

EFFECTS OF FREQUENT SUBCULTURING OF ELM CALLUS TISSUE ON GROWTH OF OPHIOSTOMA ULMI

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Abstract. The effects of the frequency of subculturing on growth of the Dutch elm disease fungus, *Ophiostoma ulmi*, on elm callus cultures were examined. Callus tissues were generated from leaf tissue of susceptible American elm, resistant American elms, and resistant Siberian elm. Half the cultures was maintained at 22° C and subcultured at six-week intervals; the other half was maintained at 4° C and not subcultured. The callus cultures were inoculated either with an aggressive or nonaggressive isolate of *O. ulmi*. The data showed absence of correlation between fungal growth on callus tissue and frequency of subculturing. This lack of correlation is attributable to lack of homogeneity in callus tissue due to induction of somaclonal variation.

American elm (*Ulmus americana*) was an important component of the North American urban environment for many years until the onset of Dutch elm disease (DED) in 1930. This disease, caused by the fungus *Ophiostoma ulmi*, has devastated the elm population.

Conventional techniques used to screen elms for resistance to DED have been time consuming and expensive; but emerging tissue culture technology may reduce resources needed for screening hosts against pathogens (1). Several researchers have shown that tissue cultures behave enough like in-vivo systems (2,4,11-14) to allow the use of the former in disease resistance tests; but others have raised concerns regarding this approach (5). Research findings suggest that temperature, inoculum concentration, medium composition, and other environmental factors can influence fungal colonization of susceptible and resistant callus tissue (6,7,13,15,17,19,20), and also affect correlations in host-pathogen interactions between callus and intact plants (10,14,15).

Elgersma and Overeem (9) observed that elms produce phytoalexins called mansonones in response to fungal infection or chemical injury. Hubbes and Jeng (16) showed that susceptible American elm callus cultures could be induced to

develop resistance to infection by *O. ulmi*. Jeng et al (18) attributed this induced resistance to accumulation of mansonones. Duchesne et al (8) reported that resistant elm, *U. pumila*, accumulated mansonones more rapidly and in higher amounts than did *U. americana*. Hubbes and Jeng (personal communication) have indicated that when callus cultures of elms are frequently subcultured, they begin to lose the capacity to produce mansonones, and thereby become susceptible to the DED fungus.

In earlier studies (6,7), tissue culture techniques were used to determine the effects of several environmental factors on growth of *O. ulmi* on callus cultures derived from susceptible and resistant elms. Our current goal is to identify the factors that affect correlations between in-vitro and in-vivo systems for consistently identifying levels of DED resistance in elms. The present study was conducted to determine if frequent subculturing of callus tissue affected fungal colonization of elm callus cultures.

Materials and Methods

Stock plants of American elm varying in susceptibility to DED and Siberian elm were propagated in the greenhouse from softwood cuttings obtained from mature trees. American elm selection A had been determined in previous tests to be susceptible to the disease while selection 8630 was highly resistant, and selection Del2 (Delaware #2) was intermediate in resistance. Siberian elm selection S was resistant in similar tests. Callus cultures were initiated from young leaves (22) on full-strength Murashige and Skoog (20) culture medium (MS), supplemented with 200 mg/l casein hydrolyzate, 8 M 6-benzylaminopurine, 0.5 M 2,4-dichlorophenoxy acetic acid, 3% (w/v) sucrose,

10% (v/v) coconut milk (Sigma Chemical Co.), and 0.7% (w/v) Difco Bacto Agar (pH 5.7). These cultures were initiated in October 1990, and maintained at 22 ± 1 C in the dark. In December 1990, the cultures were transferred to a fresh half-strength MS medium fortified with the same supplements indicated above. All of the subsequent subculturing was carried out on this medium. In February 1991, the callus tissue was scraped off the remaining leaf tissue and subcultured. Half of the plates of callus from each of the elm selections were maintained in the refrigerator at 4 C in the dark. These callus cultures were not subcultured for the duration of the experiment. The remaining callus tissue from each of the four elm selections was maintained in the dark at 22 ± 1 C and subcultured every six weeks till September on a half-strength MS medium as described above.

Eleven days before inoculation with fungal spores, small callus pieces, approximately 15 mm in diameter, were transferred from stock cultures (maintained at 22 ± 1 C or 4 C) to 60 x 15 mm Petri plates containing a half-strength fortified MS medium.

PMP1 and TN, aggressive and a non-aggressive isolates of *O. ulmi*, respectively, were grown in 100 x 15 mm Petri dishes on potato dextrose agar (PDA: Difco, Detroit, MI) covered with water-permeable cellophane, and incubated at 24 C for one week. Spores were washed from the surface of the plates with sterile distilled water and diluted to 2×10^6 spores/ml (6). Ten replicates each of calli from A, 8630, DEL2, and S were also inoculated by placing a 1.5 mm filter paper triangle containing 10 μ l of either PMP1 or TN spore suspension on the top, center of the callus (7). Appropriate uninoculated controls were also provided. Inoculations were carried out at six week intervals on June 3, July 22, and September 3, respectively.

Fungal growth was measured 72 hours after inoculation. The average diameter of the fungal colonies was determined by measuring along two axes drawn perpendicular to each other on the bottom of each Petri plate, intersecting at the filter paper triangle. The size of the filter paper triangle (1.5 mm) was subtracted from each diameter reading.

Fungal growth response was fitted to a $4 \times 2 \times 2 \times 3$ factorial analysis of variance model. The four factors were selection (4 levels), isolate (2 levels), temperature (2 levels), and inoculation date (3 levels). Comparisons of successive differences across inoculation dates were made for each selection by isolate and by temperature combination. The first of the successive comparisons was the difference between the mean growth at the July inoculation and the mean growth at the June inoculation. The second of the successive comparisons was the difference between the mean growth at the September inoculation and the mean growth at the July inoculation. Mean separation of growth rates between inoculation dates was determined by t-statistics. When appropriate F values indicated significance at $P < 0.05$.

Results and Discussion

Results are summarized in Table 1. The colonization of A callus at 22 C by both aggressive and non-aggressive isolates showed a consistent and significant increasing trend between June and September. At 4 C, growth rate of non-aggressive isolate showed a similar trend, while the growth rate of aggressive isolate decreased between June and July, but increased between July and September.

On resistant American elm 8630, the growth rates of both aggressive and non-aggressive isolates decreased significantly on callus maintained at 22 C. At 4 C, growth of the aggressive isolate did not change significantly between June and September inoculations; however, non-aggressive isolate growth increased during this period.

On DEL2 callus at 22 C, growth rate of the aggressive isolate on callus tissue decreased significantly between June and July, but increased between July and September. Growth rate of the non-aggressive isolate was unaffected during the same time period. On callus stored at 4 C, the growth rate of the aggressive isolate did not change between June and July inoculations, but decreased significantly between July and September inoculations. Growth rate of the non-aggressive isolate on callus tissue maintained at 4 C first increased between June and July and then decreased be-

Table 1. Growth of *Ophiostoma ulmi* on callus cultures developed from leaf tissue of four selections of elms.

Isolate	Temp C	Inoculation date			Successive difference	
		Jun	Aug mm	Sep	(Aug)-(Jun) mm	(Sep)-(Aug)
Selection A						
PMP1	22	7.3	9.6	12.2	2.3 ^{*a}	2.6 ^{**}
TN	22	3.1	5.9	7.6	2.9 ^{**}	1.7 ^{**}
PMP1	4	7.4	3.9	6.3	-3.5 ^{**}	2.4 ^{**}
TN	4	2.3	3.4	7.5	1.1	4.1
Selection 8630						
PMP1	22	11.6	6.2	6.7	-5.5 ^{**}	0.5 ^{**}
TN	22	5.0	4.1	0.3	-0.9	-3.8 ^{**}
PMP1	4	11.1	9.9	10.8	-1.2 ^{**}	0.9
TN	4	2.9	8.5	8.5	5.6 ^{**}	0.0
Selection Del2						
PMP1	22	8.9	1.7	6.9	-7.2 ^{**}	5.2 ^{**}
TN	22	3.6	2.4	3.3	-1.3	0.9 ^{**}
PMP1	4	7.4	8.3	1.5	0.9 ^{**}	-6.8 [*]
TN	4	1.1	6.1	2.6	5.0 ^{**}	-3.5 [*]
Selection S						
PMP1	22	4.5	2.3	2.5	-2.2 [*]	0.2
TN	22	0.6	2.8	2.3	2.2 [*]	-0.5
PMP1	4	5.3	0.0	1.6	-5.3 ^{**}	1.6
TN	4	2.6	0.0	1.4	-2.6 ^{**}	1.4

^a Differences followed by one or two asterisks are different from zero at the 0.05 or 0.01 significance level, respectively.

tween July and September.

On Siberian elm callus tissue stored at 22 C, growth rates of the aggressive isolate significantly decreased between the first and second inoculation dates, while growth rate of the non-aggressive isolate increased. A similar growth trend was observed with aggressive and non-aggressive isolates on S callus maintained at 4 C (i.e. growth of PMP1 and TN isolates decreasing significantly).

Callus tissue stored at 4 C did not proliferate and thus did not require subculturing. The assumption was that callus tissue maintained at this temperature did not undergo any genetic changes resulting in somaclonal variation. The callus tissue maintained at 22 C proliferated normally and

required subculturing every 4-6 weeks. During this period somaclonal variation in the callus tissue may have occurred extensively (3).

Hubbes (personal communication) indicated that frequent subculturing of elm callus could lead to a decrease in mansonone production when inoculated with the pathogen. Thus, frequent subculturing may increase susceptibility of elm callus tissue to *O. ulmi*, thereby causing an increase in growth rate of the fungus. No definite trends were observed in growth rates of *O. ulmi* on elm callus when stored at 22 C and subcultured or stored at 4 C and not subcultured.

These observations show that fungal growth on callus tissue is independent of the frequency of

subculturing. The assumption that maintenance of callus tissue at 4 C without subculturing will result in uniform growth rate of the fungus does not seem valid. Our data indicate that callus cultures are heterogeneous and undergo somaclonal variation. This lack of homogeneity is evident whether callus cultures were maintained at 4 C and not subcultured or maintained at 22 C and subcultured every 6 weeks. These results suggest that maintaining cultures at 4 C without subculturing does not insure increased consistency in growth rates of *O. ulmi*. Apparently, to achieve uniform results with fungus-callus interaction, one must use callus tissue soon after it is generated to avoid increased heterogeneity and inconsistent results.

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Importance to arborists

Dutch elm disease (DED) caused by fungus *O. ulmi*, is responsible for destruction of elm trees worldwide. Screening elms for resistance to DED is extremely laborious and time consuming. This study was conducted to determine if by using tissue culture technology, we could speed up identification of resistant elms. Callus cultures of susceptible and resistant elms were exposed to aggressive and nonaggressive isolates of *O. ulmi*. Callus cultures were either subcultured regularly or maintained without subculturing. Over a period of three months, it was determined that frequency of subculturing did not play any role in interaction between callus tissue and the fungus.

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Résumé. Les effets de la fréquence du repiquage de culture sur le développement des champignons pathogènes de la maladie hollandaise de l'orme, *Ophiostoma ulmi*, étaient étudiés sur des cultures de tissus d'orme à l'état de cal. Les tissus de cal provenaient de tissus de feuilles d'orme d'Amérique susceptible, d'orme d'Amérique résistant et d'orme de Sibérie résistant. La moitié des cultures était maintenue 22° C et repiquée en culture à six semaines d'intervalle; l'autre moitié était maintenue à 4° C et n'était pas repiquée en culture. Les cultures de cal étaient inoculées soit avec une isolation agressive ou soit avec une mais non agressive d'*O. ulmi*. Les données montraient une absence de corrélation entre la croissance fongique sur le tissu de cal et la fréquence de répétition du repiquage de culture. Cette absence de corrélation est attribuable à l'absence d'homogénéité dans le tissu de cal en raison d'une induction de la variation somaclonale.

Zusammenfassung. Kalluskulturen der Ulme wurden untersucht auf die Auswirkungen der Häufigkeit des Weiterkultivierens (Domestikation) auf das Wachstum des Welkepilzes *Ophiostoma ulmi*. Kallusgewebe wurde aus dem Blattgewebe anfälliger Amerikanischer Ulmen, resistenter Amerikanischer Ulmen und resistenter Sibirischer Ulmen erzeugt. Die Hälfte der Kulturen wurde bei 22° C gelagert und in sechswöchigen Abständen weiterkultiviert. Die andere Hälfte wurde bei 4° C gelagert und nicht weiterkultiviert. Die Kalluskulturen wurden entweder mit einem aggressiven oder nicht aggressiven Isolat der *O. ulmi* geimpft. Die Daten zeigten, daß kein Zusammenhang zwischen Pilzwachstum auf dem Kallusgewebe und der Häufigkeit des Weiterkultivierens besteht. Der mangelnde Zusammenhang ist auf die Inhomogenität des Kallusgewebes zurückzuführen sowie auf somaclonale Veränderungen.