Efficacy of Criteria to Identify Aggressiveness in *Ophiostoma ulmi* and Resistance in American Elm Germ Plasm

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ABSTRACT

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Criteria were evaluated to identify levels of aggressiveness of the Dutch elm disease pathogen, *Ophiostoma ulmi*, and resistance in American elms. Twenty-year-old American elms were inoculated with putative nonaggressive strains PG442 and TN and aggressive strains PMP1, H961, and 16K. Strains PMP1 and H961 were confirmed to be aggressive and strains TN, PG442, and 16K to be nonaggressive. Six-month-old American elm seedlings were inoculated with the five *O. ulmi* strains, and aggressiveness was judged by disease symptoms and fungus multiplication. Vascular discoloration and numbers of colony-forming units most closely correlated with in vivo results. By most in vitro criteria, PG442 was categorized as aggressive. Callus tissues from susceptible and resistant American elm selections were challenged with the *O. ulmi* strains. Aggressiveness was differentiated by growth on callus from both a susceptible and a resistant elm. Differences in resistance between susceptible and resistant elm selections were determined by growth of strains H961, PMP1, and PG442 but not 16K or TN. Growth and mycelial habit on agar were not reliable in identifying 16K as nonaggressive.

Dutch elm disease, caused by *Ophiostoma ulmi* (Buisman) Nannf., is the world's most devastating landscape tree disease. Host resistance is the best means of disease control. Traditional methods of identifying and testing resistant germ plasm are time-consuming and expensive. Changes in the aggressiveness of the pathogen, through mutation or introduction of new strains, may significantly affect resistant reactions in trees. The introduction of highly aggressive *O. ulmi* strains into Britain and Europe in the 1960s resulted in the loss of previously resistant elm selections as well as native trees (2,12,17). Early detection of new, aggressive fungal strains would be useful when selecting resistant germ plasm.

Gibbs and Brasier (11) established cultural criteria to differentiate aggressive and nonaggressive *O. ulmi* strains. Aggressive strains grew faster on agar with fluffy mycelial habit, whereas nonaggressive strains grew more slowly and mycelium was appressed to the agar. Schreiber and Townsend (22) reported inconsistencies between in vivo aggressiveness and cultural characteristics.

Tissue culture technology has been used successfully to identify resistance germ plasm in plant species (4,6,8,14–17). Studies with elm callus systems have been most successful in differentiating resistance levels between species such as American (*Ulmus americana* L.) and Siberian (*U. pumila* L.) elms rather than within species (9,10).

In this study, we evaluated criteria for differentiating aggressiveness of *O. ulmi* strains and the resistance levels of elm germ plasm.

MATERIALS AND METHODS

O. ulmi strains and their sources included: PMP1, an aggressive strain (Delaware, Ohio); TN, a nonaggressive strain (Tennessee); H961, an aggressive strain (Quebec, Canada); PG442, a nonaggressive strain (Portugal); and CESS16K (16K), an aggressive strain (Toronto, Canada). Aggressiveness levels of TN and PMP1 were determined in vivo in our laboratory and levels of PG442, H961, and 16K, by those providing the strains.

Spore suspensions of each isolate were inoculated into four 5-yr-old American

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elm seedlings and reisolated after 6 wk. The reisolates were used in all subsequent studies.

Field elm study. A field study was designed to determine the aggressiveness in vivo of the five *O. ulmi* strains by inoculating 20-yr-old American elms. Inoculum was produced in potato-dextrose broth in shake culture for 5 days. Conidial suspensions were centrifuged and the pellet resuspended in sterile distilled water and diluted to a concentration of 12–13 × 10^6 conidia per milliliter. Approximately 0.25 ml of the spore suspensions was inoculated through each of four to six 6-mm chisel wounds into the xylem around the circumference of the trunk, 1 m above the ground. A water control was included. Inoculations were made on 13 and 14 June 1989; treatments were arranged in a randomized complete block design with 20 replications. The percent infected elms and the percent crown exhibiting disease symptoms were recorded on 5 and 26 July 1989 and 22 June 1990.

Potted elm study. American elm seedlings were grown for 6 mo in pots in a soil:peat:perlite mix (1:1:1, v/v). *O. ulmi* strains were grown as previously described. Seedlings were inoculated by pipetting 3–5 ml of the standardized spore suspension into serum caps affixed near the base of the stem (13). Two scalpel wounds were made in the stem near the bottom of the cap. Twenty replicates per treatment and 10 sterile distilled water controls were arranged in a randomized complete block design. Seedlings were harvested after 3 mo. Leaves and bark were removed, and the length of the vascular discoloration column was measured and expressed as a percentage of stem length. The stem was then cut into eight pieces. The top 2.5 cm of each piece was plated serially onto acidified potato-dextrose agar (PDA), and the number of the piece from which the fungus grew and the maximum heights of fungus movement in the stem were recorded. The remainder of each stem was bulked and frozen at −20 C.

The frozen stem wood from each seedling was ground into sawdust in a Wiley mill and collected through a 2-mm screen (5). Then, 1 g of sawdust was distributed onto the surface of 10 100 × 15 mm petri dishes containing malt extract agar amended with 200 μg/ml of cycloheximide. *O. ulmi* colonies were counted 48 hr after incubation in the dark at 22 ± 1 C.

Tissue culture studies. Stock plants from American elm A, a 20-yr-old seedling susceptible to Dutch elm disease, and resistant American elm selections 8630 and Delaware No. 2 (Del 2) were propagated in the greenhouse from softwood cuttings. Callus cultures were initiated from young leaves on Murashige and Skoog (MS) (18) culture medium supplemented with 200 mg/L of casein hydrolysate, 8 μM 6-benzylaminopurine, 0.5 μM 2,4-diclorophenoxyacetic acid, 3% (w/v) sucrose, 10% (v/v) coconut milk, and 0.7% (w/v) Difco Bacto agar (pH 5.7). Callus tissue was routinely subcultured every 6–8 wk onto fresh, half-strength modified MS (1/2 MS) and maintained in the dark at 22 ± 1 C. Eleven days prior to inoculation, callus pieces, approximately 15 mm in diameter, were transferred from stock cultures to 15 × 60 mm petri dishes containing 1/2 MS.

Fungal strains were grown in the dark on PDA covered with water-permeable cellophane for 7 days at 22 ± 1 C. Spore suspensions were made by washing the surface of the petri dishes with sterile distilled water and adjusting the concentration to 2.0–2.7 × 10^6 spores per milliliter. Filter paper disks 3 mm in diameter were cut from 6-mm-diameter concentration disks with a paper punch. Four 1.5-mm triangles were cut from each of the 3-mm disks, sterilized, and inoculated with 20 μl of the appropriate spore suspension. After air-drying, the inoculated filter paper triangles were placed onto the centers of the calli. Controls included an inoculated triangle placed in the center of an agar dish without callus and an uninoculated triangle placed in the center of a piece of callus. There were 10 replications of the treatments and controls. After inoculation, the culture dishes were returned to 22 ± 1 C in the dark.

To determine the average diameter of the fungal colony growing from the triangle, two perpendicular axes were drawn on the bottom of the plates intersecting where the paper triangle was placed on the callus. Then, 72 hr after inoculation, the diameter of the fungal colony was determined by averaging the growth along the two axes. The size of the paper triangles was subtracted from each diameter reading.

Density ratings of the fungal mycelium on calli were made on a scale of 1–3, in which 1 = sparse mycelial growth, callus readily visible; 2 = moderate mycelial growth, callus covered by mycelium but still visible; and 3 = dense mycelial growth, callus barely or not visible through the mycelium.

Cultural characteristics. Cultural characteristics and growth rates of the five *O. ulmi* strains were compared on 1/2 MS and PDA. Stock cultures of each strain were grown on 1/2 MS or PDA. Disks 1 cm in diameter were cut from the margin of the developing colonies with a cork borer and placed in the center of a 15 × 100 mm petri dish containing 1/2 MS or PDA. There were 10 replicates per strain. After 6 days at 22 ± 1 C in the dark, the colony diameters were determined by measuring and averaging two diameters, at right angles to one another, on each dish. The appearance of the mycelial growth on the surface of the dish was rated as 1 = waxy or appressed to agar, 2 = intermediate between appressed and aerial, or 3 = aerial or fluffy (Fig. 1).

Statistical analysis. The two response variables in the field study (infected trees and foliar symptoms) and the five response variables in the potted elm study (infected trees, colony-forming units in sawdust, vascular discoloration, infected stem sections, and height of fungus movement) were each fitted to a one-factor analysis of variance model by weighted least squares. The factor consisted of five isolates. Weighted least

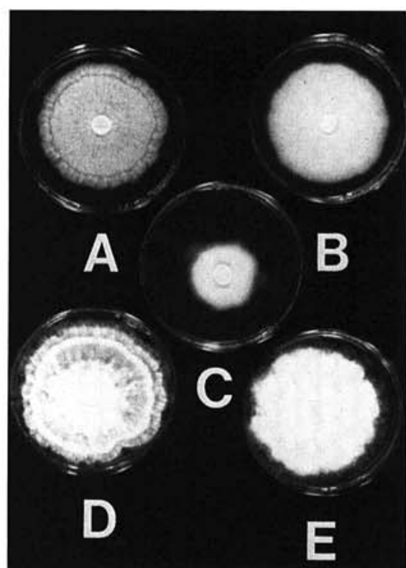

Fig. 1. Visual rating scale for mycelial growth habit of *Ophiostoma ulmi* on PDA: PG442 (B) and TN (C) = 1 (waxy mycelium appressed to agar), H961 (A) = 2 (intermediate between waxy and fluffy), and PMP1 (D) and 16K (E) = 3 (aerial or fluffy).

Table 1. Percentages of infected trees and crown symptoms in 20-yr-old American elms inoculated in the field with spore suspensions of aggressive and nonaggressive strains of *Ophiostoma ulmi* on 13 and 14 June 1989

O. ulmi	Infected elms (%)			Crown symptoms (%)		
strain	5 July 1989	26 July 1989	22 June 1990	5 July 1989	26 July 1989	22 June 1990
16K	25 a[z]	20 a	95 ab	2 a	1 a	15 a
H961	90 b	85 b	100 b	31 b	21 c	79 b
PG442	85 b	70 b	90 a	6 a	4 ab	21 a
PMP1	85 b	80 b	100 b	23 b	26 c	72 b
TN	45 a	45 a	100 b	9 a	8 b	26 a

[z] Means within a column not followed by the same letter are significantly different at $P = 0.05$ by LSD.

squares estimation was used to account for heterogeneous variance. The weights were the reciprocals of the estimated variances. All pairwise comparisons among the isolates were made using t tests. The fungal density rating and growth on callus were each fitted to a two-factor analysis of variance model by least squares. One factor was isolate with five levels and the other was tissue type with three and four levels. Weighted least squares estimation was used with the fungal density rating. All pairwise comparisons among the isolates within each strain were made using t tests. The significant differences in growth of the fungal strains in PDA and 1/2 MS were determined by standard analysis of variance with significance levels of $P = 0.05$.

RESULTS

Field elm study. Percentages of infected elms and crown symptoms are presented in Table 1. On 5 July 1989, the percentages of infected elms inoculated with H961, PMP1, PG442, TN, or 16K were 90, 85, 85, 45, and 25%, respectively. On 26 July, the percentages for these strains were 85, 80, 70, 45, and 20%, respectively. H961, PG442, and PMP1 were statistically similar, as were 16K and TN. By 22 June 1990, percentages of infected trees were 90–100%, with no statistical differences among strains. Trees inoculated with PMP1 or H961 showed percentages of crown symptoms that were similar and significantly higher than those of trees inoculated with 16K, TN, or PG442.

Potted elm study. Foliar symptoms were sporadic in all treatments and were not considered a measure of aggressiveness. Percentages of infected trees, stem length with vascular discoloration, infected stem sections, height of fungus movement, and number of colony-forming units per gram of sawdust are presented in Table 2. Strain 16K was designated as nonaggressive and differentiated from aggressive strains H961 and PMP1 by significant differences in all criteria but vascular discoloration (Table 2). Nonaggressive strains TN and PG442 also produced lower percentages of infection as measured by the criteria, but these differences were not always significant.

Tissue culture study. The growth and densities of mycelium of the *O. ulmi* strains on the surface of callus tissue are given in Table 3. On A and 8630 calli, strains H961, PG442, and PMP1 grew most rapidly, while 16K and TN grew more slowly; on Del 2 callus, H961, PG442, PMP1, and TN all grew at similar rates and more rapidly than 16K. The average growth on calli from all elm selections for PMP1 (8.7 mm), H961 (9.1 mm), and PG442 (8.8 mm) was similar and significantly more rapid than that for 16K (5.4 mm) and TN (6.6 mm). Mycelial densities of PMP1, H961, and PG442 were also similar and greater than those for either 16K or TN on calli from all elm selections. Growth of *O. ulmi* strains was most rapid on 8630 calli, followed by A and Del 2. Calli from susceptible elm A could be differentiated from calli from resistant Del 2 by the more rapid growth on the former callus source of strains PG442, PMP1, and H961 but not by either 16K or TN. Among all strains, fungal density did not vary significantly ($P = 0.05$) on calli from any elm selection (A, 1.9; Del 2, 1.7; 8630, 1.6).

Cultural characteristics. Colony growth and mycelial characteristics of the five *O. ulmi* strains on PDA and 1/2 MS are shown in Table 4 and Figure 1. On PDA, 16K grew most rapidly, followed in decreasing order by PMP1, H961, PG442, and TN. Mycelium of PMP1 and 16K was aerial or fluffy, that of H961 was intermediate, and that of TN and PG442 was waxy and appressed to the agar. On 1/2 MS, 16K again grew most rapidly, followed by H961. PG442, PMP1, and TN grew more slowly and at similar rates. The mycelial habit of 16K, PG442, and TN was waxy, that of H961 was intermediate, and that of PMP1 was fluffy.

DISCUSSION

The aggressiveness of the five *O. ulmi* strains was shown by inoculations of elms in the field. Percentages of crowns showing symptoms are more quantitative than percentages of infected trees for differentiating aggressiveness. As measured by crown symptoms, strain 16K, previously considered to be aggressive, was nonaggressive. All other strains showed levels of aggressiveness previously reported. It is of interest that PG442 was classified as aggressive by the percentage of infected trees and by most subsequent in vitro criteria. PG442 was, however, clearly nonaggressive when classified by the more quantitative criterion, percentage of crown symptoms. Because aggressiveness is defined in terms of virulence as measured by crown symptoms, this was the standard by which other criteria were judged.

Crown symptoms occurred only sporadically in potted elms and were excluded as a criterion for aggressiveness. By most criteria, 16K was least

Table 2. Aggressiveness of *Ophiostoma ulmi* strains inoculated into potted American elm seedlings[x]

	Criteria of aggressiveness[y]				
O. ulmi strain	Infected trees (%)	Median cfu/g of sawdust	Vascular discoloration (%)	Infected stem sections (%)	Height of movement in stem (%)
16K	45 a[z]	0.04 a	41 abc	15 a	23 a
H961	95 b	5.60 bc	64 c	51 bc	60 bc
PG442	75 ab	2.70 b	31 a	42 bc	54 bc
PMP1	95 b	10.00 c	60 bc	63 c	65 c
TN	80 b	1.80 b	39 ab	40 b	40 ab

[x] Twenty replicates per strain. Seedlings were harvested 3 mo after inoculation.
[y] Infected trees = trees from which *O. ulmi* was reisolated; cfu/g = colony forming units isolated per gram of sawdust from stems; vascular discoloration = length of discoloration column in xylem expressed as percentage of total stem length; infected stem sections = isolations from 2.5-cm sections along length of stem; height of movement in stem = maximum height of fungus as percentage of stem length.
[z] Values within a column not followed by the same letter are significantly different at $P = 0.05$ by LSD.

Table 3. Growth and mycelial density of *Ophiostoma ulmi* strains on callus tissue from three American elm selections

	Elm selections[w]					
	A		Del 2		8630	
O. ulmi strain	Growth[x]	Density[y]	Growth	Density	Growth	Density
16K	4.8 aA[z]	1.1 aA	3.4 aA	1.7 bB	8.0 abB	1.1 aA
H961	10.0 bAC	2.3 bAB	7.7 bB	2.4 cA	9.6 bcBC	1.9 bB
PG442	8.7 bA	1.8 bA	5.9 bB	2.4 cB	11.8 dC	2.0 bAB
PMP1	8.6 bA	2.0 bA	6.7 bB	2.0 bcA	10.8 cdC	1.9 bA
TN	6.6 aA	1.0 aA	5.9 bA	1.2 aA	7.2 aA	1.4 aA

[w] A = highly susceptible, Del 2 and 8630 = moderately resistant.
[x] Average of two diameters (mm) of the fungal colony on the surface of the callus after 72 hr.
[y] Rating: 1 = callus readily visible through mycelium, 2 = callus mostly covered, 3 = callus barely or not visible.
[z] Average of 10 replicates. Means within a column followed by the same lowercase letters and means in a row followed by the same capital letter are not significantly different at $P = 0.05$ by LSD.

Table 4. Growth ratings of *Ophiostoma ulmi* on agar media

O. ulmi strain	Colony diameter[y] (mm)	
	PDA	1/2 MS
16K	64.8 a[z]	59.7 a
H961	52.2 b	51.7 b
PG442	48.5 c	38.9 c
PMP1	58.6 d	39.3 c
TN	37.2 e	42.7 c

[y] Means of two diameters of each colony of each of 10 replicates were measured and averaged. PDA = potato-dextrose agar, 1/2 MS = half-strength Murashige and Skoog medium (18).

[z] Means within a column not followed by the same letter are significantly different at $P = 0.05$ by LSD.

aggressive. Vascular discoloration and colony-forming units most clearly differentiated aggressive and nonaggressive strains. The latter criterion had been reported to be efficacious in differentiating resistance to Verticillium wilt in maples (21). Myers and Strobel (19) also found vascular discoloration but not crown symptoms to be an efficacious criterion for measuring disease development in young American elm seedlings. Ronald and Kondo (20) were able to use crown symptoms to measure disease development in young *U. japonica* (Sarg. ex Rehd.) Sarg. seedlings. Aggressiveness of strains was most clearly differentiated by growth and density on calli from A and 8630. Nonaggressive strains 16K and TN were differentiated from aggressive PMP1 and H961 by slow mycelial growth and low density. However, PG442 showed aggressive characteristics of growth and density. This too may indicate an intermediate level of aggressiveness and support multigenic control reported by Brasier and Gibbs (1,3). The inability to differentiate strains on Del 2 callus may be due to its higher level of resistance that depressed growth of all strains.

Callus systems have been used to identify resistant germ plasm in several crops (7,8,16), including elms (9,10). In this study, mycelial density was not efficacious in differentiating resistance of any selection. Growth was sparse but most rapid on calli from 8630, followed by A and Del 2, as reported in previous studies (9,10). The growth on 8630 callus would not be expected if growth was inverse to the resistance of the explant source. The inconsistency of these results may reflect deficiencies of measuring total fungal growth. Only surface density and growth were measured.

Previously we demonstrated a positive correlation between tissue susceptibility and vertical mycelial penetration (*unpublished*) and are now investigating methods to quantify total fungal growth. Growth of H961, PG442, and PMP1 on susceptible A and resistant Del 2 callus was inversely correlated with the resistances of the explant sources. Nonaggressive strains 16K and TN grew similarly on A and Del 2. Thus, nonaggressive strains may not be suitable for in vitro resistance tests. As with other criteria, PG442 exhibited characteristics of an aggressive strain.

Gibbs and Brasier (11) established cultural characteristics to identify the aggressiveness of *O. ulmi* strains. Strain 16K, nonaggressive in vivo, exhibited aggressive cultural characteristics (Table 4, Fig. 1). Our results agreed with those previously reported (22) that correlations between in vivo and cultural criteria may be inconsistent. Mycelial habit on 1/2 MS was the only criterion that coincided with in vivo results.

Vascular discoloration and inoculum density (colony-forming units) in potted elm stems and mycelial habit on 1/2 MS agar most closely correlated with the aggressiveness of the fungal strains in the field study. Fungal growth rates and density on calli from A and 8630 were reliable in identifying 16K and TN as nonaggressive. Our results in this and previous studies (9,10) suggest that in vitro techniques are less efficacious in detecting either subtle differences in resistance within American elms or levels of aggressiveness of *O. ulmi* strains.

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