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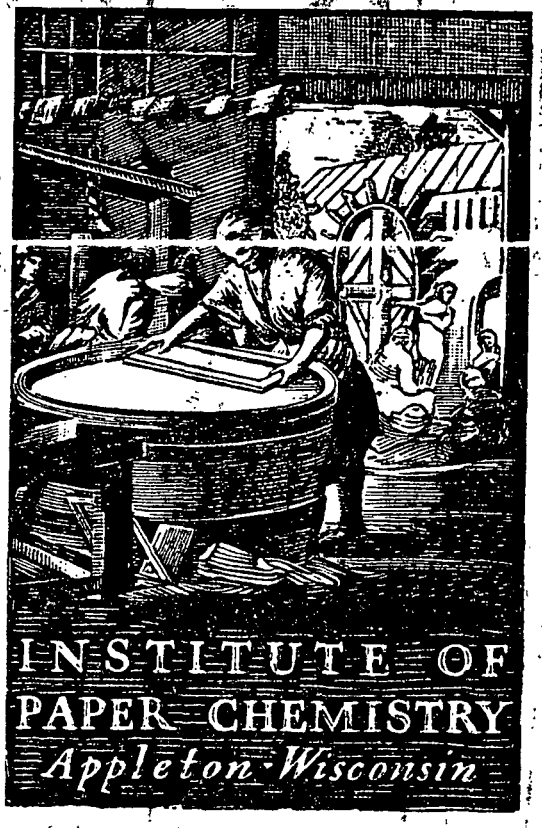

A STUDY OF THE GENETIC IMPROVEMENT OF QUAKING AND BIGTOOTH ASPEN BY SELECTION, HYBRIDIZATION, AND THE EXPLOITATION OF POLYPLOIDY

Project 2412

Report One

A Progress Report

to

LOUIS W. AND MAUD HILL FAMILY FOUNDATION

May 20, 1964

THE INSTITUTE OF PAPER CHEMISTRY

Appleton, Wisconsin

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SUMMARY

1. Selection of outstanding trees from natural stands was continued and the first selections of exceptional trees from progeny of controlled crosses were made in 1963.

2. Triploid aspen from ten sources were propagated for use in a replicated field trial designed to provide additional information on field performance and wood quality of triploid aspen.

3. Colchicine treatment of very small aspen seedlings to induce polyploidy was continued.

4. Two studies of natural variation were initiated and the description of these studies, including information on objectives and progress, are included in this report.

5. During 1963 twenty-seven parent trees were employed in the crossing program and a total of 41 crosses were attempted. Major emphasis was placed on bigtooth aspen crosses and bigtooth aspen hybrids suitable for "dry sites." Techniques for hybridizing and propagating cottonwood also received considerable attention.

6. Preliminary studies indicate several basic biochemical principles may be useful in the genetic characterization of aspen and aspen hybrids.

INTRODUCTION

Genetic improvement of forest tree species involves applying proven plant breeding techniques to forest genetics problems. The techniques most often used to improve trees include selection, hybridization, mutations, and polyploid production. Each of these techniques has certain advantages and, to insure adequate progress, most tree improvement programs are so designed that combinations of the above techniques are utilized.

Selection involves locating parent trees which have the characteristics desired and propagating these individuals vegetatively. Selection techniques are relatively simple, progress is rapid, and costs are low. Hybridization has the advantage that it is possible to produce genetic combinations that would not normally occur in nature. Hybridization consists of mating two trees of unlike genetic make-up. Certain genetic combinations may, in addition to combining desirable characteristics, produce progeny which are more vigorous than either parent.

Mutagenic chemicals, x-ray, gamma radiation, and certain forms of ionizing radiation are often employed in tree improvement programs to produce mutations and in this way increase the genetic variability of the species being studied. Polyploid production, a technique that appears to be quite promising for species within the genus Populus, is the production of individuals with extra sets of hereditary units in each cell nucleus. Triploid quaking aspen, trees in which each cell nucleus contains three sets of hereditary units instead of the normal two, grow faster and have longer fibers than normal diploid trees. These are ample reasons for considering the exploitation of such individuals for pulpwood production.

Most forest genetic programs have limited the characterization of parent trees and progeny to growth and morphological characteristics. Such an approach, although adequate for most types of studies, needs to be supplemented with more refined techniques when more fundamental studies are involved. Work is presently underway aimed toward developing biochemical techniques for the characterization of aspen and aspen hybrids.

The basic objectives of this project are to (1) find ways to increase the per acre production of usable wood, and (2) improve the quality of the wood produced. The principal techniques to be employed include selection, hybridization, and polyploid production. The report which follows describes in some detail the work carried out in this project during the period of January 1, 1963 to December 31, 1963 and illustrates the use of the techniques listed above. Also included is a description of the preliminary phases of studies underway on the biochemical characterization of aspen and aspen hybrids.

SECURING AND PROPAGATING DESIRABLE POLYPLOID AND DIPLOID ASPEN

SELECTION – OUTSTANDING TREES LOCATED DURING THE PAST YEAR

Selection of desirable parent trees is an important first step in any intensive tree improvement program. Each year thirty to forty trees are measured and evaluated as possible parent trees. A selection index system is employed and prospective trees are judged and rated numerically on the basis of rate of growth, form, and wood quality. Only trees which rank high compared with the trees presently being used by the program are employed as parent trees. Very briefly, the type of trees that we are trying to locate are individuals with rapid growth, straight stems, small branches, narrow crowns, good natural pruning, minimum stem taper (high form factor), above average wood specific gravity, and long fibers. Care is also taken to avoid the use of trees which show some evidence of susceptibility to insect and disease attack.

The next step in the evaluation of trees selected as parent trees is to judge them on how well they behave when used in experimental crosses. Approximately one of every six are later discarded because of irregular behavior as a parent tree. Described below are four outstanding individuals located during 1963 that will be used in our crossing program the first year that they flower.

Tree T-5-63

T-5-63 is a quaking aspen (*P. tremuloides*) female located in Dickenson County, Upper Michigan. This tree was reported to us by Kimberly-Clark Paper Company Foresters and is outstanding primarily because of its large size. Complete measurements have not been made on this tree but the preliminary information indicates that it is one of the largest quaking aspen we have measured. Listed below are data on the tree and Fig. 1 illustrates the size and straightness of the lower stem.

Figure 1. Kimberly-Clark Forester Roland Rouse Looking Over a Quaking Aspen Tree (T-5-63) Growing Near Iron Mountain, Michigan. T-5-63 is 25.8 Inches d.b.h., 100 Feet Tall and 70 Years Old

Total height – 100 feet

Height to 3 inch top – 85 feet

Height first live branch – 65 feet

Age – ca. 70 years

Stem straightness – good

Natural pruning – good

Branch weight – medium

Specific gravity – no data available

Fiber length (age 30) – no data available

Diameter breast height – 25.7 in.

Diameter 16.5 feet – 22.6 in.

B.h. bark thickness – .95 in.

Crown diameter – 30 ft.

No. major branches – 9

Branch angle – 65°

Form factor – 83.1

Tree G-9-63

G-9-63 is a bigtooth aspen (P. grandidentata) reported to the Institute by Wisconsin Conservation Department Forester David Lee. This tree, growing north of Bruce, Wisconsin in Rusk County, is a member of a clone of about 35 trees, all of which are above average in growth and form. The tree is a female and ranks high in our over-all list of bigtooth aspen trees. G-9-63 is growing on an above average hardwood site and for this reason we do not know how well the progeny from this tree will do on sandy, dry areas. Described below are the growth, form, and wood quality information available on the above tree.

Total height – 98 feet

Height to 3 inch top – 85 feet

First live branch – 64 feet

Age – 55 years

Stem straightness – good

Natural pruning – fair

Branch weight – fair

Specific gravity – .374 g./cc.

Fiber length (age 30) – .790 mm.

Diameter breast height – 15.6 in.

Diameter at 16.5 feet – 13.4 in.

B.h. bark thickness – .77 in.

Crown diameter – 18.5 ft.

No. major branches – 12

Branch angle – 60°

Form factor – 80.7

Tree G-8-63

This bigtooth aspen male tree was reported to the Institute by the U. S. Forest Service Foresters and is located on National Forest lands near Hiles, Wisconsin. Although the tree is not extremely tall, it is growing on a sandy, rocky site and has outgrown other nearby hardwoods. The U. S. Forest Service preferred that wood samples not be taken and so the age was estimated from the age of nearby stands. Presented below is information on growth and form of G-8-63.

Total height – 79 feet
Height to 3 inch top – 54 feet
Height first live branch – 41 feet
Age – 45-50 years
Stem straightness – good
Natural pruning – good
Branch weight – medium
Specific gravity – not available
Fiber length – not available

Diameter breast height – 21.5 in.
Diameter 16.5 feet – 17.6 in.
B.h. bark thickness – .70 in.
Crown diameter – 23.7 ft.
No. major branches – 11
Branch angle – 80°
Form factor – 77

Tree XT-Ta-14-58, S-3

The above selected tree is a triploid individual that was outstanding in form and rate of growth. The tree was produced artificially be a crossing procedure which involved crossing a good diploid quaking aspen female (T) from Upper Michigan and a tetraploid European aspen (Ta) from Sweden. The tree selected is the best of a group of progeny that were six years old from seed and had been growing in the test area for five years. The average height for this progeny group was 15.8 feet while the selected tree as shown by the measurements below had a total height of 28 feet. The sex of the tree is not known and it

is being propagated vegetatively for further testing. Figure 2 pictures S-3, the selected individual, and several other individuals from this same progeny group. Listed below is a summary of the data available on the form and growth characteristics of this tree.

Total height – 28 feet

Age – 6 years

Stem straightness – very good

Natural pruning – fair

Branch weight – medium

Specific gravity – .373 g./cc.

Fiber length (age 6) – .746

Fiber length (age 30) – 1.04

Diameter breast height – 3.2 in.

Crown diameter – 9.2 ft.

No. branches – 25

Branch angle – 55°

TRIPLOID ASPEN PROPAGATION

The search for naturally occurring triploid aspen has continued during 1963 and, as was the case in 1962, less emphasis was placed on this phase of our program. This reduced emphasis does not imply any less interest in securing new triploid individuals, but rather a switch of our efforts to the propagation and testing aspects using existing natural and artificially produced triploid trees. No new triploid clones were located in 1963, but several outstanding diploid trees, as indicated by the section on Selection, were discovered and measured.

Propagation of triploid individuals is accomplished by taking roots from selected individuals, placing the roots under suitable environmental conditions and then rooting the sprouts that result. Such a procedure is normally initiated in late fall, and the vegetatively propagated individuals are lined out in the nursery the following spring. The above propagation procedure was initiated in the fall of 1962 and ten different triploid trees and one outstanding

Figure 2. A Block of XT-Ta-14-58, a Triploid Cross in Trial X, Showing One Tree (S-3) Which Has a Total Height of 28 Feet and a Diameter at b.h. of 3.2 Inches After Five Growing Seasons in the Field. Average Height for all Individuals Was 15.8 Feet and Average d.b.h. Was 1.8 Inches

diploid tree were employed as sources of root material. Table I provides a list of the trees used and the numbers of individuals produced. Greenhouse space limitations made it necessary to limit the number of individuals in each clone.* In a number of cases many more individuals could have been produced from the root materials available.

TABLE I

PROPAGATION OF TRIPLOID CLONES

Clone Number	Location of Clone	Number of Individuals Propagated
T-2-56	Bruce Crossing, Mich. Area No. 1	53
T-36-56	Bruce Crossing, Mich. Area No. 1	149
T-71-57	Bruce Crossing, Mich. Area No. 2	191
T-7-59	Bruce Crossing, Mich. Area No. 3	216
T-9-59	Bruce Crossing, Mich. Area No. 3	115
T-38-59	Trout Creek, Mich.	178
T-43-59	Barrie Island, Lake Huron (Canada)	188
T-1-62	Chippewa Falls, Wis.	152
XT-TA-14-58 S-1	Expt. Triploid Hybrid Ripco Farm	113
XT-TA-14-58 S-2	Expt. Triploid Hybrid Ripco Farm	98
XT-12-58 S-1	Expt. Diploid Tree Ripco Farm Used as Control	113

*The term clone is used to designate a group of plants derived from a single individual by vegetative propagation.

The next step in this study will be to field plant the seven most suitable triploid materials along with the diploid control trees in a replicated, randomized block, field trial on the Ripco Experimental Farm. The Ripco Experimental Farm area was selected as a planting site because growth measurements will be available, for comparison purposes, from a number of other triploid aspen and aspen hybrids. Field planting of this trial is planned for the spring of 1965.

PRODUCTION OF ARTIFICIAL POLYPLOIDS

Polyploid trees are trees that have extra sets of hereditary units (chromosomes). Normal vegetative tissue has two sets (2n) of chromosomes in each cell nucleus and the trees are called diploids. Trees having three sets of hereditary units (3n) in the nucleus of each vegetative cell are called triploids and when four sets of chromosomes (4n) are present, the tree is called a tetraploid. A number of reports on triploid individuals in the aspen and white poplar group indicate triploids grow faster and have longer fibers than either the diploid or tetraploid trees. Tetraploid trees, although slow growing, are useful because by crossing a tetraploid individual with a normal diploid individual a large percentage of the progeny produced are triploid [tetraploid (4n) x diploid (2n) = triploids (3n)].

There are several techniques available that can be employed in producing tetraploid individuals. Of the techniques used by plant breeders, colchicine treatment of germinating seed, colchicine treatment of newly fertilized embryos, and hybridization using polyploid parents appear to be the techniques most suitable for use with species with the genus Populus. A description is given of the results obtained during 1963 in the area of colchicine treatment of germinating seed.

Colchicine Treatments

Treatment of small seedlings with the chemical colchicine causes abnormal cell division and, of the seedlings which survive, 15 to 20% have tissue which have cells with double the normal chromosome number. Colchicine-treated individuals are often quite slow growing and require special care to bring them along to a size where they will flower. The objective in producing individuals with double the normal chromosome number is that when they flower they can be used to mass produce triploid trees.

During 1963 the number of seedlings treated with colchicine was greatly decreased to facilitate completion of the chromosome count work on earlier treated materials. Table II summarizes the colchicine treatments made in 1962 and presents the completed chromosome count information for this study. A major portion of the counting work was completed during 1963. Also included in Table II are the data on the 1963 colchicine treatments made upon bigtooth aspen. The information presented in Table II substantiates our earlier preliminary conclusions that cottonwood is the least sensitive, quaking aspen and balsam poplar are moderately sensitive, and bigtooth aspen is highly sensitive to the toxic influence of colchicine. Looking at the treatment levels and the per cent polyploid individuals produced, a level of 0.4% colchicine appears to be best for cottonwood, 0.3% for quaking aspen, and 0.2% for bigtooth aspen. It has not been possible to make reasonable numbers of bigtooth aspen survive the colchicine treatment and some modification of this technique appears desirable.

Colchicine treatments have been made over the past six years and one might expect that this would mean we have a large number of individuals available for crossing studies. Losses from slow growth and reversion to diploid chromosome

TABLE II

1962 AND 1963 COLCHICINE TREATMENTS

1962 Colchicine Treatments

Plant Material	Level of Colchicine, %[a]	Number of Seedlings			Useful Polyploids[c]	
		Treated	Survived[b]	Polyploid	No.	%
XT-4-62	0.2	400	30	9	5	17
	0.3	400	17	11	4	24
	0.4	400	4	2	0	0
	0.5	400	0	0	0	0
	0.6	400	0	0	0	0
XD-0-45-62	0.4	400	124	11	7	6
	0.5	400	96	5	2	2
XG-19-62	0.4	400	0	0	0	0
XG-11-62	0.3	800	7	1	0	0
XTc-36-62	0.4	200	19	2	2	11
		1963 Colchicine Treatments				
XG-10-63	0.1	200	2	0	0	0
	0.2	200	1	0	0	0
	0.3	200	0	0	0	0
	0.4	200	0	0	0	0
XG-10-63 (2nd Series)	0.1	200	2	0	0	0
	0.2	200	1	0	0	0

[a] All treatment times were 40 hours.

[b] Individuals that survived and upon which chromosome counts were made.

[c] Seedlings having high chromosome counts and exhibiting satisfactory growth. Per cent based upon numbers of individuals which survived.

number* have reduced the number of surviving individuals. Some technique changes have been made during the past year to facilitate checking and rechecking the polyploid prospects and to improve rate growth of the treated trees in order to have them large enough for field planting in a minimum period of time. The present procedure consists of treating the seed as it is germinating, transferring the very small seedlings to a seed pot via a single sheet of tissue paper, growing the seedlings on soil for two to three months and then placing the seedlings in a nutrient solution, culture system. This latter step, which is illustrated in Fig. 3, speeds the growth and provides suitable samples for chromosome counting. Next, the best polyploid prospects are field planted, grown for three to five years, rechecked for chromosome number, and then finally forced to flower and used in crossing work.

*Diploid tissue often outgrows tetraploid tissue. In cases where a seedling is part diploid and part tetraploid tissue, upon growth, the seedling contains a higher and higher proportion of diploid tissue.

Figure 3. Colchicine Treated Aspen Growing in a Nutrient Solution to Speed Growth

STUDIES OF NATURAL VARIATION

HERITABILITY OF WOOD AND GROWTH CHARACTERISTICS OF QUAKING ASPEN

"Heritability" is a measure of the relative degree to which a characteristic is influenced by heredity as compared to environment. Heritability information, along with information on natural variation, must be available before reliable decisions can be made regarding the growth, morphological, and wood properties which would be most useful in tree improvement work.

Experimental Trial VII contains trees from experimental crosses made in 1956, 1957, 1958, and 1959. This trial was established, not only to provide growth and morphological data, but to provide heritability information on wood and fiber properties. The study involves a total of 25 quaking aspen crosses. These crosses were carried out in the Institute's greenhouse and 55 progeny from each cross have been planted in single blocks on a uniform test area at the Greenville Experimental Farm.

The plans for this trial include the standard year-by-year growth measurements for the first five years. After the trees have been in the field for five growing seasons, a complete analysis of growth and wood property information will be made. The fifth year measurements for each experimental cross will include the following:

1. Total height – all trees
2. Diameter at breast height – all trees
3. Straightness of stem – all trees
4. Branch angle, length and diameter – all trees
5. Number of live branches – all trees
6. Natural pruning – all trees
7. Specific gravity on 20 trees from each experimental cross
8. Fiber length information on 10 trees from each experimental cross
9. Fiber strength (zero-span tensile strength) information on five trees from each experimental cross
10. Pulping information including pulp yield, per cent extractives, and per cent lignin on five trees from each experimental cross.

During the past year, the fifth-year measurements listed above were made on ten of the twenty-five experimental crosses involved in this study. These data are being tabulated and a preliminary analysis is planned. The over-all plans for the study involve completing and tabulating, in the next two years, the fifth-year measurements on the remaining 15 experimental crosses. Upon completion of the measurements, the data will be composited and a complete analysis of the study will be made.

The fifth-year wood property sampling and thinning will increase the spacing from the original three by six-foot spacing to a six by six-foot spacing. This spacing will again be increased as growth requires and an additional intensive study of growth, morphological, and wood properties is planned when the trees have been in the field 15 years.

GEOGRAPHIC VARIATION IN QUAKING ASPEN

The growth characteristics and the wood properties of quaking aspen, although sampled widely as part of earlier investigations, have not been systematically studied from the "geographic variation" point of view. Studies with several species of southern pine have shown that large differences existed between geographic areas in both growth characteristics and wood properties.

Knowledge of natural variation is all important in a tree improvement program. With the need for better information on geographic variation and with the need for establishing base lines (reference lines) for judging the "wood quality"*, a study was established which would provide some of the needed answers.

*Wood quality as used in this report refers to the quality of the wood with regard to its use by the pulp and paper industry. Wood properties considered to be important include fiber length, fiber strength, specific gravity, pulp yield, lignin and extractives.

The plan adopted was to first establish study areas in five geographic locations within the state of Wisconsin and Upper Michigan. Then, depending upon the results obtained from the measurements on these five areas, the study may be expanded west to include stands in Minnesota and north to include stands in southern Ontario. The planned geographic locations within the state of Wisconsin and Upper Michigan include: (1) Madison Area, (2) Wausau, Wittenburg, and Stevens Point Area, (3) Eagle River, Woodruff and Watersmeet (Michigan) Area, (4) Iron Mountain and Norway, Michigan Area, and (5) Ashland and Cable Area. These areas when plotted on the map of Wisconsin form a "T" shaped arrangement (see Figure 4) and will provide preliminary information on north-south and east-west trends.

Five stands will be sampled in each geographic area and three trees in each of three clones will be sampled in each stand. Present plans are to limit the study to stands growing on medium-textured upland soils and to stands between 20 to 40 years of age.

The information to be taken on each stand includes:

(1) Age, form, and rate of growth information on sampled trees.

(2) Specific gravity and fiber length based on four 11-millimeter increment core samples.

(3) Soil and other site information based on soil samples taken from the A and B horizons.

(4) Pulping information* (fiber strength, pulp yield, extractives, and lignin) from one stand (9 trees) within each geographic area.

*The pulping information is to be based upon the micropulping of 4 to 6 11-millimeter increment core samples per tree.

GEOGRAPHIC VARIATION STUDY AREAS

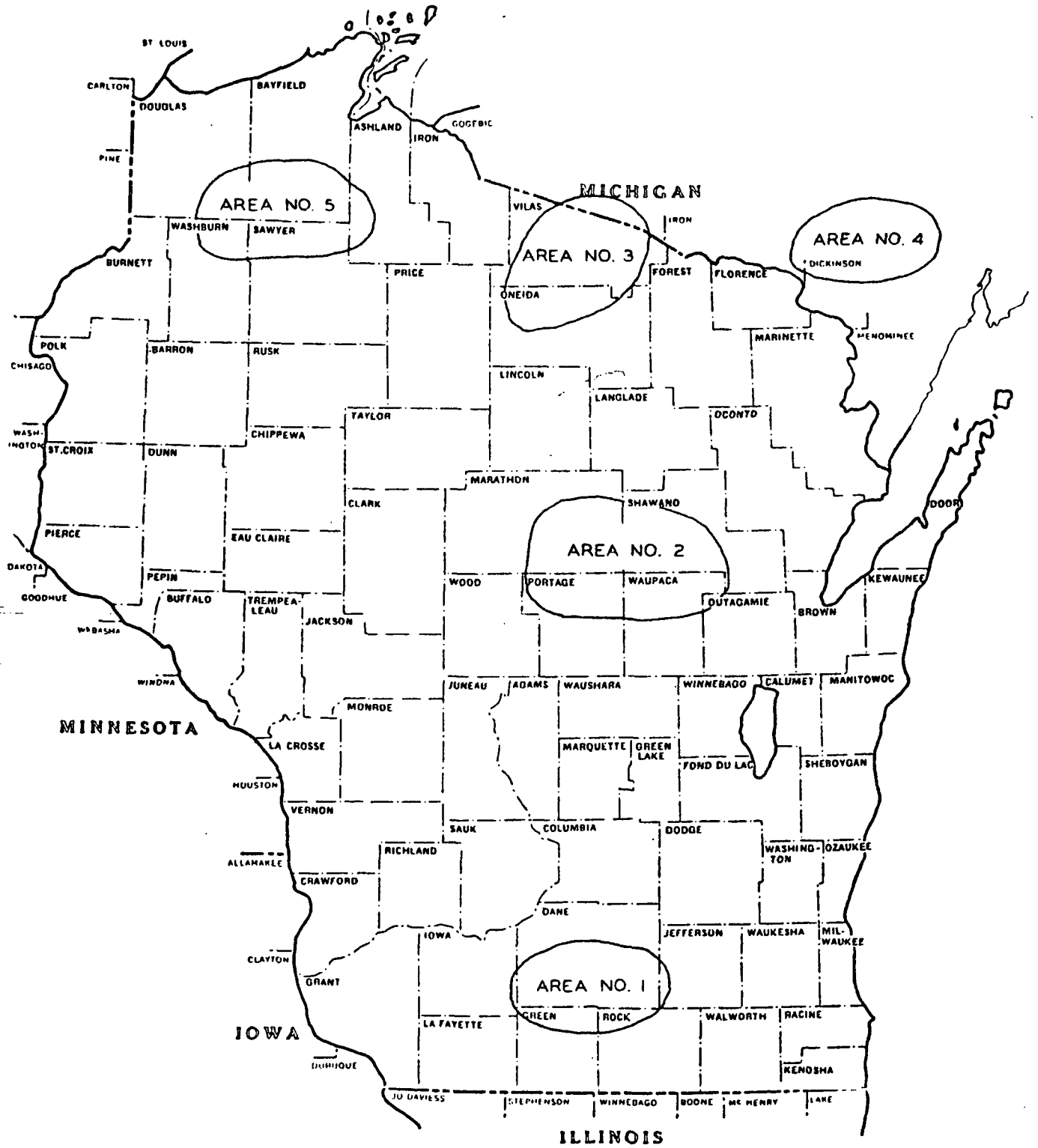


Figure 4. Location of the Study Areas to be Sampled in the Quaking Aspen Geographic Variation Study

Sampling has been underway during the past two summers and the field work has been completed in Area 2 (Wausau, Wittenburg, and Stevens Point Area), Area 3 (Eagle River, Woodruff, and Watersmeet Area) and in two of the five stands in Area 5 (Ashland and Cable Area). Considerable work is involved in the completion of observations for each geographic area. It appears that two more field seasons will be required to complete this aspect of this study.

INTRASPECIFIC AND INTERSPECIFIC CROSSING

The over-all goals of the crossing program have been to produce individuals with satisfactory growth and above average wood quality for use on three types of soil-site situations. It has been our hope to produce quaking aspen crosses* and triploid aspen suited for use on medium textured, good quality hardwood sites; bigtooth aspen crosses and bigtooth aspen hybrids for dry, sandy sites; and cottonwood selections and crosses for moist site conditions.

The crossing technique employed had its origin in Europe and has been described as the "greenhouse method" or the "cut branch technique." The procedure consists of collecting, in February or March, flower bud samples from the trees to be crossed. The male tree collections are forced to flower by placing the branch collections in a vase of tap water, changing the water daily, and daily clipping a small disk from the end of each branch. After 7 to 10 days (greenhouse temperature of 65°F.) the pollen is collected and stored over calcium chloride at 38 to 40°F. Female branch collections are handled in a similar manner with the exception that after pollination, which is accomplished by applying the pollen with a small brush, the collections are placed in ice water to further reduce the possibility of bacterial plugging of the stems. Seed development is rapid and 21 to 24 days after pollination the seed is collected, separated from the attached cotton, and ready for planting. Figure 5 illustrates the steps of pollen production, pollination, catkin development and seed production. Seed production averages as high as 150 to 300 seeds per catkin and it is not unusual to produce 20,000 seeds from a single experimental cross. Seed germination varies from 70 to 90% for good quality seed.

*The general term cross, as used in the report, refers to cross within a species and the term hybrid is used for crosses between different species.

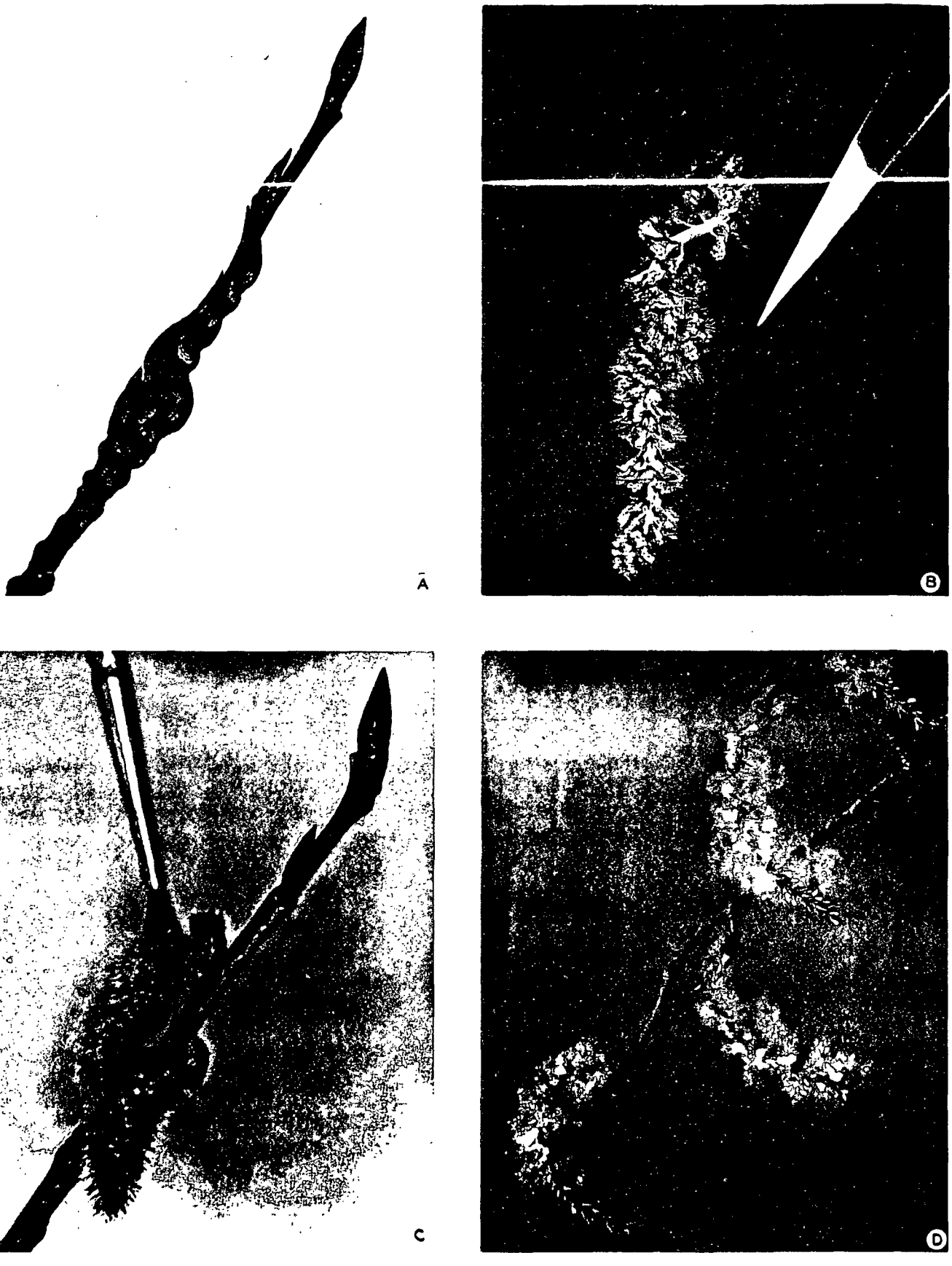


Figure 5. Illustrated Are the Steps In the Hybridization of Quaking Aspen: (a) Newly Collected Flower Buds, (b) Pollen Production, (c) Pollination of Female Flowers, (d) Cotton With Attached Seed 21 to 24 Days After Pollination

During 1963, 27 parent trees were employed in the crossing program and a total of 41 experimental crosses were attempted. Major emphasis was placed on producing bigtooth aspen crosses and bigtooth aspen hybrids. Considerable effort was also expended during the past season in developing crossing techniques for use in handling eastern cottonwood. Bigtooth aspen, quaking aspen, European white poplar, European gray poplar, bigtooth aspen hybrids, eastern cottonwood and balsam poplar (tacamahaca) were the species of Populus used as parent trees. Table III summarizes the parent trees utilized in the crossing program and Tables IV and V provide additional information on crossing success, seedling size, and seedling production.

The crosses were conducted in three series in the greenhouse during the months of February, March, and April. A large percentage of the seedlings were grown in outdoor seedbeds at the Greenville Nursery. Figures 6 and 7 illustrate seedbed production of experimental trees. The first series of crosses were made in early February and included crosses in which the parent trees were quaking aspen. The second series involved the bigtooth aspen crosses which, incidentally, normally flower later than quaking aspen and handle better if crossed later in the season. The third series of crosses involved cottonwood parent trees. This species, because it normally flowers late in the season, seems to handle best when the crosses are conducted in late April. Results of the three series of crosses are discussed in more detail below.

QUAKING ASPEN CROSSES

Quaking aspen crosses received major emphasis during the early phases of the crossing program. Differences in the performance of these early crosses are developing and outstanding individuals within the crosses are beginning to show up. Because of the large number of past quaking aspen crosses, the decision was made to

reduce the emphasis on this phase of the program pending evaluation of the results of the earlier work.

TABLE III

SUMMARY OF CROSSES AND LOCATION OF PARENT TREES

Cross No.[a]	Parents (female x male)		
XT-1-63	T-24-60 (Wausau, Wis.)	X	T-13-58 (Clintonville, Wis.)
XT-2-63	T-24-60 (Wausau, Wis.)	X	T-6-61 (Fern, Wis.)
XT-3-63	T-53-60 (Fern, Wis.)	X	T-13-58 (Clintonville, Wis.)
XT-4-63	T-53-60 (Fern, Wis.)	X	T-6-61 (Fern, Wis.)
XT-5-63	T-2-63 (Wausau, Wis.)	X	T-6-61 (Fern, Wis.)
XT-6-63	T-130-56 (Bruce Crossing, Mich.)	X	T-13-58 (Clintonville, Wis.)
XCa-T-7-63	Ca-2 (Czechoslovakia)	X	T-13-58 (Clintonville, Wis.)
XCa-T-8-63	Ca-2 (Czechoslovakia)	X	T-6-61 (Fern, Wis.)
XG-9-63	G-64 (Wausau, Wis.)	X	G-5 (Wis. Dells, Wis.)
XG-10-63	G-64 (Wausau, Wis.)	X	G-1-58 (Porcupine Mts., Mich.)
XG-11-63	G-64 (Wausau, Wis.)	X	G-4-58 (Tippy Dam, Mich.)
XG-12-63	G-64 (Wausau, Wis.)	X	G-7-62 (Boulder Junction, Wis.)
XG-13-63	G-4-57 (Wis. Dells, Wis.)	X	G-5 (Wis. Dells, Wis.)
XG-14-63	G-4-57 (Wis. Dells, Wis.)	X	G-1-58 (Porcupine Mts., Mich.)
XG-15-63	G-4-57 (Wis. Dells, Wis.)	X	G-4-58 (Tippy Dam, Mich.)
XG-16-63	G-4-57 (Wis. Dells, Wis.)	X	G-7-62 (Boulder Junction, Wis.)

Note: For footnote see end of table.

TABLE III (Continued)

SUMMARY OF CROSSES AND LOCATION OF PARENT TREES

Cross No.[a]	Parents (female x male)		
XG-17-63	G-13-60 (Millston, Wis.)	X	G-5 (Wis. Dells, Wis.)
XG-18-63	G-13-60 (Millston, Wis.)	X	G-1-58 (Porcupine Mts., Mich.)
XG-19-63	G-13-60 (Millston, Wis.)	X	G-4-58 (Tippy Dam, Mich.)
XG-20-63	G-13-60 (Millston, Wis.)	X	G-7-62 (Boulder Junction, Wis.)
XAG-G-21-63	AG-1-60 (Red Granite, Wis.)	X	G-7-62 (Boulder Junction, Wis.)
XA-G-22-63	A-2-59 (Waupaca, Wis.)	X	G-4-58 (Tippy Dam, Mich.)
XA-G-23-63	A-2-59 (Waupaca, Wis.)	X	G-7-62 (Boulder Junction, Wis.)
XA-G-24-63	A-1-62 (Orfordville, Wis.)	X	G-4-58 (Tippy Dam, Mich.)
XA-G-25-63	A-1-62 (Orfordville, Wis.)	X	G-7-62 (Boulder Junction, Wis.)
XCa-G-26-63	Ca-2 (Czechoslovakia)	X	G-5 (Wis. Dells, Wis.)
XCa-G-27-63	Ca-2 (Czechoslovakia)	X	G-1-58 (Porcupine Mts., Wis.)
XCa-G-28-63	Ca-2 (Czechoslovakia)	X	G-4-58 (Tippy Dam, Mich.)
XCa-G-29-63	Ca-2 (Czechoslovakia)	X	G-7-62 (Boulder Junction, Wis.)
XG-30-63	G-22-60 (Black River Falls, Wis.)	X	G-1-58 (Porcupine Mts., Mich.)
XD-31-63	D-7-62 (Black Creek, Wis.)	X	D-1-61 (Forest Junction, Wis.)
XD-32-63	D-7-62 (Black Creek, Wis.)	X	D-1-63 (Amherst, Wis.)
XD-33-63	D-2-63 (Sherwood, Wis.)	X	D-1-61 (Forest Junction, Wis.)
XD-34-63	D-2-63 (Sherwood, Wis.)	X	D-1-63 (Amherst, Wis.)

Note: For footnote see end of table.

TABLE III (Continued)

SUMMARY OF CROSSES AND LOCATION OF PARENT TREES

Cross No.[a]	Parents (female x male)		
XD-35-63	D-3-63 (Preble, Wis.)	X	D-1-61 (Forest Junction, Wis.)
XD-36-63	D-3-63 (Preble, Wis.)	X	D-1-63 (Amherst, Wis.)
XD-37-63	D-4-63 (Brussels, Wis.)	X	D-1-61 (Forest Junction, Wis.)
XD-38-63	D-4-63 (Brussels, Wis.)	X	D-1-63 (Amherst, Wis.)
XD-39-63	D-5-63 (Greenville, Wis.)	X	D-1-61[b] (Forest Junction, Wis.) D-1-63[b] (Amherst, Wis.)
XTc-D-40-63	Tc-1-63 (Kolberg, Wis.)	X	D-1-63 (Amherst, Wis.)
XTc-41-63	Tc-1-63 (Kolberg, Wis.)	X	Tc-2-63 (Kolberg, Wis.)

[a] X = cross, A = P. alba, Ca = P. canescens, D = P. deltoides, G = P. grandidentata, T = P. tremuloides, Tc = P. balsamifera.

[b] Pollen mixture used.

Five quaking aspen crosses (XT-1-63 thru XT-6-63) were made in 1963 and in addition quaking aspen male trees were used as parents in two crosses (XCa-T-7-63 and XCa-T-8-63) with Populus canescens (European gray poplar).

The crosses XT-1-63 through XCa-T-8-63 handled well and satisfactory seed production was obtained on most of the crosses. Seed production was least on crosses XT-3-63 and XT-4-63. This apparently is the typical seed production rate for the particular female trees involved. The first six crosses listed in Table III were conducted to produce seedlings for use in studies on methods of establishing aspen, co-operator field plantings and as a source of material for understock for grafting. Restricted flowering made it impossible for us to use

the very best parent trees and, although the parent trees employed were much above average, only crosses XT-3-63 and XT-4-63 can be recommended for large-scale field plantings.

TABLE IV

SUMMARY 1963 CROSSES

Cross No.[a]	Type Cross[b]	No. of Catkins Pollinated	No. of Catkins Collected	Amt. Seed[c]	Seeds/ Catkin[c]	Germ., %[c]
XT-1-63	C	42	39	18,385	434	99
XT-2-63	C	63	47	18,985	292	97
XT-3-63	C	30	25	5,980	187	94
XT-4-63	C	25	21	4,635	181	98
XT-5-63	C	28	28	17,230	597	97
XT-6-63	C	34	34	4,720	138	99
XCa-T-7-63	DS	38	30	1,791	47	99
XCa-T-8-63	DS	31	28	4,705	125	82
XG-9-63	DS	29	26	8,881	297	97
XG-10-63	C-DS	37	35	14,990	393	97
XG-11-63	DS	33	24	7,020	209	98
XG-12-63	DS	22	20	4,220	190	99
XG-13-63	DS	20	7	700	9	26
XG-14-63	DS	21	4	200	.1	1
XG-15-63	DS	20	8	100	.2	3
XG-16-63	DS	16	0	--	--	--
XG-17-63	DS	23	3	260	.1	1
XG-18-63	DS	23	3	340	2	14
XG-19-63	DS	29	10	1,800	13	21
XG-20-63	DS	18	8	340	.2	1
XAG-G-21-63	DS	14	7	850	11	18
XA-G-22-63	DS	50	25	3,570	1	2
XA-G-23-63	DS	60	50	4,890	19	23
XA-G-24-63	DS	109	33	3,610	17	52
XA-G-25-63	DS	47	46	1,317	20	73
XCa-G-26-63	C-DS	40	39	14,500	338	93
XCa-G-27-63	DS	50	50	8,370	134	80
XCa-G-28-63	C-DS	60	60	20,400	306	88
XCa-G-29-63	DS	60	60	11,025	177	96
XG-30-63	DS	65	1	0	--	--

Note: See end of table for footnote.

TABLE IV (Continued)

SUMMARY 1963 CROSSES

Cross No.[a]	Type Cross[b]	No. of Catkins Pollinated	No. of Catkins Collected	Amt. Seed[c]	Seeds/ Catkin[c]	Germ., %[c]
XD-31-63	B	9	3-4	84	3	12
XD-32-63	B	9	4	500	8	34
XD-33-63	B	5	3	500	110	66
XD-34-63	B	4	2	413	185	90
XD-35-63	B	11	6	1,037	133	70
XD-36-63	B	10	6	1,300	158	73
XD-37-63	B	11	2	385	58	30
XD-38-63	B	11	5	1,729	110	32
XD-39-63	B			639	121	38
XTc-D-40-63	B	9	0	--		--
XTc-41-63	B	9	2	750	240	64

[a] X = Cross, Ca = P. canescens, G = P. grandidentata, D = P. deltoides, T = P. tremuloides, Tc = P. balsamifera, A = P. alba.

[b] C = Seed for semicommercial production, DS = Dry site cross, B = crosses in black poplar group.

[c] Amount of seed, no. viable seeds/catkin collected and germination % based upon 40-mesh seed for all types of material except "XG" crosses. XG information is based upon 40 + 50 mesh seed.

BIGTOOTH ASPEN CROSSES

Genetic combinations accomplished in this rather general group include crosses made between selected bigtooth aspen parents and hybrids between bigtooth aspen and European white poplar, European gray poplar, and between bigtooth aspen and a natural aspen hybrid. The principal objective of this type of cross is the production of trees that are suitable for use on dry, sandy sites. This series of crosses (XG-9-63 through XG-30-63) is in general characterized by low seed production, low seed germination, and moderate first-year seedling growth. The exception to this general statement occurs in the crosses between European gray poplar and bigtooth aspen (XCa-G-26-63 through XCa-G-29-63). Seed production and seed

germination in these four crosses were very satisfactory and growth of the "XCa-G" crosses was outstanding with average seedling heights ranging from 2.3 to 2.8 feet.

TABLE V

SUMMARY OF 1963 SEEDLING PRODUCTION

Cross No.[a]	Total No. Seeds Planted	Total No. Seedlings Produced	No. Plantable Seedlings[b] Misc. Beds	No. Plantable Seedlings[b] Repl. Beds	Av. Height[c] All Seedlings	Av. Height[c] Plantable Seedlings
XT-1-63	2400	445	--	312	1.8	2.2
XT-2-63	2400	896	--	646	1.8	2.1
XT-3-63	4000	1000	285	306	1.6	2.1
XT-4-63	2400	1122	--	792	1.9	2.0
XT-5-63	4000	1150	303	567	1.6	2.0
XT-6-63	2400	1101	--	648	1.5	1.9
XCa-T-7-63	1600	417	--	309	2.0	2.4
XCa-T-8-63	4000	1100	283	515	1.9	2.3
XG-9-63	3200	430	73	153	1.3	1.9
XG-10-63	9600	2000	673	388	1.4	1.9
XG-11-63	4000	920	314	252	1.4	2.0
XG-12-63	3200	420	79	94	1.1	1.7
XG-13-63	700	7	--	2	1.0	2.0
XG-18-63	340	6	--	4	1.5	2.0
XG-19-63	600	41	14	12	1.7	2.3
XG-21-63	280	80	--	32	1.0	1.8
XA-G-22-63	3570	82	--	72	2.8	3.1
XA-G-23-63	6252	300	--	265	2.9	3.2
XA-G-24-63	3200	710	188	377	2.2	2.5
XA-G-25-63	1317	289	--	205	2.0	2.4
XCa-G-26-63	8100	1150	222	786	2.4	2.6
XCa-G-27-63	8000	960	400	453	2.8	2.9
XCa-G-28-63	20,700	2245	1450	795	2.6	2.7
XCa-G-29-63	9600	1700	487	904	2.3	2.5
XD-31-63	84	3	--	3	1.6	1.6
XD-32-63	500	87	--	69	2.1	2.4
XD-33-63	585	240	--	199	2.6	3.0
XD-34-63	413	212	--	159	1.9	2.3
XD-35-63	1450	212	--	138	1.9	2.5
XD-36-63	1600	280	--	215	2.0	2.3
XD-37-63	385	58	--	42	2.0	2.6
XD-38-63	2250	279	--	211	1.8	2.1
XD-39-63	639	189	--	148	2.1	2.3
XTc-41-63	750	170	--	47	1.3	1.5

[a] X = cross, A = P. alba, Ca = P. canescens, D = P. deltoides, G = P. grandidentata, T = P. tremuloides, Tc = P. balsamifera.

[b] Number of plantable seedlings 1.3 ft. or more.

[c] Av. heights based upon seedlings in replicated seedbeds, when replicated beds not available, miscellaneous seedbeds were measured.

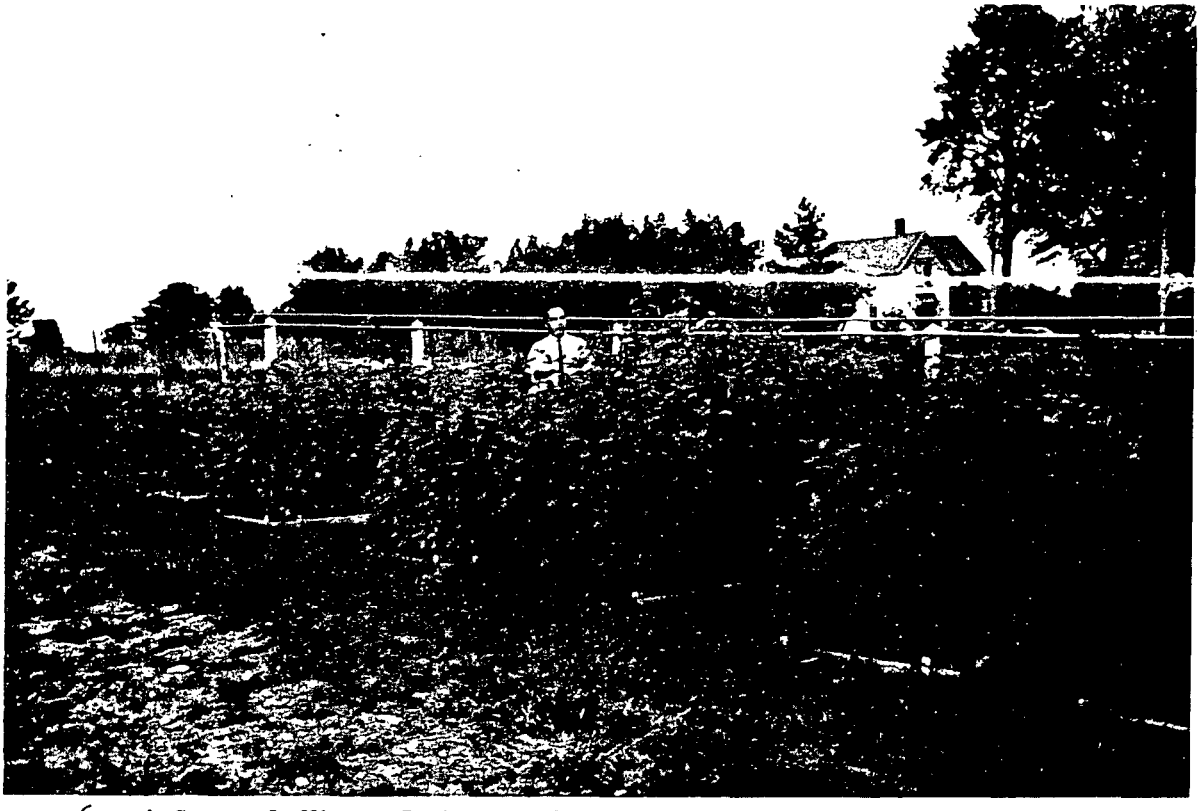


Figure 6. A General View of the Seedbeds Under One of the Three Sprinkler Lines. The Bigtooth Aspen 1-0 Seedlings (at the Left) Have a Smaller Average Height Than the 1-0, "Alba x Bigtooth" Seedlings (Center and on the Right)

Figure 7. Quaking Aspen, 1-0 Seedlings (Left Foreground) and "Canescens x Bigtooth," 1-0 Hybrid Seedlings (Right Foreground) Growing in Experimental Seedbeds

The bigtooth aspen crosses XG-9-63 through XG-20-63 were arranged in a modified diallel series and used to evaluate the crossing compatibility and overall usefulness of four male and three female bigtooth aspen parent trees. Flowering behavior, seeds and plantable seedlings produced, and first year seedling growth were used as a basis of evaluating the parent trees. Table VI clearly illustrates the superior behavior of G-64 (female) and the male trees G-5, G-1-58 and G-4-58. Figure 8 pictures G-4-58, one of the best male bigtooth aspen presently being used in our crossing program. Both G-7-62 and G-13-60 had been evaluated previously. G-7-63 which ranked as the best male of the four trees compared previously, did quite well in this comparison but ranked below the other males being compared. G-13-60, which was girdled in 1960 to induce flowering, did not behave well in 1962 and did even poorer in 1963. The girdling treatment appears to have greatly reduced the tree's vigor during the second growing season after girdling.

COTTONWOOD AND CLOSELY RELATED CROSSES

Two major problems were considered in the cottonwood crossing program. First the problem of establishing a satisfactory method for crossing cottonwood was investigated and second the evaluation of several selected male and female trees was undertaken. This latter study required crossing the trees involved and producing suitable numbers of progeny for field testing.

The investigation of the methods of handling female branch collections and/or scions during flowering and seed development involved trying several different propagation techniques. Included among the methods tried were bottle grafting, crown veneer grafting, rooting scions in sand-vermiculite, using the

TABLE VI

SUMMARY OF SEED AND SEEDLING PRODUCTION AND SEEDLING GROWTH - MODIFIED BIGTOOTH ASPEN DIALLEL CROSSING SERIES

Female Parent Trees	Male Parent Trees			
	G-5	G-1-58	G-4-58	G-7-62
G-64[a]	XG-9-63	XG-10-63	XG-11-63	XG-12-63
[b]	297	393	209	190
[c]	21	44	30	10
	1.3	1.4	1.4	1.1
G-4-57[a]	XG-13-63	XG-14-63	XG-15-63	XG-16-63
[b]	9	.1	.2	0
[c]	.1	(Discarded)	(Discarded)	--
	1.0			--
G-13-60[a]	XG-17-63	XG-18-63	XG-19-63	XG-20-63
[b]	.1	2	13	.2
[c]	(Discarded)	.2	.1	(Discarded)
		1.5	1.7	

[a] Number of seeds produced per catkin pollinated.
[b] Number of plantable seedlings (1.3 feet plus) produced per catkin pollinated.
[c] Average height of all seedlings in seedbeds.

"cut branch" techniques*, rooting and growing scions in nutrient solutions, and rooting and growing scions in agar culture media. The technique of bottle grafting scions having flower buds proved to be the most reliable method of bringing the pollinated catkins through to maturity. Crown veneer grafts were also quite successful and appeared to be worthy of further consideration. None of the other methods tried proved to be satisfactory.

A modified diallel crossing series, similar to the series described for bigtooth aspen, was established to evaluate four female and two male cottonwood trees. The scions from the four female trees, each containing a single flower bud,

*The "cut branch" technique employed involved placing the freshly collected branches in a vase of ice water, clipping off a small section of stem every other day and changing and icing the water every other day.

Figure 8. G-4-58, Which is Located Near Tippy Dam, Lower Michigan, is One of the Best Male Bigtooth Aspen Presently Being Used in our Crossing Program. The Tree Has Good Form, a d.b.h. of 18.7 Inches, a Total Height of 90 Feet and a Total Age of 52 Years

were grafted prior to pollination. Table VII provides information on the number of seeds produced per catkin pollinated, the plantable seedlings produced per catkin pollinated, and the average height of all seedlings produced. Tree D-1-63 appears to be the better of the two male trees tested and D-2-63 and D-3-63 handled the best of the four female trees tested.

Plans for handling the cottonwood seedlings include selection and propagation of the outstanding one or two per cent of the individuals produced in each cross, and the field testing in replicated trials of the remaining upper thirty to forty per cent. Additional cottonwood crosses and testing are planned for the coming two or three years.

TABLE VII

SUMMARY OF SEED AND SEEDLING PRODUCTION AND SEEDLING GROWTH – COTTONWOOD MODIFIED DIALLEL CROSSING SERIES

Female Parent Trees	Male Parent Trees	
	D-1-61	D-1-63
D-7-62	XD-31-63	XD-32-63
[a]	3	8
b	.3	7
c	1.6	2.1
D-2-63	XD-33-63	XD-34-63
[a]	110	185
b	40	40
c	2.6	1.9
D-3-63	XD-35-63	XD-36-63
[a]	133	158
b	13	22
c	1.9	2.0
D-4-63	XD-37-63	XD-38-63
[a]	58	110
b	4	19
c	2.0	1.8

[a] Number of seeds produced per catkin pollinated.
[b] Number of plantable seedlings (1.3 feet) produced per catkin pollinated.
[c] Average height of all seedlings in seedbeds.

BIOCHEMICAL CHARACTERIZATION OF ASPEN AND ASPEN HYBRIDS

OBJECTIVES

A. To extend the range of genetic markers in aspen to the biochemical level.

B. To develop rapid, efficient techniques for the detection of biochemical markers.

NATURE OF THE PROBLEM

Genetic Factors

The ultimate genetic analysis of a living cell or organism (an organized group of cells) will involve a complete chemical and physical description of the primary genetic material, DNA (desoxyribonucleic acid); it will involve a knowledge of the mechanisms essential to the transcription to RNA (ribonucleic acid) of the genetic information residing in the DNA molecule; it will further involve a knowledge of the mechanisms required to transcribe the RNA information into specific protein structures essential to the structure and function of the cell. Over and above this, a knowledge of the temporal organization of cells and a knowledge of the specific influence of environment on the above mechanisms will be essential. At the present time the bare outline of "molecular genetics" is in hand and no single cell or organism has been described genetically in the above terms. Perhaps this ultimate goal will never be achieved for any specific organism. However, as knowledge accumulates concerning the nature of the genetic process, the geneticist's ability to direct and control the evolution of living things increases dramatically. The discovery of the principles of Mendelian genetics increased tremendously the ability of man to direct and control the evolution of plants and animals. Modern eugenics, horticulture, and animal husbandry are predicated on these principles. The application of the principles of "molecular genetics" will

vastly increase the degree of direction and control which can be achieved. The important point is that an ultimate solution to the genetic process is not necessary to effect real and substantial control over the evolutionary process. The insights into the nature of "molecular genetics" achieved in the last ten years have initiated with great excitement the studies which will almost certainly culminate in a high degree of control by man of his own genetic destiny as well as control of the genetic character of the plants and animals upon which he is dependent.

The examination and translation of the primary genetic code, DNA, is a current objective of the geneticist. Out of practical necessity this work is being conducted with simpler organisms, principally viruses and bacteria. The chemical and physical problems inherent in this study are compounded many times over when higher plants and animals are considered. For this reason, a direct attack on the primary code of higher plants is not considered feasible at the present.

Similar observations can be made about the current state of knowledge concerning the RNA components of the genetic system. The absolute amounts of DNA and RNA in tissues are relatively small and the problems of removing these large polymers from the cells of higher plants without alteration are not solved.

The third major component of the genetic system is protein. Each different protein possesses in its primary structure (the order and sequence of amino acid monomeric units) a segment of the primary, DNA, code as transcribed through the RNA system. In principle, a complete knowledge of protein structure, the number of different proteins produced, and the conditions (physical and biochemical environments) essential for the synthesis of each protein would provide the basis for deducing the nature of the primary, DNA, code. Such an approach is burdened with, if not more, at least as many difficulties as the direct study of the primary, DNA, code. However, it is possible, under controlled conditions, to compare the

nature of proteins produced by two organisms (in this case, individual aspen trees) and come to a conclusion about the genetic differences or similarities which may be manifest by differences or similarities in protein composition. Protein, in addition to its specificity of structure, has the further advantage of being produced in tangible quantities, making the extraction and purification a reasonable undertaking based on current methodology. The first objective, to extend the range of genetic markers to the biochemical level, can be defined broadly as a study of protein differences between trees.

Extraction and Detection of Biochemical Markers

The critical factors to be studied are proteins and possibly certain polysaccharides. The latter, though not primarily involved in the genetic process, are high molecular weight molecules whose chemical composition and steric properties are directly controlled by proteins (enzymes). The presence or absence of a specific polysaccharide can be presumptive evidence of the presence or absence of the enzymes responsible for its synthesis and is, therefore, of interest as a genetic marker. The antigenic properties of many polysaccharides can be exploited with respect to their detection and identification.

Trees are not good sources of protein. The physiologically active regions of a tree are limited principally to the apical meristems, the cambial regions, and the leaves. Of these, the leaves are the most accessible and available in greatest quantity, especially in woody plants such as aspen. For this reason, leaves have been selected for examination in the preliminary phases of this investigation.

Certain major problems in the extraction of protein may be summarized as follows:

1. The tissue must be disintegrated (cells broken) in such a manner that the physical forces (e.g., shear, foaming, temperature rise) do not denature the protein components of the cellular extract.

2. The ionic composition, concentration, and pH of the buffer used in the extraction must be such that the proteins will be solubilized and stable. It is impossible to select any one set of conditions for the extraction and characterization of all proteins of the cell. Since solubilization of protein is necessary for physical, chemical, and biological characterization, the readily water-soluble fraction has many advantages.

3. Secondary reactions such as "browning" protease attack on other proteins, and denaturation of proteins by low molecular weight components of the extract must be avoided.

4. The solubilized protein must be removed from the cellular debris, from the low molecular weight components, and must be concentrated under mild conditions (e.g., by lyophilization, pervaporation, forced dialysis against Carbowax, or similar methods). At this stage, the efficient concentration of dilute solutions is a major problem.

Since protein is the principal nitrogen-containing high molecular weight component of the cell, total nitrogen of the extract is generally used to follow the effectiveness of the extraction. The detection and characterization of individual proteins is usually accomplished by one or more of the following techniques.

The present study involves the comparison of proteins from different sources. For this reason, methods most likely to be effective for the resolution of protein mixtures will be discussed.

Paper Electrophoresis

Although free electrophoresis has not been very successful in the separation of plant proteins, there is some hope that electrophoresis using some of the newer highly porous papers or synthetic gels (e.g., disk electrophoresis) will produce separations of interest in a comparative sense.

Ultracentrifugation

This technique will reveal differences in sedimentation characteristics of mixtures. Its value will depend upon the range of molecular weights encountered.

Adsorption Chromatography

This technique is a powerful tool in the separation of proteins, the fractionation depending upon differences in relative affinity of the protein components for the column resin. Very subtle differences in the ionic nature of the proteins will frequently result in separation.

Molecular Seive Chromatography

Columns of Sephadex and polyacrylamide gels have been used with considerable success. Fractionation is effected by differences in the degree of retardation of each component by the gel matrix. Physical factors such as size, shape, and diffusion coefficient are important in determining the extent of retardation. In dealing with heterogeneous mixtures, it is likely that certain components will adsorb directly on the gel and may be selectively removed by elution as in adsorption chromatography.

Immunological Techniques

There are several types of immunological techniques which have proven to be very powerful for detection of small differences in protein structures. The basic technique is predicated on the antigenic properties of proteins, i.e., the ability of protein to induce the formation of specific antibody protein when introduced into a suitable animal. Antibody protein will react specifically with the protein antigen which induced its formation. On reaction with antibody the antigen-antibody complex tends to precipitate. The precipitate is frequently visible to the naked eye and is an elegant means of detecting such reactions. The two most important variations of this technique are double diffusion and immunoelectrophoresis.

The double diffusion technique depends upon the fact that antigens may vary considerably in molecular weight, shape, and ionic character and may, therefore, have distinctly different diffusion coefficients. If a mixture of antigens is placed in a small circular depression in a thin layer of agar gel in a petri dish, each antigen will diffuse radially outward into the gel at different rates based on the differences in diffusion coefficients of the individual antigens. The result is a series of antigen fronts moving radially outward from the depression. Antibodies also exhibit a heterogeneity of diffusion coefficients and a similar phenomenon occurs if a mixture of antibodies are allowed to diffuse in an agar gel. If two depressions one or two centimeters apart are cut into an agar gel and a mixture of antigens is placed in one, a mixture of antibodies in the other, the antigen and antibody fronts will diffuse toward each other. When an antigen front meets its specific antibody front, a line of precipitate will form; the position of the line depends on the relative rates of diffusion of the reacting antigen and antibody. If another antigen and its homologous (specific) antibody diffuse at

relative rates different from the first antigen-antibody pair, the line of precipitate formed will be a different and equally unique position. Frequently several antigens can be detected in this manner. The limitation of the technique lies in the fact that the distances over which diffusion can be carried out are relatively short and several lines of precipitate may be too close together to be resolved. However, where positive differences are observed, the technique is simple and effective.

Immunoelectrophoresis is a refinement of the double diffusion experiment where the antigen mixture is first subjected to electrophoresis in agar or similar supporting medium to produce a linear array of antigens based on differences in electrophoretic mobility, i.e., differences in the rates of movement in an applied electrical field. After electrophoresis, a linear depression or trough is cut into the agar a short distance away from and parallel to the line of electrophoretically distributed antigens. The desired antibody mixture is placed into the trough and double diffusion allowed to proceed, forming lines of precipitate where homologous antigen-antibody systems meet. Because of the original electrophoretic distribution of antigens, lines are frequently resolved which in the simple double diffusion experiment would be too close together to be resolved.

Another useful criterion for the identification of specific proteins is the catalytic (enzymatic) property and substrate specificities of many, especially the water-soluble, proteins. The methods of assay or detection of enzymatic activity are as diverse as the reactions mediated. Basically, the disappearance of a substrate or appearance of a reaction product is measured. Changes in secondary, coupled, reactions are frequently used.

The second objective, to develop rapid, efficient techniques for the detection of biochemical markers, can be defined broadly as the design, development

and application of techniques capable of detecting genetically significant differences in the macromolecular components of aspen.

EXPERIMENTAL

Preliminary Double Diffusion Experiments

Aqueous extracts from the leaves of four different aspen trees were available at the conclusion of an academic project (1). The extracts as received were identified as follows:

Code	Species	Sex	Nitrogen Content of Extract, %, w/v
A-1	*Populus tremuloides*	male	.01
C-6	*Populus deltoides*	--	--
D-7	*Populus tremuloides*	female	.01
E-8	*Populus tremuloides*	female	--

The extracts contained the following:

0.15M Na Cl
0.045M Na_3PO_4
10% Glucose
1% Ascorbic acid
1:50,000 (w/v) phenylmercuric acetate
Water-soluble components of the excluded fraction eluted from Sephadex G-25

The academic project culminated in the demonstration of a precipitin reaction with the above extracts and homologous rabbit antiserum.

Since the above work was directly pertinent to the projected studies for Project 2412, it was decided to use all but a small amount of the residual extracts to continue the immunization of the rabbits in an effort to achieve higher antibody titers and to use the antiserum so obtained to set up double diffusion experiments against the aqueous extracts.

The rabbits were injected (1 cc. subcutaneously in the abdominal region) according to the following schedule; two rabbits were used for each extract. The dates of the injections were July 5, 8, 10, 12, 15, 17, 1963. The rabbits were bled on July 23 and 24, 1963. Approximately 40 cc. of blood was obtained from each rabbit from the marginal ear vein. The blood was refrigerated overnight, the clot removed, the serum centrifuged (Betafuge, 9RA rotor, 10,000 r.p.m., 30 min., 5°C.), 1.0 cc. of a 5% phenol solution in saline with 0.2 cc. of 0.1% merthiolate added for each 20 cc. of serum and stored at 2 to 5°C. until needed.

The leaf extracts remaining after the injections were concentrated approximately threefold by evaporation under reduced pressure at 2 to 5°C. The concentrate was dialyzed in the cold against the following buffer:

0.2M Na_2HPO_4
0.002M $MgCl_2$
12.5% Glucose
1% Ascorbic acid

The dialyzed extracts were held at 2 to 5°C. until needed.

The double diffusion experiments were carried out as follows:

Medium:	Oxoid Agar Agar No. 3	1.2%
	NaCl	.85%
	Phenylmercuric acetate	10.0 p.p.m.
	Sterilized in an autoclave at 121°C., 20 min.	

Twenty ml. of warm, liquid medium was poured into sterile 100 mm. petri plates and allowed to solidify. A sterile, 10-mm. diameter cork bore was used to cut a center well (for antiserum) and four equally spaced peripheral wells (for antigens). The peripheral wells were cut 20 mm. from the center well, center to center, giving a 10-mm. diffusion zone between wells. Antiserum was placed in the center well at zero time and antigen added to replicate plates at 0, 24, and 48 hours, Table VIII. All plates were photographed after 23 days at 20°C. Figure 9A

TABLE VIII

DOUBLE DIFFUSION EXPERIMENTS

Antiserum	Rabbit No.	Antigen Added (hours after addition of antiserum)	Antigen A-1	C-6	D-7	E-8
A-1	1	0	?[1]	?	?	0
			?	?	?	0
A-1	1	24	+	+	+	0
			+	+	+	0
A-1	1	48	+	+	+	0
			+	-*	-	-
A-1	2	0	0	0	0	0
			0	0	0	0
A-1	2	24	0	0	0	0
			0	0	0	0
A-1	2	48	0	0	0	0
			0	0	0	0
C-6	3	0	0	0	0	0
			0	0	0	0
C-6	3	24	0	0	0	0
			0	0	0	0
C-6	3	48	0	0	0	0
			0	0	0	0
C-6	4	0	0	0	0	0
			0	0	0	0
C-6	4	24	+	+	+	0
			+	+	+	0
C-6	4	48	+	+	+	0
			+	-	-	-
D-7	5	0	0	0	0	0
			0	0	0	0
D-7	5	24	0	0	0	0
			0	0	0	0
D-7	5	48	0	0	0	0
			0	0	0	0
D-7	6	0	?	?	+	0
			0	0	+	0
D-7	6	24	?	+	?	0
			+	+	+	0

Note: See end of table for footnote.

TABLE VIII (Continued)

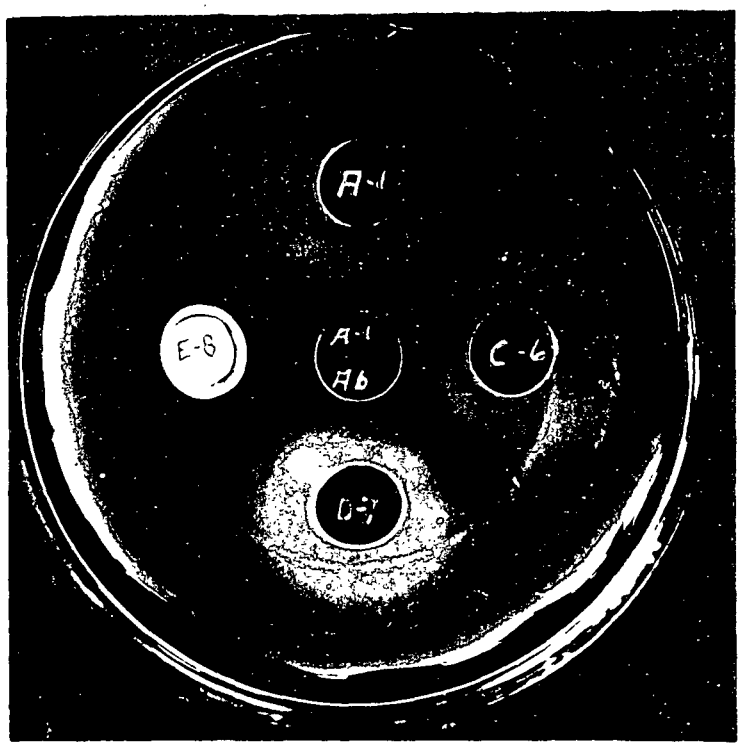
DOUBLE DIFFUSION EXPERIMENTS

Antiserum	Rabbit No.	Antigen Added (hours after addition of antiserum)	Antigen A-1	C-6	D-7	E-8
D-7	6	48	+	-	+	0
			+	-	-	-
E-8	7	0	0	0	0	0
			0	0	0	0
E-8	7	24	0	0	0	0
			0	0	0	0
E-8	7	48	0	0	0	0
			0	0	0	0
E-8	8	0	0	0	0	0
			0	0	0	0
E-8	8	24	0	0	0	0
			0	0	0	0
E-8	8	48	0	0	0	0
			0	0	0	0

[1]Second entry for each antiserum is the result obtained on a duplicate plate.
*Indicates no duplicate plate prepared because of insufficient antigen.
+Indicates one or more visible lines of antigen-antibody precipitation.
0 Indicates no visible precipitates (all plates were viewed and photographed by dark field illumination).

is an example of the appearance of plates showing precipitation zones. Note the broad precipitation zones around the wells containing A-1, C-6, and D-7 antigens and the absence of a broad zone around the E-8 well. Within the broad zones, note the presence of visible narrow bands of precipitation. After photographing, the agar gels were removed from the petri dishes and washed for several days in physiological saline solution until the broad zones of precipitate were solubilized and washed away, resolving the less soluble narrow bands of precipitate formed by specific antigen-antibody reaction. The plates were washed only long enough to resolve the precipitin bands since prolonged washing gradually solubilized them. Figure 9B shows the gel in Fig. 9A after washing. (The speckled appearance of

A.



B.

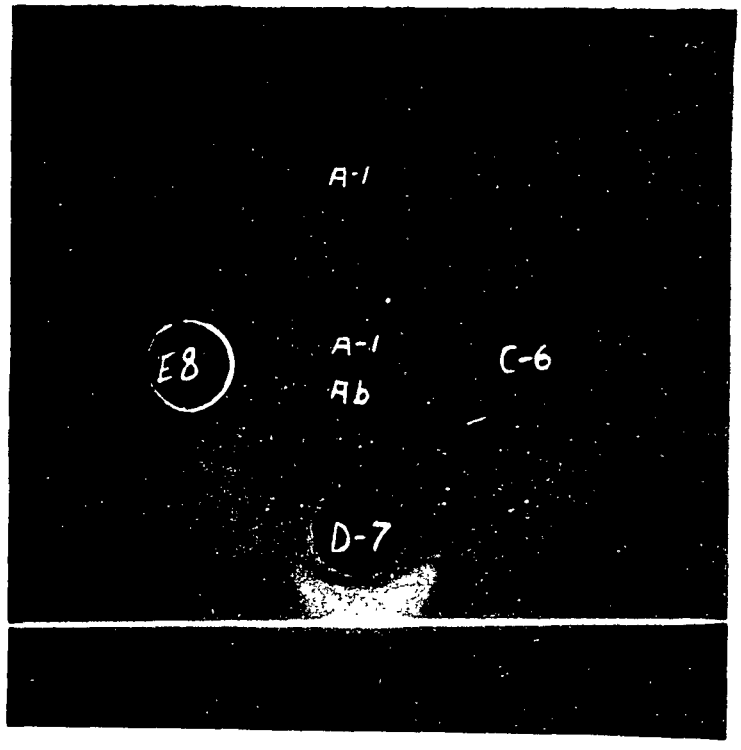


Figure 9. Typical Appearance of Double Diffusion Experiments in Agar Gel.: A., Before Washing in Saline; B., After Washing in Saline

the gel resulted from the collection of dust when inadvertently left uncovered over night.) Note the several common precipitin bands associated with A-1, C-6, and D-7. These results are typical of all conditions where positive results were obtained (see Table VIII).

In each case where positive results were obtained, only one of the two rabbits formed detectable amounts of antibodies. It was also noted that the time of addition of the antigen was critical. These results, along with the observation that E-8 antigen produced no detectable precipitate even when diffused against homologous antiserum, are consistent with the conclusion that the antigen concentration of the extracts was probably minimal and that rabbits differ in their sensitivity to the antigens used in these experiments.

Other Diffusion Techniques

The antisera obtained from the preliminary double diffusion experiments were used to explore several variations of the double diffusion experiment:

1. A piece of undifferentiated aspen tissue (Populus grandidentata) growing on a suitable nutrient agar medium (see section on Tissue Culture below) was transferred aseptically to the center of a fresh nutrient agar medium in a 100-mm. petri plate and allowed to grow in the dark at 27-29°C. After two weeks, the tissue mass had approximately doubled in size and was about 1.0 cm. in diameter. At that time, wells were cut in the agar approximately 1.0 cm. from the edge of the tissue and each well filled with an antiserum. Diffusion was carried out at 20-23°C. and after 1 to 4 weeks up to 6 precipitin bands appeared between antiserum A-1 and the tissue mass; E-8 antiserum produced no bands (consistent with the results reported in Table I); C-6 and D-7 antisera gave fewer and less distinct bands (also consistent with the qualitative results obtained in the preliminary double diffusion experiments). Three experiments of this type

were carried out with qualitatively similar results in each case. In these experiments there was a heavy but narrow band of precipitate approximately one millimeter from the edge of the wells. The precipitate formed a halo around the well. In some instances, partial halos were also observed on the side of the antiserum cup away from the center. Outside of the narrow halos of precipitate the medium remained clear except for a slight haze which is characteristic of the particular nutrient medium employed. The precipitin bands observed were light but distinct and formed in a straight line perpendicular to the line of centers between the tissue mass and the antiserum well which is characteristic of specific antigen-antibody reactions in experiments of this type.

The direct diffusion of antisera against growing tissue appears to offer promise as a rapid test procedure. It will be explored further in future work.

2. Micro double diffusion experiments were carried out according to Crowle (2). This technique, which consists of double diffusion in a thin agar gel on a microscope slide, is of interest because of the relatively simple material requirements and economy with respect to reactants. The results obtained were poor but the technique will be explored further when antisera with higher antibody titer and greater specificity is available.

3. Double diffusion in small (6 cm.) petri plates (3) produced essentially the same results as with the standard (10 cm.) petri plates and offered no advantage over the standard plates.

4. Tube diffusion experiments were carried out according to Preer, et al (4) and Becker, et al (5). These experiments involved the addition of antigen to warm, liquid agar which was poured into the bottom half of a small glass tube (3 mm. I.D. and 8 cm. long) and allowed to solidify. In the same manner, an

antiserum agar mixture was layered on top of the solidified antigen-agar layer and diffusion allowed to proceed. A general cloudy precipitate developed along with a single (approximately 1/8 inch wide) precipitin band on the antigen side approximately 1 cm. from the interface. The band did not appear to move as diffusion continued. In the previous work cited, the investigators observed the formation of sharp bands which migrated as a result of the changing relative concentrations of antigen and antibody as diffusion proceeded. Different bands migrated at different rates characteristic of different antigen-antibody systems. The single band observed in the present work did not migrate. The band was probably a mixture of the several bands observed in the double diffusion experiments. This technique will be explored further when new antiserum is available.

Protein Isolation

1. General

The general nature of the problems and techniques involved in the isolation of protein were discussed in Part II. Two other factors, ploidy and environment, must be considered in selecting a protein source in connection with genetic studies. In principle, the haploid population presents the most interesting source since the members of this population possess the independently assorted genetic factors or "genes" of the diploid parents. In aspen, however, the haploid population is confined to the megaspores (eggs) and the microspores (pollen grains), neither of which can presently be analyzed for protein because of the extremely small size (one or few cells) of the haploid individual. In rare cases, pollen grains have been induced to initiate cell division and grow as undifferentiated tissue on suitable nutrient media. Such tissue would be ideal for the detection of genetic differences through protein analysis. At the present time, the nutritional requirements for pollen tissue culture are not known. The second most

interesting protein source is the diploid individual. This source, although an order of magnitude more complex, does have the advantage of being the object of greatest practical interest at the present time. The least interesting for comparative genetic studies because of its complexity is the polyploid. Paradoxically, the aspen triploid exhibits more vigorous growth than the diploid both in the field and in laboratory tissue cultures and, as a result, has potential practical significance. In spite of the complexity of diploids and polyploids, it will be possible to work with them in a manner analogous to the clinical detection of genetic parameters at the biochemical level in humans until techniques for working with the haploid generation are in hand. Such studies will provide the basis for more refined genetic analysis as progress is made in "molecular genetics" on the one hand and plant physiology on the other.

In order for protein to serve as a reliable index of genetic parameters, conditions affecting protein synthesis must be known. In practice, these conditions are never known in any absolute sense. The only feasible approach is to study synthesis under controlled or "standardized" conditions. The degree of control can range from systematic field planting to rigorously controlled tissue culture. A dispersed single cell tissue culture growing in a completely defined synthetic medium represents the highest level of environmental control. At the present time, this type of growth can be achieved with certain plant tissues. Aspen tissue can be grown as dispersed aggregates and it is hoped that improvement of tissue culture techniques will ultimately produce the desired dispersed single cell cultures.

2. Tissue Culture

In order to explore diploid tissue culture as a source of protein for comparative genetic studies, tissue cultures were initiated from the parents and

progeny of cross number XT-12-57, Trial VII, located at The Institute of Paper Chemistry Greenville Farm Test Area.

Parents: Male: T-32-57

Female: T-30-control

Progeny: No. 1 through 55

Samples were taken during the month of October, 1963. Ten of the progeny in the 1 through 55 group were not sampled because of disease or previous cutting.

Tissue cultures were initiated by surface sterilization of young stem sections, aseptically cutting the ends to expose viable cambium of the internodal region, and placing them on a sterile nutrient agar of the following composition:

Component	mg./l.
Na_2SO_4	200
$Ca(NO_3)_2 \cdot 4H_2O$	200
$MgSO_4 \cdot 7H_2O$	360
KNO_3	80
KCl	66
$NaH_2PO_4 \cdot H_2O$	16
Ferric citrate	10
H_2SO_4 (.005 ml. concn.)	
$MnSO_4$	3
$ZnSO_4 \cdot 7H_2O$	0.5
H_3BO_4	0.5
$CuSO_4 \cdot 5H_2O$	0.025
$Na_2MoO_4 \cdot 2H_2O$	0.025
Glycine	3
Thiamine hydrochloride	0.1
Naphthalene acetic acid	0.5
Sucrose	20 g./l.
Agar	8 g./l.

Component	mg./l.
Coconut milk[a]	100 ml./l. (final volume)
Sterilized (121°C., 15 minutes in autoclave)	

[a]Heated to 60°C., cooled, and filtered (Whatman No. 1) prior to addition.

Tissue cultures from all sampled progeny were successfully initiated on the above medium and maintained for a period of three months or longer. Some of the more durable cultures are still in culture at the present. It was observed that the rate of growth of the diploid tissues was not adequate to provide a good source of protein. A further problem encountered was the nonuniformity of growth from one piece of tissue to the next. This appeared to be unorganized differentiation which produced a variety of textures and colors in the separate tissue masses. Attempts to grow the tissues in liquid medium (the above medium without agar) on a rotary shaker and in aerated submerged culture were also unsuccessful. In every case, some growth was evident but not enough to make the procedure of interest as a large-scale source of tissue.

Because coconut milk medium has been observed to induce chromosomal aberrations and polyploidy in certain tissue cultures, an attempt was made to carry the more vigorous tissues on a synthetic medium of the following composition:

Basic Medium: 8 g. agar, 20 g. sucrose and .5 p.p.m. naphthalene acetic acid made up with water to 940 ml. and sterilized, 121°C., 20 min. in the autoclave.

To this basic medium is added aseptically the following:

	Amounts/l.
Stock 1	10 ml.
Stock 2	10 ml.
Stock 3	10 ml.
Stock 4	30 ml.

Stock 1 (amounts/250 ml.)
(autoclave)

KNO_3	2 g.
Na_2SO_4	5 g.
KCl	1.6 g.
$NaH_2PO_4 \cdot H_2O$	.46 g.
$MgSO_4 \cdot 7H_2O$	13.5 g.
$MnSO_4 \cdot H_2O$	125 mg.
$ZnSO_4 \cdot 7H_2O$	54 mg.
H_3BO_3	38 mg.

Stock 2 (amount/250 ml.)
(autoclave)

$Ca(NO_3)_2 \cdot 4H_2O$	6.75 g.
ferric citrate	75 mg.
glycine	75 mg.
stock 2A	10 ml.
thiamine	25 mg./100 ml.
pyridoxine . HCl	25 mg./100 ml.
nicotinic acid	125 mg./100 ml.

Stock 3 (amounts/500 ml.)
(cold sterilization)

inositol	5 g.
choline chloride	.5 g.
hypoxanthine	125 mg.
stock 3A	50 ml.
riboflavin	
Ca pantothenate	120 mg./500 ml.
biotin	12 mg./500 ml.

Stock 4 (amount/600 ml.)
(cold sterilize)

asparagine	.4 g.
aspartic acid	.12 g.
arginine	.16 g.
cystine	.03 g.
histidine	.052 g.
isoleucine	.21 g.
leucine	.31 g.
lysine.HCl	.31 g.
methionine	.26 g.
phenylalanine	.05 g.
valine	.26 g.
glutamic acid	1.0 g.
proline	.1 g.
threonine	0.13 g.
tryptophane	.08 g.
tyrosine	.8 g.

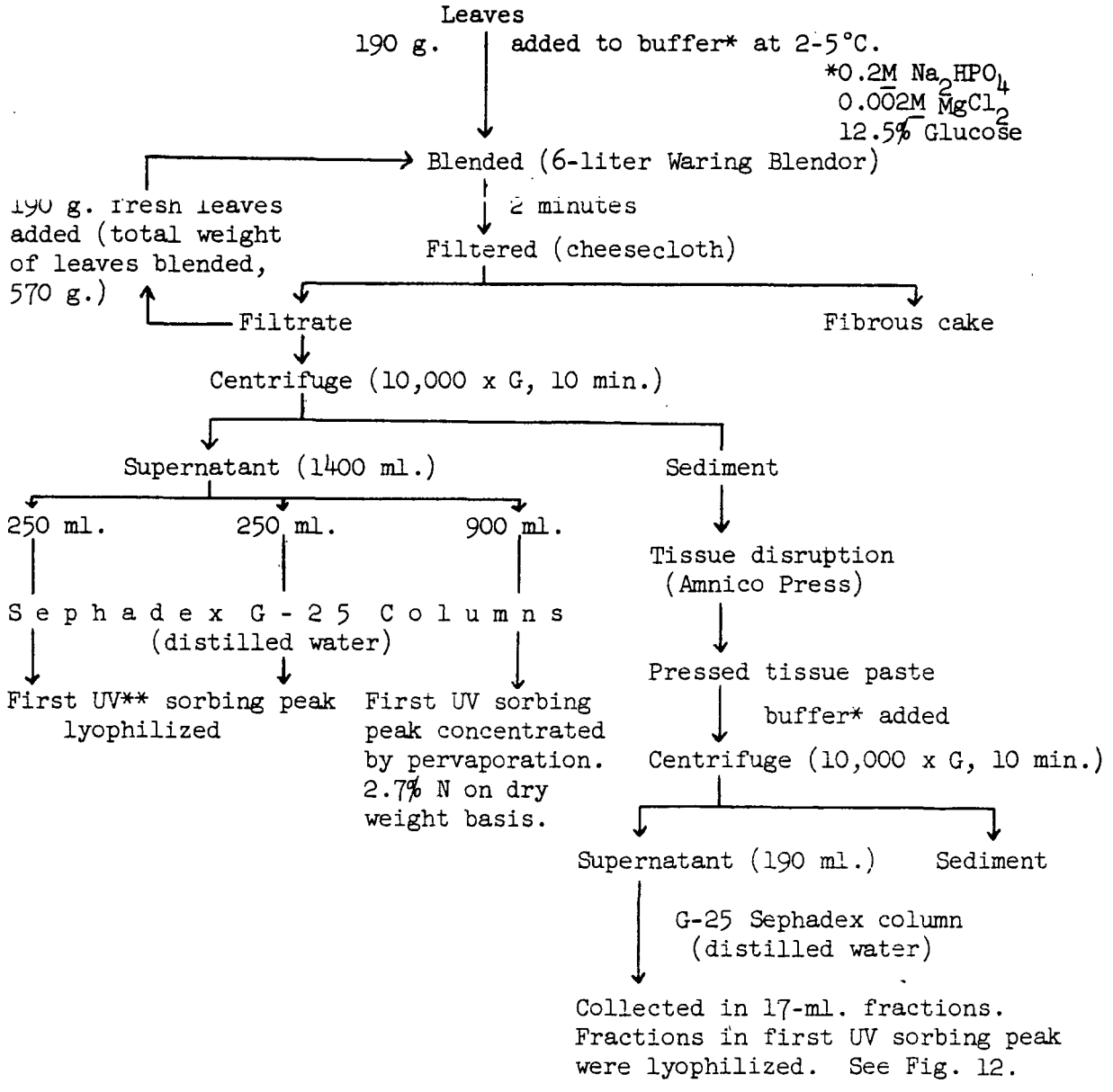
adjusted to pH 5.0 with HCl

Most of the tissues grew on the synthetic medium but less well than on the coconut medium both in solid and liquid culture.

At the present time, the most promising source of tissue is a strain of triploid aspen which can currently be cultured at the rate of about 1.2 g. per liter of coconut milk medium per day. This culture is available through Dr. Martin Mathes of the Forest Genetics Group. A large-scale shaker, designed to handle 36 three-liter flasks, is being constructed and will be used to provide a steady supply of fresh triploid tissue for protein analysis and further immunochemical work. The triploid tissue is being maintained in liquid coconut milk medium on a rotary shaker until large-scale operation is possible.

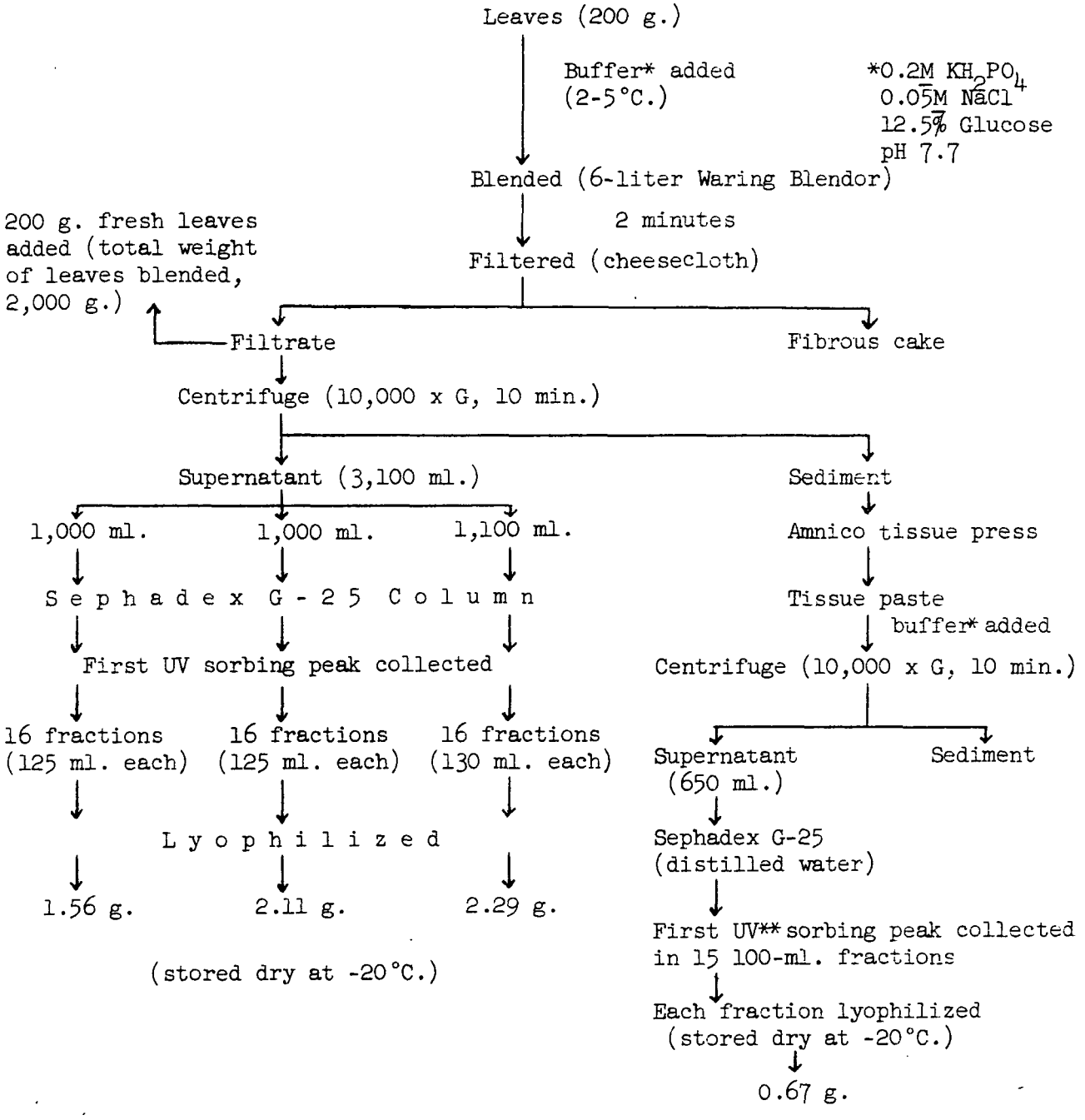
3. Leaf Protein

Leaves were collected in August and September, 1963 from clones of Populus tremuloides (triploid), P. canescens (diploid) and P. alba (diploid) at The Institute of Paper Chemistry Greenville Farm Test Area. The leaves were stored at -10 to -20°C. until they were used. In order to continue the immunochemical work described in Part III, A, the water-soluble, high molecular weight components of P. tremuloides were obtained by the fractionation procedure shown in Fig. 10 and 11. Figure 12 indicates the distribution of high molecular weight (above 3-5,000 molecular weight) ultraviolet-absorbing components of the extract. Similar plots were obtained in every case for the components in the excluded fractions (components not retarded) on Sephadex G-25. After lyophilization, the product was a light brown and readily soluble in buffer. This high molecular weight fraction was approximately 0.6% of the original fresh weight of the leaves. The protein content of this fraction (assuming all nitrogen to be protein nitrogen) is given in Fig. 10 and 12. The sedimentation characteristics of the material in the ultracentrifuge (Spinco, Model E) is shown in Fig. 13. Three complex



**265 mμ

Figure 10. Preliminary Extraction of High Molecular Weight Water-Soluble Components of Populus tremuloides



**265 mμ

Figure 11. Preparation of High Molecular Weight Water-Soluble Components of Populus tremuloides for Immunochemical Studies

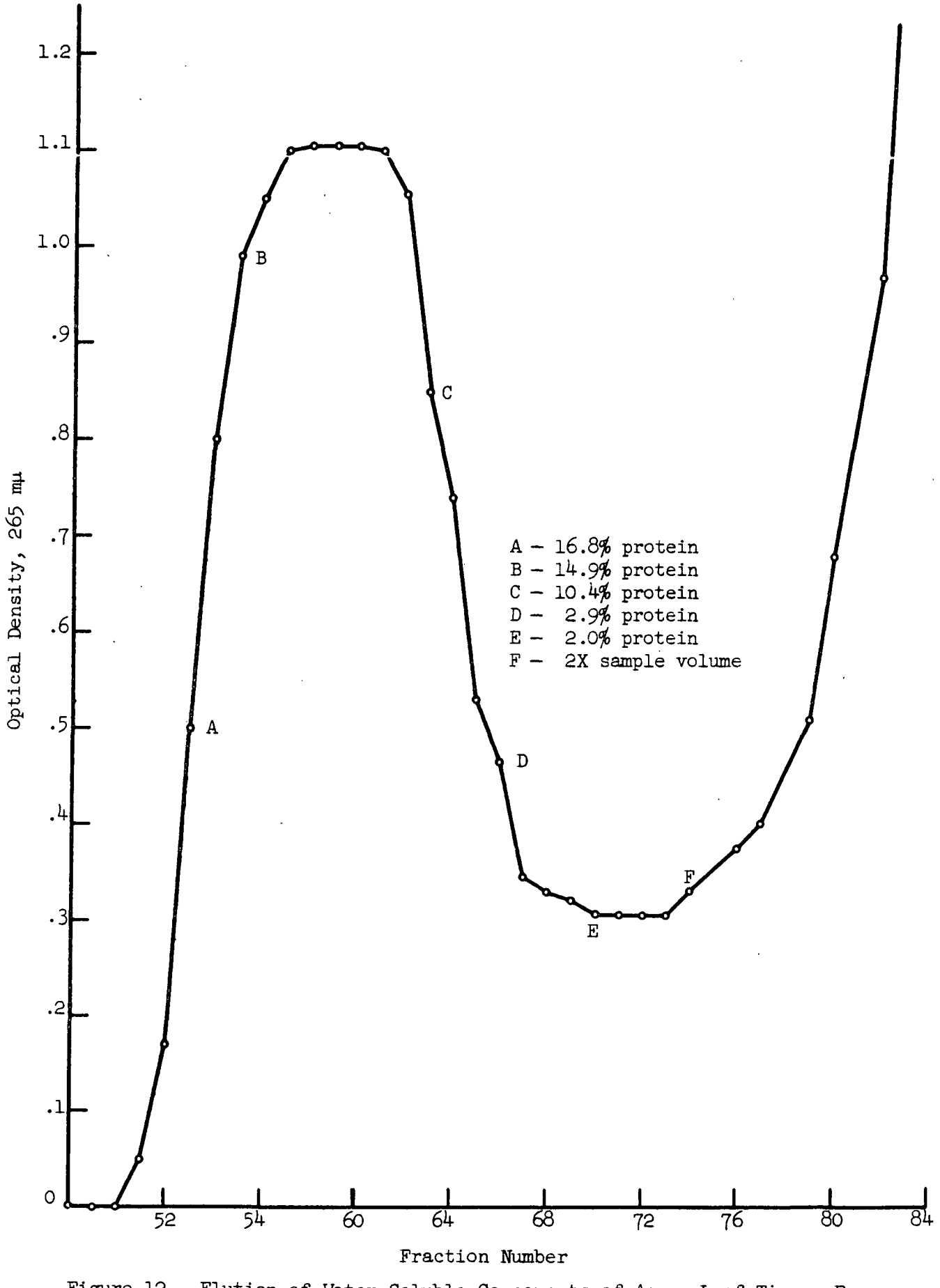
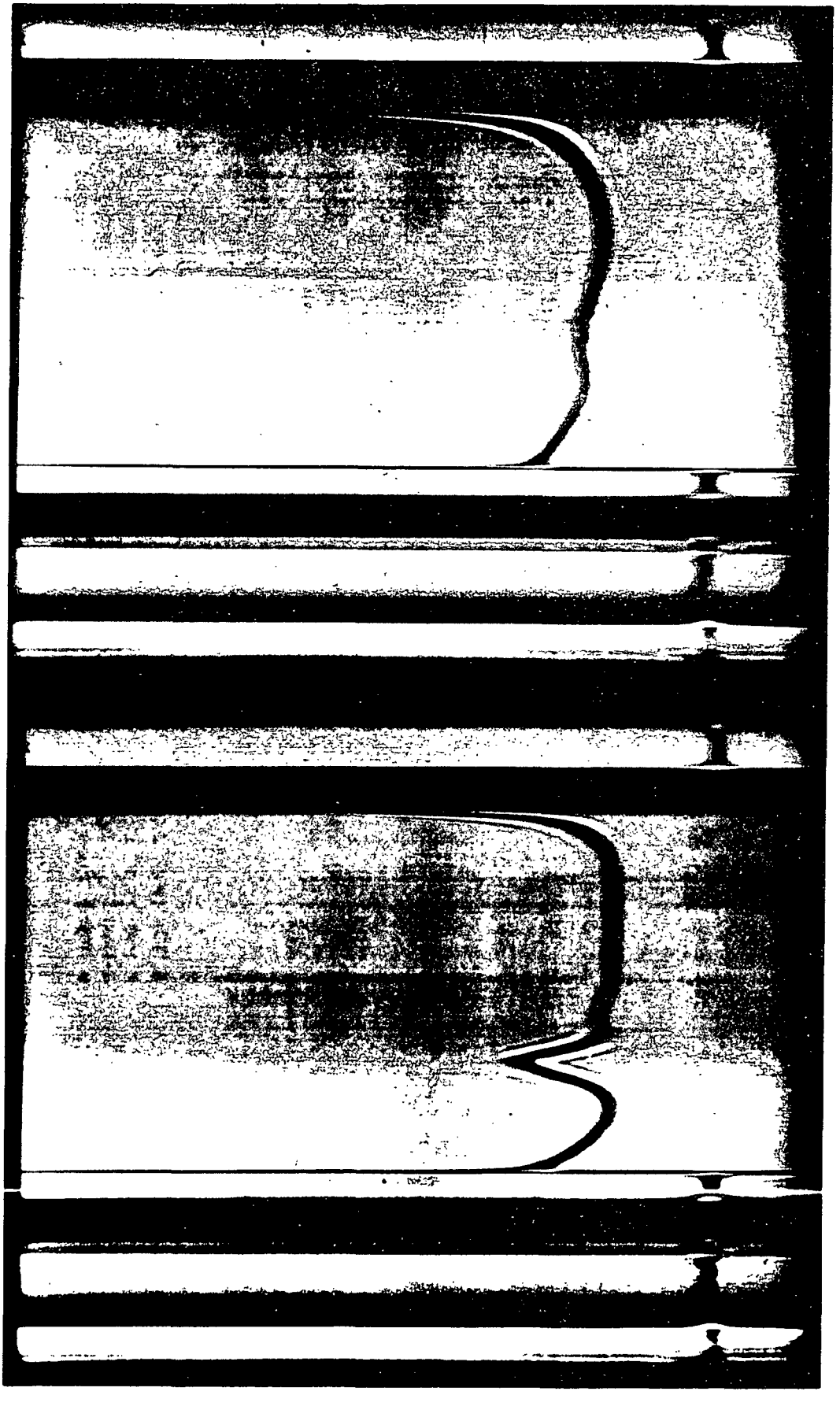


Figure 12. Elution of Water-Soluble Components of Aspen Leaf Tissue From Sephadex G-25



A

B

Figure 13. Sedimentation of Water-Soluble Components of P. tremuloides (Model E Ultracentrifuge): A., 6 Minutes; B., 35 Minutes at 56,100 r.p.m.

boundaries were resolved by electrophoresis (Spinco, Model H, electrophoresis-diffusion instrument).

A problem of adsorption was encountered on the Sephadex G-25 columns in the above work. The adsorbed substance was brown and difficultly eluted, so there remained a question of protein residuals on the column which might elute with subsequent samples from a different source. In order to circumvent the Sephadex step, the P. canescens supernatent, Fig. 14, was subjected to an ethanol precipitation step, followed by dialysis. The yield after dialysis was approximately 0.3% of the fresh weight of the leaves. Some of the precipitate did not go into solution during dialysis. The yields from both the Sephadex and ethanol fractionation procedures were adequate for further immunochemical studies.

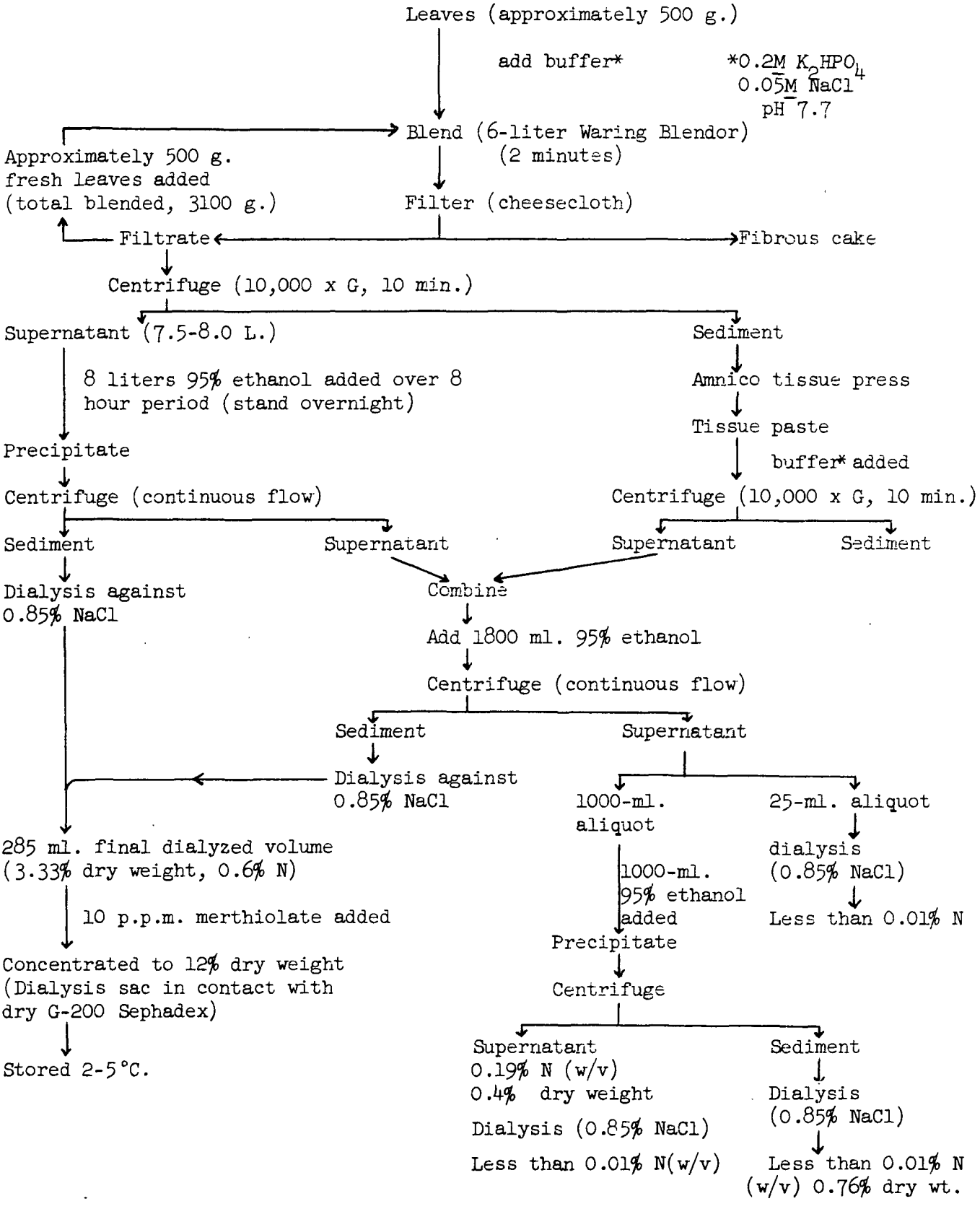


Figure 14. Alcohol Fractionation of Water-Soluble Components of Populus canescens

PLANS FOR 1964

During 1964 the emphasis of Project 2412 will be directed toward four principle areas of investigation. These areas of investigation will include: (1) selection and hybridization, (2) production of artificial polyploids, (3) studies of natural variations with special emphasis on "wood quality," and (4) biochemical characterization of aspen and aspen hybrids.

Selection and hybridization studies will continue to emphasize the production of trees suitable for use in so-called "dry site" plantings. A considerable amount of effort will also be directed toward the production of cottonwood crosses suitable for use on "wet site" areas in central and north central Wisconsin. Final proof of the success of these crosses and hybrids produced under the sponsorship of Project 2412 lies in the survival, growth, and wood quality of these materials in field trials. The field testing aspects of the Institute's "over-all program" are handled under the auspices of Project 1800, a closely allied project dedicated to aspen genetics and tree improvement work.

Production of polyploids, particularly tetraploids and triploids, are of both practical and academic interest. Investigations in the area of polyploid production will stress the use of the techniques of: (1) colchicine treatment of immature embryos, and (2) hybridization using polyploid parents, as possible methods of producing desirable tetraploid individuals. Large-scale production of triploid individuals will be investigated further using pollen from a tetraploid tree growing in Sweden.

Two investigations presently underway in the area of natural variation include: (1) heritability of wood and growth characteristics of quaking aspen, and (2) geographic variation of quaking aspen. Work on these studies is to be

continued and will provide information on natural variation and heritability of growth and "wood quality" characteristics. Completion of these investigations is expected in 1965.

Projected biochemical studies for 1964 are as follows:

A. Tissue culture: A program is underway to provide a sustained yield of Populus tremuloides tissue in shake flask culture. An effort will be made to obtain well-dispersed cell growth by the manipulation of the hormonal, nutrient, and enzymatic environment of the tissue.

B. Leaf protein studies: Work will be continued on the isolation of leaf proteins and their employment in immunochemical studies of differences between individual trees.

C. Immunochemical techniques: The leaf protein currently in hand and protein obtained from tissue culture when it becomes available will be used for the immunization of rabbits and the antisera so obtained used to continue the development and application of immunochemical techniques to the problem of genetic characterization of aspen at the biochemical level.

D. The general direction of the work will be to isolate specific proteins in sufficient quantity for immunochemical studies. The use of specific proteins will provide antisera of greater specificity and higher titers for comparative studies.

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FUTURE PUBLICATIONS

1. Benson, M. K., and Einspahr, D. W. Control of vegetative competition in aspen plantings. Submitted to Tree Planter's Notes.

2. Einspahr, D. W., and Benson, M. K. Production and evaluation of aspen hybrids. Accepted for publication in J. Forestry.

3. Gardner, H. S., and Einspahr, D. W. Micropulping small wood samples. Presented at the TAPPI Second Forest Biology Conference, Nov., 1963. To be published in Tappi.

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