BIOTECHNOLOGY AND GENETIC OPTIMIZATION OF FAST-GROWING HARDWOODS

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BIOTECHNOLOGY AND GENETIC OPTIMIZATION OF FAST-GROWING HARDWOODS

Final Report

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ABSTRACT

A biotechnology research program was initiated to develop new clones of fast-growing Populus clones resistant to the herbicide glyphosate and resistant to the leaf-spot and canker disease caused by the fungus Septoria musiva. Glyphosate-resistant callus was selected from stem segments cultured in vitro on media supplemented with the herbicide. Plants were regenerated from the glyphosate-resistant callus tissue. A portion of plants reverted to a glyphosate susceptible phenotype during organogenesis. A biologically active filtrate was prepared from S. musiva and influenced fresh weight of Populus callus tissue. Disease-resistant plants were produced through somaclonal variation when shoots developed on stem internodes cultured in vitro. Plantlets were screened for disease symptoms after spraying with a suspension of fungal spores. A frequency of 0.83 percent variant production was observed.

Genetically engineered plants were produced after treatment of plant tissue with Agrobacterium tumefasciens strains carrying plasmid genes for antibiotic resistance. Transformants were selected on media enriched with the antibiotic, kanamycin. Presence of foreign DNA was confirmed by Southern blot analysis. Protoplasts of poplar were produced but did not regenerate into plant organs.

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SUMMARY

This research and development program was initiated to address two major goals: the production of poplar trees resistant to the herbicide glyphosate, and production of plants with increased resistance to attack by the fungus Septoria musiva, the causal agent of a leaf-spot and canker disease. Additionally, genetic engineering was applied to poplar and attempts were made to culture and regenerate protoplasts derived from poplar tissues.

Poplar trees for the study were obtained from the Northeast through an arrangement with Canadian forestry professionals in Quebec province. Various isolates of the pathogenic fungus were obtained from a pathologist in the northern great plains region.

A selection method obtained the glyphosate resistance. Tissue cultures of poplar were transferred to media containing various concentrations of glyphosate. Occasionally, small colonies of tissue flourished in the presence of the herbicide. These colonies were assumed to have developed resistance and were induced to produce shoots, some of which were converted to whole plants. The plants were tested for resistance by treatment with glyphosate and were found to be susceptible to typical commercial concentrations of the herbicide used to control weeds. Thus, the selection of callus tissues, which grew in the presence of glyphosate, did not produce a plant with the herbicide-resistant trait.

Two approaches were taken to produce poplars with increased ability to resist attack from the fungus Septoria musiva. The first approach was similar to that employed to develop glyphosate resistance and was based on in vitro selection

against a crude toxin obtained from cultures of the fungus. A biologically active extract was obtained from media in which the fungus had grown. The extract inhibited growth of poplar tissue when it was added to culture medium. This was a clear demonstration that in vitro selection was possible; however, the biologically active compound was difficult to produce, was essentially uncharacterized, and was not necessarily involved in disease development in the whole plant. Thus, a second opportunity for development of plants was investigated.

Previous work with poplar tissue cultures indicated that certain regenerated plants differed from the original donor plants. New variations were apparently created as a result of the tissue culture process. Such variation is termed somaclonal variation. Since variation can arise in tissue culture, large numbers of plants were regenerated from tissue cultures of several poplar clones. The plants were screened in the greenhouse by applying a spore suspension obtained from cultures of the fungus. Plants whose leaves showed no symptom development were retained as apparent variants and subjected to further treatment with fungal spores. Treatment of over 14,000 plants produced 153 plants with altered reaction to the fungal spores. A relatively high frequency of variant production was observed: however, disease development in field plantations is likely to be more complex than the simple, leaf symptom tests conducted in the greenhouse. Plant material was sent to a collaborator in New York State for observation of field performance of some of the new variants.

Recently, genetic engineering has emerged as an exciting new technology with the potential to improve plant performance through the incorporation of genes from different organisms into plants. New genes are typically introduced into plant

tissue with the aid of a bacterium that carries the particular gene and can "inject" the gene into plant cells. The bacteria employed in genetic transfer are usually from the Agrobacterium family. The poplar work involved incorporating new genes for antibiotic resistance into cells of poplar tissue using Agrobacterium to carry the "new" genes. Genetically altered tissues were selectively cultured and new plants were eventually regenerated from these tissues. DNA samples from leaves of these plants were analyzed for the presence of genes from the bacterium. The results showed that the new genes were present in the plant tissues and that transfer of foreign genes was successful. Thus, the work demonstrated the feasibility of poplar improvement using genetic engineering methods.

Protoplasts, plant cells without cellulose walls, were produced from callus and leaf tissue. The protoplasts were cultured to produce small colonies of tissue but no organs were developed from these colonies. This successfully completed program has provided new plant material from clones well-adapted to the region, and has demonstrated the feasibility of applying genetic engineering methods to some of these same clones. The utility of cell selection methods for whole-plant improvement was not demonstrated using the herbicide-resistant trait.

Section 1

INTRODUCTION

In response to proposal request number ER-96-85, Native Plants Incorporated (NPI) proposed a project for New York State Energy Research and Development Authority (NYSERDA), the Gas Research Institute (GRI) and the New York Gas Group (NYGAS). The successful proposal described a series of tasks designed to apply the techniques of modern biotechnology the following goals:

(i) creation of Populus clones with resistance to the herbicide glyphosate;

(ii) creation of Populus clones with resistance to the leaf spot and canker disease caused by the fungus Septoria musiva;

(iii) development of an efficient protocol for introduction of "new" genes into poplar clones using genetic engineering technology and

(iv) investigation of the feasibility of developing poplar plants from isolated protoplasts.

Much progress was made during the first year and was documented in the first status report submitted in 1987. Building on this foundation effort during the second year has focused on the following areas:

- development of glyphosate resistance;
- development of a transformation (genetic engineering) system;
- development of Septoria resistance, and
- protoplast isolation and culture.

The following sections describe the work, the results, and the significance of the findings in relation to the general field of plant biotechnology.

Section 2

COLLECTION OF BIOLOGICAL MATERIAL

Twenty-five hardwood cuttings of each of 18 Populus clones were obtained from Mr. Brian Barkley of the fast-growing forest technology development group of the Ontario Ministry of Natural Resources. The following clones were received from the Canadian group:

DN 1	Populus euramericana cv."Allenstein"
DN 10	P. euramericana cv. Robusta
DN 17	P. euramericana cv. Robusta
DN 19	P. euramericana cv. Blanc de Poitou
DN 52	P. euramericana cv. Q-36-Q
DN 66	P. deltoides (D 36) x nigra (N3)
DN 74	P. deltoides (D 35) x nigra (N3)
DN 93	P. euramericana 37/61
DN 125	P. deltoides (D 58) x nigra (N1 and N2 mixed)
DN 133	P. deltoides (D 56) x nigra (N1 and N2 mixed)
DN 134	P. deltoides (D 56) x nigra (N1 and N2 mixed)
DN 173	P. euramericana cv. Dorskamp (925)
DTAC 32	P. deltoides x tacamahaca
JAC 4	P. tacamahaca x deltoides
NE 52	P. maximowiczii x berolinensis cv.Plantierinensis
NM 2	P. nigra x maximowiczii MAX-1
NM 4	P. nigra x maximowiczii MAX-3
NM 6	P. nigra x maximowiczii MAX-6

In addition to this material, five poplar clones were obtained from Mr. W. Berguson of the University of Minnesota. These clones were collected based on their susceptibility to infection by the fungus Septoria musiva. The plant materials obtained included:

o	NE 206	Extremely susceptible
o	NE 345	Moderately susceptible
o	L 296	Extremely susceptible
o	DN 29	Very susceptible
o	Raverdeau	Very resistant

On receipt, cuttings were established as plants in NPI research greenhouses where they were grown up to provide additional cuttings for whole-plant studies and to provide explants for in vitro cultures.

Four isolates of the pathogenic fungus S. musiva were obtained from Dr. J.M. Krupinsky, with the United States Department of Agriculture in Mandan, North Dakota. Isolates 83-5848, 83-5872, 83-5913 and 83-5948 were cultured onto maintenance media.

Section 3

COMPREHENSIVE LITERATURE SEARCH

A computer-assisted search of published and abstracted literature used the following keywords:

1. Poplar or Cottonwood or *Populus*
2. 1 and *Septoria musiva*
3. 1 and *Agrobacterium*
4. 1 and tissue culture or *in vitro*
5. 1 and glyphosate
6. 1 and herbicide
7. plant transformation

The search revealed some 6000 titles. Approximately 70 of these appeared relevant and were obtained through university libraries. A bibliography is included with this report in section 10.

Section 4

ESTABLISHMENT OF CULTURES AND OPTIMIZATION OF TISSUE CULTURE PROTOCOLS

Previously published work with tissue culture and micropropagation of Populus spp. indicated that various approaches to plant regeneration have been successfully applied (Mathes 1964, Winton 1970, Venverloo 1973, Chalupa 1974, Christie 1978, Mehra and Cheema 1980, Ahuja 1984, Douglas 1985). Three plant-regeneration protocols were optimized for application in this project: direct development of shoots from stem segments; development of shoots from callus tissue produced on stem segments and production of shoots from in vitro cultured buds. The first two methods are represented diagrammatically in Figure 4-1.

CALLUS CULTURE AND ADVENTITIOUS SHOOT FORMATION

Optimal conditions for both callus formation and shoot regeneration were determined for the poplar clones. Stem sections from young green shoots, 10-30 cm behind the apex, were disinfested by hypobaric soaking in a 10 percent solution of commercial bleach for 10 minutes. Segments were cut into 2-3 mm slices and placed into petri plates containing 20 ml aliquots of MS medium (Murashige and Skoog 1962) supplemented with various concentrations of plant growth regulators. Direct adventitious shoot production was stimulated by culture of stem segments on a medium supplemented with 0.3 mg per liter benzyladenine (BA) while callus production was induced by culture on a medium enriched with 0.3 mg per liter BA and 0.2 mg per liter 2,4-dichlorophenoxyacetic acid (2,4-D). When callus tissue was transferred to medium supplemented with either 0.2 or 0.3 mg per liter BA, shoots were produced from the callus. Experiments were carried out with stem segments of several clones and results indicated that different clones had

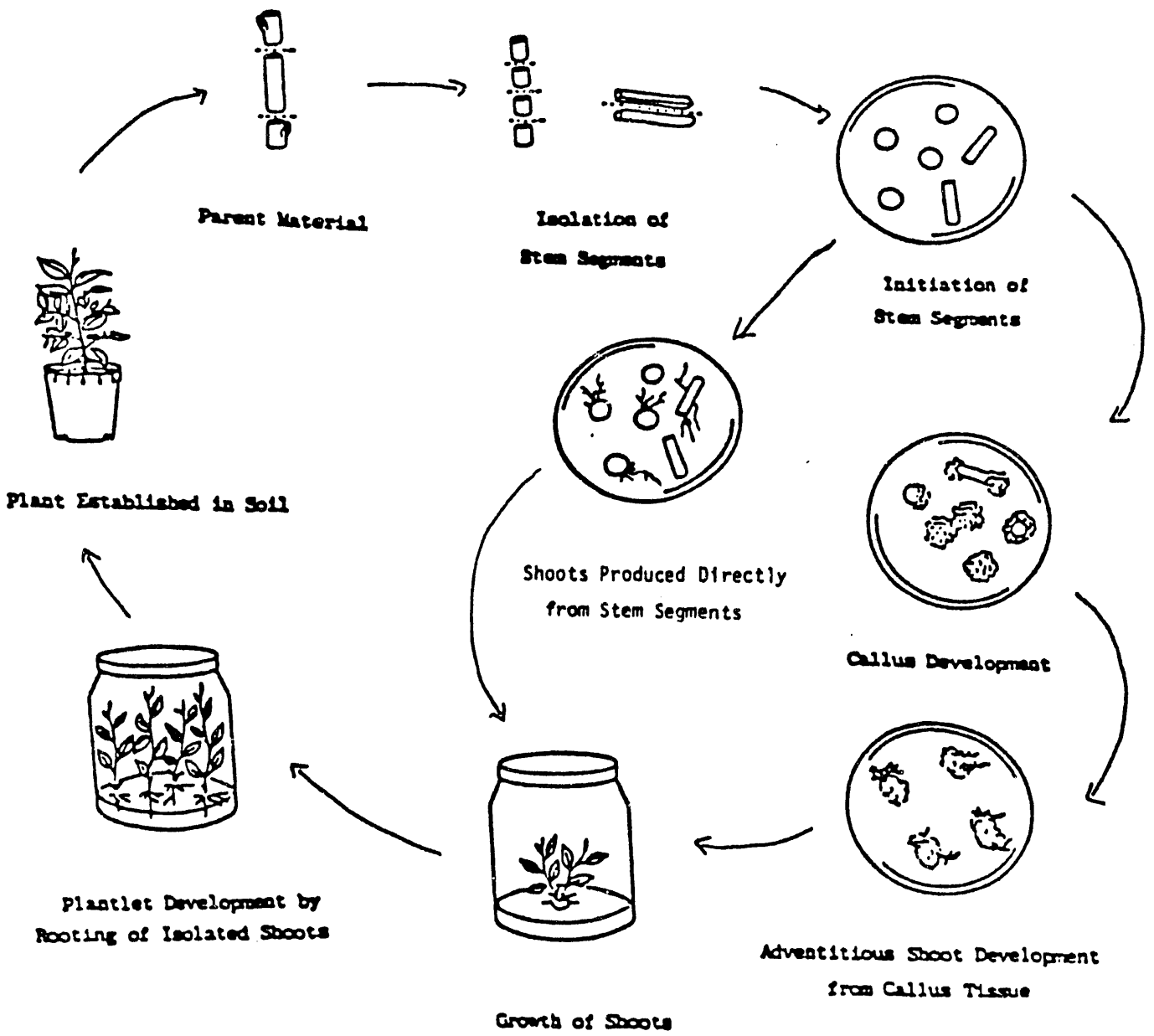

Figure 4-1. Diagram of Plant Regeneration Routes from Populus Stem Tissue

different capacities to produce shoots and callus. However, such a large collection of plant material was established that only those clones that were most responsive to *in vitro* manipulation were selected. These clones provided the raw material for the subsequent work with glyphosate and *S. musiva*.

MICROPROPAGATION OF POPLAR

Axillary or lateral buds, and shoot-tips from soft, rapidly growing shoots, provide the best material for initiation of shoot-tip cultures for micropropagation. This process is represented in Figure 4-2. Stems were collected from stock plants. Leaves were removed and stem segments were disinfested. After rinsing, nodal segments and shoot tips were removed and placed into culture vessels containing aliquots of medium. The basal culture medium consisted of the salts and organic constituents of the woody plant (WP) medium (Lloyd and McCown 1980) supplemented with BA 0.1-0.5 mg/L., sucrose at 3 percent, and agar at 0.6-0.7 percent. The pH of the medium was adjusted to 5.75 prior to autoclaving at 121 C for 30 minutes.

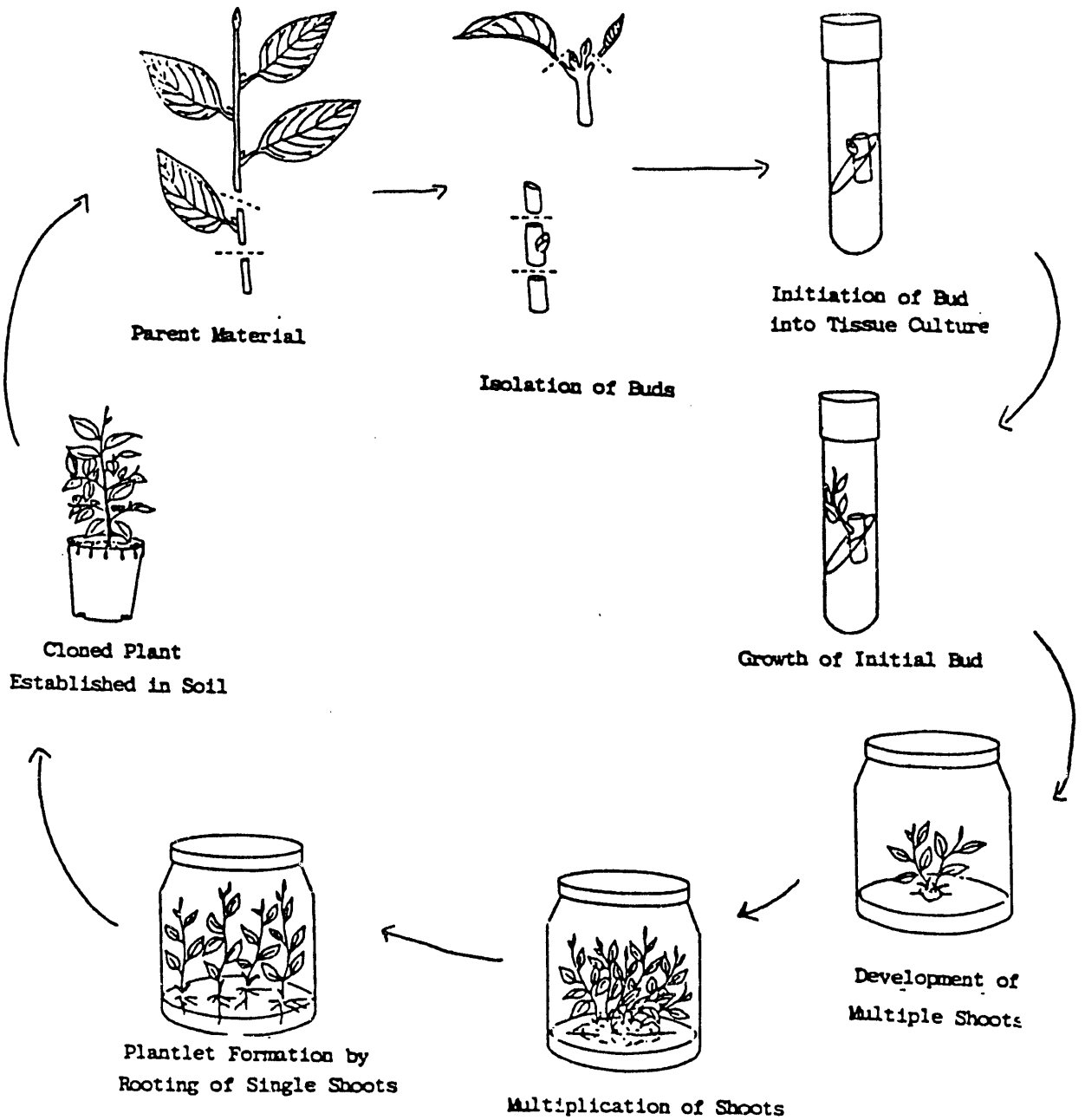

Figure 4-2. Diagram of Micropropagation from Shoot Buds of Populus

Cultures were incubated at 23-27 C under 16 hours of radiation from a source that emitted 30-90 microeinsteins per square meter per second.

Shoot growth from existing buds was evident after two-four weeks in culture. After 25-35 days, the plant material was transferred to a fresh aliquot of similar medium. Shoot proliferation in cultures of Populus spp. occurred through the enhancement of axillary branching in buds present on the original explant. This type of growth resulted in the formation of a mass of stem tissue from which several elongating shoots arose. These clumps of shoots and associated basal tissue were the basic units used for in vitro multiplication. A typical culture is shown in Figure 4-3.

These "clumpy" structures formed from the initial explant during the second, third, or fourth in vitro cycle. After the formation of such structures, multiplication was achieved by division of the large clumps into several smaller units that were transferred to separate culture vessels. At the same time, elongated shoots that formed on the original clump were removed and either dissected into three-four node segments and placed on aliquots of multiplication medium, or the shoots were used to develop plantlets.

Rooting of individual shoots was achieved by one of two methods. Using the first method, plantlet formation was achieved by rooting shoots in vitro on WP medium supplemented with indolebutyric acid at one-three mg/liter. Shoots at least 1.5 cm long were removed from multiplying cultures, small leaves were removed from the bottom third of the stem and the shoot was stuck into the rooting medium. Roots appeared on the stems within two-three weeks.

Figure 4-3. Multiplying shoot-tip culture of clone NM4 after two months incubation

When root growth was apparent, plantlets were transferred to soil and cultivated under nursery conditions. Plantlets were removed from the culture vessels and potted into containers filled with a well-draining, pasteurized medium. After a thorough watering, the containers were placed in a warm, humid, shaded, environment to start the acclimatization process. Acclimatization typically involves integrated manipulation of temperature, light intensity and relative humidity. By close observation of the plants coupled with appropriate manipulation of the

environment, acclimatization was achieved during a two-four week period.

The second method of plantlet formation from in vitro-derived shoots involved rooting and simultaneous soil acclimatization. Shoots at least 1.5 cm long were removed from multiplying cultures and transferred directly to containers filled with a potting medium. Direct soil transfer of unrooted shoots was a useful technique in micropropagation of poplar, but a high degree of environmental control was required to ensure that the majority of shoots formed roots and became successfully established as plants. Micropropagation protocols were established for most of the clones collected.

Section 5

DEVELOPMENT OF GLYPHOSATE RESISTANCE

Novel plant mutants have been isolated by the application of selective growth conditions to cultured cells (Chaleff 1981) and unique phenotypes have been isolated by selection of plant tissue *in situ* (Carlson 1984). Herbicide-resistant, mutant cells have been identified in tobacco, celery and carrot by cultivation of cells in media containing various levels of herbicide (Chaleff 1983; Merrick and Collin 1981; Nafziger et al. 1984). In particular, glyphosate-tolerant plants were regenerated from cells selected *in vitro*. Induced tolerance to the herbicide glyphosate was expressed in regenerated tobacco plants (Singer and McDaniel, 1985).

An extensive series of experiments was initiated to determine the responses of *in vitro*-cultured *Populus* tissues to glyphosate. Since uptake and metabolism of herbicides can be influenced by target tissue and stage of plant development, the effects of glyphosate on callus growth and shoot regeneration were examined to determine the optimal range of glyphosate for use in selection studies. These experiments were conducted with clone DTAC-32 and clone NM2. Dose responses were generated by *in vitro* culture of stem explants on media enriched with 0, 10, 100, 300, 1000, and 3000-micromolar glyphosate. After 30 days in culture, callus growth and shoot regeneration were evaluated in 12 replicates of each clone in each glyphosate concentration in each medium. Results are presented in Table 5-1.

Table 5-1

EFFECT OF GLYPHOSATE ON WEIGHT OF CALLUS FORMED BY STEM SEGMENTS OF CLONES DTCA32 AND NM2

Micromolar Concn. of Glyphosate	Fresh Weight per Explant (g)[a/] NM2	DTAC32
0	0.21 a	0.36 a
10	0.13 b	0.15 b
100	0.08 bc	0.12 bc
1000	0.03 c	0.07 bc
3000	0.03 c	0.05 bc
10000	0.01 c	0.03 c

[a/] Means within a column followed by a different letter are significantly different by Duncan's multiple range test at p=0.05.

Since the two clones differed in susceptibility to glyphosate, it was not possible to choose a single concentration to induce a 50 percent or 95 percent growth inhibition.

A comparative study was undertaken to determine the sensitivity of cultured Populus microshoots to glyphosate. Shoot-tip cultures of clone NM6 were transferred to multiplication medium enriched with 0, 100, and 300 micromolar glyphosate. Multiplication and shoot quality were assessed after 30 days of culture, when microshoots were subcultured onto fresh aliquots of similar medium and evaluated after a further 30-day cycle.

Table 5-2

EFFECT OF GLYPHOSATE ON SHOOT GROWTH AND QUALITY IN CULTURES OF CLONE NM6

Glyphosate Concentration (micromolar)	Growth and Quality Index [a/] 1st CYCLE	2nd CYCLE
0	4.0	4.0
100	2.9	0
300	1.8	0

[a/] Shoot quality was assessed on a scale of 0-4 where 0=dead and 4=healthy growth

Results are presented in Table 5-2. and indicate that 100-micromolar glyphosate concentrations consistently inhibited growth over the first 30 days and death of the explants during the second 30-day period. Thus, microshoot growth in vitro is more glyphosate-sensitive than callus growth or shoot regeneration.

It is important to know the glyphosate sensitivities of greenhouse-grown plants of the clones included in the selection program. It is essential to determine the appropriate range of glyphosate concentrations with which to screen regenerated clones selected in vitro. Studies were initiated to determine tolerance of greenhouse-grown plants. Uniform plants of clone DN66 were sprayed with 0, 0.2, 2.0, and 20.0 millimolar glyphosate solutions. Plant quality and overall toxicity symptoms were evaluated after two weeks. The results indicated that treatment with 2.0, 10.0 and 20.0 millimolar glyphosate was an appropriate range of concentrations for application in future studies.

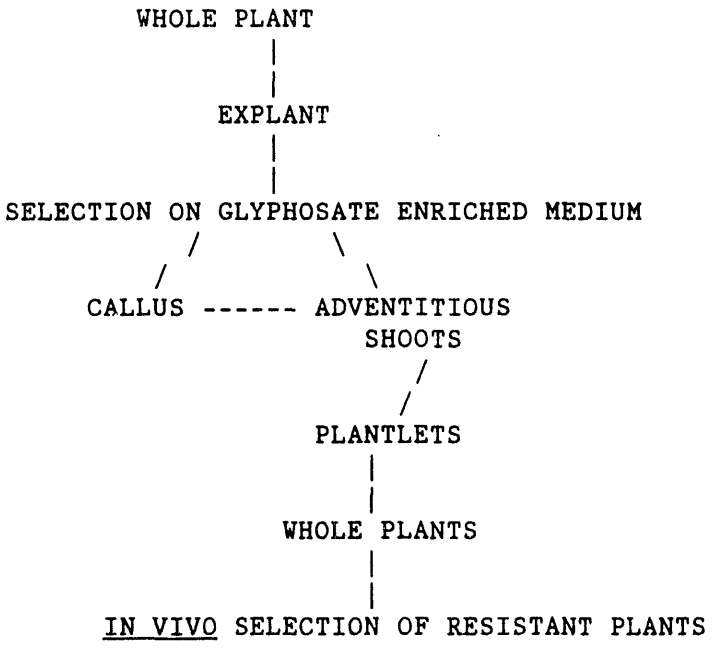

Figure 5-1. Protocol for Selection against Glyphosate

Using information obtained from initial experiments, a general protocol for selection of glyphosate-resistant cells and tissues was developed and is represented diagramatically in Figure 5-1. Clones NM2, NM4 and DN66 were selected as target clones based on three considerations: capacity for callus production; capacity for adventitious regeneration of shoots, and response to glyphosate in the culture medium. Stem cross-sectional segments were placed on callus-forming and shoot-forming media containing 0, 100, or 300 micromolar glyphosate. Shoot and callus formations in each clone were evaluated after 60 days of culture by observation of 24 replicate stem segments per glyphosate concentration. Glyphosate-resistant callus was formed in the three clones.

Clones NM2, NM4, NM6, and DN66 were selected as target clones based on three considerations: capacity for callus production; capacity for adventitious regeneration of shoots, and response to glyphosate in the culture medium. Stem cross-sectional segments were placed on callus-forming and shoot-forming media containing 0, 100, or 300 micromolar glyphosate. Shoot and callus formations in each clone were evaluated after 60 days of culture by observation of 24 replicate stem segments per glyphosate concentration. Glyphosate in the culture medium completely inhibited adventitious shoot production in the four clones. Herbicide-resistant callus was formed at the lower concentration from explants of all clones, but only NM4 explants produced callus at the higher concentration (Tables 5-3 & 5-4).

Table 5-3

MEAN SHOOT NUMBER[a/] REGENERATED PER EXPLANT FROM STEM SEGMENTS OF FOUR POPULUS CLONES FROM MEDIA ENRICHED WITH GLYPHOSATE

	Concentration of Glyphosate (micromolar)		
Clone	0	100	300
DN66	4.9 c	0	0
NM2	2.7 ab	0	0
NM4	1.8 a	0	0
NM6	3.0 b	0	0

[a/]Means calculated from 24 replicates; those within columns followed by different letters are significantly different at p=0.05 by Duncan's New Multiple Range Test.

Table 5-4

MEAN CALLUS GROWTH[a/] FROM STEM SEGMENTS OF FOUR POPLAR CLONES CULTURED ON MEDIA ENRICHED WITH VARIOUS LEVELS OF GLYPHOSATE

	Concentration of Glyphosate (micromolar)		
Clone	0	100	300
DN66	0.12 a	0.25 a	0.0 a
NM2	1.90 b	1.33 b	0.0 a
NM4	3.16 c	1.80 b	1.05 b
NM6	1.54 b	0.30 a	0.0 a

[a/]Mean scores calculated from 24 replicates per treatment.
[b/]Means within columns followed by different letters are significantly different at p=0.05 by Duncan's New Multiple Range Test.

Calli from clones NM2, NM4, and DN66 were maintained by monthly subculture onto similar medium over a period of five months. Samples of this herbicide-resistant callus tissue were transferred to shoot-production media supplemented with 100-micromolar glyphosate. After six weeks, shoot regeneration was observed from calli of clones NM2 and DN66 grown on media enriched with 100 micromolar glyphosate. No shoots regenerated from calli of clone NM4 or calli of clones NM4 and DN66 that originated on media containing 300-micromolar glyphosate. Mean number of shoots per callus clump and total numbers of shoots produced are presented in Table 5-5.

Table 5-5

MEAN[a/] SHOOT REGENERATION FROM POPLAR CALLUS AFTER FIVE MONTHS OF GROWTH ON GLYPHOSATE-ENRICHED MEDIA

	Concentration of Glyphosate (micromolar)		
Clone	0	100	Total Shoots
NM2	0.87 a	0.0	27
DN66	1.16 a	0.0	7
NM4	0.0	0.0	0

[a/]Means calculated from at least six replicates; those within columns followed by different letters are significantly different at p=0.05 by Duncan's New Multiple Range Test.

After regeneration on media containing herbicide, microshoots from a particular callus clump were assigned a label and transferred to aliquots of multiplication medium containing no herbicide. Propagation was allowed to proceed for 12 or 16 weeks to increase the pool of glyphosate-resistant material.

Samples of shoots from seven of the glyphosate-resistant callus lines were transferred to multiplication media containing either 0, 25, 50, 75, or 100 micromolar glyphosate. Shoots regenerated from non-selected callus were transferred to similar media as a control for the experiment. After six weeks, the numbers of shoots produced by each primary explant were counted from each replicate vessel for each shoot-line in each medium treatment. The data were summarized using analysis of variance techniques (Table 5-6).

Table 5-6

ANALYSIS OF VARIANCE FOR DATA FROM GLYPHOSATE TOLERANCE IN SELECTED SHOOTS

Source	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Origin	7	78.2	11.2	3.4
Treatment	4	1260.6	315.1	96.2
Origin * Trt	28	86.3	3.1	0.9
Error	300	983.2	3.3	
Total	339	2408.3		

Single Degree of Freedom Contrasts for Effect Termed-Origin

Contrast	Mean Square	F Ratio
non-selected vs B111	22.3	6.8
non-selected vs B113	48.1	14.7
non-selected vs A113	17.0	5.2
non-selected vs A112	3.3	1.0
non-selected vs B119	2.0	0.6
non-selected vs A114	7.4	2.2
non-selected vs B116	0.4	0.1

Single Degree of Freedom Contrasts for Effect-Termed Treatment

Contrast	Mean Square	F Ratio
Linear	999.9	305.1
Quadratic	248.0	75.7

Figure 5-2 shows the response of shoots from a selected line compared with a control line. A significant portion of variability observed in the experiment was attributable to effects of the origin of the primary explant. A set of comparisons between control shoots and those which arose on glyphosate-resistant callus were constructed. These contrasts indicated that only three of the original shoots, from selected calli, produced significantly more microshoots on glyphosate-enriched media, than shoots produced from non-selected tissue. The mean numbers of shoots produced from explants of the various lines are presented in Table 5-7 and indicate that shoots from lines B111, B113, and A113 retained some degree of glyphosate resistance compared with shoots from non-selected callus.

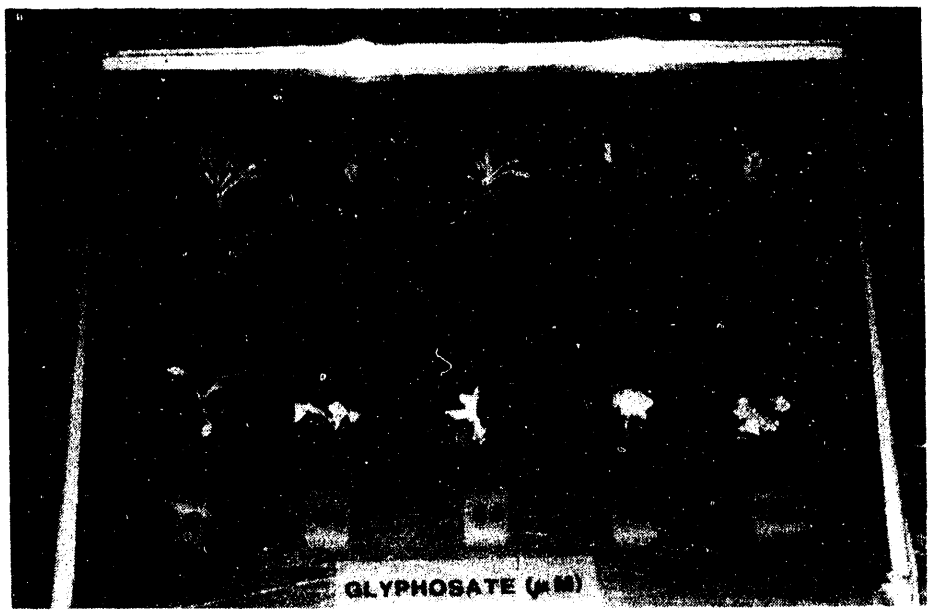

Figure 5-2. Effects of glyphosate in the culture medium on growth of shoots regenerated from resistant and susceptible callus

Table 5-7

EFFECT OF ORIGIN OF PRIMARY SHOOT (HERBICIDE TOLERANT OR CONTROL, NON-SELECTED, CALLUS TISSUE) ON MULTIPLICATION OF POPLAR SHOOTS AFTER SIX WEEKS CULTURE ON MEDIA AMENDED WITH VARIOUS CONCENTRATIONS OF GLYPHOSATE

Callus Origin of Primary Shoot	Mean Number of Shoots[a/]
Non-selected callus	1.5
Tolerant callus B111	2.7
Tolerant callus B113	3.0
Tolerant callus A113	2.5
Tolerant callus A112	1.9
Tolerant callus B119	1.7
Tolerant callus A114	2.1
Tolerant callus B116	1.6

[a/] Mean numbers of shoots per explant averaged over glyphosate concentrations and replications.

The effects of glyphosate treatments were independent of the callus-origin of the primary explant. Mean shoot numbers summarized in Table 5-8 show that increasing concentrations of glyphosate inhibited multiplication of shoots regenerated from both resistant and susceptible callus.

Table 5-8

EFFECTS OF GLYPHOSATE CONCENTRATION ON NUMBER OF SHOOTS PRODUCED FROM A SINGLE PRIMARY SHOOT AFTER SIX WEEKS OF CULTURE ON MEDIA SUPPLEMENTED WITH VARIOUS CONCENTRATIONS OF HERBICIDE.

Concentration of Glyphosate	Mean Number of Shoots[a/]
0	5.7
25	2.7
50	1.0
75	0.7
100	0.6

[a/]Mean numbers of shoots per explant averaged over lines and replications.

The shoots, which regenerated from glyphosate-resistant callus, were transferred to root-inducing medium prior to transfer to greenhouse conditions. After a period of acclimatization, plantlets were allowed to grow to produce enough material for whole-plant evaluation of herbicide tolerance. Plants resulted from 11 of the 18 original callus lines which were selected as glyphosate resistant (Table 5-9). Plantlets produced from NM2 non-seleted callus were included as controls. Cuttings were removed from the plants after about four months in the greenhouse. These cuttings were rooted and plants were allowed to become established. After three months, samples of plants representing each line of glyphosate-resistant callus were taken for treatment with herbicide.

Table 5-9

INVENTORY OF POPULUS SHOOTS REGENERATED FROM CALLUS TISSUE PRODUCED ON MEDIA CONTAINING 100 MICROMOLAR GLYPHOSATE

Clone	Callus Line	Number of Shoots	Number of Plants
NM2	A111	18	19
NM2	A112	9	
NM2	A113	2	
NM2	A114	24	
NM2	B110	3	6
NM2	B111	5	10
NM2	B112	60	12
NM2	B113	24	
NM2	B114	125	45
NM2	B115	10	
NM2	B116	58	46
NM2	B117	13	2
NM2	B118	160	36
NM2	B119	56	20
DN66	B121	3	
DN66	B122	11	3
DN66	B123	12	
DN66	B124	7	
DN66	B125	85	17
TOTAL		629	216

Samples of plants from each of 11 lines and non-selected control plants were treated with a spray application of glyphosate at each of the following concentrations: 0, 200, 2000, and 20,000 micromolar. After 10 days the plants were evaluated for the effects of glyphosate. All plants that received the highest concentration of herbicide were defoliated, blackened and appeared extremely distressed. A majority of plants were dead. The plants that received the lowest concentration of herbicide (200 micromolar) were unaffected by the treatment. Plants exposed to the intermediate concentration of glyphosate occassionally exhibited necrotic spots on the leaves but were not significantly distressed by the herbicide application. The nonselected control plants behaved in a similar manner to tne treated plants.

Experimental creation of glyphosate-resistant Populus clones has generated some interesting results. Samples of calli that were originally produced in the presence of 300 micromoles of glyphosate have consistently failed to produce shoots when transferred to media known to promote adventitious shoot production. Samples of glyphosate-resistant calli formed in the presence of 100 micromoles did regenerate shoots when transferred to appropriate medium. Thus, the conditions from which calli were selected exerted a strong influence on the subsequent morphogenic potential of the callus. This observation suggests that the ability to tolerate higher concentrations of herbicide involves changes in the same mechanisms that control cellular differentiation.

When shoots developed from glyphosate-resistant callus were multiplied on media containing no herbicide, some of the shoots reverted to the susceptible phenotype whereas others remained resistant to the herbicide. The loss of resistance after removal of selective pressure indicates that the original resistance was an unstable epigenetic change that was maintained only in the presence of the herbicide. Those shoots that exhibited increased glyphosate tolerance can be assumed to be stable variants produced by of selective pressure.

The growth of shoots and plantlets selected as glyphosate-resistant was observed to be considerably slower than that of non-selected shoots or plantlets. This observation indicates that the development of glyphosate resistance is not a simple independent trait but is rather a complex trait with effects that are manifest on the plant phenotype as a whole. The effect observed is an undesirable reduction in growth that may seriously restrict the use of the primary objective of the

selection; i.e., glyphosate-resistant Populus clones.

Plants that regenerated from glyphosate-resistant callus may appear to have more tolerance to spray application of glyphosate herbicide, particularly the DN66-selected clone B125. A DN66 control was not included in the evaluation; therefore, the observed level of tolerance, although it appears significantly higher than the original level observed in an earlier screening, is not conclusively demonstrated. The NM2-selected material does not seem to possess more tolerance over control plants. The concentrations of glyphosate applied in the evaluation 0.2, 2.0, and 20 mM overlap the recommended application rates of the herbicide. Concentrations between 2 and 25 mM are recommended for annual weeds and concentrations between 13 and 50 mM are recommended for perennial weeds. Poplars are killed by 20 mM of glyphosate. It must be concluded that practical tolerance to normal applications of the herbicide glyphosate was not achieved as a result of in vitro selection of glyphosate-tolerant callus tissue.

Section 6

GENETIC TRANSFORMATION USING AGROBACTERIUM TUMEFASCIENS

A most exciting methodology for improving the genotype of silviculturally important plant species uses genetic engineering to introduce novel genes into plants. Specific genes have been cloned that confer traits such as insect resistance, herbicide tolerance, viral resistance and increased protein content. Such genetic engineering of single genes can complement a traditional breeding program or a somaclonal variation program in which multigenic traits, such as high biomass production, have been maximized. When this work was initiated there were no published reports of the application of genetic engineering technology in forest tree species. However, during the course of this project, an article was published describing the expression of "new" genes in transformed tissue of poplar (Fillati et. al. 1987).

Genetic engineering (transformation) in plants is typically accomplished by insertion of a fragment of DNA containing the gene(s) of interest into a specific strain of a bacterium, Agrobacterium tumefasciens or Agrobacterium rhizogenes(An et. al., 1985; DeBlock et. al., 1984). The engineered bacterial strain is then used to infect host plant cells, and thereby shuttles the desired DNA sequence into the plant genome. The bacterial strain also contains a gene conferring a selectable marker (typically an antibiotic resistance) that is inserted into the host plant and enables transformed cells to be selected from a mixed population of cells (An et. al., 1985). Plant tissue is placed on a medium enriched with antibiotic and only those cells that are transformed (i.e., whose genome contains the gene(s) donated by the bacterial strain) survive. Survivors are then

regenerated into whole plants (DeBlock et. al., 1984). The steps involved in genetic engineering of poplars are shown in Figure 6-1.

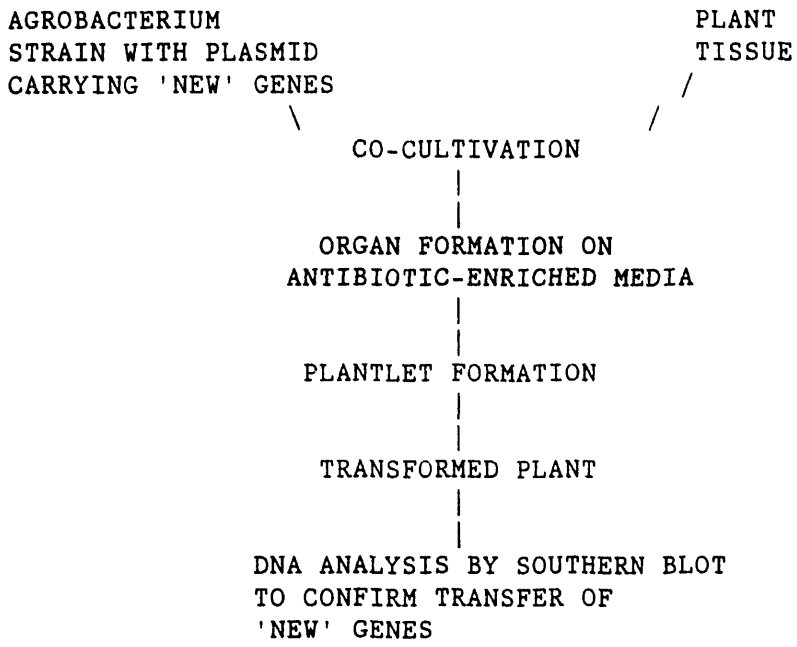

Figure 6-1. General Protocol for Genetic Engineering of Poplar

SENSITIVITY TO THE SELECTABLE MARKER KANAMYCIN

In order to develop a generally applicable transformation system for Populus, genes were transfered that confer resistance to the antibiotic, kanamycin, since this trait can be used as a selectable marker. For optimal selection of transformed tissues, the kanamycin concentration that inhibits 80-100 percent of shoot regeneration in non-transformed tissues should be used. Thus, experiments were conducted to determine the sensitivities of in vitro cultured poplar stem segments to the antibiotic, kanamycin. Disinfested stem segments were placed on shoot regeneration medium containing 0, 100, 300, 1000, and 3000 mg per liter kanamycin. Ten replicate segments were cultured in each treatment. Medium

containing 300 mg per liter kanamycin inhibited shoot production by 89-96 percent whereas 100 mg per liter kanamycin caused 85-87 percent inhibition. The results showed that stem segments were sensitive to and affected by kanamycin. Concentrations of 100 and 300 mg per liter antibiotic were established as optimal levels for selection in subsequent transformation experiments.

To determine the effects of kanamycin on multiplication in shoot-tip cultures, microshoots were placed in jars containing multiplication medium supplemented with either 0, 100 or 300 mg per liter kanamycin. Five replicates, each containing three explants, were used for each treatment. Multiplication was assessed and compared to controls after 30 days of culture.

The cultures were transferred to similar media and maintained for a further 30-day period after which similar data were collected. Results are presented in Table 6-1, and show that multiplication was inhibited by the presence of 100 mg/L kanamycin. Thus, multiplication of shoots on kanamycin-enriched medium can provide additional preliminary evidence for genetic transformation.

Table 6-1

EFFECT OF KANAMYCIN ON MULTIPLICATION IN CULTURES OF NM6 SHOOTTIPS CULTURES WERE MAINTAINED ON A 30-DAY CYCLE

Concentration of Kanamycin mg\l.	Multiplication Rate 1st Cycle	2nd Cycle
0	2.1	3.8
100	1.5	1.1
300	1.0	1.0
500	1.0	1.0
1000	0.9	1.0

SELECTION OF INFECTIVE A.TUMEFASCIENS STRAINS

For effective gene transfer by A. tumefaciens, the vector strain must have a host range which includes the plant to be genetically engineered. Therefore experiments were conducted to determine the strains of A. tumefasciens, that infected the clones collected. Ten strains of the bacterium were tested by co-culture with microshoots. Auxin-independent callus formation in stem segments that were exposed to the bacteria was used as the assessment criterion of an infective strain. Infection occurred with the following strains: A136 pTi octopine strain; A281 pTi 542; A208 pTi T37, and A281 pTi B0542.

TRANSFORMATION OF POPLAR

Transformation was achieved by immersion of stem segments in a suspension of actively growing bacteria prior to culture on shoot regeneration medium. After three-five days the segments were transferred to similar medium containing both kanamycin, a selective agent, and cephalotoxime, a bacteriocide. In a series of experiments, several factors, including concentration of bacterial suspension, duration of incubation with stem segment, and particular strain of Agrobacterium were assessed in order to optimize the transformation process Table 6-. Shoots or callus which survived and grew on kanamycin-enriched media were assummed to be putative transformants. Figure 6-2 shows the callus production from transformed tissue and the lack of callus formation on the non-transformed tissue.

Table 6-2

Inventory of Poplar Samples for Which Genetic Transformation Was Confirmed by Southern Blot Analysis

Code	Clone	Agrobacterium Strain	Number of Shoots
B12	NM6	GV3850 #1 pGA472	8
B15	NM6	GV3850 #1 pGA472	15
B16	NM6	GV3850 #1 pGA472	10
B22	NM6	GV3850 #1 pGA472	20
B24	NM6	GV3850 #1 pGA472	4
B25	NM6	GV3850 #1 pGA472	14
B27	NM6	GV3850 #1 pGA472	10
B28	NM6	GV3850 #1 pGA472	4
B211	NM6	GV3850 #1 pGA472	15
B212	NM6	GV3850 #1 pGA472	8
C27	NM6	GV3850 #3 pGA472	10
C28	NM6	GV3850 #3 pGA472	4
C29	NM6	GV3850 #3 pGA472	15
C211	NM6	GV3850 #3 pGA472	25
C212	NM6	GV3850 #3 pGA472	10
C214	NM6	GV3850 #3 pGA472	20
AA01	NM6	A281	8
AB01	NM6	A348 pTiA6C35+	8
AB02	NM6	A348 pTiA6C35+	10
AC01	NM6	A348 pTiA6C19+	10
AC02	NM6	A348 pTiA6C19+	4
AC03	NM6	A348 pTiA6C19+	8
AC04	NM6	A348 pTiA6C19+	8
BB03	NM6	GV3850 #3 pGA472	10
CC01	NM6	A348 pTiA6C35+	8
CC02	NM6	A348 pTiA6C35+	4
CC22	NM6	A348 pTiA6C35+	4
CC23	NM6	A348 pTiA6C35+	10
CC24	NM6	A348 pTiA6C35+	12
CD21	NM6	GV3850 #3 pGA472	8

Figure 6-2. Effect of Genetic Transformation on Callus Tissue Growth from Poplar Stem Segments Cultured In Vitro in the Presence of Kanamycin

Shoots that were produced in transformation experiments were assigned a label and transferred to multiplication medium for cloning. A complete list of transformed plant material is in Table 6-2.

CONFIRMATION OF GENE TRANSFER

The identity of some of these putative transformants was confirmed by examination of the plant DNA for the presence of a foreign gene (Southern blot analysis). Samples of tissue (2g fresh weight) from transformed and control plants were frozen in liquid nitrogen, lyophilized, and DNA was extracted using a modification of plant DNA minipreparation method originally obtained from S. Dellaporta (personal communication, E.S.W.). DNA was cleaved into fragments by the enzyme HinD III, and analyzed by Southern Blot analysis on nitrocellulose paper using radiolabeled plasmid GA472 as a probe. Analysis of the callus clones selected as resistant to

kanamycin, indicated that over 80 percent of these clones contained DNA from the Agrobacterium plasmid in their genomic DNA. Thus, successful transformation of Populus tissue was confirmed.

When such putatively transformed shoots had achieved an appropriate quantity of biomass, a sample of tissue was removed for Southern Blot analysis to confirm the presence of foreign genes. The procedure of Dellaporta et. al.(1983) was used to prepare plant DNA. The DNA samples were digested with restriction enzyme HinD III and subsequently analyzed using procedures described by Fillatti et. al. (1987). An X-ray photograph from electrophoresis gels after hybridization with radioactive DNA probes is presented in Figure 6-3. In the figure, lanes labelled Pop. Cal. none and Pop. Sh. none, represent DNA digests from control callus tissue and shoots that were not previously exposed to A. tumefasciens. The occurrence of dark bands in the majority of the Populus digests that correspond to some of those in the series of dilutions of the standard digest, indicates that plasmid DNA was incorporated into the Populus genome and that genetic transformation occurred.

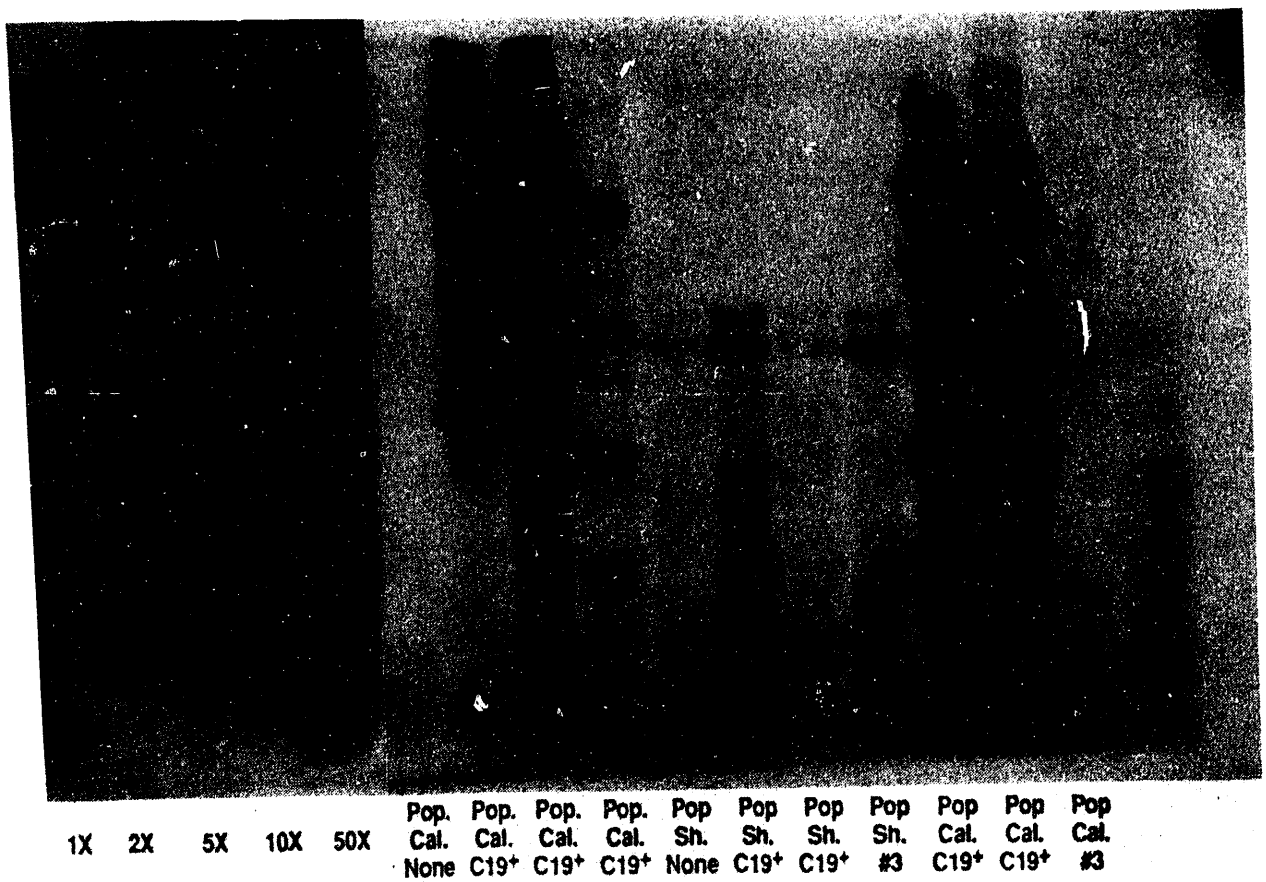

Figure 6-3. Southern Blot of Digested DNA from Control and Transformed Poplar Probed with Radiolabeled DNA from Plasmid PTiA6C19+. Fragments of Foreign DNA Inserted into Poplar Appear as Dark Bands, Some Corresponding to Plasmid Fragments. Lanes with no Dark Bands Represent Digests From Control Plants

DEVELOPMENT OF IMPROVED STRAINS OF A. TUMEFASCIENS FOR GENETIC ENGINEERING

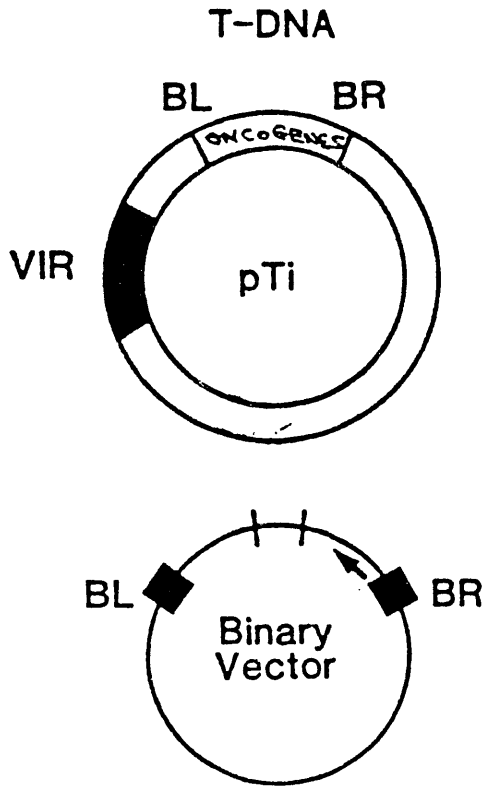

Figure 6-4. Representation of wild-type (upper) and binary vector (lower) plasmids of A. Tumefasciens

The two plasmids (self-replicating, circular strands of DNA) in the bacterial strains are shown in Figure 6-4. The transfer DNA is located between the left (BL) and right (BR) border regions. These fragments of DNA can be incorporated into the host plant genome. The portion of DNA labelled "vir" in Figure 6-4 is necessary to promote infection but is not transferred

Transformation was observed at fairly high efficiency: however, the strains of Agrobacterium contained an "armed" wild-type Ti plasmid (i.e., a plasmid carrying "oncogenes" which code for enzymes that produce plant hormones) as well as the mini-binary vector plasmid GA472. This vector system has worked well for producing transgenic plants in Nicotiana, Brassica, and Petunia species. However, it was not efficient in obtaining transgenic poplar plants because of the high frequency of co-transformation with transfer DNA from the wild-type Ti plasmid. This phenomenon of co-transformation occurred frequently following infection of poplar with Agrobacterium in all of the experimental conditions that were used. Since the oncogenes (tumor-inducing genes) were transferred as well as the genes for antibiotic resistance, the transformed cells produced hormone-autonomous callus, from which plants were difficult to regenerate. While some plants have been obtained, a more efficient method to obtain transgenic plants may use the binary vector Agrobacterium strains containing "disarmed" Ti plasmids with deleted or mutated oncogenes. Two "disarmed" strains were obtained but these strains did not contain the mini-Ti plasmid with genes for kanamycin resistance. Therefore, triparental mating techniques were used to insert the mini-Ti plasmid into the disarmed Agrobacterium strains.

IMPROVEMENT OF TRANSFORMATION SUCCESS

Using the basic protocol for transformation developed during the first year, several studies were initiated to determine the experimental variables that exerted the greatest influence on the frequency with which transformed shoots were produced. To determine the effects of Agrobacterium strain and kanamycin concentration in the regeneration medium, two strains of bacteria were co-cultivated with stem segments of clone DN66 which were then transferred to media containing 0, 100, or 300 mg/l kanamycin. The results of the experiment are presented in Table 6-3.

Table 6-3

EFFECT OF AGROBACTERIUM STRAIN AND KANAMYCIN CONCENTRATION ON SHOOT PRODUCTION FROM STEM EXPLANTS OF DN66

Conc. of Kanamycin mg\l	Mean Number of Shoots[a/] Agrobacterium Strain None	C	X
0	4.0 a	6.3 b	8.1 c
100	1.0	1.0	1.0
300	0.0	0.0	0.0

[a]/Means calculated from 25 replications; those within rows followed by different letters are significantly different at p=0.05 by Duncan's New Multiple Range Test.

According to these results, the effects of bacterial strain are mediated by the concentration of kanamycin in the regeneration medium since a strain effect was apparent only when shoots regenerated on media containing no kanamycin. Kanamycin concentration inhibited the regeneration of shoots and completely prevented regeneration at 300 mg/l.

In a second experiment, the influence of concentration of inoculum three bacterial strains was determined by co-cultivation of stem segments of clones DN66 and NM6 followed by transfer to regeneration medium containing either 0 or 100 mg/l kanamycin. The results summarized in Table 6-4 show that effects of strain and inoculum concentration were detectable only in the absence of the antibiotic.

Table 6-4

EFFECT OF AGROBACTERIUM STRAIN, CONCENTRATION AND KANAMYCIN IN THE CULTURE MEDIUM ON MEAN NUMBER OF SHOOTS PRODUCED FROM STEM SEGMENTS OF NM6 AND DN66 POPLARS

	Mean Number of Shoots[a/]		
Agrobacterium Strain	Inoculum Concn. cells\ml X 10^8	Kanamycin Concn. mg\l	
		0	100
None		8.6 c	0.6 a
C	0.5	4.1 a	0.9 a
	0.25	9.0 c	1.3 a
S	0.3	5.3 a	1.5 a
	0.15	4.3 a	1.3 a
U	0.6	7.9 bc	1.6 a
	0.3	6.0 b	0.7 a

[a/]Means calculated from 20 replicates; those within columns followed by different letters are significantly different at p=0.05 by Duncan's New Multiple Range Test.

Since cultivation in the presence of a selective marker (kanamycin) is necessary to distinguish shoots that regenerate from transformed tissue, apparently bacterial strain and concentration of inoculum did not influence the production of transformed shoots.

The effect of co-cultivation time on the production of transformed shoots was determined when stem segments of NM6 were inoculated with one of four bacterial strains, co-cultivated for either four or seven days, and transferred to regeneration medium containing either 0 or 100 mg/l kanamycin. Neither bacterial strain nor length of co-cultivation period influenced the production of transformed shoots on media supplemented with kanamycin (Table 6-5).

Table 6-5

EFFECT OF AGROBACTERIUM STRAIN, CONCENTRATION, CO-CULTIVATION TIME, AND KANAMYCIN IN THE CULTURE MEDIUM ON MEAN NUMBER OF SHOOTS PRODUCED FROM STEM SEGMENTS OF NM6

			Shoot Number[a/]	
Agrobacterium Strain	Inoculum Concn. cells\ml X 10^8	Co-culivation Time in Days	Kanamycin Concn. mg\l	
			0	100
None		4	2.3	0.1
		7	0.9	0.1
A	0.2	4	2.4	0.0
		7	0.9	0.1
C	0.2	4	1.9	0.0
		7	0.7	0.0
S	0.3	4	0.6	0 . 0
		7	2.2	0.1
U	0.3	4	0.8	0.0
		7	0.5	0.0

[a/]Means calculated from at least 13 observations per treatment.

Shoot formation in the stem segments occurred at very low frequency on media containing the antibiotic.

When four strains of bacteria, each applied at two inoculum concentrations were co-cultivated with stem segments of clone DN66 at either 0 or 100mg/l kanamycin, production of transformed shoots was not influenced by bacterial strain or concentration of inoculum. The results summarized in Tables 6-6 and 6-7 confirmed that kanamycin concentration in the regeneration medium exerted a strong effect on shoot regeneration and that no effects were attributable to the presence of any strain of A. tumefasciens.

Table 6-6

EFFECT OF AGROBACTERIUM STRAIN ON MEAN NUMBER OF SHOOTS PRODUCED FROM STEM SEGMENTS OF DN66

Agrobacterium Strain	Mean Number of Shoots[a/b/]
None	2.9 a
A	2.9 a
C	3.4 a
S	3.2 a
U	4.4 a

[a/]Means calculated from at least four observations per treatment.

[b/]Means followed by different letters are significantly different at p=0.05 by Duncan's New Multiple Range Test.

Additional experiments assessed the effect of a second antibiotic, geneticin, as a selective agent in regeneration medium and the assessed influence of a new medium formulation reported by Filatti et. al. (1987) on regeneration of transformed shoots from Agrobacterium-treated stem segments of DN66 and NM6. However, no shoot regeneration was observed in either experiment; therefore, no measure of the effects of the variables was obtained.

Table 6-7

EFFECT OF KANAMYCIN IN THE CULTURE MEDIUM ON SHOOT PRODUCTION FROM AGROBACTERIUM-TREATED STEM SEGMENTS OF CLONE DN66

Kanamycin Concn.	Mean Number of Shoots[a/]
0	5.1 b
100	1.3 a

[a/]Means calculated from at least 57 observations; those followed by different letters are significantly different at p=0.05 by Duncan's New Multiple Range Test.

DISCUSSION AND CONCLUSIONS

The transformation experiments conducted during the course of this work indicate that overall success in the production of transformed shoots and eventually plants is governed by the selection system choice, in this case the concentration of kanamycin, the clone and particular tissue used for the regeneration of shoots. Other factors, such as particular strain of infective A. tumefasciens, concentration of inoculum, and length of co-cultivation period, have no significant effect on the production of transformed shoots. Thus, careful selection of tissue from a clone capable of high-frequency adventitious shoot production, appears to be the best route to ensure success in transformation studies. The feasibility of achieving genetic transformation in Populus clones which are high biomass producers in the Northeast region, has been very successful. Application of this technology using genes conferring important silvicultural traits is possible and will be achieved when these genes are available for incorporation into Agrobacteria.

Table 6-8

List of A. Tumefasciens Strains Currently Held by NPI

NPI Code	Plasmid	Mini Plasmid
A	GV3850 #1	pGA472
B	GV3850 #2	pGA472
C	GV3850 #3	pGA472
D	GV3850 #4	pGA472
E	EHA 101	
F	ACH 5 pTi tch 5	
G	A136 pTi (octopine strain)	
H	A281	
I	A348	
J	A519 pTi (succinamopine strain)	
K	A208 pTi (nopaline)	
L	A277 pTiB6-806	
M	Bo542	
N	A348 pTiA6C19+	
O	A348 pTiA6C19-	
P	A348 pTiA6C35+	
Q	A348 pTiA6C35-	
R	A348 pTiA6 #3	
S	GV385	
U	EHA 101 LUXA	
V	EHA 101 LUXB	
W	EHA 101 pGA472	
X	472	

Section 7

DEVELOPMENT OF SEPTORIA MUSIVA RESISTANCE

Septoria musiva has been identified as the cause of a leaf spot and canker disease of poplar plantations in temperate humid climates (Krupinsky and Johnson 1985). The development of symptoms associated with Septoria diseases is complex but is thought to be mediated in part by toxin(s) produced by the pathogen (King et al 1983). In some plant pathogen interactions, where toxins are produced by a fungus, it has been possible to select tissues and cells in vitro with altered susceptibilities to toxins or crude, biologically active, culture filtrates (Behnke 1980, Brettel and Thomas 1980, Gengenbach et al 1977, Helgeson and Deverall 1983). In many cases, plants produced from these selected cells and tissues have shown greater resistance to the pathogen.

Based on the published information, a general approach to the development of Septoria-resistant poplars was devised and is represented diagrammatically in Figure 7-1. Since the proposed screening procedure depended on biolgical activity in fungal culture filtrates, or preparations therefrom, some preliminary work was necessary to establish that any activity in a crude toxin preparation could be correlated with development of disease symptoms in poplar plants, organs, or tissues.

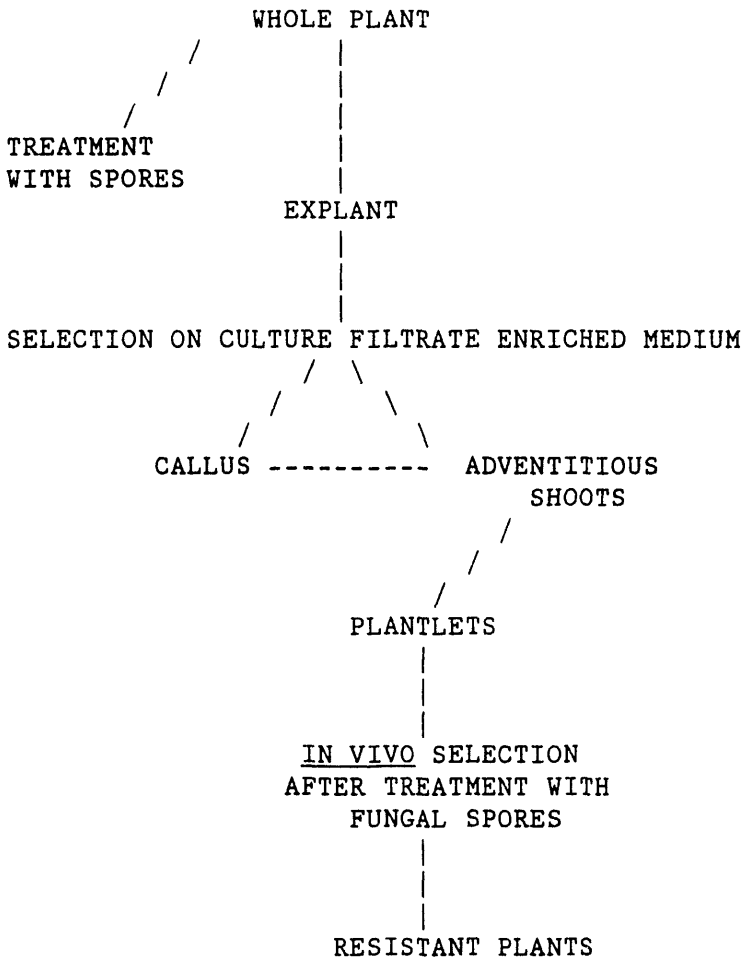

Figure 7-1. Protocol for Selection Against Septoria

WHOLE PLANT INOCULATION

To investigate the response of poplar clones to four S. musiva isolates, plants of clones NM2, NM4, NM6, DN10, DN19, DN74, DN134 and JAC4 were sprayed with spore suspensions (10^6 spores per ml) of four fungal isolates. Water was applied to a set of control plants. Immediately after treatment, plants were covered with plastic bags to provide high humidity conditions conducive to rapid infection. Plastic bags were removed after 72 hours. Plants were examined 10, 20 and 30 days after inoculation when the extent of foliar symptom development was scored on a rating scale (Table 7-1).

Table 7-1

ARBITRARY SCALE FOR THE EVALUATION OF SEPTORIA MUSIVA SYMPTOMS IN GREENHOUSE PLANTS

Mean Number of Lesions	Category Value
0	0
1-4	1
5-12	2
13-24	3
25-50	4
51-100	5
>100	6

The data were analyzed by analysis of variance. Significant effects of clone, isolate and time were observed as well as significant second-order interactions. The means are presented in Tables 7-2, 7-3, 7-4, and 7-5.

Table 7-2

EFFECT OF FUNGAL ISOLATE ON MEAN CATEGORY VALUE

Isolate	Mean Value[a/]
83-5872	2.2 c
83-5848	3.4 d
83-5948	1.8 b
83-5913	2.4 c
Water (control)	0.6 a

[a/]Data were averaged over eight clones and three evaluations; those followed by different letters are significantly different by Duncan's New Multiple Range Test at p=0.05.

Table 7-3

EFFECT OF CLONE ON THE DEVELOPMENT OF DISEASE SYMPTOMS IN GREENHOUSE GROWN PLANTS. DATA WERE AVERAGED OVER FIVE ISOLATES AND THREE EVALUATION TIMES

Clone	Mean Value[a/]
NM2	2.9 d
NM4	1.4 a
NM6	2.9 d
DN10	2.4 c
DN19	1.7 ab
DN74	2.0 b
DN134	2.0 b
JAC4	1.4 a

[a/]Means followed by different letters are significantly different by Duncan's New Multiple Range Test at p=0.05.

Table 7-4

EFFECT OF NUMBER OF DAYS POST-INOCULATION ON DEVELOPMENT OF FOLIAR DISEASE SYMPTOMS IN GREENHOUSE-GROWN POPLARS. DATA ARE AVERAGE CATEGORY VALUES FROM EIGHT CLONES AND FIVE TREATMENTS.

Days to Evaluation	Mean Value[a/]
10	0.7 a
20	2.2 b
30	3.4 c

[a/]Means followed by different letters are significantly different by Duncan's New Multiple Range Test at p=0.05.

Table 7-5

EFFECT OF CLONE AND FUNGAL ISOLATE ON THE SEVERITY OF FOLIAR SYMPTOMS IN GREENHOUSE-GROWN POPLARS. DATA ARE AVERAGED OVER THREE EVALUATIONS.

	Mean Foliar Rating[a/]				
	S. Musiva Isolate				
Clone	83-5872	83-5848	83-5948	83-5913	Water
NM2	3.8 cd	3.8 bc	1.3 a	4.3 c	1.1 a
NM4	0.6 a	3.5 bc	1.6 a	0.8 a	0.4 a
NM6	4.1 d	4.3 c	1.6 a	3.8 c	0.5 a
DN10	3.0 c	4.3 c	2.5 b	1.8 b	0.8 a
DN19	0.5 a	2.5 a	1.8 ab	3.5 c	0.3 a
DN74	1.5 b	3.3 ab	2.0 ab	2.3 b	1.1 a
DN134	2.6 c	3.0 ab	1.8 ab	2.2 b	0.5 a
JAC4	1.3 ab	2.8 a	1.7 ab	0.6 a	0.3 a

[a/]Means within a column followed by a different letter are significantly different by Duncan's New Multiple Range Test at p=0.05.

Leaf lesions on four clones are shown in Figure 7-2. In general, the clones could be grouped into three broad categories: i.e., least susceptible (NM4, DN19 and JAC4), intermediate (DN74 and DN134), and most susceptible (NM2, NM6 and DN10). Isolates could also be grouped: 83-5848 most virulent: 83-5872 and 83-5913 intermediate, and 83-5948 least virulent. Disease symptoms generally increased in severity with time after inoculation. However, significant interactions pointed to exceptions to these generalities: DN19 appeared particularly susceptible to isolate 83-5913, while clone NM4 appeared resistant to all isolates except 83-5848.

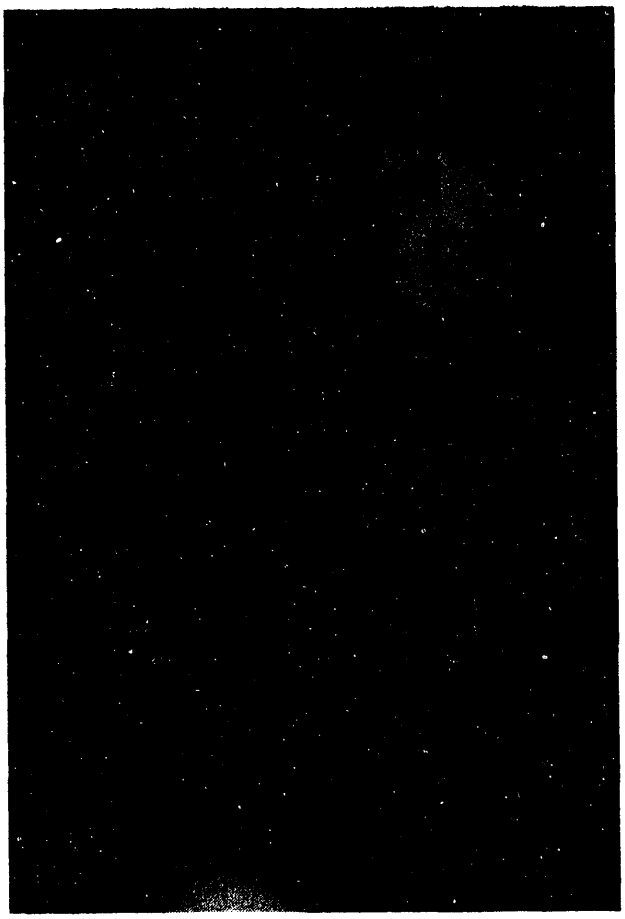

Figure 7-2. Lesions on Leaves from four Poplar Clones after Inoculation with a Spore Suspension of S. Musiva

DETACHED LEAF, STEM AND PETIOLE INOCULATION

To evaluate the response of isolated organs to infection with S. musiva spores, petiole and stem segments (approx. 3cm long) from clones NM2, NM4, NM6, DN10, DN19, DN74, DN134 and JAC4 were placed on water agar. One 10-microliter droplet of a spore suspension (10^6 spore per ml) was placed on the center of each segment. Suspensions were prepared from spores of isolates 83-5872 and 83-5913. The presence or absence of lesions was scored 16 days after inoculation. The percentage of segments that developed disease-like lesions are presented in Table 7-6. The data indicate that petiole infection with isolate 83-5872 produced symptoms that tended to correlate with data from the greenhouse study: however, data from the stem tissue and from isolate 83-5913 showed no correlation with the whole plant study.

Table 7-6

EFFECT OF CLONE AND ISOLATE ON PERCENT OF STEM AND PETIOLE SECTIONS THAT EXHIBITED LESIONS 16 DAYS AFTER TREATMENT WITH A SUSPENSION OF S. MUSIVA SPORES

	Fungal Isolate			
	83-5872		83-5913	
Clone	Petiole	Stem	Petiole	Stem
NM2	100	0	20	0
NM4	30	0	0	0
NM6	50	0	25	0
DN10	80	30	0	0
DN19	20	0	0	0
DN74	40	0	0	0
DN134	20	0	20	0
JAC4	40	80	0	0

Leaf discs from the same clones were inoculated with 10 microliters of spore suspensions of all fungal isolates. The effect of leaf age on the development of disease lesions was determined by comparing discs from young leaves (third below apex) and from old leaves (seventh below apex). Results are presented in Tables 7-7 and 7-8 that show leaf age has an influence on the development of lesions, since younger leaves in general seemed more susceptible to infection and colonization than older leaves.

The data also indicated to point to the interactive effects of clone and fungal isolate on the development of disease symptoms. The pathogen-host interaction in this particular system appears to be very complex, since manifestation of symptoms is influenced by genotype of the plant age and developmental stage of the target tissue and by the particular strain of fungus.

Table 7-7

EFFECT OF CLONE AND ISOLATE ON LESION AREA, MEASURED IN MM^2, IN DISCS FROM OLDER EXPANDED LEAVES AFTER TREATMENT WITH S. MUSIVA SPORE SUSPENSIONS

	Mean Lesion Area[a/]			
	Isolate			
Clone	83-5848	83-5872	83-5948	83-5913
NM2	0.8 a	3.2 b	0.6 a	4.1 d
NM4	6.2 e	8.5 e	16.3 e	2.8 c
NM6	3.3 d	9.2 f	8.0 d	8.6 e
DN10	1.9 b	3.1 b	2.4 b	0.2 a
DN19	3.7 d	7.6 d	2.1 b	2.9 c
DN74	2.7 cd	1.0 a	3.0 c	1.6 b
DN134	0.4 a	3.8 c	0.1 a	0 a
JAC4	2.3 bc	8.5 e	2.6 bc	0 a

[a/]Means within a column followed by a different letter are significantly different by Duncan's New Multiple Range Test at p=0.05.

Table 7-8

EFFECT OF CLONE AND ISOLATE ON LESION AREA, MEASURED IN MM^2, IN DISCS FROM YOUNGER EXPANDING LEAVES AFTER TREATMENT WITH S. MUSIVA SPORE SUSPENSIONS

	Mean Lesion Area[a/]			
	Isolate			
Clone	83-5848	83-5872	83-5948	83-5913
NM2	8.9 abc	27.6 c	4.8 a	0
NM4	10.5 abc	0.7 a	14.2 ab	0
NM6	4.9 a	3.4 a	5.0 a	0
DN10	0.6 a	1.1 a	4.9 a	0
DN19	11.0 bc	33.6 c	19.5 b	0
DN74	3.8 ab	0.2 a	8.3 a	0
DN134	17.2 c	9.9 ab	9.2 a	0
JAC4	7.3 abc	11.8 b	10.5 ab	0

[a/]Means within a column followed by a different letter are significantly different by Duncan's multiple range test at p=0.05.

BIOLOGICAL ACTIVITY OF SEPTORIA CULTURE FILTRATES

Several liquid media were evaluated for in vitro culture of fungal isolate 83-5848. Three media were compared: a simple V8 medium (200ml V8 juice and 3g $CaCO_3$ per liter); a medium described by Bousquet et. al. 1980 (B), and a medium described by Kent and Strobel 1976 (KS). Aliquots (2 ml) of spore suspension were added to one-liter batches of medium in three-liter flasks that were agitated on a rotary shaker (95 rpm) at 26°C under 16 hours of light. Cultures were harvested after three weeks of incubation when the KS medium was observed to have stimulated the best proliferation of fungal mycelium. Preliminary evaluation of crude culture filtrates from the flasks using a leaf disc assay indicated that filtrates from both KS and B media were capable of producing disease-like lesions. Since the KS medium promoted better growth of fungal mycelium this was selected as the medium for further production. A typical example of a Septoria liquid culture is presented in Figure 7-3.

Liquid cultures were harvested by filtration through a coarse-sintered glass funnel. A spore-free filtrate was concentrated 10-fold by rotary evacuation, lyophilized and dialyzed against cold tap water. The dialysate was lyophilized for storage. Generally, approximately 2g of crude extract was obtained from each two liters of culture medium.

Crude extracts were obtained from isolates 83-5913, 83-5848 and 83-5872. Samples of two of the extracts were suspended in sterile water and 10 microliter aliquots were applied to leaf discs of clones NM2, NM4, NM6, DN10, DN19, DN74, DN134 and JAC4. Aliquots of spore suspensions were also applied to discs. After 20 days,

leaf discs were inspected for presence of lesions. The percent ages of discs with lesions are presented in Table 7-9.

Figure 7-3. S. Musiva in Liquid Culture Medium (Fungal Mycelium Is Visible as Sediment)

Table 7-9

EFFECT OF FUNGAL CULTURE FILTRATE EXTRACT AND SPORES ON PERCENTAGE OF LEAF DISCS THAT DEVELOPED DISEASE-LIKE LESIONS. DATA WERE TAKEN AFTER 20 DAYS INCUBATION.

	Percent Leaf Discs with Lesions			
	Isolate Number			
	83-5913		83-5848	
Clone	Spore	Extract	Spore	Extract
NM2	100	100	40	80
NM4	60	100	100	0
NM6	100	80	100	60
DN10	20	100	80	20
DN19	80	100	100	20
DN74	40	80	60	20
DN134	60	100	100	20

Since culture filtrates were found to possess biological activity, filtrate extracts were applied to in vitro cultures of Populus tissues and organs to assess the potential for use of such extracts as screening agents for the identification of materials with enhanced pathogen tolerance or resistance. Extracts were dissolved in 95 percent ethyl alcohol and aliquots of solutions were added to autoclaved media while still hot. These aliquots were added so that alcohol never accounted for more than 10 ml of any liter of medium. Extracts of isolates 83-5948 and 83-5913 were added to culture medium. Callus tissue (0.5g) from clone NM4 stems and leaves was transferred to the enriched medium and reweighed after 16 days of incubation. Data from this study were analyzed. The source of the callus did not exert an effect; however, the presence of fungal-culture filtrate extract from isolate 83-5913 significantly affected callus growth (Table 7-10).

Table 7-10

EFFECT OF FUNGAL CULTURE FILTRATE EXTRACTS ON FRESH WEIGHT OF CALLUS TISSUE FROM CLONE NM4 AFTER 16 DAYS INCUBATION (INITIAL EXPLANT WAS 0.5G)

Extract Isolate Number	Mean Fresh Weight[a/](g)
83-5913 (0.5g\L)	1.7 b
83-5913 (1.0g\L)	1.0 a
83-5848 (1.0g\L)	1.5 ab
Control	1.9 b

[a/]Means followed by different letters are significantly different by Duncan's New Multiple Range Test at p=0.05.

Experiments were performed with shoot-tip cultures of clones NM2 and NM6 grown in the presence of fungal extracts when microshoots were transferred to enriched multiplication medium. Development of disease-like symptoms on cultured

microshoots was assessed after 21 days incubation. After analyzing the data, no significant differences in necrotic spot development could be ascribed to clone or to the presence of fungal culture filtrates. When attempts were made to repeat these initial observations two problems emerged. The first was associated with a difficulty in maintaining sterility in media supplemented with culture filtrates; and second, the addition of ethyl alcohol to culture media exerted a negative effect on control cultures. A different method of sterilization was investigated for the culture filtrate extracts. Arrangements were made with a local hospital to expose samples of extracts to ethylene oxide gas at 27°C for four hours. These sterile samples were incorporated into tissue culture media and effects of the culture filtrates were reassessed. Samples of callus tissue (0.5g) from clones NM6 and DN134 were transferred to media containing 0, 0.1, 0.25 and 0.5 g/L of extracts from three isolates. After four weeks, the callus was reweighed and data were analyzed. Results for the experiment with NM6 are presented in Table 7-11 and for DN134 in Tables 7-12 and 7-13. DN134 callus was affected significantly by both origin of the extract and by concentration in the culture medium, whereas NM6 callus growth was independent of the source of the extract but was influenced significantly by concentration.

Table 7-11

EFFECT OF CONCENTRATION OF FUNGAL CULTURE FILTRATE EXTRACT ON FRESH WEIGHT OF NM6 STEM CALLUS

Concentration of Extract (g/l)	Fresh Weight of Callus (g)[a/]
0.0 g\L	1.78 bc
0.1 g\L	1.99 c
0.25 g\L	1.48 ab
0.5 g\L	1.34 a

[a/]Data are means of 15 observations and are pooled over isolates, those followed by different letters are significantly different by Duncan's New Multiple Range Test at p=0.05.

Table 7-12

EFFECT OF ORIGIN OF CULTURE FILTRATE EXTRACTS ON FRESH WEIGHT GAIN OF DN134 STEM CALLUS

Extract Origin	Fresh Weight of Callus[a/]
83-5848	1.65 b
83-5872	0.99 a
83-5913	1.11 a
Control	1.60 b

[a/]Data are means of 15 observations, those followed by different letters are significantly different by Duncan's New Multiple Range Test at p=0.05.

Table 7-13

EFFECT OF CONCENTRATION OF S. MUSIVA CULTURE FILTRATE PREPARATION ON FRESH WEIGHT OF DN134 STEM CALLUS

Concentration of Extract (g\l)	Fresh Weight of Callus (g)[a/]
0.0	1.60 c
0.1	1.47 c
0.25	1.24 b
0.5	1.03 c

[a/]Data are means of 15 observations, those followed by different letters are significantly different by Duncan's New Multiple Range Test at p=0.05.

Thus, the data clearly showed that growth in disorganized poplar tissue cultures was influenced by exposure to an extract from filtered media in which S. musiva was grown.

A further study was initiated to evaluate the effects of liquid, concentrated, spore-free, Septoria culture filtrates on fresh weight gain of callus tissue of clones NM6 and DN134. Isolates of the fungus were cultured in KS medium. Culture filtrate was prepared and concentrated from four liters to 200 mls by rotary evaporation at 36°C. The concentrate was added to callus maintenance medium at 0, 0.5, 2.5, 5.0, 12.5, 25.0, 50.0, and 100.0 mls per liter of medium prior to sterilization by autoclaving. The concentrations of culture filtrate in the plant culture medium were 0, 1, 5, 10, 25, 50, 100, or 200 percent of the original in the fungal culture medium. Approximately 0.5 g of fresh callus tissue was transferred to each aliquot of solid medium and grown for 28 days. Fresh weight of the callus tissue was measured at 14 and 28 days after transfer to the enriched

medium. Since fresh weight changed little during the first 14 days, the weight gain during the second period was calculated for each callus sample and was summarized by analysis of variance.

Effects of clone, Septoria isolate and concentration were significant. The effects of isolate and concentration were dependent on each other since a signifcant interaction was observed among the factors. Results presented in Tables 7-14, 7-15, 7-16 and 7-17 show that mean fresh weight gain was greatest in clone NM6 and, in general, addition of between 1 and 10 percent fungal filtrate concentrate was stimulatory whereas 100 percent or more was inhibitory. Isolate 5913-104C at 10 percent level stimulated the greatest fresh weight increase and isolate 5848-104A was inhibitory only when present at 200 percent level.

The slight stimulation of growth produced by incorporation of a non-dialysed, fungal, culture-filtrate concentrate into a callus maitenance medium, may be a function of increased availibility of nutrients such as sugars or amino acids. The effect may be due to the production of compounds that in low concentrations are stimulatory to plant growth but are inhibitory at higher concentrations. It is conceivable that the biologically active crude toxin produced by the fungus acts as a stimulant at low concentrations and as an inhibitor at higher concentrations.

Table 7-14

EFFECT OF CLONE ON FRESH WEIGHT GAIN IN POPULUS CALLUS CULTURED IN MEDIA AMENDED WITH LIQUID, FUNGAL, CULTURE FILTRATES FROM SEVERAL SEPTORIA ISOLATES

Clone	Fresh Weight Gain (g)[a/]
NM6	0.44 b
DN134	0.25 a

[a/]Means calculated from 140 observations per clone; those followed by different letters are significantly different at p=0.05 by Duncan's New Multiple Range Test.

Table 7-15

EFFECT OF SEPTORIA ISOLATE ON FRESH WEIGHT GAIN IN POPULUS CALLUS CULTURED IN MEDIA AMENDED WITH LIQUID, FUNGAL, CULTURE FILTRATES

Isolate	Fresh Weight Gain (g)[a/]
5848 104A	0.39 b
5872 104B	0.30 a
5913 104C	0.36 ab
None	0.34 ab

[a/]Means calculated from 70 observations; those followed by different letters are significantly different at p=0.05 by Duncan's New Multiple Range Test.

Table 7-16

EFFECT OF DOSE OF LIQUID, FUNGAL, CULTURE FILTRATE ON FRESH WEIGHT GAIN OF *POPULUS* CALLUS CULTURED IN MEDIA SUPPLEMENTED WITH SUCH FILTRATES PREPARED FROM *SEPTORIA MUSIVA* CULTURES

% Liquid Filtrate	Fresh Weight Gain (g)[a/]
0	0.34 bc
1	0.42 cd
5	0.46 de
10	0.54 e
25	0.31 b
50	0.27 b
100	0.29 b
200	0.17 a

[a/]Means calculated from at least 14 observations; those followed by different letters are significantly different at p=0.05 by Duncan's New Multiple Range Test.

Table 7-17

EFFECT OF *SEPTORIA* ISOLATE ORIGIN AND DOSE OF LIQUID CULTURE FILTRATE ON FRESH WEIGHT GAIN OF *POPULUS* CALLUS CULTURED *IN VITRO* ON MEDIA SUPPLEMENTED WITH FILTRATES

	Mean Fresh Weight Gain (g)[a/]		
	Septoria Isolate		
% Liquid Filtrate	5848 104A	5872 104B	5913 104C
1	0.44 b	0.37 cde	0.45 bc
5	0.40 b	0.46 de	0.54 cd
10	0.48 b	0.49 e	0.71 d
25	0.43 b	0.20 abc	0.29 ab
50	0.43 b	0.09 a	0.28 ab
100	0.33 ab	0.29 bcd	0.26 ab
200	0.22 a	0.15 ab	0.13 a

[a/]Mean values calculated from at least 14 observations; those within columns followed by different letters are significantly different at p=0.05 by Duncan's New Multiple Range Test.

An experiment was initiated to assess the effects of extracts of filtrates of Septoria isolates on fresh weight gain of callus tissue of clones NM6 and DN134. This experiment included a replication of a study detailed in the first-year report. Solid, dry extracts were prepared from culture media of six Septoria isolates by filtration, evaporation, lyophilization, dialysis and further lyophilization. The powdered extracts were dissolved in minimum quantities (less than 10 ml) of alcohol and added to plant tissue culture media at 0, 0.25, 0.5, 1.0, and 2.0 mg/l. Extracts were added to molten agar media (i.e. above 46°C). Approximately 0.5 g of callus was transferred to each replicate vessel of each medium treatment and allowed to grow for 28 days. Fresh weight of each callus was measured and recorded after 14 and 28 days. The weight gain was calculated as the final weight minus weight at 14 days. Data on fresh weight gain were summarized by analysis of variance.

Effects of Septoria isolate, concentration of extract, and clonal origin of the callus were significant determinants of callus weight gain. (Tables 7-18, 7-19, 7-20, and 7-21) Extracts of all Septoria isolates were inhibitory compared to controls containing no extract. Extracts of isolates 5848-103B, 5872-104B, and 5913-104C were similar and more inhibitory than extracts of isolates 5848-102A, 5848-103A, and 5848-104A. In general, NM6 callus explants gained more fresh weight than those of DN134 when exposed to the fungal extracts, indicating that clone DN134 was more sensitive to the extracts than NM6. The responses of the two clones to increasing doses of filtrate extracts differed. Callus growth of explants of clone DN134 was least in the presence of either 1.0 or 2.0 mg/l, and was greater in the presence of 0.5 mg/l than with 0.25 mg/l. Callus of clone NM6 was inhibited by all doses and most strongly inhibited by the higher doses.

Table 7-18

EFFECT OF CLONE ON FRESH WEIGHT GAIN IN POPULUS CALLUS CULTURED IN MEDIA AMENDED WITH FUNGAL, CULTURE FILTRATE EXTRACTS FROM SEVERAL ISOLATES OF SEPTORIA MUSIVA.

Clone	Fresh Wieght Gain (g)[a/]
NM6	0.54 b
DN134	0.38 a

[a/]Means calculated from at least 160 observations; those followed by different letters are significantly different at p=0.05 by Duncan's New Multiple Range Test.

Table 7-19

EFFECT OF SEPTORIA ISOLATE ON FRESH WEIGHT GAIN IN POPULUS CALLUS CULTURED IN MEDIA AMENDED WITH FUNGAL, CULTURE FILTRATE EXTRACTS

Isolate	Fresh Weight Gain (g)[a/]
5848 102A	0.61 b
5848 103A	0.54 b
5848 103B	0.37 a
5848 104A	0.58 b
5872 104B	0.29 a
5913 104C	0.36 a
None	0.76 c

[a/]Means calculated from at least 14 observations; those followed by different letters are significantly different at p=0.05 by Duncan's New Multiple Range Test.

Table 7-20

EFFECT OF DOSE OF FUNGAL, CULTURE FILTRATE EXTRACT ON FRESH WEIGHT GAIN OF POPULUS CALLUS CULTURED IN MEDIA SUPPLEMENTED WITH SUCH FILTRATES PREPARED FROM SEPTORIA MUSIVA CULTURES

Filtrate Extract (mg\l)	Fresh Weight Gain (g)[a/]
0.0	0.76 d
0.25	0.51 bc
0.5	0.53 c
1.0	0.42 ab
2.0	0.37 a

[a/]Mean calculated from at least 20 observations; those followed by different letters are significantly different at p=0.05 by Duncan's New Multiple Range Test.

Table 7-21

EFFECT OF DOSE OF CULTURE FILTRATE EXTRACT AND CLONE ON FRESH WEIGHT GAIN OF POPULUS CALLUS CULTURED ON MEDIA SUPPLEMENTED WITH SEPTORIA MUSIVA EXTRACTS

	Mean Fresh Weight Gain (g)[a/]	
	Clone	
Filtrate Extract (mg\l)	DN134	NM6
0.0	0.74 d	0.78 c
0.25	0.39 b	0.64 b
0.5	0.53 c	0.53 ab
1.0	0.35 ab	0.48 a
2.0	0.26 a	0.49 a

[a/]Means calculated from at least 21 observations; those within columns followed by different letters are significantly different at p=0.05 by Duncan's New Multiple Range Test.

SOMACLONAL VARIANT PRODUCTION AND FUNGAL SPORE SCREENING

Most of the effort of creating disease-resistant clones was spent regenerating shoots and subsequently screening the population for somaclonal variants. Clones DN10, DN17, DN66, DN134, NM2, NM4, and NM6 were used as sources of explant material. Stem segments were removed from stock plants, disinfested by soaking in a dilute solution of commercial bleach, and thin sections of stem were transferred to tubes containing culture medium supplemented with growth regulators (PL2). After approximately 30 days, adventitious shoots were removed from the segments and the basal shoot-producing tissue was transferred to a fresh aliquot of similar medium. Shoots were transferred to a medium enriched with indolebutyric acid where roots formed within 21 days. Plantlets were transferred to soil conditions and maintained in a high humidity environment during acclimatization to ex vitro conditions.

When regenerated plants were between four and eight inches tall, with well-developed foliage, the plants were sprayed with a suspension of Septoria spores at 10^6 spores per ml prepared as a mixture from all isolates. Spores were harvested from cultures of infective Septoria isolates maintained on solid KS media in petri plates. Fungal isolates were maintained for up to three months in vitro when spores from these cultures were then inoculated into leaves of greenhouse stock plants. Fungal inocula were taken from diseased lesions for culture in vitro to ensure that infective cultures of the fungus were always maintained.

A total of 9600 stem segments of the various clones produced 11400 shoots that were transferred to rooting media. They produced 10300 plantlets in the greenhouse.

Treatment of these plants is progressing. Plants that failed to show disease symptoms after the first treatment with spores were re-inoculated. Plants which showed no symptoms after the second treatment were selected as putative somaclonal variants with altered reaction to Septoria musiva. Only 83 plants have survived the second round of selection from the more than 10,000 tested. This rate of 0.83 percent variant production seems high when typical mutation frequencies are expected to be around 0.001 percent or 0.0001 percent. Some of the plants selected in the greenhouse will likely revert to the susceptible phenotype as the plant grows since epigenetic changes resulting from the tissue culture phase will tend not to influence the response to disease pressure.

During the final year of the program, additional plants were regenerated and exposed to fungal spore treatments. A further 4,000 plantlets were screened to expose 70 altered phenotypes. All suspected disease-resistant types were exposed to at least three treatments with fungal spores in greenhouse tests. Altered phenotypes were produced in the following clones: NM2, NM4, NM6, DN10,DN66, and DN134.

The results of the somaclonal study seem almost incomprehensible with such a high frequency of variant production (approxomately 1 percent). However, the plants have yet to be screened for disease interaction under field conditions in the northeast where the full use of the new phenotypes can be fully assessed. Further evaluation of these somaclones seems desirable and samples of several of the new types were sent to a collaborator at SUNY in Syracuse for further testing.

Section 8

PROTOPLAST TECHNOLOGY

Manipulation of isolated plant protoplasts offers a new approach to genetic improvement of plants, particularly through creation of somatic hybrids between plant species where formation of natural, sexual hybrids is not possible. A general procedure for the isolation and culture of protoplasts is represented in figure 8-1. Experiments were initiated to assess the potential for protoplast isolation from the poplar clones collected.

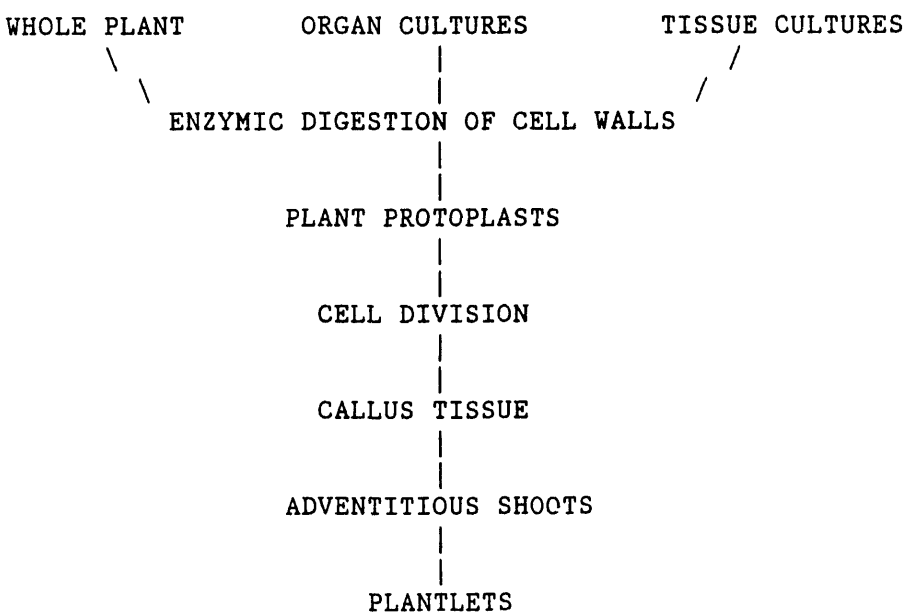

Figure 8-1. General Protocol for Isolation and Culture of Protoplasts

Initial work focused on screening mixtures of enzymes in combination with varying incubation periods in order to define a precise and reproducible protocol. The

method developed for the production of viable protoplasts from poplar tissue involved the enzymic digestion of cell walls from finely chopped callus tissue by exposure to a mixture of 0.5 percent cellulysin and 0.1 percent macerase at 25° for six-eight hours. Protoplasts were collected by filtration, centrifugation in a sucrose gradient, and transferred to a modified K8p (Kao and Michayluk 1975) medium where cell wall formation and cell division were observed.

A method for isolating of viable protoplasts of clone NM6 was developed during the first year of the program. This method was applied to callus tissue and to leaf tissue of clone NM6 to provide material for study. Several enzyme mixtures were used for callus tissue and all treatments yielded viable protoplasts that were capable of growth up to the microcallus stage. When leaf tissue from shoot-tip cultures was used for isolation, a reddish-brown coloration was observed during cell wall digestion. This colored compound, perhaps phenolic in nature, drastically decreased the viability of any protoplasts and was not suppressed when enzyme concentrations and mixtures were altered or when the osmoticum was varied.

Experiments were initiated with callus-derived protoplasts of clone NM6 to identify a suitable combination of plating density and media that promoted growth and development. A personal communication from Professor McCown at the University of Wisconsin indicated that ammonium ions (NH^4) in the culture medium could be toxic to Populus protoplasts and that the auxin 2,4-D also exerted a toxic effect. Media were formulated without NH^4, and with no 2,4-D, but containing one of the auxins NAA, IAA, or NOA. No matter which particular combination of medium components was used, regeneration beyond the stage of microcalli was never observed.

SECTION 9

DISCUSSION AND CONCLUSIONS

Poplar plants are probably the most widely used woody plant in applying modern biotechnology to forestry species. One reason the genus Populus has been chosen is that many clones are available that are responsive to in vitro manipulation. Poplar was a wise choice for this type of study.

Considerable progress was made toward the goals of this research program. The quest for glyphosate-resistant poplars apparently resulted in no useful tolerance to the herbicide in any of the clones. Resistance to micromolar concentrations of the herbicide was observed in cultures of disorganized cells, but such resistance was not transmitted in a stable, useful manner to plants which regenerated from such callus.

While a transformation system for the genetic manipulation of Populus has been reported by others, it is unclear that complete plants were produced from transformed tissue. We have successfully produced a number of plants from transgenic poplar tissue and have confirmed that "new" genes were incorporated into regenerated plants. Plants resulting from transformation experiments will be grown to provide cuttings for field planting in the final year of the program.

The development of genetic transformation technology based on a disarmed binary vector system in Agrobacterium tumefasciens will greatly enhance the applicability of this fundamental genetic engineering method. Excellent results were obtained by the production of transformed plants, confirming that foreign genes were incorporated into the Populus genome. Production of genetically transformed poplar

plants is quite feasible but is labor-and resource-intensive. This work drew on talented and skilled scientists who brought a large body of experience and expertise to the solution of technical problems. As a result, this program indicates that the transformation system is still a research tool and cannot be regarded as a generally applicable routine laboratory procedure for any Populus clone. The use of the genetic transformation procedure will be greatly enhanced when genes that alter important silvicultural traits are identified and available to the scientific community for incorporation into plasmid DNA of Agrobacteria.

The information concerning the fungal strain and plant-clone specificity is indicative of the complexity of the fungus-plant interaction. Development of clones with some degree of increased resistance to attack by the pathogenic fungus S. musiva has proceeded along two distinct paths. An in vitro methodology was investigated based on selection against an extract of the fungus or fungal culture medium. This approach was valid when biological activity in culture extracts was demonstrated and such extracts influenced the growth of callus tissue of two clones. However, the protocol for extraction and collection of the active compounds, a crude toxin, was resource-intensive. Manipulation of the extract in order to successfully incorporate the hydrophobic substance into an aqueous culture medium was not always routine and straightforward. Nevertheless, a crude toxin was shown to be present in culture filtrate extracts and was shown to exert a biological response on poplar callus. The use of the selection system was limited also by an inability to promote high-frequency plant regeneration in any selected callus.

A second approach to the creation of resistant plants involved screening a large number of regenerated plants for altered susceptibility to infection by spores of

S. musiva. Variant phenotypes were detected at a frequency of 1.0 percent. These variants failed to develop typical disease lesions after at least three treatments with suspensions of fungal spores. The screening of plants in the greenhouse to spores of the pathogen is likely to provide a more stable and applicable resistance than the screening of callus tissue, since the disease is transmitted and dispersed as spores whereas the crude toxin screen has a less obvious natural analogy. Further evaluation of the variant plants will be undertaken through cooperation with scientists at SUNY.

Development and application of protoplast technology has been included in the program since such methodology is critical in the development of novel hybrids that cannot be generated by conventional breeding. These potential hybrids may include novel unions of willow or alder with poplar. Much progress was made with the protoplast techniques and dividing callus has resulted. However, no plants were regenerated from the callus.

Section 10

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