Induction of haploid parthenogenesis in aspen by postpollination treatment with Toluidine-blue*

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The experimental efficiency of haploids in higher plants and their use in forest-tree-breeding Programmes have been discussed for some time. Experimental work on the artificial induction of haploid parthenogenesis has been particularly active in the Genus Populus. The present study with poplar material from the Section Leuce war, undertaken to determine if haploid embryogeny can be induced by pollen with low or no germinability; or if pollen-tube growth in the stigmatic tissue additionally stimulates embryogeny prior to fertilization. While Stettler (1968 b) suggested the latter possibility, several other references described the use of pollen of weak germinability (Kopecki 1960), of foreign species (Valentine et al. 1968, Stettler 1968 a and b), or of pollen exposed to a Cobalt-60 source (Stettler and Bawa 1971, Valentine et u 1968, Winton and Einspahr 1968). These and other references reviewed by Stettler and Bawa (1971) indicate that in the Genus Populus haploid parthenogenesis occurs spontaneously or can be induced in various ways.

In the present study the induction was carried out with pollen inactivated by the basic dye Toluidine-blue-0 (MERCK) (TB). The chemical inactivation of male gametes using TB had first been described by Briggs (1952) and Edwards (1954), who had induced pseudogamy in mice and frogs. Later Rogers and co-workers applied this principle to higher plants and tested the effects of Toluidine and other Phaenothyacine and Acridine derivatives on pollen. They noticed the inactivation of the mitosis of generative nuclei in pollen cultures in vitro (Rogers and Ellis, 1966; Gearhart and Rogers, 1969) and experimented with TB treated pollen to induce haploid parthenogenesis in *Lycopersicum* and Zea mays (Al-Yasiri, 1967; Al-Yasiri and Rogers, 1971). While the outcome was negative, Winton later repeated the method with Populus tremuloides, using TB treated P. alba pollen, and obtained one haploid seedling (Winton pers. comm.).

Material and Methods

To facilitate the discrimination of parthenogenetic haploids from generatively produced diploids in the expected progenies, parents were chosen that were easily distinguished by the leaf undersurface. The female parents, P. tremula from a Baltic provenance and P. tremula X tremuloides hybrids, all had glabrous leaves. Whereas the male parents, P. alba, all had densely pubescent leaves (Fig. 1). We expected haploid seedlings of parthenogenetic origin to be glabrous, and diploid seedlings from fertilized ovules to have the pubescence typically found in hybrids between aspen and white poplar.

Figure 1. — Leaf undersurface of P. ***tremula*** (a) (glabrous) and P. ***alba*** (b) (pubescent)

Suitable female trees were available from the breeding material of the Institute. Four trees of Populus tremula were chosen from an East Prussian provenance plantation in Wedesbüttel (We 1; We 5; We 6; We 93). Most probably this plantation was established in 1940 with a single tree progeny collected in an indigenous stand near Tapiau. Progenies from cross experiments within this restricted population had been known for severe inbreeding depressions, although they are wellknown in other combinations (Langner per. comm.). The hybrids between P. tremula and P. tremuloides (IA; Th 1290) are described by Seitz (1963). The rationale behind contrasting females from a "pure" species with those of hybrid origin was to see wether differences in heterozygosity would have an impact on haploid frequency. Pollen samples from P. alba were kindly delivered from Italy and Yugoslavia. Each pollination was made with a mix from several samples. During the 3-year study some of the original maternal trees had to be replaced by others, mostly because of the lack of flowers, in one instance a tree's use was discontinued (hybrid IA) because it showed a tendency to develop some hermaphroditic flowers.

TB was used in acqueous solution. In 1970, pollen was mixed with three different concentrations of TB, 5, 10, and 15 ppm, and applied as a slurry to the receptive female catkins with the aid of a brush (Winton pers. comm.). In 1971 and 1972, the catkins were first pollinated with untreated pollen, then sprayed with a 10 ppm TB solution with an atomizer after varied intervals. Treatments with pollen alone (P. tremula or P. alba) and TB spray alone, served as controls. All experiments were carried out on cut

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* In memoriam Professor Dr. Klaus Stern.

branches in running water in climatically controlled isolation chambers at 10 hrs. photoperiod and 18° C temperature.

The effects of both treatments on pollen germinability and pollen tube growth in situ were examined microscopically in fluorescent light. Pollinated stigmas were sampled 24 hrs. after the application of the pollen/TB slurry (1970) and from the control, and after the spraying of the TB (1971) respectively. Pollen germination and length of the pollen tube were assessed in 3 stigmatic flaps sampled from 6 to 9 catkins per tree and treatment. The stigmatic flaps were stained with a fluorochromatic fractionn of Waterblue (BAYER) according to the method of Linskens and Esser (1957). — In the later experiments this method was replaced by an easier fluorochrome preparation with Chinablue (MERCK) in alcaline medium (Preil und Reimann-Philipp 1969). Both preparations gave satisfactory results. The microscopical observation was made with a ZEISS photomicroscope using a mercury vapor lamp (HBO 200) as a source of ultraviolett light with a BG 12 exciter filter and No. 50 barrier filter. The microphotographs were taken with an AGFAPAN 25 professional film.

The selection of the glabrous maternal seedlings started as soon as the 3rd or 4th leaf had been developed. Due to the heterophylly of the Genus it was possible to confirm this selection from leaves of the 2nd flush during the following years.

To determine the chromosome number suitable material was fixed from the youngest leaves beginning in June. In cases were the material was unsatisfactory because of poor growth the fixations were repeated from leaves of the second flush during the following years. The leaves were fixed in Alcohol : Acetic Acid 3 : 1 and stained in Acetic Orcein (Orcein synthetic (GURR) : Orcein natural (GURR) : 1 : 1; Rommel pers. comm. 1973). Extraneous plasmatic inclusions hindering a distinct image of the metaphase chromosomes, were dissolved by carefully heating the material in a drop of 30% Acetic Acid (Rommel und Zweigert 1972).

Results and Interpretation

Pilot Experiments 1970: The response of pollen germination to the TB treatment was observed after the application of the pollen/TB slurry in 1970 *(Table 1)*. Control pollinations with the untreated pollen mixture revealed that long pollen tubes had penetrated into the female tissue, although some individual differences between the 3 female trees were evident. By contrast, pollen germination was very poor, and only a few, short pollen tubes were observed after the application of the slurry in all TB concentrations, but the fewest in the highest concentration. Similar results were found in the pollen/H_2O slurries suggesting that pollen germination was inhibited more by the effects of moisture than by the chemical nature of TB.

Catkin survival and seed set were very poor in these experiments including those from control pollinations. Accordingly, *Table 2* summarizes those cases where positive results were obtained. Seed set failed entirely in the case of 5 ppm and 15 ppm TB/slurries whereas in the 10 ppm treatment a total of 7 seedlings were raised, four of which had glabrous leaves, thus suggesting parthenogenetic origin.

Table 2. -- Numbers of seedlings obtained from treating catkins with pollen/TB slurry in 1970 4).

parent ♀	Pollen/TB slurry, 10 ppm (♂ *P. alba*) No. catkins	No. seeds	seedlings Total No. 3)	seedlings maternal No.
We 5 1)	5	3	3	1
We 5 control 5)	26	24	24	—
We 93 1)	4	2	—	—
We 93 control 5)	5	29	29	—
Th 1290 2)	20	4	—	—
IA 2)	25	4	4	3

1) *P. tremula*

2) *P. tremula* × *P. tremuloides*

3) comprises both hybrid and maternal phenotypes

4) Because of abnormally low seed set no frequencies were calculated

5) controlled pollination without TB treatment

1971/1972 Experiments: From the pilot experiments in 1970 it was obvious, that the TB treatment of pollen reduced pollen germination and tube growth. Consequently the results corresponded with those from other experiments in which pollen of low germinability had been used to induce haploid parthenogenesis. Yet, other experiments by Stettler and co-workers seemed to suggest that pollen germination and tube growth were essential for catkin maturation and embryogeny (Stettler 1968 b; Stettler and Bawa, 1971, and unpublished results). Assuming that pollen-tube growth in the female tissue would stimulate embryogeny even before the fusion of male and female gametes it was decided to test a treatment in which the pollen would be first permitted to develop a tube into the stigma before being exposed to TB. If properly timed, a post-pollination treatment with TB might still inactivate the male gametes in time to prevent fertilization. This stratagem was tested in the 1971/1972 experiments.

Table 3 summarizes the 1971-data of pollen germination and pollen tube growth resulting from the TB treatment of pollinated stigmas after four different intervals. In all classes the pollen had germinated and pollen tubes had penetrated into the stigmatic tissue before the TB spray inhibited further growth. But pollen-tube length varied considerably, depending on the interval between pollination and TB treatment *(Fig. 2 a—c)*. In the series with the shortest intervals (6 hrs.) pollen germination and tube growth was poorest, whereas in the series with the longest

Table 1. — Pollen-tube growth at 24 hrs. after treating stigmas with pollen slurries. Numbers indicate the percentages of pollen grains having long (l), short (s), or no (o) tubes.

♀ parent	5 ppm TB l	s	o	10 ppm TB l	s	o	15 ppm TB l	s	o	H_2O slurry l	s	o	control l	s	o
	Pollen/TB slurries (♂ *P. alba*)														
We 5 1)	—	0.6	99.4	0.2	—	99.8	—	0.2	99.8	—	0.6	99.4	66.6	0.3	33.1
Th 1290 2)	—	1.9	98.1	—	0.06	99.0	—	--	100	—	—	100	42.2	15.0	43.8
IA 2)	—	2.4	97.6	—	0.3	99.7	—	--	100	—	0.6	99.4	41.7	2.0	56.3

1) *P. tremula*

2) *P. tremula* × *P. tremuloides*

Table 3. — Pollen-tube growth at 24 hours after treating pollinated stigmas with TB spray (10 ppm) in 1971. Numbers indicate the percentages of pollen grains having long (l), short (s), or no (o) tubes.

♀ parent	Interval between pollination and TB spray 6 hours l	s	o	12 hours l	s	o	18 hours l	s	o	24 hours l	s	o
Th 1290[2]	19.6	14.2	66.2	30.5	21.5	48.0	19.5	14.8	65.8	52.7	47.3	—

[2] *P. tremula* × *P. tremuloides*

interval (24 hrs.) all pollen grains had either a short or long tube. Little difference was noted, however, between 6, 12, and 18 hrs. intervals.

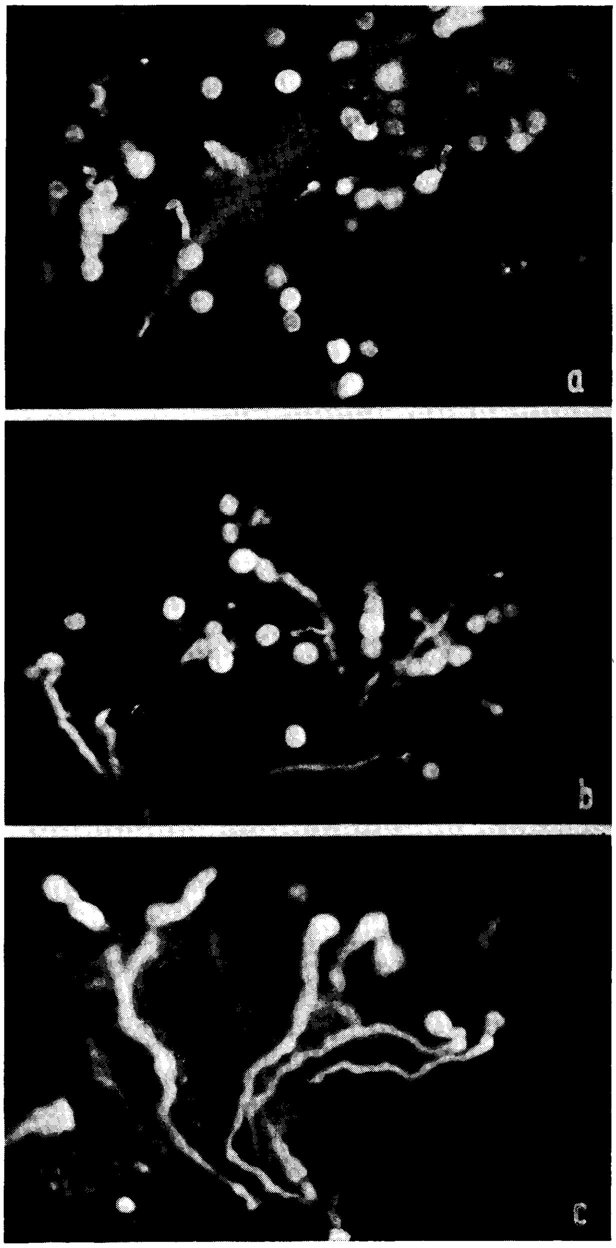

Figure 2 a—c. — Control of pollen germination after different intervals of TB treatment a) 6 hrs., b) 12 hrs., c) 24 hrs. after pollination.

The number of seedlings obtained from these experiments are summarized in *Table 4 a and b.* In both years no seed was obtained from the 6-hour interval treatment indicating that inactivation had happened too soon, although some long pollen tubes had been observed in the respective germination test. On the other hand, only hybrid seedlings were obtained from the 24-hour-interval treatment, indicating that fertilization had already happened before the TB spray was effective. However, in both years, substantial numbers of maternal seedlings were obtained from the 12-hour and 18-hour-interval treatments.

Table 4. — Number of Seedlings obtained from post-pollination TB treatment

♀ parent	Internal between pollination and TB spray treatment 12 hours Total[3] No.	Maternal No.	%	18 hours Total[3] No.	Maternal No.	%
a) 1971 data						
Th 1290[2]	507	30	5,92	1027	90	8,7
b) 1972 data						
Th 1290[2]	14	6	43	44	9	20,4
We 1[1]	708	166	23,4			
We 6[1]	61	8	13,1			
We 93[1]	376	104	27,7			

[1] *P. tremula*
[2] *P. tremula* × *P. tremuloides*
[3] comprises both hybrid and maternal phenotypes

For various reasons only one tree could be used throughout all three years, Th 1290. While it had failed to give any seed in the 1970 pilot study it was responsive in the two subsequent years to the 12 hour and 18-hour interval treatments, but more so in 1972 than in 1971. The factors responsible for this year-to-year variation are unknown and may well have to do with exogenous influences independent of the study. Nevertheless it is of interest to note that this tree which gave the highest frequencies of maternal seedlings was a hybrid.

Due to the post-pollination TB-treatment seeds of zygotic as well as parthenogenetic origin would develop in the same capsule. Thus, from the beginning the haploid embryos would be subject to a selection of fitness, particularly as the heterozygosity of the dioecious Genus *Populus* would cause a wide variability in which many recessive genes would express themselves among the haploid embryos and seedlings. *Table 5* gives the data on seedlings survival obtained in four progenies until transplantation before and after the selection of the maternal phenotypes during the first season. As the experimental design based on the selection of a leaf character, no respective data were obtained during embryo development and the early growth of the seedlings. But a wide variability of seedlings survival and other characters were noted between the four progenies. While the maternal seedlings originating from the hybrid TH 1290 were very homogenous remarcable differences were noted between and within the *P. tremula* progenies, such as growth rate and type, leaf shape, and susceptability to diseases. These results may correspond with former observations of inbreeding depression in cross experiments within the Wedesbüttel population (Seitz pers. comm.). *Figures 3 a—f* demonstrate the wide variation of growth rate within the phenotypic maternal seedlings from the We 1 progeny.

Tradescantia, but the application of this principle to induce haploid parthenogenesis in *Lycopersicum* and *Zea mays* failed to produce viable embryos (Al-Yasiri 1967; Al-Yasiri and Rogers 1971). The authors attributed this to the lack of an endosperm as result of the prevention of double fertilization. The present results differ from these earlier experiments and may be due to the fact that poplars, as all *Salicaceae*, have an inconspicuous nuclear endosperm which may not be vital for further embryo development. Similar observations had been earlier made by Seitz (1952/53) and were also described by Stettler and Bawa in haploidy-induction experiments with *P. trichocarpa* (1971). Furthermore, in the Genus *Populus* haploid parthenogenesis had been induced occasionally before, after pollination with pollen of weak germinability, irradiated pollen with good germinability, and remote hybridization. It may be concluded therefore that the phases during which the essential stimulation of the responsive egg cell can take place, may be relatively long (although in absolute terms no longer than probably 2 days). Within this period some egg cells may already be stimulated by the mere pollination of the stigma, thus giving rise to some haploid embryos. The high frequency of maternal seedlings after the late application of TB in the experiments 1971 and 1972 confirmed the assumption on which the experiments in both years had been based: The pollen tubes growing into the pistils stimulated the potential condition in the unfertilized egg cell for embryo development before the fertilization took place. The delayed application of TB then inactivated the male gametes and consequently prevented the fusion of the male and female nuclei to form the diploid zygote, thus resulting eventually in a haploid embryo of partenogenetic origin. The choice of the parents in the described experiments, using the trait glabrous/pubescent permitted an efficient selection of the maternal, phenotypic seedlings during the first season of the progenies.

Table 5. — Survival of seedlings obtained from the 1972 post-pollination TB-treatment.

♀ parent	Number of seedlings raised[3])	Seedlings surviving through transplantation at 6 weeks No.	%	Transplanted seedlings with maternal phenotype No.	%	Surviving maternal seedlings at 5 months No.	%
We 1[1])	708	672	94.9	136	20.2	82	60.1
We 6[1])	61	44	72.1	1	2.2	1	—
We 93[1])	376	340	90.5	19	5.5	14	73.7
Th 1290[2])	58	44	75.9	15	34.1	10	66.7

[1]) *P. tremula*
[2]) *P. tremula* × *P. tremuloides*
[3]) comprises both hybrid and maternal phenotypes

Table 6. — Chromosome counts made in samples of maternal and hybrid phenotypic seedlings from four 1972-progenies[3])

♀ parent	Hybrid seedlings 2n	unknown	Total No.	Maternal seedlings n	aneuploid	2n	unknown	Total No.
We 1[1])	11	5	16	18	5	1	43	67
We 6[1])	2	1	3	1	—	—	—	1
We 93[1])		not determined		3	2	—	9	14
Th 1290[2])		not determined		7	—	—	7	14

[1]) *P. tremula*
[2]) *P. tremula* × *P. tremuloides*
[3]) figures indicate seedlings numbers in different cytological categories

Table 6 summarizes the chromosome counts from the 1972 material made in 1973 and 1974. Although only few of the hybrid seedlings were checked, all of them had diploid chromosome numbers. By contrast, most of the maternal phenotypic seedlings were haploid or chimaeras in which case different degrees of aneuploidy were observed in the same tissue. These findings are also in line with the observations about varying viability summarized in *Table 5*. While in the best progeny (We 1) only 67 maternal seedlings survived the first year, 43 were too small and weak for fixation. Of the remaining 24 seedlings in which chromosome numbers could be counted, 18 were haploid in 1973, 1 was diploid, and 5 revealed to be chimaeras with nuclei of 19 to 28 chromosomes in the same tissue (*Figures 4 a—c*). All results were confirmed in 1974. This cytological situation may be caused by different degrees of mitotic irregularities such as chromatide segregation, and/or faulty chromosome distribution during anaphase. If, however, regular chromosome doubling occured early enough during the embryo development, maternal diploid seedlings may emerge from it. Otherwise such irregularities will cause mixoploidy with varying chromosome numbers in different parts of the tissue. The degree of irregularity as well as the severity of chromosome loss may be partly responsible for the inviability of seedlings. The remaining samples of maternal phenotype seedlings gave either inconclusive counts or offered no cell divisions for assessment. Finally some seedlings were too sub-vital to permit the sampling of tissue.

Conclusions

Observations described by Gearhart and Rogers (1969) showed the suppression of the generative nuclear division after TB had been added to pollen cultures in vitro of

As shown by the cytological examination haploids were only found among the maternal phenotypes. With regard

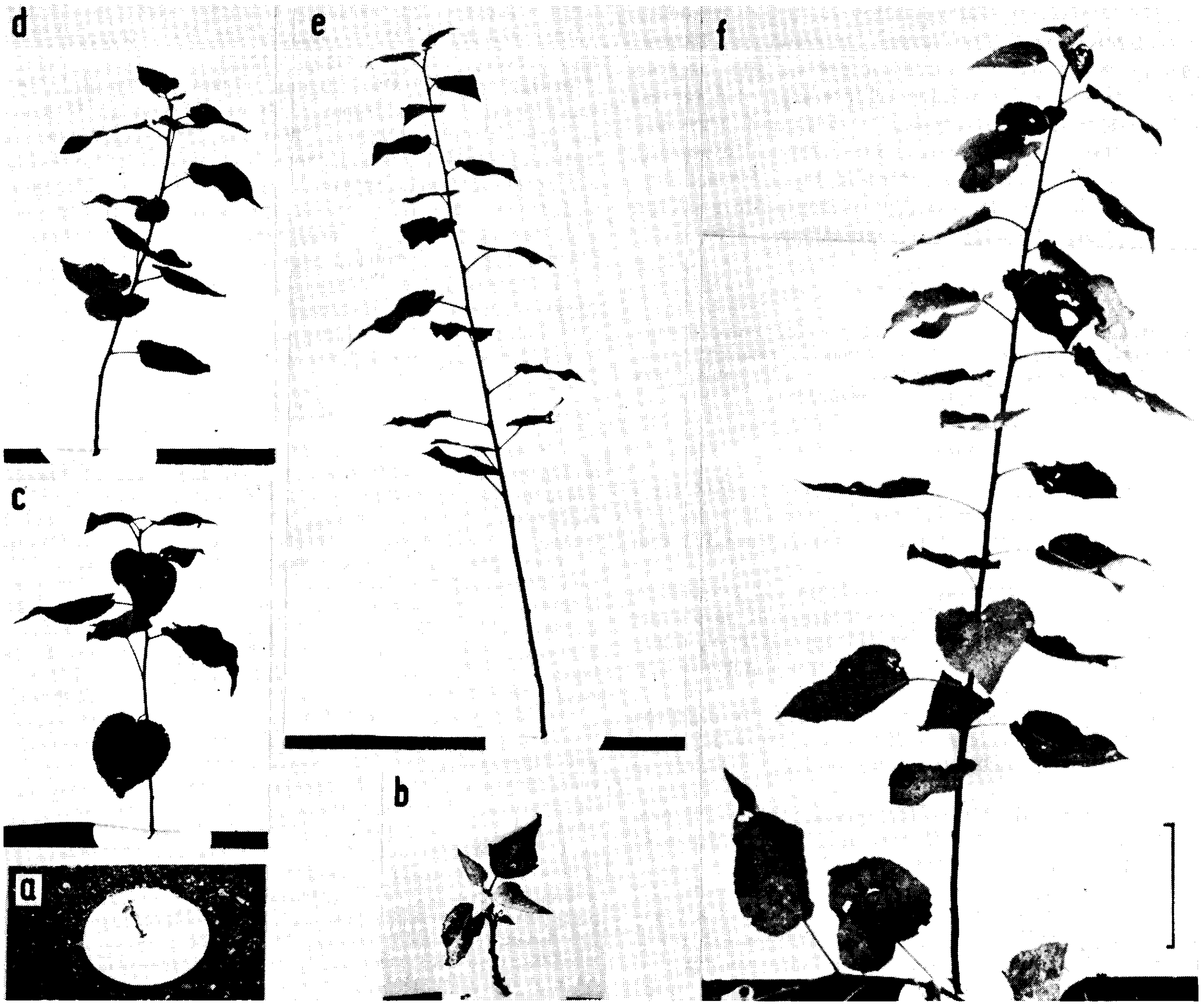

Figure 3 a—f. —Variation of growth rate within the phenotypicmaternal seedlings from the We 1 progeny (———— 10 cm).

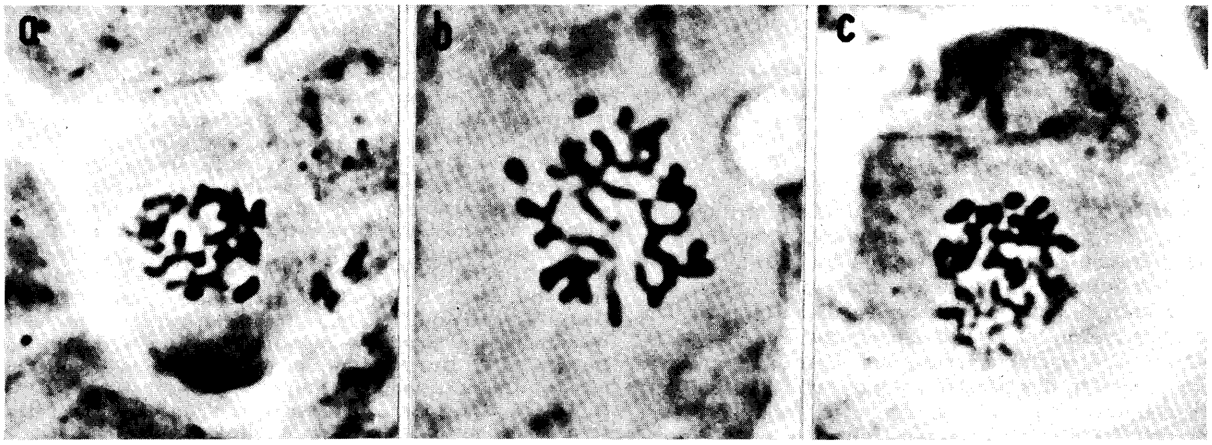

Figure 4 *a*—c. — Chromosome numbers counted in 3 maternal seedlings from the We 1 progeny a) haploid (9=19), b) aneuploid, c) diploid (2n = 38).

to the observation of mixoploidy in the maternal seedlings similar findings have been reported elsewhere. In tree species mixoploidy has been observed after experimentally increased as well as reduced ploidy. Stettler and Bawa (1971) and Winton and Einspahr (1968) described mixoploidy resulting from haploid seedlings in *Populus,* while in Gymnosperms species similar observations were made frequently in artificial polyploids in the Genus *Larix* (Illies 1966 a and b) as well as spontaneously in *Picea* and *Chamaecyparis* (Illies 1952, 1969) and in *Thuja* (Pohlheim 1968, 1971). A closer study of this phenomenon in the present material is in progress (Illies and Amenda).

Finally it can be concluded from the present results that the procedure offers a practical method to increase the production of haploid parthenogenesis in *Populus,* thus making it feasible to consider the haploid approach in a breeding programme. By screening a large number of individuals it should be possible to find such females which are particularly responsive to it, although allowance should be made for the year-to year variation in factors underlying this responsiveness. The severe selection for fitness, beginning as early as the development of the haploid embryo, would reduce the number of seedlings of potential merit in a breeding programme to a minimum. Furthermore, the method would give efficient access to the cryptic variation of recessive genes inherent in the parental material, thus contributing to a better understanding of the genetic architecture of forest tree populations.

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Summary

Experiments were carried out, to induce haploid parthenogenesis in *Populus tremula* and *P. tremula* × *P. tremuloides*, with pollen, that was inactivated by the basic dye Toluidine-blue (TB).

To facilitate the detection of haploids in the progenies, pollen from *P. alba* was used. Thus, seedlings of parthenogenetic origin were expected to be of the maternal, glabrous, phenotype, while diploid hybrids from zygotic origin were expected to be pubescent.

Initial pilot experiments involved the treatment of pollen with different concentrations of TB in acqueous solution and the subsequent application of this pollen/TB slurry to receptive stigmas.

Microscopic study of the pollen germinability in situ showed that pollen-tube growth was inhibited which also resulted in an extremely low seed set.

The subsequent experiments used a modified technique in which the TB solution was applied as a spray at a variable interval after pollination. By that time, pollen tubes had penetrated the female tissue before being inactivated by the dye. These second experiments resulted in a much higher frequency of maternal seedlings.

The cytological examination of the maternal seedlings revealed primarily haploids, some aneuploids and one diploid maternal seedling, whereas all hybrid seedlings were found to be diploid.

Key words: Populus Leuce; induction of haploid parthenogenesis; haploid, maternal, phenotypic seedlings; post-pollination treatment; basic dye Toluidine-blue.

Zusammenfassung

In Versuchen zur Induktion der haploiden Parthenogenese bei *Populus tremula* und *P. tremula* × *P. tremuloides* wurde der basische Farbstoff Toluidin-blau (TB) zur Inaktivierung des Pollens verwendet. Um das Auffinden der haploiden Sämlinge parthenogenetischen Ursprungs in den Nachkommenschaften zu erleichtern, wurde Pollen von *P. alba* benutzt. Die parthenogenetischen Sämlinge mußten dann dem Phänotyp der Mutter entsprechend glattblättrig sein und sich von den rauhblättrigen, aus regulärer Befruchtung der Zygote entstandenen Hybriden leicht unterscheiden lassen.

In Vorversuchen wurde wässrige TB-Lösung verschiedener Konzentration (5, 10, 15 ppm) in Pollen/Toluidin Suspensionen auf die bestäubungsfähigen Narben aufgetragen. Wie die mikroskopische Kontrolle des Pollens auf anschließend fixierten Narbenlappen zeigte, war die Pollenkeimung fast vollständig ausgefallen. Der niedrige Samenansatz entsprach dieser Beobachtung.

In den folgenden Versuchen wurde das Toluidin in verschiedenen Zeitabständen nach der regulären Bestäubung auf die Blüten gesprüht. Wie die Pollenkontrolle ergab, hatte der Pollen vor Auftragen des Toluidins gekeimt, und die Pollenschläuche waren in das Gewebe der Narben eingedrungen, ehe ihr weiteres Wachstum inaktiviert wurde. Von den angezogenen Sämlingen waren bis zu 20% glattblättrig und entsprachen damit dem Phänotyp der Mutter.

Die zytologische Überprüfung der glattblättrigen Sämlinge ergab in der Mehrzahl Haploide, einige Aneuploide und einen diploiden Sämling, während die phänotypisch hybriden Sämlinge diploid waren.

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