Induction of Polyploidy in Pines by means of Colchicine Treatment

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Since the discovery of the colchicine treatment as a method of inducing chromosome doubling in plants, many artificial polyploids have been made in agricultural plants. So far, though, few efforts have been made to induce polyploidy in forest trees.

Mirov and Stockwell (1939) were probably the first to succeed in inducing chromosome mutation in woody plants through the use of colchicine.

Hirayoshi (1942) induced polyploidy in several woody plants by treating seeds and growing buds of seedlings with a 0.1-0.2 percent colchicine solution. Toda (1942) induced tetraploidy and sectorial chimaeric octoploidy in Rhus succedanea through treating cotyledons with a 0.4 percent colchicine mixed with lanolin. Jensen and Levan (1941) induced polyploidy in Sequoia gigantea by germinating seed on filter paper moistened with a 0.2 percent solution of colchicine. Fujii (1946) demonstrated that soaking germinated plumules in a 0.3-0.4 percent colchicine solution is a feasible means of inducing polyploidy in Robinia pseudoacacia. Nagai (1946) induced tetraploidy in X Pinus densithunbergii by soaking seeds in a 0.4 percent colchicine solution for 20 days. Ando (1949) induced tetraploidy in Chamaecyparis obtusa by soaking seeds in a 0.4 percent colchicine solution for 24 hours, germinating them on moist filter paper, and coating the surface of the germinated seed with the same solution for 20 days. Nabeshima (1949) obtained a tetraploid of Paulownia *tomentosa* by soaking the seeds in 0.4 percent colchicine solution for 24 hours and then germinating the seeds on filter paper moistened with 0.025 percent colchicine solution until germination.

All of the foregoing reports refer to chromosome mutations effected immediately after the treatment. It is well known that in trees polyploid tissues arising from treatment often revert to the normal diploid condition. This may mean that the polyploid cells or tissues are usually swamped by the faster-growing diploid tissues surrounding them.

This paper reports on experiments conducted for the purpose of inducing polyploidy in several species of pine by means of colchicine treatment. Materials, facilities, and equipment needed for the investigation were made available at the Institute of Forest Genetics by the California Forest and Range Experiment Station of the United States Forest Service, under the United States Information and Educational Exchange Program during the period from April to November 1951. The author wishes to express his hearty thanks to Mr. F. I. Righter, Dr. J. W. Duffield, and Dr. N. T. Mirov of the Forest Service and Mr. R. Z. Callaham of the Bureau of Entomology and Plant Quarantine, United States Department of Agriculture, for their advice and help in carrying out the investigations.

Seed-Treatment Experiments

Materials and Methods

Seeds of the following six pines were used: Pinus *ponderosa*, P. jeffreyi, P. contorta var. latifolia, X P. *attenuradiata*, X P. densithunbergii, and P. lambertiana. The seeds had been collected during 1940—1945 and stored at 35°—40° F. until used. The seeds of P. ponderosa, P. *jeffreyi*, and P. lambertiana were stratified for 80 days, and the other seeds were stratified for 20 days, before treating them. Two colchicine treatments were employed, as follows:

Treatment A: Soaking seeds in 0.2 percent colchicine solution for 1, 2, 3, 4, 5, and 6 days respectively. One hundred seeds of each species were subjected to each treatment.

Treatment B: Seeds which had started to germinate in moist sand were placed on filter-papers in petri dishes containing 0.2 percent or 0.4 percent colchicine solution. Each seed was arranged so that half of it was submerged in the solution during the treatment which was for one of the following periods: 4, 5, 6, 7, 8, 9, 10 days.

After treatment the seeds were sown in pots of sand in the greenhouse in early May 1951. At the same time, untreated seeds were sown in the same way for comparison.

After completion of germination. all of the pots were removed to a lath house.

Results

Treatment *A*

The seeds began to germinate one week after sowing. The germination percentages for each treatment are given in table 1. No marked abnormality in the germination of treated seeds was observed, but the data suggested that

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Table 1
Germination percentage of seeds 3 weeks after beginning of germination*)

Species	Soaking in 0.2 percent colchicine for						
	1 day	2 days	3 days	4 days	5 days	6 days	Control
	Percent germinated						
P. ponderosa	77	69	84	55	54	70	57
P. *jeffreyi*	12	6	6	4	3	3	11
P. contorta var. latifolia	10	18	9	23	17	13	14
P. lambertiana	8	10	8	8	0	6	2
× P. densithunbergii	85	90	66	94	77	54	86

*) × P. *attenuradiata* was not used in this test.

some of the treatments may have stimulated germination of *P. ponderosa* seeds. Seedling abnormalities suggestive of polyploidy were not observed; in fact, all seedlings appeared to be normal when compared with the controls.

Treatment B

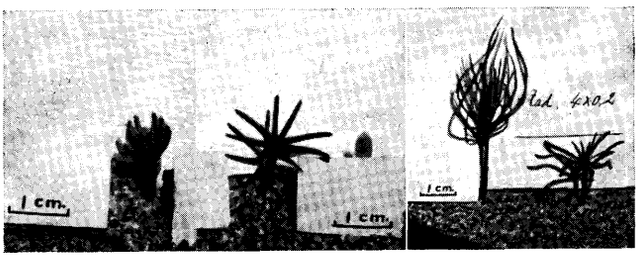
Abnormalities suggestive of polyploidy were observed among all the species included in the tests of treatment B.

Morphological abnormalities:

Morphological abnormalities of cotyledon seedlings were differentiated into three classes:

1. VERY ABNORMAL: Stem, dwarfed and swollen; cotyledon, short and swollen. (Fig. 1.)

2. MODERATELY ABNORMAL: Stem, dwarfed but not swollen; cotyledon, short and swollen. (Figs. 2 and 3.)



Figs. 1—3 (left to right): — Fig. 1. Very abnormal *P. ponderosa* obtained from Treatment B (6 days in 0.4 percent colchicine solution). 17 days after sowing. — Fig. 2. Moderately abnormal *P. ponderosa* obtained from Treatment B (6 days in 0.2 percent colchicine solution). 16 days after sowing. — Fig. 3. × *P. attenuradiata* obtained from Treatment B (4 days in 0.2 percent colchicine solution). Left: normal. Right: moderately abnormal. 17 days after sowing.

Table 2
Numbers of abnormal seedlings abtained from different dosages of colchicine, applied in Treatment B*)

Colchicine dosage		Number of seed sown	Number of germinated seed at 4 weeks after sowing					
Concentration, percent	Duration, days		Very abnormal	Moderately abnormal	Slightly abnormal	Total abnormal	Normal (unaffected)	Died dued to defective root
Pinus ponderosa								
0.2	4	11	1	1	1	3	5	1
	5	11	—	1	8	9	—	—
	6	7	1	2	—	3	—	2
	7	12	1	—	1	2	—	1
	8	11	1	1	2	4	—	1
	9	13	5	1	1	7	1	4
0.4	4	10	3	—	—	3	1	3
	5	9	1	1	1	3	—	—
	6	10	4	—	1	5	—	3
	7	10	3	—	1	4	—	3
	8	10	4	—	—	4	—	3
	9	10	4	—	1	5	—	3
Pinus jeffreyi								
0.2	4	6	—	—	—	—	2	—
	5	6	—	—	1	1	3	—
	6	5	—	—	1	1	2	—
	7	7	1	2	—	3	1	1
	8	6	—	—	—	—	1	1
	9	6	—	—	—	—	1	—
0.4	4	4	—	—	—	—	2	—
	5	6	—	—	—	—	3	—
	6	6	—	—	—	—	—	—
	7	8	—	—	2	2	—	1
	8	8	1	—	2	3	1	1
	9	8	1	—	—	1	1	—
× *Pinus attenuradiata*								
0.2	4	10	4	1	4	9	1	2
	5	13	4	—	4	8	—	2
	6	12	4	—	4	8	—	2
	7	12	4	1	2	7	1	2
	8	12	3	—	1	4	3	3
	9	10	—	—	—	—	—	—
	10	10	—	—	—	—	2	—
0.4	4	10	3	—	5	8	—	1
	5	12	5	—	—	5	—	2
	6	10	2	—	—	2	1	1
	7	10	—	—	—	—	—	—
	8	7	1	2	1	4	—	—
Pinus contorta var. *latifolia*								
0.2	4	12	9	—	—	9	—	5
	5	12	3	—	1	4	—	3
	6	12	7	—	—	7	—	5
	7	12	8	—	—	8	—	7
	8	12	—	—	—	—	—	—
0.4	4	12	5	1	—	6	—	2
	5	12	7	—	—	7	—	7
	6	12	9	—	—	9	—	8
	7	12	—	—	—	—	—	—

*) × *P. densithunbergii* and *P. lambertiana* were not used in this test.

3. SLIGHTLY ABNORMAL: Stem, normal; cotyledon, shortened.

The numbers of such abnormal seedlings obtained by various dosages are given in table 2.

Almost all the VERY ABNORMAL seedlings died sooner or later because of defective root systems. Most of the MODERATELY ABNORMAL and SLIGHTLY ABNORMAL seedlings, however, developed to considerable size by late September, and some of them continuously produced conspicuously thick, short, bluish, primary leaves which, upon cytological examination, were found to exhibit polyploidy. Most of these polyploid seedlings were approximately normal in height growth (See Figs. 4 to 7 and table 3).

Chromosomal abnormalities

Growing needles were collected between 9:30 and 10:30 a. m. in July, and between 10:00 and 11:00 a. m. in September. Special care was taken not to injure the bases. Needle-bases were immediately soaked in saturated paradichlorobenzene solution for ten hours to shorten the long, tangled chromosomes. Next the needle-bases were removed to normal HCl for 4 hours to digest away the middle lamella holding the cell together. They were then fixed in CARNOY's fluid for 24 hours, and from these materials, aceto-carmin smear preparations were made.

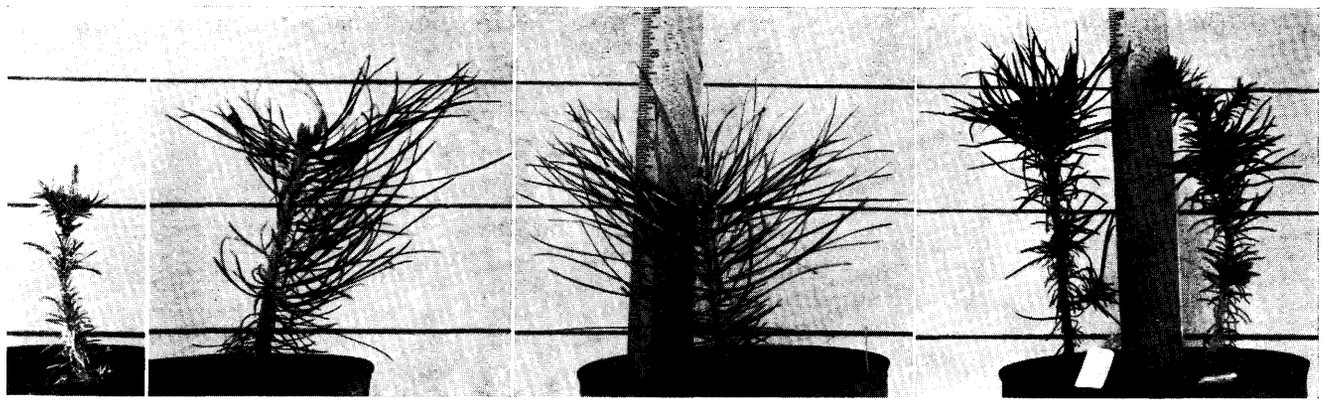
The chromosome numbers observed are given in table 3.

The diploid chromosome number (2n) of *Pinus* is 24, as all investigations so far have shown (RICHENS, 1945, SATO, 1949). In this experiment, a number of seedlings were tetraploids, although some of them were mixoploids, consisting of tetra-, tri-, and diploid, tissues (Table 3, Figs. 8 to 11). Most of the polyploid seedlings maintained the polyploid characters up to July 1952, but a few of them reverted to the diploid condition. In *P. jeffreyi* and × *P. attenuradiata* no complete tetraploidy was observed. No surviving polyploids of *P. contorta* var. *latifolia* were obtained because of very high mortality of abnormal seedlings, which presumably were polyploids. Although the relative distributions of cells of different chromosome number in the mixoploids are not clarified, some *P. ponderosa* mixoploids were sectorial chimaeras, having both dwarfed and normal primary leaves (Fig. 5).

Table 3
Chromosome numbers, morphology, and height of treated seedlings.

Seedling number	Colchicine dosage percent/days	Date of sowing month/day/year	Somatic chromosome number		Height in inches		Leaf morphology	
			Middle of July-'51	Late Sept.-'51	Late Sept.-'51	Late Nov.-'52	Late Sept.-'51	Late Nov.-'52
P. ponderosa								
1	0.2/8	5/8/51	48	48	2.0	3.5	S. T. B.	S. T. B.
2	0.2/8	5/8/51	48	24	2.0	—	Nor. (reverted)	—
3	0.2/9	5/13/51	48	48	2.0	5.5	S. T. B.	S. T. B.
4	0.2/4	5/8/51	48	48	2.5	4.0	S. T. D.	S. T. D.
5	0.2/4	5/8/51	48	48	2.5	4.5	S. T. D.	S. T. D.
6	0.2/4	5/8/51	48,24	48, 24	3.0	5.5	S. T. D.	Nor./ S. T. D. (chimaera)
7	0.4/6	5/15/51	48,24	48, 24	2.3	4.0	S. T. D.	Nor. (reverted?)
8	0.2/6	5/5/51	48	48, 24	2.3	5.5	M. T. D.	M. T. D.
9	0.4/4	5/8/51	48	48	3.5	5.8	S. T. B.	S. T. D.
11	0.2/6	5/17/51	48	48	1.5	3.0	S. T. D.	S. T. D.
12	0.2/7	5/13/51	48,24	48, 24	3.3	7.5	Nor./ S. T. D.	Nor./ S. T. D. (chimaera)
13	0.4/7	5/16/51	48,24	48, 24	2.0	4.3	S. T. D.	Nor. (reverted?)
15	0.4/5	5/9/51	48,24	48, 24	1.0	4.3	S. T. D.	Nor. (reverted?)
16	0.4/5	5/9/51	48,24	48, 24	2.0	4.0	S. T. D.	S. T. D.
18	0.2/4	5/9/51	48,24	48, 36, 24	3.3	4.8	S. T. D.	Alm. Nor. (reverted)
21	0.4/6	6/16/51		48	1.5	2.5	S. T. B.	S. T. B.
22	0.4/6	6/16/51		48, 24	1.5	3.0	S. T. D.	S. T. D.
23	0.4/6	6/16/51		48, 36, 24	2.0	3.8	S. T. B.	S. T. B.
24	0.4/6	6/16/51		48,	2.0	4.5	S. T. D.	S. T. D.
25	0.4/6	6/16/51		48,	2.0	4.3	S. T. D.	S. T. D.
26	0.4/6	6/16/51		48, 36, 24	2.0	Died	S. T. D.	—
27	0.4/6	6/16/51		48, 36, 24	2.5	4.0	Nor./ S. T. D.	Nor./ S. T. D. (chimaera?)
28	0.4/6	6/16/51		48, 36, 24	2.0	3.3	Nor./ S. T. D.	Nor. S. T. D. (chimaera?)
29	0.4/6	6/22/51		48, 36, 24	1.5	3.5	S. T. B.	S. T. B.
30	0.4/6	6/22/51		48, 36, 24	1.5	3.5	S. T. B.	S. T. B.
31	0.4/6	7/25/51				2.8	S. T. B.	S. T. B.
32	0.4/6	7/25/51				2.3	S. T. B.	S. T. B.
33	0.4/6	6/26/51				4.5	S. T. B.	S. T. B.
34	0.4/6	7/25/51				5.3	S. T. B.	S. T. B.
Control		5/9/51	24	24	3.5	6.8	Nor.	Nor.
× *P. attenuriadiata*								
1	0.2/4	5/10/51	48, 36	48, 36	4.5	7.3	S. T. D.	S. T. D.
2	0.2/4	5/10/51	48, 36, 24	24	4.5	Died	Nor. (reverted)	—
3	0.2/4	5/10/51	48, 36, 24	48, 36, 24	3.5	Died	S. T. D.	—
4	0.2/4	5/10/51	48, 36	48, 36	4.0	8.5	S. T. D.	S. T. D.
8	0.2/6	5/15/51	48, 36	48, 36	4.0	9.5	S. T. D.	S. T. D.
10	0.2/5	5/11/51	48, 36, 24	48, 36, 24	3.5	6.3	M. T. D.	M. T. D.
12	0.2/6	5/15/51	48, 36, 24	48, 36, 24	2.3	4.8	M. T. L.	M. T. L.
16	0.4/4	5/10/51	48, 36, 24	48, 36, 24	4.0	8.5	M. T. D.	Nor. (reverted?)
Control		5/10/51	24	24	4.0	6.5	Nor.	Nor.
P. jeffreyi								
1	0.4/4	5/15/51	48, 24	48, 24	2.0	3.3	S. T. D.	S. T. D.
Control		5/15/51	24	24	3.5	4.8	Nor.	Nor.

Abbreviations: S: short, M: moderate long, T: thick, B: bluish, D: dark green, L: light green, Nor: normal.

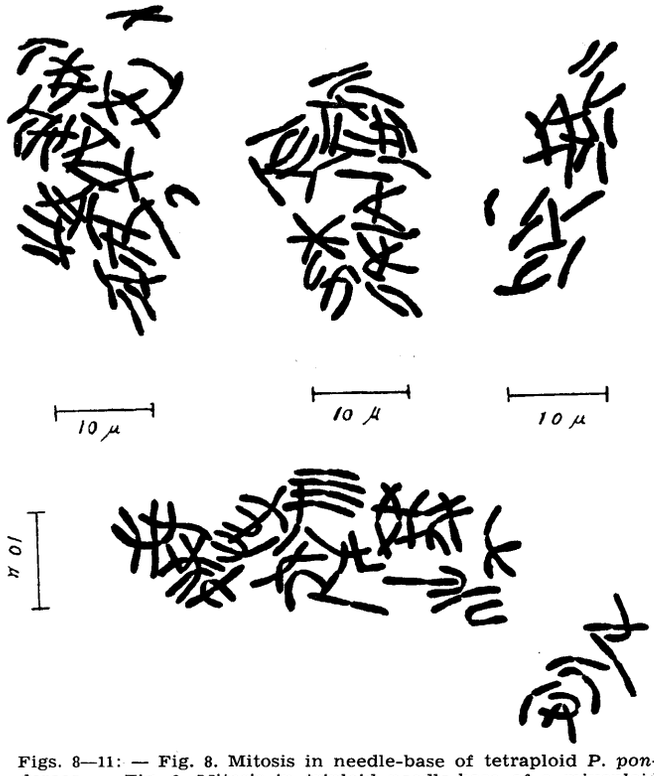


Figs. 4—7 (left to right): — Fig. 4. Tetraploid *P. ponderosa* obtained from Treatment B (6 days in 0.2 percent colchicine solution). Age: 18 months. — Fig. 5. Sectorial chimaeric (mixoploid) *P. ponderosa* obtained from Treatment B (7 days in 0.2 percent colchicine solution). Short leaves are tetraploid; long leaves are diploid. Age: 18 months. — Fig. 6. Diploid *P. ponderosa* unaffected by Treatment B. Age: 18 months. — Fig. 7. Mixoploid (3n, 4n) × *P. attenuradiata* seedlings obtained from Treatment B (4 days in 0.2 percent colchicine solution). Age: 18 months.

Table 4
Size and number of stomata in tetraploid, mixoploid, and diploid pine seedlings.

Seedling	Length of guard cell				No. of stomata per sq. 0.1 mm			
	Real value μ		Ratio [4])		Real value μ		Ratio [4])	
	Primary leaf	Secondary leaf	Primary leaf	Secondary leaf	Primary leaf	Secondary leaf	Primary leaf	Secondary leaf
P. ponderosa								
Tetraploid (ave. in 7 plants)	61	—	122	—	18	—	60	—
Tetra, di-mixo. [1]) (ave. in 4 plants)	54	—	108	—	24	—	80	—
Diploid (ave. in 2 plants)	50	—	100	—	30	—	100	—
× *P. attenuradiata*								
Tetra-, tri-, mixo [2]) (ave. in 2 plants)	73	63	155	134	16	17	59	59
Tetra-, tri-, di-mixo. [3]) (ave. in 2 plants)	60	—	128	—	23	—	85	—
Diploid (ave. in 2 plants)	47	47	100	100	27	29	100	100

[1]) Mixoploid consisting of tetraploid and diploid tissues.
[2]) Mixoploid consisting of tetraploid and triploid tissues.
[3]) Mixoploid consisting of tetraploid, triploid, and diploid tissues.
[4]) Ratios are expressed as percent of diploid.



Figs. 8—11: — Fig. 8. Mitosis in needle-base of tetraploid *P. ponderosa*. — Fig. 9. Mitosis in triploid needle-base of a mixoploid (3n, 4n) *P. ponderosa*. — Fig. 10. Mitosis in needle-base of diploid *P. ponderosa*. — Fig. 11 (below). Mitosis in tetraploid needle-base of a mixoploid (3n, 4n) × *P. attenuradiata*.

Anatomical abnormalities

Length and number of stomata. The length of guard cells and the number of stomata per unit area were determined for tetraploids, mixoploids, and diploids. Length of guard cells of primary and secondary leaves of each abnormal seedling were measured and number of stomata per 0.1 sq. mm. were counted.

Tetraploid *P. ponderosa* seedlings and tetra-, tri-, mixoploid × *P. attenuradiata* seedlings had fewer and longer stomata than the diploid forms of those plants (Fig. 12). Mean values for the stomata of the polyploid and diploid forms of those plants are presented in table 4.

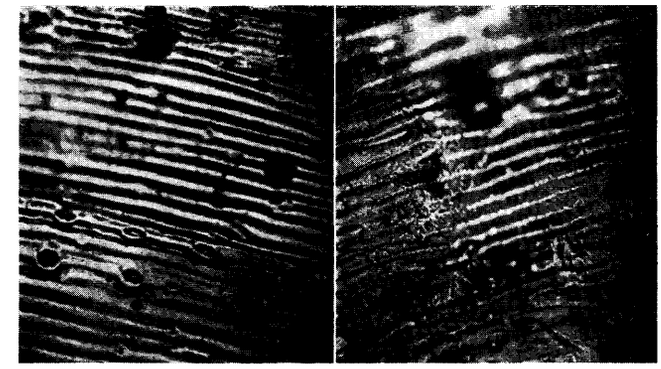


Fig. 12. Primary needles of tetraploid (left) and diploid (right) *P. ponderosa* seedlings, showing that the tetraploid has larger and fewer stomata than the diploid.

According to data in table 4, the difference in stomata size between polyploid and diploid seedlings seems to be greater in × *P. attenuradiata* than in *P. ponderosa;* and in both species, the difference in number of stomata appears to be more conspicuous than the difference in size of stomata between polyploid and diploid seedlings.

Endodermal abnormalities. The anatomical properties of needle tissue of the polyploid seedlings were observed in transverse sections of leaves prepared by freehand sectioning and stained with fast green.

In both primary and secondary leaves of *P. ponderosa* and × *P. attenuradiata,* larger cells and tissues were observed in polyploids than in diploids, and irregularity of the endodermis was observed as a conspicuous character of the polyploid seedlings (Fig. 13). In neither size nor form were the endodermal cells uniform, and the arrangement of these cells in the transverse section was abnormal, forming an irregular circle. This is in striking contrast to the uniformity in size, and the regularity in arrangement, of the corresponding cells in the diploid seedlings.

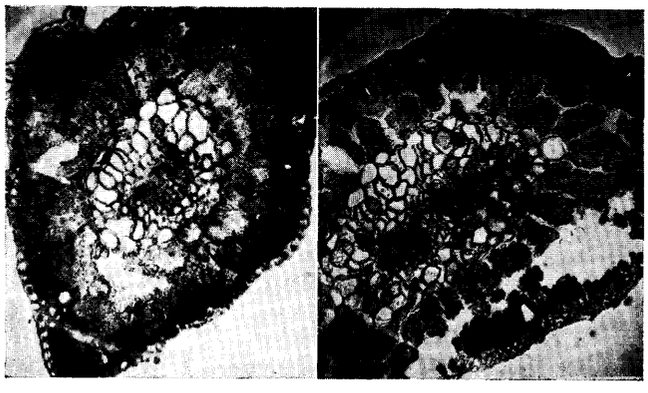
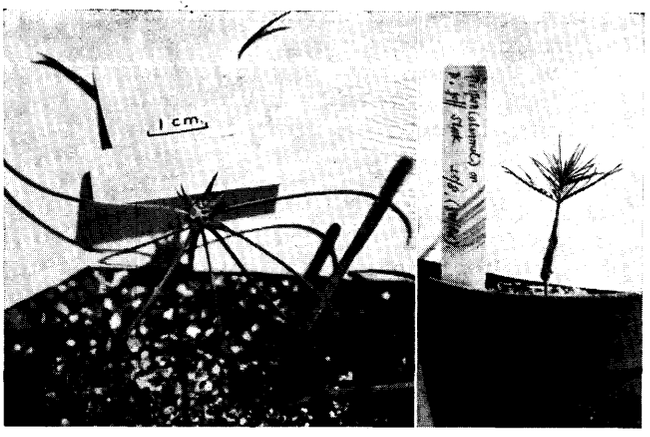


Fig. 13. Cross section of primary and secondary leaves of a mixoploid (3n, 4n) × *P. attenuradiata* showing irregular arrangement and variation in size of endodermal cells. Left: Primary leaf. Right: Secondary leaf.

Shoot-Treatment Experiments

Materials and Methods

Seedlings in the cotyledon stage from untreated seeds of *Pinus ponderosa, P. jeffreyi, P. lambertiana, P. contorta* var. *latifolia,* × *P. attenuradiata* and × *P. densithunbergii* were used for shoot-treatment experiments. Tips of hypocotyls of 20 seedlings of each species were treated with 0.2 percent colchicine solution by dropping the solution on the tips, when the primary leaves were about to appear, in such a way that the tips always were moist from the solution.



Figs. 14—15: — Fig. 14 (left). Abnormal (short) primary leaves growing from affected tips of pine seedlings after treatment with 0.2 percent colchicine solution. *P. ponderosa* after treatment with 30 drops of solution. — Fig. 15 (right). Moderately abnormal *P. ponderosa* seedling, obtained from Treatment B, grafted on normal *P. jeffreyi* seedling. 50 days after grafting.

Results

In *P. ponderosa, P. jeffreyi,* × *P. attenuradiata* and × *P. densithunbergii,* thirty drops of colchicine solution were needed to check the growth of primary leaves; whereas, fifteen drops of colchicine solution were enough to check the growth of primary leaves of *P. contorta* var. *latifolia* and *P. lambertiana.* Short, swollen primary leaves, suggestive of polyploidy, developed from the treated tips in the strongly affected seedlings (Fig. 14). However, almost all those seedlings later produced normal primary leaves, and only a few seedlings retained abnormality up to November 1952 (Table 5). They proved to be mixoploids, consisting of tetraploid and diploid tissues.

Grafting Experiments

As previously mentioned, all of the VERY ABNORMAL seedlings from colchicine-treated seeds died from lack of an effective root system. Mirov (1940) reported a similarly high mortality among polyploid pine seedlings.

As death appeared to be due to weak and inadequate roots, some of the polyploids were grafted to healthy stock of untreated seedlings in an effort to preserve the polyploids.

Materials and Methods

VERY ABNORMAL and MODERATELY ABNORMAL *P. ponderosa* seedlings obtained from seeds treated for 6

Table 5
Chromosome number, morphology, and height of shoot-treated seedlings.

Seedling number	Colchicine dosage percent/no. of drop	Date of sowing month/day/year	Somatic chromosome number in late September-'51	Height in inches in late November-'52	Leaf morphology in late November-'52
			P. ponderosa		
35	0.2/30	5/9/51	48, 24	3.5	S. T. D.
			× *P. attenuradiata*		
21	0.2/30	5/6/51	48, 24	6.8	M. T. D.
22	0.2/30	5/6/51	48, 24	4.0	M. T. D.
23	0.2/30	5/6/51	48, 24	4.0	S. T. D.
			P. jeffreyi		
5	0.2/30	5/6/51	48, 24	6.0	Nor./S. T. D. (Chimaera?)

Abbreviations: S: short, M: moderate long, T: thick, B: bluish, D: dark green, L: light green.

days in 0.4 percent colchicine solution were selected as scions immediately after germination. Healthy seedlings (in the cotyledon state) of normal *P. ponderosa* and *P. jeffreyi* from untreated seeds were used as stocks.

Grafting was done by means of the clefting technique. Latex film (38 percent solids of gum of *Hevea brazilensis* in NH_4OH) was used as banding-tape to bind the scion to the stock at the point of union. After grafting, seedlings were covered with a glass jar or canvas bag.

As a control, grafts of normal seedlings were made.

Results

Grafts of VERY ABNORMAL seedlings failed, presumably due to meristematic discrepancy between the scions and stocks. Successful grafts of MODERATELY ABNORMAL seedlings were obtained fairly readily (Fig. 15), and grafts of normal seedlings were readily obtained.

Many of the grafted VERY ABNORMAL seedlings lived for from two to four weeks after grafting, but then they all died.

The latex film proved satisfactory as a binding material.

Experiments with Excised Embryos

Embryos were excised in an attempt to combine embryo culture with colchicine treatment. This combination is considered the most effective method of subjecting pine seeds to colchicine treatment.

Materials and Methods

Both dormant embryos from dry seeds and active embryos from stratified seeds of *P. jeffreyi* were used. In mid-July, seeds were sterilized by soaking them in saturated aqueous bromine solution for five minutes. After this the embryos were extracted from the endosperm (female gametophyte) and placed on culture medium under aseptic conditions.

Culture media employed were as follows:

(1) 1.5 percent aqueous agar gel.

(2) 1.5 percent agar in KNOP's solution.

(3) 1.5 percent agar in KNOP's solution and 5 percent sucrose.

(4) 1.5 percent agar in KNOP's solution, 5 percent sucrose and 1 p.p.m. indolebutyric acid.

(5) 1.5 percent agar in KNOP's solution, 5 percent sucrose, 30 percent unautoclaved coconut milk and 6 p.p.m. 2,4-D.

(6) 1.5 percent agar in KNOP's solution, 5 percent sucrose, 30 percent unautoclaved coconut milk and 12 p.p.m. 2,4-D.

(7) 1.5 percent agar in KNOP's solution, 5 percent sucrose, 20 percent unautoclaved coconut milk.

(8) 1.5 percent agar in KNOP's solution, 5 percent sucrose and 30 percent unautoclaved coconut milk.

(9) 1.5 percent agar in KNOP's solution and 20 percent unautoclaved coconut milk.

(10) 1.5 percent agar in KNOP's solution and 30 percent matured endosperm extract[2]).

(11) 1.5 percent agar in KNOP's solution and 30 percent unmatured endosperm extract[2]).

Glass tubes, 0.7 inch in diameter and 3.5 inches in length, were used as containers. Ten cultures were made for each culture medium. The tubes were placed in an inoculation chamber in the laboratory where the temperature in the daytime fluctuated between 27° and 33° C. In order to avoid high mid-summer temperatures, the tubes were later removed to a basement where the temperature remained around 27° C. Here, they were kept in a large glass container with water at the bottom in order to avoid extreme evaporation and transpiration.

Results

Embryos Excised from Dormant Seeds

Extracted embryos from dormant seeds started to germinate on every culture medium 6 days after planting, but no development of the radicle was observed on any culture medium. Callus formations at the base of the hypocotyl were fair on all of the media except 1 and 2, and were particularly good on media to which 2,4-D was added.

No germinated embryo continued growth after reaching a stemlength of about a half inch and cotyledon length of about .4 inch.

All of the germinated embryos dried up after two months.

Particularly swollen stems were observed on seedlings grown in culture media to which 2,4-D was added.

No positive effect of coconut milk and endosperm extract on the growth of embryos was found.

Embryos Excised from Stratified Seed

On each culture medium, embryos from the stratified seeds started to germinate one or two days after planting.

On plain agar, and agar with KNOP's solution, the embryos gave rise to stem and cotyledons but not to radicles. After development of a 0.4-inch-long hypocotyl and 0.4-inch-long cotyledons, the embryos made no further growth. After 2 months they died.

On all media except plain agar and agar with KNOP's solution, the embryos showed better growth, and many embryos rooted within one week after planting.

But those embryos, both rooted and unrooted, also ceased to grow after they developed 0.7-inch to 0.9-inch-long stems, 0.5-inch-long cotyledons, and 0.3-inch-long roots (when rooted).

Conspicuously swollen stems and twisted cotyledons were observed on seedlings grown on the culture media to which 2,4-D had been added.

The embryos developed somewhat better on media containing endosperm extract or coconut milk than on the other media.

All of the germinated embryos, however, dried up within two months after germination.

In this case, the cause of cessation of growth and eventual death of the embryos is not clear.

Experiments with Embryos in Excised Endosperms

Inasmuch as the embryos developed to the cotyledon stage when cultured in endosperm extract, it seemed reasonable to suppose that polyploidy could be induced readily by culturing seeds having seedcoats and integuments removed, in a solution of colchicine. Accordingly some tests were set up to test this hypothesis.

Materials and Methods

Stratified *P. jeffreyi* seeds were soaked in a saturated bromine solution for 5 minutes and rinsed with sterilized water. Then the seedcoats and integuments were removed under aseptic conditions.

[2]) Endosperm extract was obtained by crushing the endosperm of stratified seed in KNOP's solution under aseptic conditions. Sedimentfree milk-white suspensions were used.

The embryos with endosperms were soaked in colchicine solution as follows: (1) 0.4 percent solution for two days, and (2) 0.2 percent solution for three days. Five seeds were subjected to each of the treatments.

Culture media used were 1 percent plain agar, and 1 percent agar with KNOP's solution. Again, 0.7-inch × 3.5-inch glass tubes were used as containers.

Plant materials were placed on culture media on June 15 and kept in an inoculation chamber in the laboratory. Five duplications were made in each case.

Results

Only one embryo in each treatment germinated within 7 days after planting. They developed to the cotyledon stage within ten days after germinating.

Both seedlings had swollen hypocotyls and cotyledons just as did the VERY ABNORMAL seedling obtained from colchicine treated seed.

Both seedlings continued their growth, but very slowly. One of them started to produce primary leaves after completion of germination. By July 12, the seedlings grew to the following sizes:

Treatment	Length of cotyledon	Length of hypocotyl	Length of root	Length of primary leaf
	inches			
0.4 percent colchicine for two days	0.7	0.9	2.0	0.1
0.2 percent colchicine for three days	0.7	0.9	1.0	—

The two seedlings were transplanted to small cans of sand and placed on a table under diffused light in the laboratory. They were watered with KNOP's solution. Although they continued to live in good condition, they made no further growth.

In August, the seedlings were accidentally exposed to strong sunlight for several hours, and within a few days they died.

Discussion and Conclusions

Sensitivity of Pine Embryos to Colchicine

One to six days soaking of stratified pine seeds in colchicine solution was not enough to induce significant abnormality in embryos. Rather, there was a stimulating effect on the germination of ponderosa pine seeds. SCHRÖCK (1951) observed a similar effect of colchicine on the germination of 5-months-old birch seeds as well as on the growth of seedlings from these seeds. These facts seem to indicate certain dosages of colchicine may have a stimulating effect on plant growth.

Soaking one side of the germinating seeds in colchicine solution on filter paper was effective in producing polyploid seedlings. It can be seen from tables 2 and 3 that seeds of small size, such as *P. contorta* var. *latifolia*, × *P. attenuradiata*, are more sensitive to colchicine dosages used than large seeds such as those of *P. ponderosa* and *P. jeffreyi*.

The dosages which produce abnormal seedlings most effectively do not coincide with the dosages which produce the greatest number of polyploids which can survive. The results of these different dosages are summarized as follows:

Species	Most effective dosages for producing	
	Abnormal seedling	Developed polyploid
P. ponderosa	0.2% for 6 days or 0.2% for 9 days	0.2% for 4 days or 0.2% for 6 days
P. jeffreyi × *P. attenuradiata*	0.2% for 7 days 0.2% for 4 days or 0.4% for 8 days	— 0.2% for 4 days
P. contorta var. *latifolia*	0.2% for 4 days or 0.4% for 9 days	—

It should be noted, however, that the percentages of polyploids obtained were 18 percent for *P. ponderosa* and 20 percent for × *P. attenuradiata*. No polyploids resulted from treatment of *P. jeffreyi* and *P. lambertiana* because of their relative insensitivity to the drug, and no polyploids of *P. contorta* var. *latifolia* capable of surviving were produced.

Stability of Induced Polyploidy

The polyploids obtained fall into four cytological classes: i. e., perfect tetraploids *(P. ponderosa)*, mixoploids consisting of 3n and 4n tissues (× *P. attenuradiata)*, mixoploids consisting of 2n, 3n and 4n tissues *(P. attenuradiata, P. ponderosa)*, and mixoploids consisting of 2n and 4n tissues *(P. jeffreyi, P. ponderosa)*. In the first class, the entire embryo was completely and uniformly affected by colchicine. In the other classes, the effect was not complete and uniform throughout the embryo.

The different kinds of polyploids were not equally stable; some of them reverted to the diploid condition, but most of them maintained the polyploid condition up to November 1952. The reversion of mixoploids to the diploid condition apparently was caused by resumption of active diploid cell-division which, suppressed at first, eventually submerged the slowly-dividing tetraploid, and triploid, cells. However, it is to be particularly noticed that most of the polyploids remained stable, whereas instability has usually been observed in colchicine induced mutants of woody plants (SATO 1949).

Value of Shoot-Treatment with Colchicine to induce Polyploidy

Treatment of pine shoots with colchicine apparently is not as effective as seed-treatment in inducing stable polyploidy since chiefly mixoploids, which show a very strong tendency to revert to the diploid condition, are produced. These results may be due to failure of shoot treatment to affect the whole meristem of the growing point.

Morphological Appearance and Growth of Induced Polyploids

The common peculiarities in morphological appearance of the artificially produced polyploids are: Thickened and shortened needles and bluish color of needles. Height growth of the polyploid seedlings was not inferior to that of ordinary seedlings, contrary to reports which have been presented regarding many induced polyploids (STEBBINS 1950). Recently S. CHIBA (1950) reported that all the natural tetraploids of *Cryptomeria japonica* found in a forest nursery were gigas forms. These examples are, perhaps, sufficient to indicate that dwarfness is not necessarily an invariable result of polyploidy in conifers. It is noteworthy that some of the mixoploids showed sectorial chimaeric outer appearance consisting of two parts; i. e., a diploid long-leaved part and a tetraploid short-leaved part.

Anatomical Properties of Induced Polyploid Pines

Size and number of stomata. — It is well known that stomata of polyploids are larger than stomata of diploids, ANDO (1949), BERGSTRÖM (1940), FUGII (1946), HIRAYOSHI (1942), MÜNTZING (1936), NABESHIMA (1949), NAGAI (1946), STEBBINS (1950). The results of this study (Figs. 12 and 13) are in agreement with previously reported observations. Some studies have shown, however, that stomata-size is not always proportional to the chromosome number. JOHNSSON (1940) observed that diploid *Populus tremula* had stomata as large as the triploids and even larger, and pointed out that the calculated mean stomata size cannot constitute an accurate characteristic because the length of the stomata varies much within a leaf. CHIBA (1950) observed similar phenomena in natural polyploids of *Cryptomeria japonica,* and pointed out the impossibility of distinguishing triploids from tetraploids or diploids by stomata length. Similarly, DUFFIELD (1943) reported that the size and distribution of stomata are not reliable indices of naturally-occuring polyploidy in *Acer rubrum.* Those facts seem to indicate that the size and number of stomata are not reliable indicators of polyploidy in wild populations. Nevertheless, the size and number of stomata are characters upon which a preliminary selection of polyploids among treated plants may be based.

Aberration of endodermis. — The observed variability in size of endodermal cells and the uneven arrangement of these cells in leaves of polyploids (Fig. 13) may be caused by the unequal increase in cell-size of this tissue resulting from colchicine treatment. These irregularities were less distinguishable in the tetra-, di-, mixoploids of *P. ponderosa* and × *P. attenuradiata* than in the tetraploids and in the tetra-, tri- mixoploids of those species. Whether or not abnormality of the endodermis in needles can be an accurate indicator of polyploidy, however, has not been established.

The Possibility of Grafting Very Abnormal Polyploid Seedlings

Failure to obtain successful grafts of very abnormal seedlings, despite the high success obtained in grafting moderately abnormal seedlings, may result from the meristematic discrepancy between the swollen stem of the polyploids and the normal stem of the diploid.

A comparative anatomical study of the meristems would doubtless give rise to suggestions for obtaining successful grafts of very abnormal seedling. Such a study will be undertaken in the future.

Pine Embryo Culture

Excised embryo-culture. — Embryos extracted from dormant seeds failed to produce roots, but embryos from stratified seeds did produce roots. This difference may have resulted from failure of some root-producing factors to be transmitted to the embryo from the endosperm in dormant seeds, whereas such factors were transmitted to the embryo from the endosperm in stratified seeds. The failure of the stratified embryos to produce roots on culture media without sucrose or coconut milk or endosperm extract is considered due to too-low osmotic pressure of the media, as HANNIG observed (See BRUNNER, 1932); but whether their failure to continue growth was due to lack of some factor or to uncontrolled environmental conditions has not been determined. Although these experiments failed to demonstrate that embryo culture, combined with colchicine treatment, can induce polyploidy in pines, they did demonstrate that extracted embryos of stratified pine seeds may root readily.

Treatment of embryo in excised endosperm. — Very abnormal (probably polyploid) seedlings were obtained by treating seeds from which seed-coats and integuments were removed with a solution of colchicine. This method should be an effective means of inducing polyploidy.

Summary

Stratified seed of various pine species were (1) soaked in colchicine solutions or (2) germinating seeds were partly soaked in colchicine solutions for different periods; and hypocotyls of seedlings were treated with drops of a colchicine solution. Soaking ungerminated seeds produced no abnormalities but may have stimulated germination. Treatment of germinating seeds produced various kinds of polyploids which are described. Small seeds were more sensitive to colchicine than large seeds. Some polyploid plants were stable; others reverted to the diploid condition. Shoot-treatment was not as effective as seed-treatment in producing stable polyploids. As many polyploids had weak roots different types were grafted to normal seedlings. Grafts of very abnormal plants failed, but grafts of moderately abnormal seedlings succeeded. Very abnormal seedlings resulted from subjecting extracted embryos to colchicine treatment.

Zusammenfassung

Titel der Arbeit: *Erzeugung von Polyploidie bei Kiefern durch Colchicin-Behandlung.* — Ältere, abgelagerte Saat (z. T. schon Ernte 1940; Lagertemperatur 1,5 bis 4,5° C) verschiedener Kiefernarten *(Pinus ponderosa, jeffreyi, contorta* var. *latifolia, attenuradiata, densithunbergii, lambertiana)* wurde als Material für Colchicin-Experimente benutzt. Bei der Behandlungsweise A sind die Samen direkt in einer 0,2%igen Colchicin-Lösung für 1 bis 6 Tage eingequollen worden. In Abwandlung davon wurden in der Versuchsgruppe B die Samen zuerst in feuchtem Sand vorgekeimt und erst dann in Petrischalen mit 0,2- oder 0,4%igen Colchicin-Lösungen gebracht (benutzte Lösungsmenge so groß, daß die Samen halb eintauchten; Behandlungsdauer 4 bis 10 Tage). Als dritte Variante wurde die Vegetationspunktbehandlung von Keimlingen mit Colchicin-Lösungen (0,2%) versucht, und dabei ist die Zahl der aufgesetzten Tropfen verändert worden. — Die Behandlungsweise A brachte keine morphologischen Anomalien hervor, sie stimulierte dagegen die Keimung der Samen. Die Behandlung nach B hatte verschiedenartige Polyploide zur Folge, deren morphologische, anatomische und zytologische Besonderheiten beschrieben werden. Kleinere Samen waren gegen Colchicin-Einwirkung empfindlicher als größere. Manche der erzeugten Polyploiden erwiesen sich als stabil, andere kehrten zum diploiden Status zurück. — Die Sproßspitzenbehandlung der Keimlinge schien für die Herstellung stabiler Polyploider nicht so wirksam zu sein, wie die Samenbehandlung. — Da viele dieser experimentell hergestellten Polyploiden eine schlechte Bewurzelung zeigten, wurden die verschiedenen aufgetretenen Typen auf normale gleichalte Sämlinge gepfropft. Eine derartige Pfropfung gelang in keinem Falle bei morphologisch sehr anomalen Pflanzen, bei weniger anomal aussehenden Sämlingen war sie jedoch leicht erreichbar. — Die stärk-

sten Anomalien zeigten die Sämlinge, wenn die aus den Samen extrahierten Embryonen mit Colchicin-Lösungen behandelt worden waren. Entsprechende Embryo-Kulturmethoden sind mit Einzelheiten beschrieben worden.

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(Aus der züchterischen Tätigkeit der Landesforstverwaltung Schleswig-Holstein)

Über zwei Typen der Japanlärche (Larix leptolepis Gord.) in Schleswig-Holstein

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Die Jap. Lärche ist in Schleswig-Holstein zum ersten Mal etwa um das Jahr 1880 und später von der Jahrhundertwende an auf größeren Flächen angebaut worden. Ihre Gesamtfläche beträgt zur Zeit etwa 4000 ha, von denen über 1000 ha nach 1945 aufgeforstet worden sind. Die Besichtigung ihrer Bestände hat zu der Feststellung geführt, daß auf bestimmten Flächen *außerordentlich verschiedenartige Typen* der *Larix leptolepis* zu finden sind. In der forstlichen Literatur hat WECHSELBERGER (1) das Vorkommen und frühzeitige Fruchten eines besonders ungünstig aussehenden Typs der Jap. Lärche in Nordwestdeutschland vermerkt. JÄGER (2) unterscheidet nach der Aststellung 2 Typen und erwähnt außerdem einen dritten mehr oder weniger krummwüchsigen Typ mit oft nach unten gebogenen Ästen. Über die *Merkmale und das Vorkommen zweier besonders charakteristischer Typen* in Schleswig-Holstein wird nachstehend berichtet.

Besichtigt wurden etwa 300 ha zur Saatgutanerkennung angemeldeter Bestände. Während in einigen Forstrevieren des Landes aus bestimmten Zeiten der Aufforstung überwiegend einheitliche Bestände erwachsen sind, fällt in den Beständen der ehemaligen Prov.-Forstverwaltung ein Gemisch verschiedener Typen auf.

Die beiden Vorkommen unterscheiden sich schon durch ihre *Bestandesform* voneinander. Während die ersteren hauptsächlich größere zusammenhängende Flächen bilden, sind die letztgenannten fast ausschließlich auf kleineren Flächen und Streifen an den Gestellen gepflanzt worden. Dabei zeigen die zahlreichen Außenränder die typische Entwicklung tief beasteter Randstämme; die im Inneren der Jagen an den schmalen Holzabfuhrwegen stehenden Reihen hingegen sind in fast völligem Schluß erwachsen.

Die stellenweise verwirrende Vielfalt der Formen, die noch durch Beimischung anderer Arten der Gattung *Larix* erhöht wird — *L. Gmelini* (RUPR.) syn. *L. dahurica* TURCZ., *L. Gmelini japonica* (REG.) PILG. syn. *L. kurilensis* MAYR, *L. Gmelini olgensis* syn. *L. koreensis* oder *L. Gmelini* var. *coreana* NAKAI —, konnte die Erscheinung nicht überdekken, daß auch unter völlig gleichen Umweltbedingungen einem überwiegend wiederkehrenden *feinen Typ*, der in den Reinbeständen vorherrscht, ein durch viele Variationen erkennbarer extrem *grober Typ* gegenübersteht. In dieser von Anfang an augenscheinlichen Wahrnehmung fand sich mit den fortschreitenden Untersuchungen der Schlüssel, den größten Teil des vorhandenen Formengemischs der Jap. Lärche in den genannten Beständen ihrem Habitus nach aufzugliedern.

Beide Typen kommen sowohl in ihren extremen Besonderheiten rein vor, als auch, mehr oder weniger erkennbar, da, wo diese in der Mitte der Variationsbreite verschwimmen. Jedoch lassen sich die Zwischenformen im allgemeinen auf diese beiden *Grundtypen* zurückführen. Es muß infolge mangelnder Kenntnis des natürlichen Verbreitungsgebiets dieser Holzart dahingestellt bleiben, ob es sich bei diesen beiden Typen um Vertreter verschiedener Rassen oder nur um extreme Glieder der allgemeinen Variationsbreite einer einzelnen Rasse handelt.

Die beiden Typen lassen sich wie folgt beschreiben:

1. *Grob-starrer Typ:* in der Kronenform annähernd rhombisch, mit sperriger grober Beastung, im Kronenraum leer, mit einer fast linearen, kurz, dicht und starr benadelten, unterseits kahlen Verzweigung; in extremster Form beinahe araucarienähnlich (Abb. 1 links).

2. *Fein-weicher Typ:* Krone pyramidenförmig, dicht beastet, fein, voll und hängend verzweigt, weich und locker benadelt; im Gesamthabitus als „Spargelkrauttyp" zu bezeichnen (Abb. 1 rechts).

Bei Nebeneinanderstellung der beiden Typen ergibt sich noch eine Reihe feinerer, zum Teil außerordentlich deutlicher Unterschiede. Beim fein-weichen Typ sind leichte *Stammkrümmungen* verhältnismäßig häufig, während sie beim grob-starren Typ seltener vorkommen. Die *Rinde* des grob-starren Typs ist, jedenfalls in den ersten Jahrzehnten, gröber als bei dem fein-weichen Typ, später