USE OF PROTOPLAST, CELL, AND SHOOT TIP CULTURE IN AN ELM GERMPLASM IMPROVEMENT PROGRAM

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Abstract

An elm germplasm improvement program was established using three distinct approaches: (1) development of protoplast regeneration protocols with the goal of attempting somatic hybridization between *Ulmus americana* and disease resistant hybrids; (2) evaluation of the extent of somaclonal variation in plants regenerated from protoplasts; and (3) selection in vitro of variants from *Ulmus americana* callus lines exposed to toxin and culture filtrates of *Ceratocystis ulmi*. Plants were regenerated from protoplasts of the Dutch elm disease resistant hybrid *Ulmus* x 'Pioneer', but repeated attempts to regenerate *Ulmus* x 'Homestead' failed. Somaclonal variation has been described in a population of plants regenerated from 'Pioneer ' elm protoplasts and this variation can be fixed in clones through asexual propagation. *Ulmus americana* selections with field level resistance to Dutch elm disease demonstrate resistance in vitro as evidenced by callus growth on media containing culture filtrates of *Ceratocystis ulmi*. Attempts to select variants of previously susceptible *Ulmus americana* clones have been frustrated by the inability to regenerate plants from callus of adult phase *Ulmus americana*.

1. Introduction

Research directed toward the elucidation of the anatomical, physiological, biochemical, and genetic interactions between the hosts and causative agents of Dutch elm disease has occupied the interests of foresters, botanists, horticulturists, and plant breeders for decades. The role of *Ceratocystis ulmi* and the pathotoxin it produces, cerato-ulmin, has been recognized as a significant part of the disease syndrome; however, other factors such as juvenile vigor, tree stress, feeding by the elm bark beetle, host resistance, other putative pathotoxins, and other disease syndromes (phloem necrosis) are acknowledged to mitigate the rapidity of the decline leading to ultimate death of infected trees (Strobel and Lanier, 1981; Takai et al., 1983).

Disease resistance has been identified in certain *Ulmus* species and in some *Ulmus* hybrids like 'Pioneer' and 'Homestead' (Townsend and Masters, 1984 a,b). Individual trees of *Ulmus americana* with high levels of resistance or tolerance have been identified and clones of these trees have been released or subjected to advanced testing (Townsend, 1981).

Direct introgression of disease resistance into *Ulmus americana* is prevented because of sexual incompatibility between the tetraploid *Ulmus americana* and the diploid resistant European and Asian species (Dermen and May, 1966). Novel approaches using modern tissue culture techniques have attempted to use cell selection, protoplast hybridization, and anther culture to overcome this barrier.

The results described in this report are the outcome of a cooperative project between the USDA Nursery Crops Research Laboratory and the Department of Horticulture at Ohio State University that was organized to develop a protocol for regeneration of plants from *Ulmus* protoplasts, screen cell cultures for somaclonal variation with regard to pathotoxin susceptibility, and grow and evaluate tree populations to test for field level resistance to Dutch elm disease.

2. Materials and methods

2.1. Tissue culture procedures

Actively elongating shoot tips or recently expanded leaves of the *Ulmus* hybrids 'Pioneer' and 'Homestead', and the *Ulmus americana* selections 'Amer', '#8630', and 'Delaware #2' were surface sterilized in 0.5% (v/v) sodium hypochlorite for 15 min and rinsed 2 times in sterile distilled water. Shoot tips were trimmed to 1 cm in length and leaf tissue was trimmed to about 1 cm^2. Except where noted, a modified Murashige and Skoog (Murashige and Skoog, 1962) medium containing 200 mg/l casein hydrolysate, 100 mg/l myo-inositol, 3% sucrose, and 0.6% Difco Bacto agar (pH 5.7) was used. Growth regulators and other additives or modifications were as indicated in Table 1. Protoplasts were isolated and cultured as described previously (Sticklen et al., 1985, 1986). Tissue cultures were maintained in a growth room at 22 C $\pm$ 2 C under 40 $umol/m^2/sec$ of cool white fluorescent light for 16 hrs per day (callus cultures for protoplast isolation and pathogen culture filtrate screening experiments were grown in the dark).

Microcuttings (about 2 cm in length with well expanded leaves) taken from actively multiplying cultures were rooted in Redi-Earth (finely screened peatmoss and vermiculite) in foil containers with clear plastic lids. Microcuttings were rooted under the conditions described for growth of tissue cultures. Rooted microcuttings were transplanted into plastic trays containing Redi-Earth, placed on a shaded intermittent mist bench in the greenhouse for 7 days (6 sec mist every 6 min; light intensity, 360 $umol/m^2/sec$), followed by 7 days on a shaded greenhouse bench (light intensity, 600 $umol/m^2/sec$) and were grown on in the greenhouse under standard practices of fertilization, repotting, and pest control thereafter (Fink et al., 1986).

2.2 Culture filtrate screening experiments

Culture filtrates of aggressive and nonaggressive isolates of *Ceratocystis ulmi* were prepared from centrifuged, ultrafiltered (0.45 um Nalgene filter) suspensions of 8 day cultures growing in Salemink's liquid medium (Takai, 1978). Culture filtrates were introduced into the half strength MS callus culture medium at 1, 5, 10, 20, and 50% (v/v). Appropriate controls having identical dilutions of Salemink's nutrient broth medium were tested. Fresh weight increase of 0.5 g calli of 'Amer', '#8630', and 'Delaware #2' was determined at the end of 8 weeks.

Terminal stem cuttings (10 to 15 cm in length) taken from new growth on greenhouse grown plants of the same cultivars were assayed for wilting symptoms after 24-48 hrs of being held in distilled water, Salemink's nutrient broth, 50% culture filtrate, or undiluted culture filtrate. A subjective scale was used where 0 represented no symptom expression and 3 represented severe wilting, necrosis, and leaf curl.

2.3 Somaclonal variation studies

A population of 168 plants of *Ulmus* x 'Pioneer' regenerated from protoplasts was grown on in a growth chamber (25 C, 200-300 $umol/m^2/sec$ of cool white fluorescent light, 16 hr days) according to accepted horticultural practices of fertilization, watering, and pest control. This population of regenerants was described by several quantitative features (rate of shoot elongation, increase in caliper, leaf length to width ratios, and number of leaf serrations). Shoot elongation rates were the average rates taken on 5 dates in two growth flushes, and stem caliper was measured at the fifth node from the shoot tip of actively growing plants.

Two-node cuttings of most of these regenerants were rooted, and the 5 most heavily rooted cuttings in each clone were potted and returned to the growth chamber under the same conditions as described previously and were evaluated by the same quantitative parameters (these populations were referred to as ramets).

Phenotypic characters of the 3 populations were compared; regenerants, a random sample of 40 of the 168 plants regenerated from protoplasts; ramets, groups of 5 rooted

cuttings derived from 37 of the 40 regenerants, and a group of 5 'Pioneer' elm plants derived from rooted cuttings. Statistical procedures included analysis of variance and correlation analysis.

3. Results

Considerable attention was devoted to characterizing the morphogenic responses of *Ulmus* x 'Pioneer'. 'Pioneer' has a proven high level of resistance to Dutch elm disease, and was chosen as the logical model for attempted somatic hybridization with *Ulmus americana* after repeated attempts to regenerate *Ulmus* x 'Homestead' met with failure (Sticklen et al., 1985, 1986).

Protoplasts isolated from friable leaf-derived callus of 'Pioneer' elm underwent colony formation and meristems subsequently differentiated on this callus when it was exposed to a pulse of 30 uM benzyladenine (BA) in the light (Table 1). These meristems were overgrown by additional callus if subcultured to media containing 10 to 30 uM BA. However, if the meristem containing callus was subdivided into small pieces about 2 mm in diameter) and transferred to medium containing 2 uM BA, callus growth was arrested in favor of shoot elongation. When these shoots had elongated to about 2 cm in length, they could be rooted under nonsterile conditions in a peat-vermiculite medium under high humidity, and subsequently grown on in the greenhouse or growth chamber.

Within 2 or 3 weeks of growth resumption in the growth chamber, differences in the growth rate among the population of plants regenerated from protoplasts (the regenerants) were evident. These differences were noted visually and were quantified by growth rate histograms (Fig. 1). Length increase of the individuals varied from 0.15 to over 1.45 mm per day.

Whether this variation was a characteristic of the individual or whether this variation was due to influences outside of the individual (such as poor rooting, damage during transplanting, or nonapparent disease infestation) was an important issue. If this variation appeared fixed in the clonal progeny of the individuals, it would support the notion of somaclonal variation among the regenerated plants. To test this hypothesis, nodal cuttings were taken from the regenerants and the variation among these ramets was compared to the variation of the regenerants and to rooted cuttings from the 'Pioneer' stock plants. The range of variation in both shoot length and caliper increase was lower in the ramet population. The small population of 'Pioneer' rooted cuttings served as a relative base for comparison; however, the small number of plants did not allow for an accurate comparison of the growth rate distribution.

A better estimator of the tendency to fix variation, either genetic or epigenetic, is obtained by examining the correlation of the relative rank of the regenerants and the ramets with regard to several morphological features. In other words "do the slowest or fastest growing regenerants produce the slowest or fastest growing ramets?" All correlations were positive, indicating a tendency to clonal fidelity of the growth parameters (Table 2). When the two extreme ramet populations for growth rate were segregated and analyzed separately, the correlation was significant at $p=0.001$ with a correlation coefficient of 0.89. Furthermore, if the ramet populations had included all 168 of the original regenerants, the ability to establish subclones would have been enhanced. The extreme dwarfs were so slow growing that they did not yield nodal cuttings, or in some cases these cuttings failed to root.

The demonstration of somaclonal variation among protoplast derived plants validated the need for an efficient screening system that could be applied at the cell or callus culture level. Two key steps in the development of such a screening procedure were the isolation of a culture filtrate which induced symptoms at the whole plant level in susceptible clones without affecting resistant clones and the demonstration that the in vitro response of callus from genotypes of known resistance or susceptibility to culture filtrate containing media paralleled the response at the whole plant level. When culture filtrate was prepared from aggressive isolates of *Ceratocystis ulmi*, a wilt symptom was induced in cuttings from susceptible American elm clones. The culture filtrate induced wilting, leaf rolling, and

necrosis in the susceptible clone 'Amer' while the resistant clone '#8630' failed to develop symptoms.

When this culture filtrate was incorporated into the culture media for *Ulmus americana* the growth rates of Dutch elm disease resistant and susceptible genotypes were affected differentially (Fig. 3). Callus growth of the susceptible genotype 'Amer' was inhibited by the incorporation of 20 to 50% culture filtrate in a dose-dependent fashion. Additionally, incorporation of culture filtrate into the medium reduced the cell viability (as estimated by the triphenyl tetrazolium chloride reduction assay) of the susceptible clone 'Amer' while the resistant clones, #8630 and Delaware #2, were unaffected (Fig. 4). These results indicated the potential for using culture filtrate as a screening tool for putative somatic hybrids or somaclonal variants of *Ulmus americana* derived from protoplast or callus culture.

4. Discussion

The research reported here suggests several avenues which may be followed leading to the development of Dutch elm disease resistant *Ulmus americana*. The original intent of this project was to develop a protocol for somatic hybridization of *Ulmus americana* and either 'Pioneer' or 'Homestead' elm. We have documented the regeneration of plants from 'Pioneer' elm, but protocols for fusion and regeneration of the putative somatic hybrids were not attempted. We did develop a screening system which might facilitate separation of disease resistant heterokaryons following fusion; however, the reliability of the culture filtrate in protoplast systems must yet be demonstrated.

The existence of somaclonal variation in a population of 'Pioneer' elms regenerated from protoplasts suggests that callus or protoplast culture might be one logical approach to elm germplasm improvement. This approach would be contingent upon *Ulmus americana* displaying a similar level of somaclonal variation upon regeneration from callus. The next step in this project will involve characterizing the level of disease resistance of the population regenerated from protoplasts to determine whether somaclonal variation with regard to disease resistance is encountered in the same manner as morphological variation.

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Table 1 - Optimal culture media used in obtaining various morphogenic responses of *Ulmus sp.*

Source tissue	Morphogenic response	Medium	Optimal growth regulator concn Auxin	Cytokinin
'Pioneer' protoplasts	Cell division; colony formation	KM 8p	1.0 uM 2,4D + 2.5 uM NAA	1.0 uM zeatin
'Pioneer' protoplast-derived callus	Meristem differentiation	MS	--------	30 uM BA
'Pioneer' callus with meristems	Shoot elongation	MS	--------	2 uM BA
Greenhouse grown 'Pioneer' leaves	Friable callus	half strength MS	2.5 uM 2,4D	1.0 uM kinetin
in vitro 'Pioneer' leaves	Shoot proliferation	MS	--------	2.0 or 4.0 uM BA
'Pioneer' shoot tips	Shoot proliferation	MS	--------	2.0 uM BA
Ulmus americana leaves	Callus initiation	MS + 10% coconut milk	0.5 uM 2,4D	8.0 uM BA
Ulmus americana callus	Callus proliferation	half strength MS + 10% coconut milk	0.5 uM 2,4D	8.0 uM BA
Ulmus americana callus	Meristem differentiation	--------	--------	--------

Table 2 - Correlation between numerical rank of 37 regenerants and 37 ramet populations with regard to several morphological features.

Feature	Correlation coefficient (r)
Number of leaf serrations	0.48
Leaf length by width ratio	0.52
Stem caliper increase	0.66
Stem length increase rate	0.41
Paired comparison of guard cell length of fastest and slowest growing ramets	0.89

Figure 1. Population histogram of shoot growth rates of regenerants, ramets, and 'Pioneer' plants.

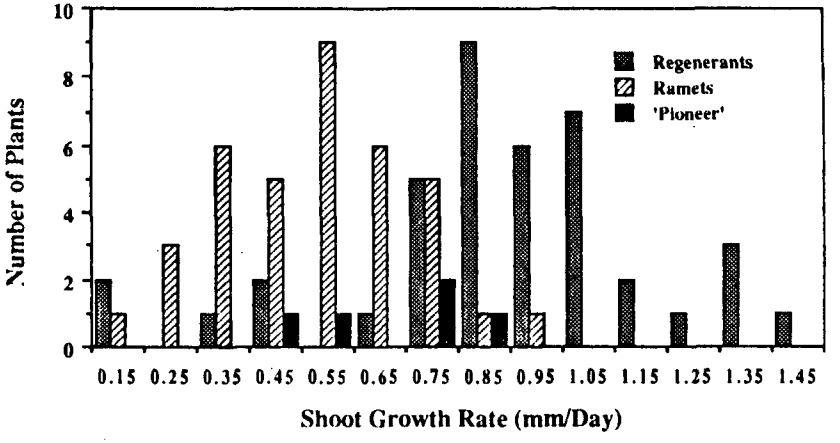

Figure 2. Population histogram of stem caliper at fifth node from the shoot tip of actively growing shoots of regenerants, ramets, and 'Pioneer' plants.

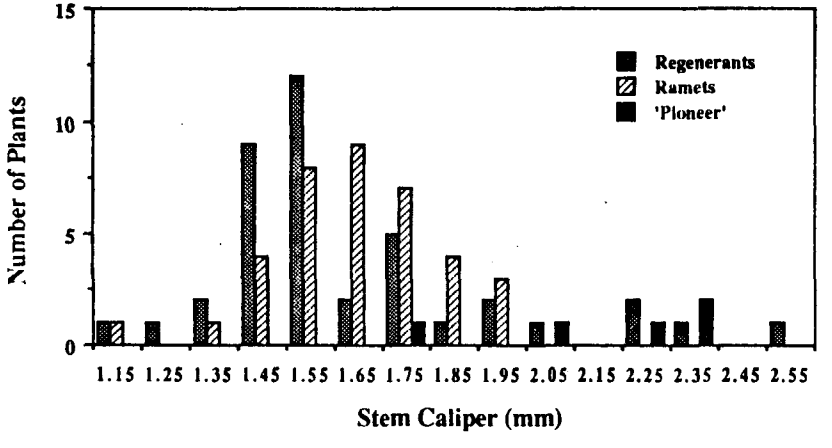

Figure 3. Effect of *Ceratocystis* culture filtrate on fresh weight increase of callus from susceptible ('Amer'), moderately resistant ('Delaware #2'), and resistant ('#8630') clones of American elm.

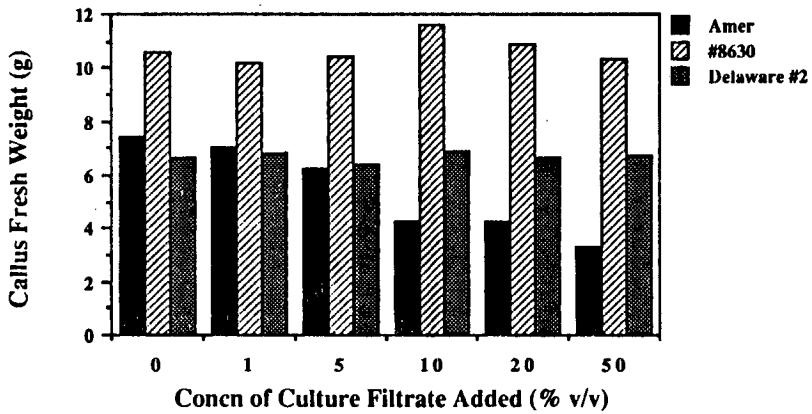
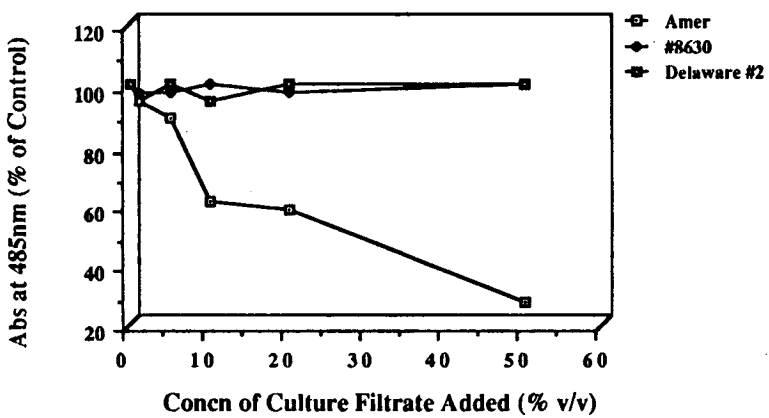
Figure 4. Effect of increasing concentrations of *Ceratocystis* culture filtrate on cell viability of American elm selections with varying degrees of resistance (see Figure 3) as assessed by triphenyl tetrazolium chloride reduction.