HRI's Mission:

To direct, fund, promote and communicate horticultural research, which increases the quality and value of ornamental plants, improves the productivity and profitability of the nursery and landscape industry, and protects and enhances the environment.

Effect of Elm Selection, Explant Source and Medium Composition on Growth of *Ophiostoma ulmi* on Callus Cultures[1]

Subhash C. Domir,[2] Lawrence R. Schreiber,[2] Jann M. Ichida,[2] and Steven M. Eshita[3]
USDA-ARS
U.S. National Arboretum
Ohio Research Site
359 Main Road
Delaware, OH 43015

Abstract

We examined the effects of elm selection, explant source and media composition on growth of the Dutch elm disease (DED) fungus *Ophiostoma ulmi* on callus cultures. Calluses were generated from leaf and stem tissue of an American elm (*Ulmus americana* L.) seedling (A), susceptible to the disease; an American elm selection 8630, resistant to the disease; and a Siberian elm (*U. pumila* L.) seedling, also resistant to DED. Calluses were generated on modified Murashige-Skoog (MMS) medium, either with (MMSC) or without coconut milk. Explant source did not affect the fungal growth rate on the callus. Rate of *O. ulmi* growth on American elm A callus was similar on both media; on Siberian and 8630, fungal growth rate was more rapid on callus cultured on MMS than on MMSC. However, in the absence of callus tissue, *O. ulmi* growth on MMSC medium was more than five times as rapid as it was on MMS. We observed significant interactions between explant source and selection, and between medium and selection. Fungal growth was always more rapid on American A, and American 8630 then on Siberian. Scanning electron microscopy revealed heavy fungal sporulation on American A, slight on Siberian and none on American 8630. High performance liquid chromatography analysis showed that the secondary metabolic profiles were distinguishable for callus tissue versus explant tissue, but were similar for calli generated from different explant sources.

Index words: tissue culture, Dutch elm disease, disease resistance, *Ulmus americana*, scanning electron microscopy, high performance liquid chromatography

Significance to the Nursery Industry

Dutch elm disease (DED) has destroyed elm trees extensively all around the world. Using conventional techniques to screen elms resistant to DED is a long and arduous process. This study was conducted to develop a model system for accelerating identification of disease resistant elms. Callus cultures of susceptible and resistant elms were initiated on two different modified Murashige-Skoog media, one with and one without coconut milk. Leaf and stem tissue were used as explant sources. Explant source did not affect the growth of the DED fungus on callus cultures. However, media composition did affect fungal growth depending upon the elm selection. These results suggest that in tissue culture studies, environmental and genetic factors must be taken into account in order to correlate results with intact plants.

Introduction

Tissue culture systems offer unique opportunities to study host-pathogen interactions. Although concerns have been expressed regarding positive correlations between tissue culture and *in-vivo* systems (3), such correlations have been demonstrated (1, 2, 7, 8, 9, 10). Fungal colonization of susceptible and resistant callus is affected by temperature, inoculum concentration, and media (9, 11, 12, 14, 15). Control of medium composition and other environmental factors can influence correlations in host-pathogen interactions in callus and intact plants (6, 10, 11).

In an earlier study (4), we reported the influence of temperature and inoculum concentration on fungal colonization of callus from susceptible and resistant elms. This study describes the influence of elm selection, media composition, and explant source on the growth of *O. ulmi* on elm callus cultures.

Materials and Methods

Callus cultures were developed from young leaf and stem tissue from American elm (*Ulmus americana* L.) (A), susceptible to DED; American 8630 (8630), an American elm resistant to DED; and Siberian elm, (*U. pumila* L.) (S), an elm species resistant to DED (17). Trees were maintained in a greenhouse under 16-hour day length. Two media were used to initiate the callus cultures. The first (MMS) contained Murashige and Skoog (15) salts supplemented with 200 mg/l casein hydrolyzate, 8 um 6-benzyladenine, 0.5 um 2,4-dichlorophenoxyacetic acid, 3% (w/v) sucrose, and 0.7% (w/v) Difco Bacto agar (pH 5.7). The second (MMSC) had the same ingredients with the addition of 10% (v/v) coconut milk. Callus cultures were routinely subcultured every 6–8 weeks onto fresh half-strength MMS or MMSC medium. The cultures were maintained in the dark at 22 ± 1°C (72 ± 2°F). The cultures were maintained for six months before inoculations were carried out. Fifteen days before inoculation with fungal spores, small callus pieces, approximately 15 mm (0.6 in) in diameter, were transferred from stock cultures to 60 × 15 mm (2.4 × 0.6 in) Petri

[1]Received for publication October 4, 1991; in revised form December 26, 1991. Portion of this work was funded by a grant from the Horticultural Research Institute, 1250 I St. N.W., Suite 500, Washington, DC 20005. The authors thank Dr. T.L. Graham and Mrs. Jacquelyn Thomas for their assistance in experimental work.

[2]Plant Physiologist, Plant Pathologist, and Microbiologist, resp.

[3]Microbiologist, USDA-FS, N.E. Forest Experiment Station, Delaware, OH.

plates containing either half-strength MMS or MMSC medium.

PMP1, an aggressive *O. ulmi* isolate, was grown in 100 × 15 mm (4.0 × 0.6 in) Petri dishes on potato dextrose agar (PDA: Difco, Detroit, MI) covered with water-permeable cellophane, and incubated at 24°C (76°F) for one week. Spores were washed from the surface of the plates with sterile distilled water and diluted to 2 × 10^6 spores/ml. Ten replicates each of calluses from A, 8630, and S elm leaf and stem tissue were inoculated by placing a 1.5 mm (0.06 in) on each side filter paper triangle containing 20 ul of a spore suspension on the top, center of the callus (4). Controls included uninoculated filter paper triangles placed on the callus tissue. Inoculated filter paper triangles were placed on half-strength MMS and MMSC media plates to compare fungal growth on the two media. Appropriate controls with uninoculated filter paper triangles were also provided. Fungal growth was reported as percent of callus diameter. The average diameter of the calluses and fungal colonies were determined by measuring along two axes drawn perpendicular to each other on the bottom of the Petri plate, intersecting at the filter paper triangle. The diameter of the calluses and fungal colonies were measured 72 hours after inoculation. The size of the filter paper triangle (1.5 mm; 0.06 in) was subtracted from each diameter reading. Fungal growth was analyzed using analysis of variance (ANOVA), following arc sin transformation. Analyses of variance with associate 'P' values were obtained using the software program from the Statistical Analysis System (SAS) (18). Comparison of fungal growth on the two media was carried out using the Duncan-Waller multiple range test.

Calluses from all treatments were sampled 72 hours after inoculation for scanning electron microscopy (SEM) examination. Samples were taken from the point of inoculation to the edge of the *O. ulmi* growth front and fixed for 18 hours at 4°C (7°F) in 3% glutaraldehyde buffered to pH 7.2 in 0.05 M cacodylate buffer. Samples were rinsed three times in the same buffer for 4 hours at 4°C (7°F), dehydrated in a graded ethanol series, critical-point dried with CO_2, mounted and coated with gold (200–300 nm thickness) with a Hummer V sputter coater. The specimens were examined using a Leica 962 scanning electron microscope at 14.5 kV.

High performance liquid chromatography (HPLC) analysis of soluble aromatic secondary metabolites from tissues was carried out using the method of Graham (5) with the following modifications. Extractions used 0.2 ml of 80% ethanol per 0.1 g tissue. For analysis, NovaPak C_{18}, 8 mm × 10 cm (0.32 × 4 in) Radial-Pak cartridge (Waters, Milford, MA) was used with a flow rate of 2.5 ml/min and output monitoring at 236 nm via a Waters 490 detector.

Results and Discussion

The ANOVA of fungal growth on callus is presented in Table 1. Multiple comparisons among various treatments were made using least squares means. Rates of fungal growth were significantly different among the three selections. *O. ulmi*, grew much slower on Siberian elm than on either A or 8630 (Figs. 1 and 2). SEM studies indicated that fungal sporulation was greatest on A, less on S and lacking on 8630 (Fig. 3A, B, C). In addition, mycelial growth was less dense on S (Fig. 3C) than on either A (Fig. 3A) or 8630 (Fig. 3B).

Table 1. Analysis of variance for fungal growth on calli from three elm selections inoculated with *Ophiostoma ulmi*.

Source of Variation	DF	MS	F	P
Selection	2	19127.13	15.19	0.0001***z
Medium	1	11874.79	9.43	0.0027*
Explant	1	1705.45	1.35	0.2471
Selection × Medium	2	8055.00	6.40	0.0024*
Selection × Explant	2	6844.00	5.44	0.0056*

zAsterisks (* and **) indicate significant differences at P = .01 and P = .001, respectively.

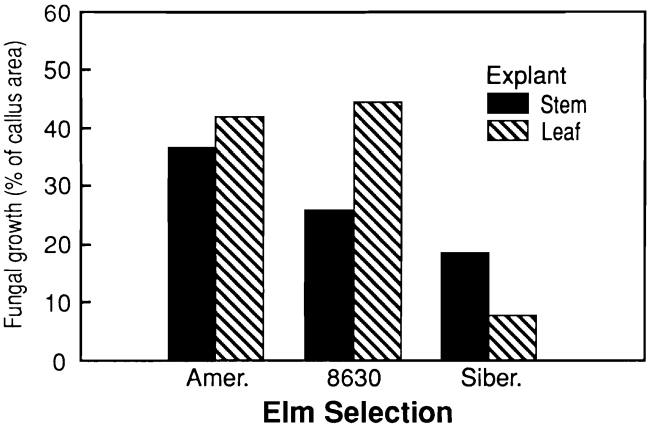

Fig. 1. Growth response of fungus *O. ulmi* on callus generated from leaf and stem tissues of three elm selections: susceptible American (A), resistant American (8630), and resistant Siberian (S), 72 hours after inoculation. Callus tissue was generated on modified Murashige-Skoog medium with (MMSC) and without (MMS) coconut milk. Fungal growth, measured as colony diameter, is expressed as percent of callus diameter.

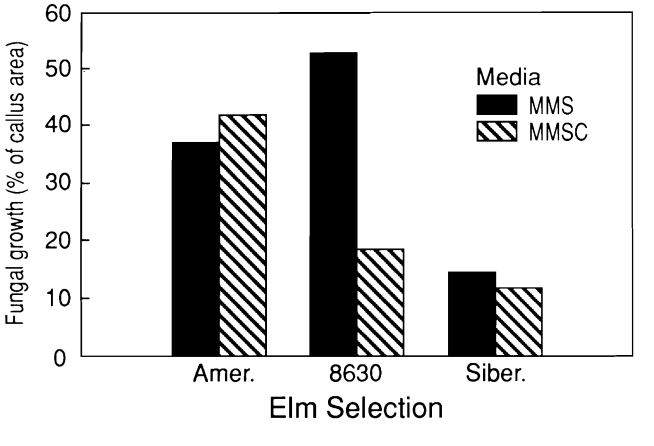

Fig. 2. Growth response of fungus *O. ulmi* on callus generated from elm leaf and stem explants of susceptible American (A), resistant American (8630), and resistant Siberian (S). Callus tissue was generated on two media, MMS and MMSC. Diameter of fungal colony, measured 72 hours after inoculation, is expressed as percent of callus diameter.

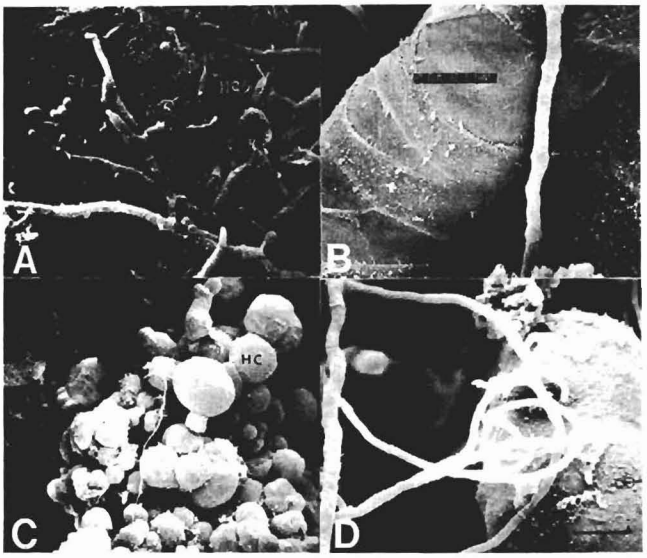

Fig. 3. Scanning electron micrographs of *Ophiostoma ulmi* on callus tissue from elm selections 72 hours after inoculation. (A, B, C on MMSC medium, D on MMS medium). A.) Susceptible American elm (A). Abundant conidia (C) and mycelium attached to host cells (HC). B.) Resistant American elm (9630). Sparse mycelium (arrow) weakly associated with host tissue cells. Note lack of sporulation. C.) Siberian elm. Sparse fungal colonization and sporulation on resistant callus tissue. D.) Resistant American elm (8630) on MMS medium. More fungal growth is evident on callus generated on MMS medium than on MMSC medium (Fig. 3B). Bars represent 10 um.

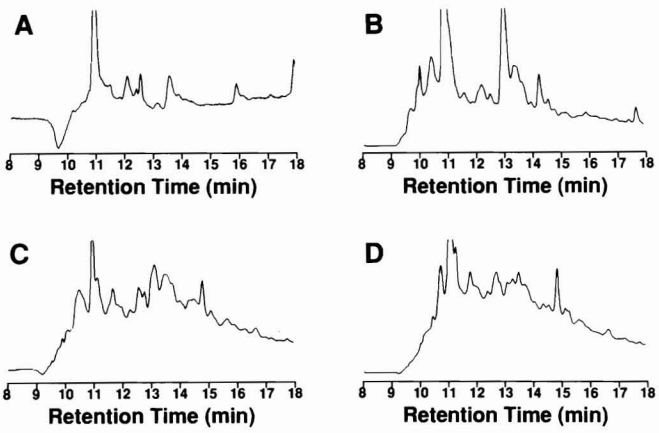

Fig. 4. HPLC metabolite profiles of tissues from susceptible American elm. A, callus generated from leaf tissue; B, leaf mesophyll; C, leaf midrib; D, stem. Metabolites were extracted from tissues and HPLC-analyzed as described in "Materials and Methods."

Explant source had no overall effect on the rate of fungal growth. However, the interaction between selection and explant source was significant (Table 1). Thus, the fungus grew more slowly on callus generated from stem rather than from leaf tissue of A and 8630, but more rapidly on stem than leaf callus of S (Fig. 1). No difference in fungal density was seen by SEM on callus from leaf or stem.

The fungal growth rate on callus tissue from A or S cultured on either MMS or MMSC media was not significantly different, while fungal growth rate on 8630 cultured on MMSC was significantly less than on MMS (Fig. 2). SEM observations indicated that hyphal colonization on 8630 callus was also less on MMSC than on MMS (Fig. 3B and D). A significant interaction was observed among calluses from different selections and the different media (Table 1).

In all instances fungal density and sporulation were less on 8630 and S than on A (Fig. 3A, B, C, D).

The average colony diameter of *O. ulmi* in the absence of callus on MMS and MMSC was 4 and 22 mm respectively, a significant difference.

The HPLC profile of extract from callus derived from leaf tissue of A (Fig. 4A) indicated that some, but not all, of the soluble aromatic secondary metabolites characteristic of leaf mesophyll, leaf midrib, and stem (Fig. 4B, 4C, and 4D, respectively) were expressed. A major metabolite, common to all four samples, eluted at 11 minutes. The explant tissues yielded chromatograms with features distinct from each other, as well as from the leaf-derived callus. However, chromatograms from leaf- and stem-derived callus showed that they produced secondary metabolities similar in nature (data for stem derived callus not shown).

Haberlach et al. (6) reported that increasing the kinetin concentration of a medium increased colonization of resistant tobacco callus by the fungus *Phytophthora parasitica* var. *nicotianae*. The type of cytokinin affected the extent of fungal colonization. This effect was reversed by an increase in indoleacetic acid (an auxin) concentration. However, Miller et al. (15) showed that decreased kinetin concentration increased development of *Phytophthora megasperma* on alfalfa callus. We found that coconut milk, a rich source for growth hormones, stimulated fungal growth in the absence of callus. However, it reduced the rate of fungal growth on callus tissue from 8630 but not on callus tissue from A or S. This confirms the finding of others (6, 15) that the effects of media on fungal growth on callus varies with the tissue from which it was derived.

We investigated the use of stem tissue as an explant source for callus since DED is a vascular and not foliar disease. However, the data indicated that overall explant source did not significantly affect the rate of fungal growth on callus tissue. There was significant interaction between explant source and selection. Thus, fungal growth was greater on callus derived from stem tissue than leaf tissue of S. No significant differences in fungal growth were observed on callus derived from either leaf or stem explant of A or 8630. However fungal density was greater on A than on 8630 as reported in our earlier study (4). This suggests that significant genetic differences exist among the three selections that may overshadow the response to explant source.

The callus from A leaf tissue expressed a secondary metabolite profile partially similar to leaf mesophyll, midrib and stem tissues. This might explain why fungus colonization of the callus derived from the leaf and stem explant sources was not significantly different.

This study suggests that the specificity of the genotype of the callus source is important in determining the rate of fungal growth. The inherent genetic make up of the source plant relating to disease resistance carries over to the callus derived from it even though environmental and cultural factors affect the fungal growth. Thus, rate of fungal growth over callus from A was more rapid than over that from S despite all environmental and cultural variables tested. This and other studies (9, 11, 12, 13, 14, 15) have indicated that under controlled culture conditions, the elm callus culture system may serve to identify resistant and susceptible germ-

plasm. Additional studies with intact plants are being carried out to further corroborate our results. Also, other chemical studies must be conducted to quantify the fungus in order to further strengthen our conclusions.

Literature Cited

1. Budde, A.D. and J.P. Helgeson. 1981. Phytoalexins in tobacco callus tissue challenged by zoospores of *Phytophthora parasitica* var. *nicotianae*. Phytopathology 71:206.

2. Deaton, W.R., G.T. Keyes, and G.B. Collins. 1982. Expressed resistance to black shank among tobacco callus cultures. Theor. and Appl. Genet. 63:65–70.

3. Dixon, R.A. 1980. Plant tissue culture methods in the study of phytoalexin induction. *In* Tissue Culture Methods for Plant Pathologists. Ingram, D.S. and Helgeson, J.P. (Ed.), p. 185. Blackwell Scientific Publications, Oxford.

4. Domir, S.C., L.R. Schreiber, and J.M. Ichida. 1991. Factors affecting host-pathogen interaction between elm callus cultures and *Ophiostoma ulmi*. J. Environ. Hort. 9:211–215.

5. Graham, T.L. 1991. A rapid, high resolution high performance liquid chromatography profiling procedure for plant and microbial aromatic secondary metabolites. Plant Physiol. 95:584–593.

6. Haberlach, G.T., A.D. Budde, L. Sequeira, and J.P. Helgeson. 1978. Modification of disease resistance of tobacco callus tissue by cytokinins. Plant Physiol. 62:522–525.

7. Helgeson, J.P., J.D. Kemp, G.T. Haberlach, and D.P. Maxwell. 1972. A tissue culture system for studying disease resistance: the black shank disease in tobacco callus cultures. Phytopathology 62:1439–1443.

8. Helgeson, J.P., G.T. Haberlach, and C.D. Upper. 1976. A dominant gene conferring disease resistance to tobacco plants is expressed in tissue cultures. Phytopathology 66:91–96.

9. Helgeson, J.P., A.D. Budde, and G.T. Haberlach. 1978. Capsidiol: a phytoalexin produced by tobacco callus tissue. Plant Physiology 61:58 (suppl.).

10. Holliday, M.J. and W.L. Klarman. 1979. Expression of disease reaction types in soybean callus from resistant and susceptible plants. Phytopathology 69:576–578.10.

11. Huang, J.S. and C.G. Van Dyke. 1978. Interaction of tobacco callus tissue with *Pseudomonas tabaci*, *P. pisi* and *P. fluorescens*. Physiol. Plant Path. 13:65–72.

12. Ingram, D.S. 1976. Growth of biotropic parasites in tissue culture. *In* Physiol. Plant Path. Heitefuss, R. and Williams, P.H. (Ed.) pp. 743–759. Springer-Verlag, Berlin.

13. Keen, N.T. and R. Horsch. 1972. Hydroxyphaseollin production by various soybean tissues: a warning against use of "unnatural" host-parasite systems. Phytopathology 62:439–442.

14. Latunde-Dada, A.O. and J.A. Lucas. 1986. Influence of temperature on host resistance and fungal sensitivity to medicarpin in Lucerne callus infected with *Verticillium albo-atrum*. Physiol. and Molec. Plant Path. 28:89–97.

15. Miller, S.A., L.C. Davidse, and D.P. Maxwell. 1984. Expression of genetic susceptibility, host resistance, and nonhost resistance in alfalfa callus inoculated with *Phytophthora megasperma*. Phytopathology 74:345–348.

16. Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant 15:473–497.

17. Pijut, P.M., S.C. Domir, R.D. Lineberger, and L.R. Schreiber. 1990. Use of cultural filtrates of *Ceratocystis ulmi* as a bioassay to screen for disease tolerant *Ulmus americana*. Plant Sci. 70:191–196.

18. SAS User's Guides: Statistics version 5 edition. 1985. SAS Institute, Inc. Cary, NC. 1240 pp.