IN VITRO CULTURE OF AMERICAN ELM ANTHERS

by M. Keith Redenbaugh, graduate student, SUNY College of Environmental Science and Forestry, Syracuse, NY 13210; David F. Karnosky, forest Geneticist, the Cary Arboretum of the New York Botanical Garden, Millbrook, NY 12545; and Robert D. Westfall, research associate, SUNY College of Environmental Science and Forestry, Syracuse, NY 13210

ABSTRACT. The objectives of this study were to examine the suitability of 14 nutritive media and five stages of microspore development for the culture of American elm anthers. Flower buds of branches from two trees were forced in the laboratory during February, March, and April. Anthers from these trees were plated on a total of 1262 petri dishes (five anthers per dish) to test the different media and the various development stages. Cultures were screened for production of aggregates of enlarged, multinucleate microspores. The frequency of formation of these aggregates ranged from 0 to 50 percent depending on the stage of anther development and on the particular medium. Attempts to induce cell division in the enlarged microspores have not yet been successful.

The Dutch Elm Disease has seriously affected the utility of the American elm (*Ulmus americana* L.) as a shade and ornamental tree. Attempts to hybridize American elm with disease-resistant Asian elms have not been successful (Townsend 1975). A major reason for this failure is probably the difference in chromosome number. All elm species have 28 chromosomes except for American elm, a natural tetraploid with 56 chromosomes. The possibility exists, therefore, that an equalization of the chromosome number could facilitate an interspecific hybridization program. One approach has been to double the chromosome number of the 28-chromosome elm species. This has been successfully accomplished by treating Siberian elm (U. *pumila* L.) seedlings with colchicine (Dermen and May 1966). In a second method, the chromosome number of American elm could be reduced to 28 by anther culture. This method can either produce a plantlet directly from the microspores or induce haploid callus formation. The callus is then available for shoot induction or for use in somatic cell hybridization.

Cultured anthers of a number of species have produced plantlets and callus tissue (Sunderland 1974). However, no successful attempts to produce callus tissue or plantlets from American elm anthers have been reported in the literature, Whole plants have been propagated from callus derived from American elm hypocotyl tissue (Durzan and Lopushanski 1975).

This study was designed to evaluate different media and stages of flower development for haploid callus formation. Additionally, tree-to-tree variation in anther culture response was examined for eight American elm trees.

MATERIALS AND METHODS

Ten trees from two general areas in Syracuse, New York were chosen as sources of anthers. Five trees (numbers 14, 58, 98, 121, 151) were from the Ulmus Test Area (Progress Report: Forest Tree Improvement at State University College of Forestry, Syracuse, New York, 1962, pages 54-60). The other five trees (numbers 43-313-2, 15-139-1, 15-155-1, 16-120-1, and 15-142-1) were selected from street locations provided by Dr. Paul Manion's urban tree survey of Syracuse. Two trees (121 and 151) were used to examine media and stages of development differences. Anthers from the other eight trees were used for studying tree-to-tree differences. From early February to early March branches were brought directly into the laboratory to force flower development. Dehiscence occurred within five days. After March ninth branches were stored at 4°C to retard natural flower development. This provided material into early April.

Table 1 lists the 14 media examined in this study. These media were chosen from published accounts which reported successful anther or elm culture, or which were recognized for their basic utility in plant tissue culture research. For cases where it was used, agar was autoclaved separately from other compounds. Several chemicals were added after autoclaving by cold sterilization methods (Cornwall syringe with Gelman filters). Autoclaved activated charcoal was thoroughly mixed in the appropriate media just prior to agar gelling. An automatic pipette was used to fill the 3.5 cm petri dishes to a uniform level. The filled dishes were stored in the original, plastic, sterile shipping bags.

The forced flower buds were excised from the branches, disinfested in 6% sodium hypochlorite for five minutes, and subsequently rinsed in autoclaved distilled water. Anther dissection on plates containing old media was done immediately after disinfestation. The anthers were popped out of the anther

Table 1. Sources of the Media (and Their Additives) Used in Testing the in vitro Growth of American Elm Anthers.

SOURCE	MEDIA.	ADDITIVES				
		Agar	Coconut Water	Activated Charcoal	An.agnostakis Comsounds1	Other
Durzan and Lopushanski 1975	1	0.5%	---	---	---	---
Gresshoff and Do,' 1974	2	0.8%	---	---	---	---
Murashige and Skoog Shoot Multiplication Medium (Gibco)	3	1.0%	10%		+	---
Kitsch and Nitsch 1969	4	0.8%	10%	---	---	---
	5	0.8%	10%	0.2%	___	---
	6	0.8%	10%	0.2%	---	0.02mg/1 Kinetin
	7	0.8%	10%	0.2%	---	0.002mg/1 Zeatin
	8	---	10%	---	---	---
	9	0.8%	---	---		2
	10	0.8%	---	0.2%	---	2
Reinert and White1956	11	0.8°	---	---	---	---
White 1963	12	1.0%	10%	---	+	3
	13	1.0%	10%	0.2%	+	3
	14	---	10%	---	+	3

[1] Anagnostakis compounds: 0.2% asparagine, 0.5ppm thiamine, and 800ppm yeast extract.

[2] Additives suggested by David Thompson (personal communication-): 2mg/1 Kinetin and 2mg/1 IAA.

[3] 0.001mg/1 copper sulfate, 1.0mg/1 IAA, 5.0mg/1 ferric sulfate.

sack with alcohol-sterlized forceps and. placed, with the connective side down, on the appropriate media. The petri dishes were then sealed in a large plastic container and incubated without light at room temperature.

The anthers were plated at the following five stages of development: pollen mother cell, tetrad, uninucleate, pollen grain, and late pollen grain. Pollen mother cell refers to a premeiotic state (2n). Tetrad is just after meiosis with the microspores joined in clusters of four (n). The uninucleate-stage consists of individual-microspores prior to the first mitosis. Pollen grains have undergone mitosis. Late pollen grains simply refer to a state several hours after the pollen grain stage. The stage of flower development was determined by squashing and staining anthers in Giemsa stain (Karnosky and Setliff 1977).

In the study of media and developmental stage differences, two plates of each medium were used for each of the two study trees and five stages of development. Five whole anthers were placed on each plate. In addition two media, Nitsch and Nitsch with kinetin and Nitsch and Nitsch without agar, were plated with isolated microspores. Microspores were isolated by crushing the anthers with a forcep and spreading them over the media.

The examination of tree-to-tree differences was begun after high aggregate frequencies were found on several media and at two stages of development (uninucleate and pollen grain). Two of these media (Nitsch and Nitsch with zeatin and White with charcoal) were used to test variation in response for the eight trees. Five anthers from each tree were plated on each medium at each stage of flower development. Again, there were two replicates of each plating.

Each anther was scored for aggregate production. The media and stage of development investigation contained 6340 anthers while 800 anthers were plated in the tree-to-tree variation study. Responses for these two groups were organized into 3-way contingency tables. Aggregate frequency differences among contingency table components were tested with the G-statistic (Sokal and Rohlf 1969).

RESULTS AND DISCUSSION

Callus tissue is generally defined as a non-differentiated mass of dividing cells. In this investigation, masses of cells were observed to emerge from inside the anther through the line of dehiscence

for certain combinations of media, stages of development, and tree sources. These aggregates of cells occurred in two general shapes: smooth clumps and irregular, filamentous protuberances. Samples from smooth clumps were examined cytologically and found to be composed of enlarged, multinucleate pollen grains that had not undergone cell division. The latter type of aggregate was not examined due to its rarer occurrence and the desire not to destroy any possible callus cultures. Because no cell division was observed, the structures were labeled "aggregates" rather than callus. Of the 7140 anthers plated, 11% contained aggregate formations.

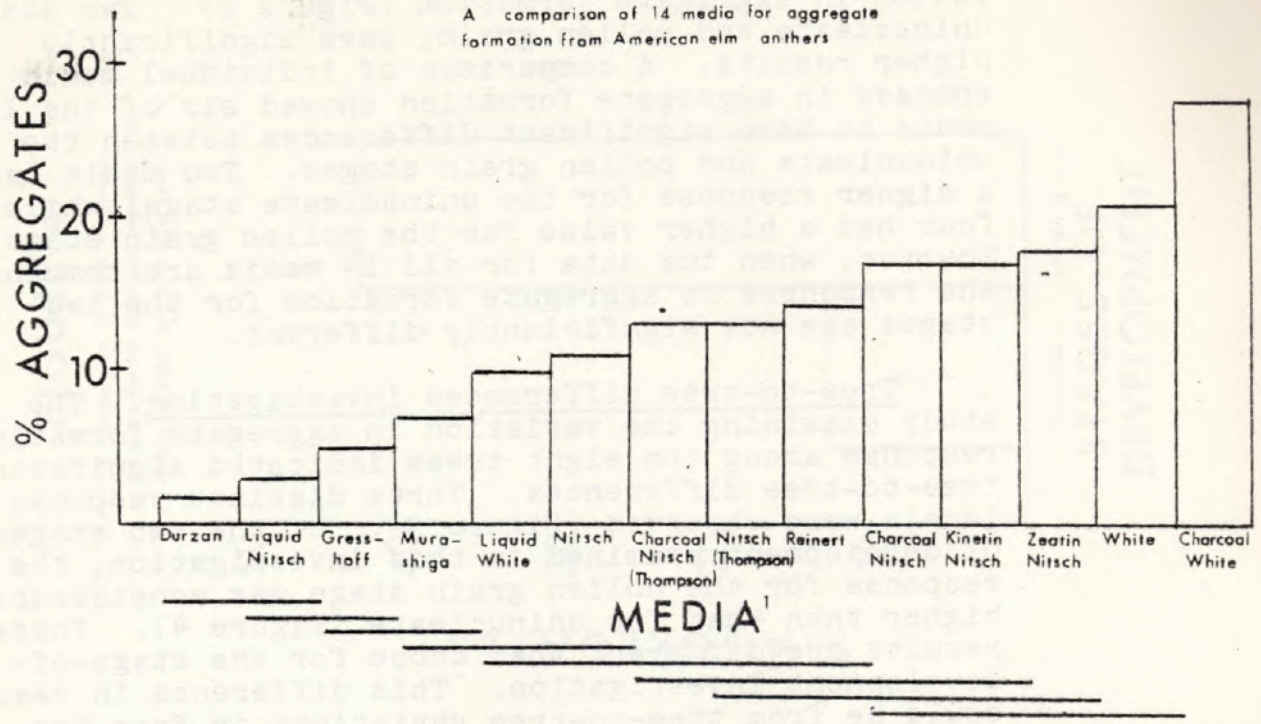
Media differences investigation. The examination of media differences showed the highest percentage of aggregate formation (28%) on White's medium with 0.2% activated charcoal. The figure is significantly higher than the results for the other 13 media. A comparison of the 14 media indicated that the Nitsch and Nitsch and the White's media gave the highest percentages of aggregate formation (Figure 1). All values were based on results from the five stages of development. The low results for the two liquid media (Nitsch and Nitsch, and White) were greatly affected by excessive spillage. The 3.5 cm petri dishes were not suitable for liquid cultures. The medium that gave the lowest results (Durzan and Lopushanski) maintained the anthers in their original color, shape, and size throughout the experiment. On all other media, the anthers gradually became brown with widespread dehiscence or shriveling.

The 0.2% activated charcoal provided significant increases of aggregate formation in two of the three cases where it was used (White, and Nitsch and Nitsch). This corresponds with published results (Anagnostakis 1974, and Wang and Huang 1976) in which charcoal stimulates plantlet production. These authors suggest that the activated charcoal absorbs unidentified toxic chemicals.

Microspores were isolated from the anthers in order to closely follow aggregate/callus formation and to remove the problem of anther wall callusing. However, the microspores failed to undergo any development on the two media tested. One of these media, Nitsch and Nitsch with kinetin, had a fairly high aggregate formation (17%) when whole anthers were used (Figure 1). This suggests endogenous substances supplied by the anther wall that are missing in the medium upon which the isolated microspores are placed. This problem has been overcome by using an anther extract produced by boiling and grinding anthers with subsequent centrifugation to give a supernatent which can then be filtered sterilized and added to the basal medium for anther culture. Datura plantlets have been produced using this method (Nitsch 1977).

FIGURE 1

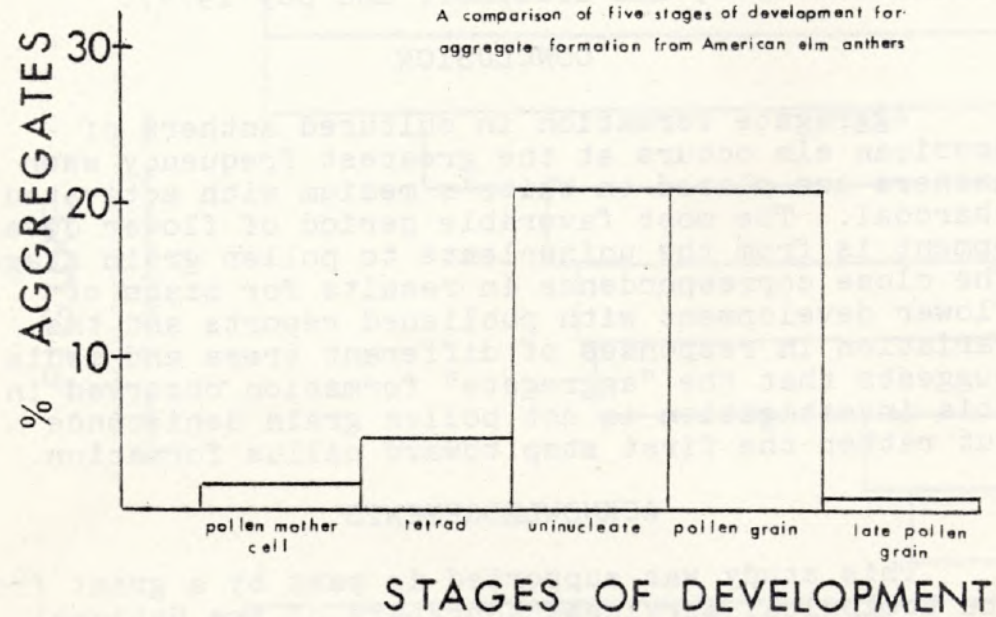
A comparison of 14 media for aggregate formation from American elm anthers



[1] Components connected by bars are not significantly different

FIGURE 2

A comparison of five stages of development for aggregate formation from American elm anthers



Stage of development investigation. The five stages of flower development showed substantial variation in aggregate formation (Figure 2). Two stages, uninucleate and pollen grain, gave significantly higher results. A comparison of individual media responses in aggregate formation showed six of the 14 media to have significant differences between the uninucleate and pollen grain stages. Two media had a higher response for the uninucleate stage, while four had a higher value for the pollen grain stage. However, when the data for all 14 media are combined, the responses to aggregate formation for the two-stages are not signficiantly -different.

Tree-to-tree differences investigation. The study examining the variation in aggregate formation response among the eight trees indicated significant tree-to-tree differences. Three distinct response levels were observed (Figure 3). Of the two stages of development examined in this investigation, the response for the pollen grain stage was considerably higher than that for uninucleate (Figure 4). These results are different than those for the stage-of-development investigation. This difference in results could be from tree-to-tree variations or from the cold storage treatment (since all anthers used in this particular examination were held at 4°C). Although there are some contradictions in results, they correspond very closely with most published reports which show that the uninucleate to pollen grain interval gives the best response whether in callus activity or plantlet formation (Sunderland 1977, Wernicke and Kohlenbach 1976, and Gresshoff and Doy 1974).

CONCLUSION

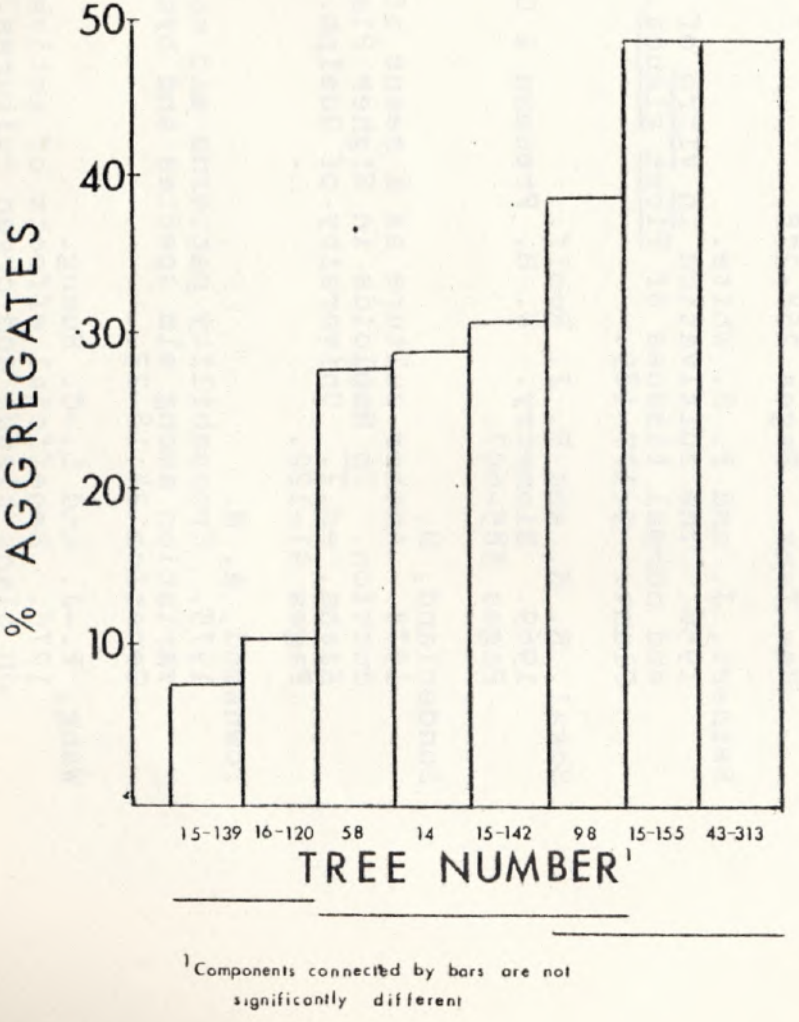
Aggregate formation in cultured anthers of American elm occurs at the greatest frequency when anthers are plated on White's medium with activated charcoal. The most favorable period of flower development is from the uninucleate to pollen grain stage. The close correspondence in results for stage of flower development with published reports and the variation in responses of different trees and media suggests that the "aggregate" formation observed in this investigation is not pollen grain dehiscence but rather the first step toward callus formation.

ACKNOWLEDGEMENTS

This study was supported in part by a grant from the Ecological Services Laboratory of the National Capital Parks Service. The authors appreciate the helpful suggestions and assistance of Ms. Sande Anagnostakis, Mr. Dan Apple, Dr. Paul Manion, and Mr. David Thompson.

FIGURE 3

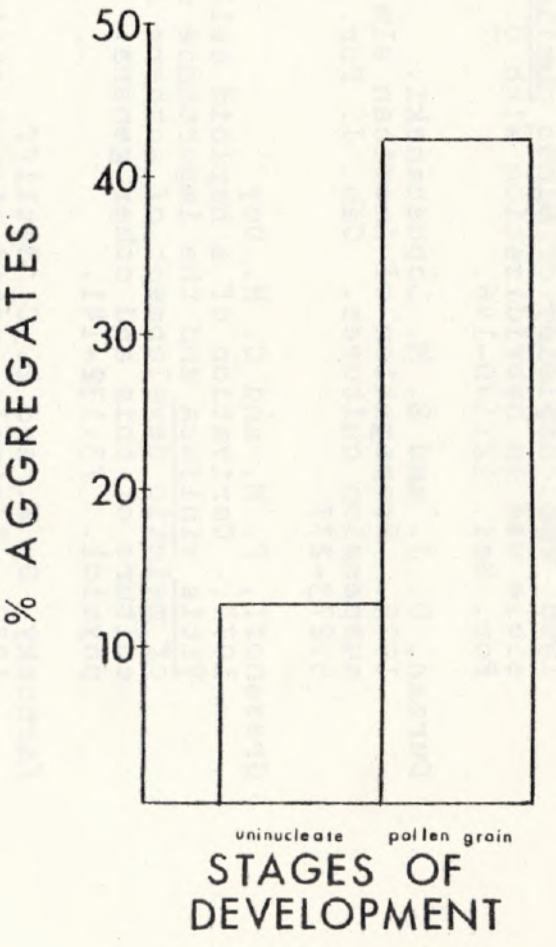
A comparison of aggregate formation from anthers of eight American elm trees



[1] Components connected by bars are not significantly different

FIGURE 4

A comparison of two stages of development for aggregate formation from anthers of eight American elm trees



LITERATURE CITED

Anagnostakis, S.L.
1974. Haploid plants from anthers of tobacco--enhancement with charcoal. Planta 115:281-283.

Dermen, H. and C. May.
1966. Colchiploidy of Ulmus pumila and its possible use in hybridization with U. americana. For. Sci. 12:140-146.

Durzan, D. J. and S. M. Lopushanski.
1975. Propagation of American elm via cell" suspension cultures. Can. J. For. Res. 5:273-277.

Gresshoff, P. M. and C. H. Doy
1974. Derivation of a haploid cell line from Vitis vinifera and the importance of the stage of meiotic development of anthers for haploid culture of this and other genera. Z. Pflanzenphysiol. 73:132-141.

Karnosky, D. F. and E. C. Setliff
1977. A simple, rapid giemsa staining technique for routine study of tree chromosomes. Can. J. For. Res. 7:-435-436.

Nitsch, C.
1977. Culture of isolated microspores. In Plant Cell, Tissue, and Organ Culture (J. Reinert and Y. P. S. Bajaj, eds.). Springer-Verlag. New York. Pages 268-278.

Reinert, J. and P. R. White.
1956. The cultivation in vitro of tumor tissues and normal tissues of Picea glauca. Physiol. Plant. 9:177-189.

Sokal, R. R. and F. J. Rohlf.
1969. Biometry. W. H. Freeman & Co. San Francisco. Pages 585-607.

Sunderland, N.
1974. Anther culture as a means of haploid induction. In Haploids in Higher Plants (K. J. Kasha, ed.7 University of Guelph. Guelph. Pages 91-122.

Townsend, A. M.
1975. Crossability patterns and morphological variation among elm species and hybrids. Silvae Genetica 24:18-23.

Wang, P.-J. and L.-C. Huang.
1976. Beneficial effects of activated charcoal on plant tissue and organ cultures. In Vitro 12:260-262.

Wernicke, W. and H. W. Kohlenbach.
1976. Investigations on liquid culture medium as a means of anther culture in Nicotiana. Z. Pflanzenphysiol. 79:189-198.

White, P. R.
1963. The Cultivation of Animal and Plant Cells. 2nd ed. Ronald Press Co. New York.