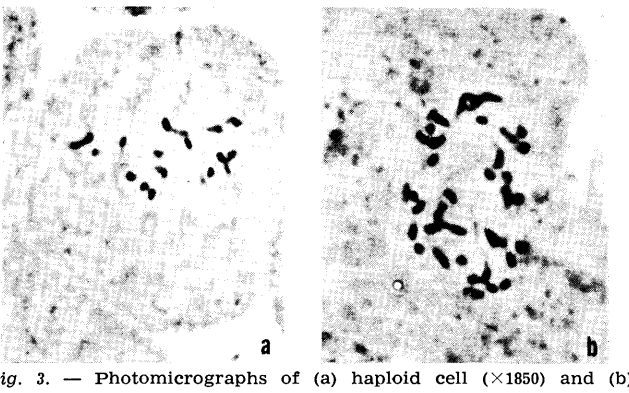

Fig. 3. — Photomicrographs of (a) haploid cell (×1850) and (b) diploid cell (×1750) from embryo squashes.

Table 5. — The number of 28-day-old embryos found in six pistils from each female parent/treatment combination.*)

Types of embryos found	Irradiation dose of "mentor" pollen											
	20 kR				40 kR				80 kR			
	Mix ratio foreign: „mentor"				Mix ratio foreign: „mentor"				Mix ratio foreign: „mentor"			
	4:1	1:4	0:1	Total	4:1	1:4	0:1	Total	4:1	1:4	0:1	Total
	a	b	c		d	e	f		g	h	i	
Haploid							1	1	1			1
Diploid	69	43	80	192	7	1	15	23	1	2	1	4
Uncountable	16	21	35	72	2	1	8	11	4	5	1	10

*) Each number is the total from all four female parents.

some separation was not always perfect, we had a number of embryos with counts ranging from 17 to 22. To distinguish them from the more reliable haploids and from the diploids, we labeled them "probable haploids". It is conceivable, although improbable, that they were aneuploids. All cases where chromosome numbers could not be determined due to poor staining, chromosome clumping, or loss of embryo during staining were categorized as uncountable *(Tables 5 and 6).*

Consistent morphological differences were shown between different types of embryos. Four weeks after pollination, diploid embryos from control branches had well developed cotyledons, plumule and radicle, and filled almost all the space within the ovule. Diploid embryos resulting from experimental treatment were either heart shaped or globular and filled less than half of the ovule space. Haploid embryos were generally smaller than diploid embryos, but showed more variation in size. Many haploids were globular and filled less than a quarter of the ovule. Other haploids were heart shaped but filled almost all the space available in small ovules.

Frequencies of embryos found

The variation in the number of potentially functional ovules per catkin was significantly affected by the female parent and the treatment, both at the 0.1 percent level of significance, as well as by interaction of the two at the 5 percent level, as determined by an analysis of variance. The treatment effect was largely due to the irradiation dose, low doses giving greater numbers of potentially functional ovules than high doses. Accordingly, we expected to find more embryos among the low-dose treatments.

Table 6. — Numbers of 28-day-old embryos found in two responsive female parents.

Female parent	Types of embryos found	Irradiation dose of "mentor" pollen								
		40 kR				80 kR				
		Mix ratio foreign: "mentor"			Total	Mix ratio foreign: "mentor"			Total	Grand Total
		4:1	1:4	0:1		4:1	1:4	0:1		
		d	e	f		g	h	i		
213	Haploid	1	2	3	6	3		1	4	10
	Probable haploid		1	2	3	8		7	15	18
	Total haploids	1	3	5	9	11		8	19	28
	Diploid	3	2	12	17	5	3	6	14	31
	Uncountable	8	3	22	33	9	9	16	34	67
	Sample examined[1])	1500	1600	1886	4986	640	850	2204	3694	8680
	Percent haploid success[2])	.067	.187	.265	.181	1.719		.363	.514	.323
214	Haploid									
	Probable haploid					3	1		4	4
	Total haploids					3	1		4	4
	Diploid	9	12	18	39	5	8		13	52
	Uncountable	1	3	8	12	4	7		11	23
	Sample examined	280	1000	660	1940	880	782	51	1713	3653
	Percent haploid success					.341	.128		.234	.109

[1]) Estimated number of potentially functional ovules examined; the mean number of potentially functional ovules per pistil was determined from a sample of three pistils in each treatment; the mean was then multiplied with the number of pistils actually examined.
[2]) (Total haploids · 100)/sample examined.

A preliminary screening of six pistils from each female parent/treatment combination verified this expectation. For each female, the three 20 kR treatments gave markedly more embryos than the 40 and 80 kR treatments *(Table 5)*. These embryos, however, were either diploid or uncountable, and we had no way of determining their origin. They could have resulted from hybridization, fertilization by "escape" "mentor" pollen, outside contamination, apomixis, or diploidization of haploids. The first alternative seemed most likely for the treatments with foreign pollen, least likely for those without. In contrast, we found one haploid each in two of the six treatments with high doses.

The results from this preliminary screening suggested that haploids were more likely to be found among the material from the 40 and 80 kR treatments *(Table 5,* columns d—i). Rather than sampling material from all nine treatments *(Table 5,* columns a—i) at a moderate intensity, we decided to sample the material from the six most promising treatments (d—i) at a high intensity. Thus, an estimated 22,000 ovules were examined. Results of this study are tabulated for females Nos. 213 and 214 in which haploids were found *(Table 6)*. Haploid success is expressed as the percentage of the number of potentially functional ovules (i. e. ovules available for embryogeny) sampled. To the extent that the ovules sampled were a random sample of those initially available our calculated values are the best estimate of the actual frequency of haploid parthenogenesis.

As in the 1966—67 study, tree No. 213 showed the highest response to haploid induction treatments, with an average success rate of 0.323 percent, which is about three times as high as that of tree No. 214 with 0.109 percent. Treatment differences were also conspicuous but were subject to sampling error because of the small numbers. In both trees, treatments with 80 kR "mentor" pollen were more successful than those with 40 kR "mentor" pollen; in both trees also the most successful treatment was a 4 : 1 mix of "mentor" pollen given 80 kR *(Table 6,* column g). Of great interest is the fact that 41 percent of the 32 haploids were produced by treatments using "mentor" pollen alone, showing that foreign pollen is not a requisite for haploid induction.

No trends were obvious regarding the occurrence of diploid embryos. However 36 of 83 diploid embryos were from crosses using mentor pollen alone. This is roughly the same proportion, 43 percent, as that found in the haploids and supports the hypothesis that many diploids may have originated as haploids, then doubled their chromosome number spontaneously and, thus, became homozygotes. However, alternate explanations such as contamination, apomixis, etc. are not ruled out.

The two trees, Nos. 210 and 215, yielding no haploids were sampled at the same intensity as the two responsive females and were otherwise similar in most parameters studied.

Additional observations on haploid embryos

Several observations were made on haploid embryos that have a bearing on the evaluation of success. They relate to the endosperm, to the spontaneous diploidization of haploids and to the relationship between haploid and diploid embryos in the same pistil.

Black cottonwood, as all poplars (Graf, 1921; Grehn, 1952; Seitz, 1952), has an inconspicuous nuclear endosperm which is almost entirely consumed by the end of embryogeny, but still can be identified easily at 28 days after pollination. Endosperms were found in almost all diploid embryos but were absent in most haploids regardless of whether they had been induced by "mentor" pollen alone or by a pollen mix. This demonstrates that in black cottonwood, embryogeny can proceed without an endosperm. The smaller size of most haploid embryos may have been due partly to this absence of a nutritive tissue; unfortunately, we did not have enough haploids *with* an endosperm to examine this possibility. The absence of an endosperm was also noted in 5 of 36 diploids induced by "mentor" pollen alone. This is evidence that these five probably originated as haploids but underwent chromosome doubling early in embryogeny.

Among the 32 haploid embryos, we found two having a few diploid cells, again suggesting spontaneous chromosome doubling.

The final question to receive attention was whether haploid success is independent of diploid success. Conceivably, embryos may enhance the growth of other embryos in the same pistil as well as the growth of the pistil itself. On the other hand, embryos that share the same pistil may compete for available metabolites, thus hindering each other's growth. In the first case, haploid success would be contingent upon, in the second case, opposed by diploid success. To answer this question, we kept records from all embryos of a given pistil. All pistils examined from trees Nos. 213 and 214 were classified as containing one or more diploids ("diploid success"), one or more haploids ("haploid success"), both ("haploid/diploid success"), or no embryos at all ("no success"). Uncountable embryos were counted as diploids. A Chi-square test, based on 540 pistils, indicated that the two kinds of success were independent. Since the data were skewed towards "no success" the results more likely favor antagonism between diploidy and haploidy rather than synergism between the two. Haploids typically occurred singly; in only two cases did we find haploid embryos co-existing with more than one diploid embryo. In contrast, about 40 diploids were found in typical control pistils. Thus, while we have evidence that diploid embryos survive crowding, we have virtually none that haploids do if crowded by diploids. It may well be that additional haploid embryos were lost during early embryogeny as a consequence of diploid competition.

DISCUSSION AND CONCLUSIONS

Cytology

In the course of this study we have examined hundreds of haploid cells at various mitotic stages. At metaphase, chromosomes appeared as dots or short rods *(Fig. 3)*. At late prophase or early metaphase they were more elongate, showing, in the case of a few chromosomes, two chromosomal arms. The number of such metacentrics and submetacentrics was always less than nine. Our observations are in good agreement with those made by Smith (1943) and Seitz (1952) but in disagreement with those of Kopecky (1960) whose figure shows almost all the chromosomes to be metacentric or submetacentric. In our judgement, many of the apparent metacentrics and submetacentrics in Kopecky's photomicrograph of a haploid *P. alba* are pairs (not necessarily homologous) rather than single chromosomes. Cells similar to that shown in his micrograph were commonly observed in our diploid control material and were probably the result of poor chromosome separation. Karyotype differences between *P. alba* and *P. trichocarpa* may exist but, according to Smith's (1943) comparative study of the genus *Populus*, are not large enough to account for the difference in interpretation.

Chromosome Doubling

The four mixoploids containing both haploid and diploid cells, are most easily explained by the hypothesis that they had originated as haploids but underwent chromosome doubling in part of their tissue. This phenomenon has long been described, e. g. by EAST (1930) in *Fragaria vesca* and has also been suggested by WINTON & EINSPAHR (1968) who reported mixoploids in *P. tremuloides*. POHLHEIM (1968) noted that several branches of the haploid *Thuja gigantea* f. *'gracilis'* BEISSN. spontaneously changed to diploidy. Theoretically, a diploid cell in a haploid plant, originating from a faulty mitosis, could become established and give rise to a cell line with a faster growth rate than its haploid neighbor cells, and eventually dominate the meristem. This phenomenon is more likely to occur in long-lived plants than in annuals; in fact, it may be frequent enough that special treatments for chromosome doubling are not needed whenever haploidy is used as an intermediate step to homozygosity. By contrast, the phenomenon may call for remedial measures such as periodical pruning, whenever stable haploids are desired.

In this light, some of the diploids with maternal-species phenotype, notably the forma *"verrucosa"* and the two healthy, but stunted plants, are probably homozygotes. This is further suggested by the absence of an endosperm in several diploid embryos produced by irradiated "mentor" pollen alone. Among alternative hypotheses to account for maternal phenotypes, contamination by normal black cottonwood pollen is the most probable but the possibility of apomixis cannot be eliminated.

Frequency of Haploid Parthenogenesis

Frequency estimates are crucial to evaluate the efficiency of the haploid breeding method. Yet, both components of the frequency estimate, number of parthenogenotes produced and number of available ovules from which they were recruited, are burdened with errors. It is safe to say that our estimated number of haploid parthenogenotes is conservative because it was based on cytological evidence and, therefore, omitted haploids that (1) died prior to sampling; (2) survived but had no dividing cells, or (3) had dividing cells but were stained poorly and thus, uncountable. The number of available ovules from which haploids were recruited is more difficult to assess. Positive and negative errors introduced by catkin mortality, ovule diagnosis, and sampling, may cancel each other. If so, the overall frequency estimates would underestimate the total frequency.

Estimates of haploid frequencies are summarized for the most responsive tree, No. 213, in *Table* 7. Clearly, haploids were much more frequent among embryos than among seedlings. The most likely explantations for this differences are that our screening for haploids was far more effective in embryos than in seedlings and there was a true decrease in the number of haploids due to selection between the embryo and seedling stage.

How do these frequencies compare with those reported for other material? In reports on haploids in *P. alba* (KOPECKY, 1960) and in *P. tremuloides* (WINSTON & EINSPAHR, 1968) not enough data were given to compare with our study. In herbaceous angiosperms, haploid seedlings have been experimentally induced at frequencies of 0.017 percent in *Datura* (SATINA, BLAKESLEE, and AVERY, 1937), 0.26 percent in *Solanum* (JORGENSEN, 1928; KOSTOFF, 1942), and in selected strains of cultivated species at frequencies averaging 1—5 percent (KIMBER & RILEY, 1963), in a particular case at 35 percent (RILEY, 1963).

Table 7. — Estimated frequencies of haploids in tree No. 213.*)

Observations	Sampling based on Seedlings		Embryos	
	1966	1967	1967	1968
Numberof haploids	3	1	1	11
Estimated number of functional ovules sampled	9,971	15,555	104	640
Percent haploids	0.030	0.006	0.962	1.719

*) Data are from the most successful individual treatment in a given year.

Clearly, these estimates compare favorably with ours. This is not entirely unexpected since our material came from wild populations of an obligate outcrossing species. Efficiency calculations (NEI, 1963; STETTLER, BAWA, and LIVINGSTON, 1969) nevertheless indicate that in black cottonwood the haploid breeding method has research merit even at the low frequencies reported, because large numbers of ovules can be handled in a small greenhouse area. Of course, much efficiency will be gained if the majority of haploid embryos can be grown into seedlings.

Induction Technique

Mixes of pollen from distantly related species with highly irradiated pollen from the maternal species, were successful in inducing haploid parthenogenesis in black cottonwood. However, irradiated "mentor" pollen alone, at 40 or 80 kR, not only stimulated catkin maturation and ovule growth, but produced haploids, too. In fact, the absence of an endosperm in many haploids produced by a pollen mix suggests that the foreign pollen may not have participated at all in the crucial events associated with haploid parthenogenesis.

As we have found that embryogeny in black cottonwood can proceed without a functional endosperm it seems reasonable to explore further improvements in the induction technique with "mentor" pollen alone or, in fact, with purely physical or chemical stimuli. This would eliminate hybrid embryos from the system which probably detract from, rather than contribute to, haploid success.

Additional improvements are to be sought in the appropriate use of *in-vitro* techniques. Haploid embryos are smaller than diploid embryos, and perhaps many of them fail to germinate, or else die shortly after germination. If they were transferred to a nutrient medium during final embryogeny or early seedling stages many would probably survive. We have tested such a system with diploid embryos with some success. Embryo culture might be quite efficient, since ovules containing haploid embryos can often be reconized by their smaller size.

Experimental Strategy

The three-step strategy adopted, (1) to screen randomly picked females for individuals responsive to the haploid-induction technique of remote hybridization; (2) to perfect the induction technique on the responsive females; and (3) to test the perfected induction technique on a larger number of females randomly picked from a wide array of natural populations, seems to be justified by the results. From among the original nine trees we found one moder-

ately and one highly responsive (Nos. 214, 213, respectively) on which the induction technique can be further improved. Three trees (Nos. 210, 212, 215) deserve consideration as putative responsive females.

Little was gained by distinguishing among parent populations between small, isolated, and large, continuous populations. This may be so because the particular populations were not markedly different in their degree of homozygosity, or because the trees were not representative of the populations from which they were picked. The outcome does not, however, question the underlying principle that initial studies on haploid induction are more efficiently conducted on inbred, than on outbred, material (CHASE, 1953; PIESCH, 1968).

Finally, although our results show that haploid *gynogenesis* can be induced in a tree species with a reasonable degree of efficiency, they do not preclude that haploid *androgenesis*, that is the formation of sporophytes from male gametes, may not be more efficient. The recently developed *in-vitro* technique by which NITSCH & NITSCH (1969) raised tobacco plants from immature pollen grains in large numbers, may hold much promise and should be intensively tested on selected forest-tree material.

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Abstract

In 1966—67, nine female Black cottonwood trees, from nine geographically separate populations in the State of Washington, were treated with pollen from *P. alba, P. canescens, P. grandidentata, P. tremuloides,* and *P. deltoides.* Foreign-species pollen was mixed with irradiated (100 kR) "mentor" pollen of the maternal species in a ratio of 2 : 1. In addition to hybrid seedlings found, five females produced haploid and/or diploid seedlings of maternal-species phenotype. One such diploid carried a leaf syndrome (forma *"verrucosa"),* suggestive of homozygosis at one locus if not at all loci. In 1968, four of the five responsive females were treated with mixes of foreign pollen *(P. canescens, P. tremuloides)* and irradiated "mentor" pollen (20, 40, 80 kR) in ratios of 4 : 1, 1 : 4, 0 : 1, respectively. Embryos were sampled 28 days after pollination and examined cytologically. Haploids were found among the progeny of two female parent trees at average frequencies of 0.109 and 0.323 percent, in the most successful combination at 1.719 percent. Irradiated "mentor" pollen alone was capable of haploid induction, too, and may have been the causative agent in the pollen mixes, as judged by the absence of an endosperm in most haploids. Four mixoploids were found, suggesting that spontaneous chromosome doubling may take place during embryogeny. The implications of the results on further improvements, and on ultimate practicality, of haploid breeding are discussed.

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