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Research note

Establishment and characterization of American elm cell suspension cultures

Steven M. Eshita*, Joseph C. Kamalay, Vicki M. Gingas & Daniel A. Yaussy
*USDA Forest Service, Northeastern Research Station, 359 Main Rd., Delaware, Ohio 43015, USA (*requests for offprints; Fax: (740) 368-0152; E-mail: seshita@fs.fed.us)*

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Abstract

Cell suspension cultures of Dutch elm disease (DED)-tolerant and DED-susceptible American elms clones have been established and characterized as prerequisites for contrasts of cellular responses to pathogen-derived elicitors. Characteristics of cultured elm cell growth were monitored by A_{700} and media conductivity. Combined cell growth data for all experiments within a genotype showed relatively low variances and between-genotype contrasts during repeated passages showed no significant differences. Subculturing exponentially growing cells at 8–14 day intervals, within readily measured parameters of media conductivity (4.95–4.2 mmhos) and cell concentration ($\geq$ 1.4 A_{700}), consistently resulted in repeatable profiles of elm cell growth and minimized lag phase. Culture cells were essentially homogeneous after 5 subculture passages and their overall appearance was stable. We conclude that the described procedure resulted in consistent cultures suitable for elicitor treatment experiments.

Abbreviations: A_{700} – absorbance at 700 nm; DED – Dutch elm disease; ln – natural logarithm; MS – Murashige and Skoog medium; MSE – mean square error; nm – nanometer; RGR – relative growth rate; μM – micromolar

The American elm (*Ulmus americana* L.) has been ravaged by Dutch elm disease (DED) in cities and forests of North America. Some of the surviving trees have significant levels of tolerance to DED, as characterized in tree selection programs at the U.S. National Arboretum (Townsend et al., 1995). A reliable *in vitro* test for DED-tolerance would augment efforts at *in planta* contrasts of DED-tolerant versus DED-susceptible germplasm. Contrasts of cellular responses to pathogen-related elicitors could lead to the identification and characterization of disease resistance mechanisms. However, for non-*americana* elm genotypes, poor correlations have been reported between *in vivo* DED-resistance and callus growth in the presence of fungal culture filtrate (Diez and Gil, 1998a), and between the former and phenylalanine ammonia-lyase activity in co-cultures of cell suspensions and *Ophiostoma novo-ulmi*, the DED pathogen (Diez and Gil, 1998b).

Callus cultures of *U. americana* have been used to study elm cell phytoalexin responses to the DED pathogen (Yang et al., 1993), and correlation between *in vivo* DED-resistance and callus growth in the presence of fungal culture filtrate has been reported (Pijut et al., 1990). However, in our hands callus cultures exhibited a relatively large degree of variation and high sensitivity to factors which were difficult to control (Domir et al., 1992; Domir et al., 1994), favoring the development of a cell suspension culture system.

Cell suspension cultures offer potential advantages for the study of elicitor-induced defense responses. In such a simplified system, plant cells which are synchronously challenged with an inducer uniformly undergo physiological reactions and mount a concerted response. Cell growth under controlled conditions, without the complexities of the intact tree, allows assessment of defense metabolism mediated by the cultured cells (Dixon, 1985). The purposes of this in-

vestigation were to determine conditions needed to establish vigorous suspension culture growth, to initiate cultures from different American elm clones, and to determine the extent of within-genotype and between-genotype variations and the level of batch-to-batch culture variability.

American elms used for initiation of elm suspension cultures included a seedling approximately 2 years old, ramets of two mature DED-tolerant selections, New Harmony (formerly #680) and Valley Forge (formerly Delaware-3), and ramets of two mature DED-susceptible trees, NA57845 and WT-2. Rooted cuttings of each clone were greenhouse-grown under standard conditions. Newly flushed leaves 2 to 8 cm in length were surface disinfected in 0.5% sodium hypochlorite and 0.1% Sigmaclean (Sigma Chemical Co., Cat. # S 4107, St. Louis, MO, USA) for 10 min, and rinsed twice in sterile distilled water. One to 1.5 cm-wide foliar cross-sections were excised, each containing midvein and laminar tissue. Explants were cultured on modified MS (Murashige and Skoog, 1962) medium containing 3% sucrose, 5.4 μM naphthylacetic acid, 4.4 μM benzyladenine and 0.48% (w/v) 'Agargel' (Sigma Chemical Co., Cat. # A 3301) in Petri plates (100 $\times$ 15 mm) and incubated in darkness at 22 °C or under cool white fluorescent illumination (40 μmol m^{-2} sec^{-1}) at 28 °C. Subsequent incubations were in darkness because of poor cell growth in the light.

Friable, cream-colored calluses formed on the wounded vein tissue of the mature tree explants after 8 days in dark culture. In contrast, callus formation was not apparent for several weeks on laminar tissues from seedlings. Callus could be used to initiate suspension cultures for at least 3–4 weeks after formation.

Suspension cultures were initiated from callus in a stepwise series of volume increases at 25 rpm on a gyrorotatory shaker, subculturing when a thick suspension of cells was achieved. Initially, twenty-five mg clumps of callus tissue were placed in 1 ml of liquid MS medium in 22 mm, 12-well culture plates (#25815-12, Corning Glass Works, Corning, NY, USA). After 3 weeks, each 1 ml suspension was added to 3 ml of liquid medium in 35 mm 6-well plates (Corning #25810-6). After an additional week, each 4 ml suspension was transferred to a 125-ml baffled culture flask containing 30 ml of liquid medium. Cells from some genotypes, including New Harmony, proliferated readily when callus was transferred directly to suspension culture flasks.

After experimenting with several sampling and monitoring techniques to measure culture growth, A_{700} and media conductivity were chosen for ease of use and reliability. Two or 3 replications were sampled for each time point in each experiment. To determine suspension absorbance, cells were diluted with distilled water and A_{700} was read in a Beckman DU7400 (Beckman Instruments, Inc., Fullerton, CA, USA) or a Novaspec (Pharmacia-LKB, Piscataway, NJ, USA) spectrophotometer. To determine changes in medium conductivity one ml of suspension was centrifuged at 4 °C at 16 000 $\times$ g for 5 min to clear cells and debris. Conductivity of the cleared medium was measured with a YSI Model 35 Conductance Meter and #3417 dip cell (Yellow Springs Instrument Co., Yellow Springs, OH, USA).

Transfer methods, including culture intervals from late lag to early stationary phase, and inoculum densities were examined to determine which conditions resulted in predictable cell growth. Different methods resulted in wide variations in the duration of the lag phase of the subsequent cultures. Subculture transfers just after mid-log phase resulted in the lowest durations of lag phase (data not shown). The volume of culture transferred also had a significant effect on lag phase duration, as shown in Figure 1. Cell growth entered log phase significantly earlier when a larger volume of cells was subcultured. We observed that the use of 2 ml inoculum from just beyond mid-log phase of cell growth minimized the lag phase for each of the cell lines.

Overall cultured cell biology was similar for every elm genotype. Examination of proliferating cells at various phases in the suspension culture initiation indicated that elm cultures were essentially homogeneous after 5 subculture passages. The overall appearance of the cell populations was retained throughout growth, as single cells or loose cell clusters.

Media conductivity measurements, although indirect indicators, represent a rapid, simple method of determining the growth phase of plant cell suspension cultures (Hahlbrock and Kuhlen, 1972). As expected, medium conductivity decreased inversely with the increase of cell number and A_{700}, from 6 mmhos to a minimum of 2–2.5 mmhos. Unexpectedly, media conductivity of suspension cultures of each elm genotype increased after reaching a minimum (Figure 1), in contrast to the results seen with other cultured plant cells (Hahlbrock and Kuhlen, 1972; Kwok et al., 1992). This conductivity increase, seen in late log or early stationary phase, did not coincide

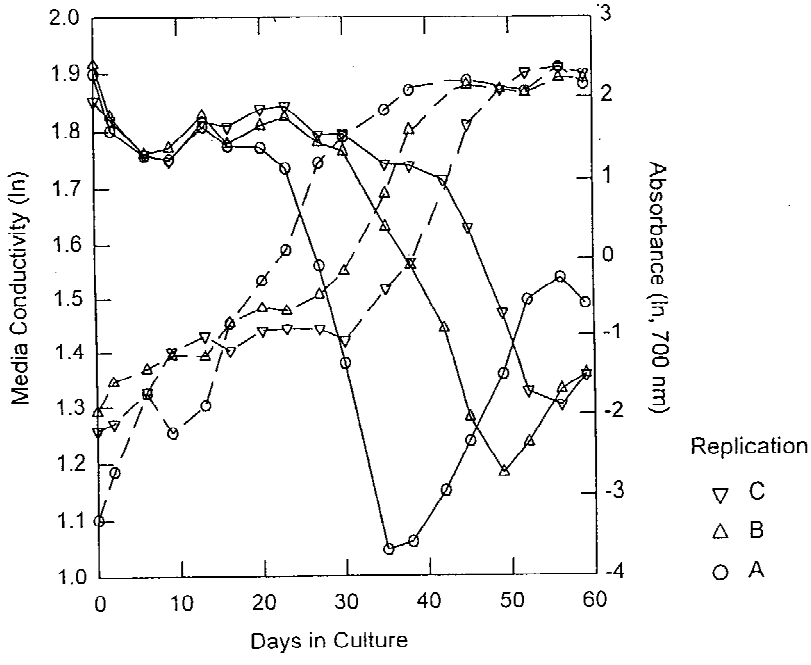

Figure 1. Growth characteristics of 'New Harmony' American elm suspension cultures. Data points represent the ln A_{700} (broken lines) and ln mmhos of media conductivity (solid lines) of three replications initiated from late log cells at the same time but using different subculture volumes. Cultures represented in curves A (○), B (Δ), and C (▽) were initiated with 1.0 ml, 0.5 ml, and 0.25 ml, respectively.

Table 1. Contrasting growth parameters of elm suspension cultures[1]

Genotype	Passage[2]	Suspension absorbance			Media conductivity		
		RGR[3]	r^2	MSE	RGR	r^2	MSE
New Harmony	A1	0.152	0.975	0.055	N.A.		
	A2	0.110	0.972	0.051	−0.029	0.957	0.004
	A3	0.090	0.974	0.053	−0.063	0.963	0.003
	B1	0.173	0.982	0.037	−0.039	0.944	0.008
Seedling	A1	0.083	0.881	0.363	−0.042	0.936	0.003
	A2	N.A.			−0.033	0.941	0.006
	A3	0.066	0.919	0.130	−0.034	0.857	0.009
	B1	0.112	0.976	0.050	−0.041	0.941	0.004
New Harmony[4]	(all 4)	0.126	0.958	0.086*	−0.038	0.890	0.011*
Seedling	(all 4)	0.083	0.906	0.224	−0.037	0.921	0.005
New Harmony vs. Seedling[5]		0.102	0.923	0.176	−0.037	0.898	0.008

[1]Growth parameters derived from fitted models for each passage were contrasted with f-tests. An * indicates significant differences at $\alpha = 0.05$.
[2]The first three passages (A1, A2, and A3) used cells from one initiated cell line and the fourth passage (B1) was from a cell line separately initiated from the same genotype.
[3]Weighted mean relative growth rate for the fitted models.
[4]Model used to test differences between passages within the New Harmony genotype.
[5]Model used to test differences between the New Harmony and the Seedling genotypes.

with a significant loss of cell viability, assessed by fluorescein diacetate staining, but may be an indication of altered physiological status. Cells subcultured in mid-stationary phase were significantly slower to enter growth than were cells from any time during the log phase. Notably, New Harmony cells and medium showed detectable browning immediately after entry into stationary phase while browning of the other genotypes was delayed. The media conductivity increase was significantly higher in the New Harmony media than that of the elm seedling or of the other elm genotypes.

We contrasted the cell growth results of four independent passages using cells from disparate sources: a mature, DED-tolerant American elm (New Harmony) and a DED-susceptible elm seedling. In each case the first three passages (A1, A2, and A3) used cells from one initiated cell line and the fourth passage (B1) was from another cell line separately initiated from the same genotype. Conventional comparisons of relative growth rates are assessed at a single point along the growth curve of a culture. By modeling the growth curves of the different cultures, the entire curves may be compared, not just the inflection points. Growth models were constructed as modified Richards functions (Richards, 1959) using data from the two independent methods: light scattering of cell suspensions (A_{700}) and conductivity of cell-free media.

Mean relative growth rates (RGR), corrected r^2 values, and mean square error (MSE) values were calculated for each model for growth rate comparisons (Table 1). The fitted models for the four New Harmony passages were significantly different from each other for both A_{700} and media conductivity, while the models for the seedling passages were not statistically different. However, data points of the New Harmony passages have smaller variances, as noted by the MSEs in Table 1, giving the models a narrower confidence band which results in a much stricter test of similarity. Even with differences in the individual New Harmony passages, the model of the combined passages achieved a very good fit attaining a 0.958 r^2 value, indicating that the model explains 95.8 percent of the variation in the data points of all passages. There were no significant differences between the combined passage models of the two genotypes. Contrasts of growth models using data from either the direct cellular assay (absorbance) or the indirect assay (media conductivity) resulted in identical conclusions. We conclude that changes in absorbance and conductivity followed a reproducible pattern within a growth cycle and between growth cycles for each American elm genotype. Similar results were seen for models based on cell concentration using hemacytometer counts (not shown).

The number of cultured cell lines maintained by continuous subculturing has been expanded to include additional DED-tolerant and DED-susceptible genotypes. Data for cell growth parameters of the cell lines from additional American elm genotypes were superimposed on the consensus growth curve models with no significant change in variance. We intend to describe cellular components of DED-tolerance by contrasting cellular reactions to secretions and elicitors derived from *Ophiostoma novo-ulmi*, the DED fungus. Significant distinctions in growth rates and other cellular parameters during routine growth of subculture replicates or of the different elm genotypes would preclude such comparisons. We conclude that this culture method will be useful for studies on the molecular interaction of American elms and *O. novo-ulmi*.

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