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USE OF CULTURE FILTRATES OF *CERATOCYSTIS ULMI* AS A BIOASSAY TO SCREEN FOR DISEASE TOLERANT *ULMUS AMERICANA*

PAULA M. PIJUT[a,]*, SUBHASH C. DOMIR[b], R. DANIEL LINEBERGER[a,]** and LAWRENCE R. SCHREIBER[b]

[a]*Department of Horticulture, The Ohio State University, Columbus, OH 43210 and* [b]*USDA-ARS, Nursery Crops Research Laboratory, Main Road, Delaware, OH 43015 (U.S.A.)*

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Callus cultures of elm (*Ulmus americana* L.) derived from Dutch elm disease susceptible, intermediate-resistant, and resistant genotypes were exposed to the culture filtrates of three pathogenic isolates of *Ceratocystis ulmi*, the causal agent of Dutch elm disease. Callus fresh weights, cell viability, and reactions of stem cuttings were determined after exposure to various concentrations of the filtrates. Calli from the susceptible elm failed to increase in fresh weight and lost viability after exposure to media containing culture filtrate. Calli from the resistant and the intermediate-resistant elms exhibited growth rates and maintained viability similar to controls not exposed to culture filtrate. Stem cuttings of the susceptible elm wilted after exposure to the culture filtrate. The symptoms were similar to wilt seen with the disease. Cuttings from the resistant elm had no disease symptoms whereas, the intermediate elm had some leaf chlorosis. Callus screening may thus be useful for selection of *Ulmus* germplasm for Dutch elm disease resistance.

Key words: Ulmus americana; Ceratocystis ulmi; elm callus; culture filtrate; dutch elm disease

Introduction

Dutch elm disease, caused by *Ceratocystis ulmi* (Buisman) C. Moreau [1] (*Ophiostoma ulmi*), is a serious vascular wilt disease of elm trees in Europe and North America. The principal host in North America is the American elm, *Ulmus americana* L. The most practical means for suppressing this disease is the exploitation of polygenetically controlled host resistance [2]. Breeding programs aimed at improving disease resistance of elms require years of concerted effort in the development and evaluation of germplasm for relative resistance to Dutch elm disease. Current methods of screening for resistance involve inoculating elm seedlings with the pathogen and ranking disease severity. Results with greenhouse or field trials may vary with environmental conditions and require ample space and time.

Screening and selection of plant tissues in vitro for resistance to fungal toxins or culture filtrates has been successful for several species [3 – 10]. *C. ulmi* produces a toxin, cerato-ulmin, which has been characterized as the phytotoxin significantly involved in the development of Dutch elm disease [11,12]. A wilt-inducing glycopeptide (peptidorhamnomannan) has also been characterized and seems to be present in all cultures of *C. ulmi* [13]. The purified toxin, cerato-ulmin, elicits morphological and physiological symptoms in American elm similar to those induced by *Ceratocystis* [14]. Toxic symptoms of elm cuttings include reduction in transpiration, increase in leaf respiration, wilting,

Address for correspondence: Subhash C. Domir, USDA-Agricultural Research Service, Nursery Crops Research Laboratory, 359 Main Road Delaware, OH 43015, U.S.A.
*Present address: USDA-Forest Service, North Central Forest Experiment Station, 1992 Folwell Ave., St. Paul, MN 55108, U.S.A.
**Present address: Department of Horticulture, Clemson University, Clemson, SC 29634, U.S.A.
Abbreviations: MS, Murashige and Skoog.

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chlorosis, and electrolyte leakage from leaf tissue [15].

The objective of this study was to determine whether a reliable tissue culture screening system to detect *C. ulmi* resistance in elms could be established.

Materials and Methods

Plant material

Softwood cuttings from three *U. americana* trees (15—30 years old), located at the USDA-ARS Nursery Crops Research Laboratory (Delaware, OH), were rooted and established as stock plants in the greenhouse. A typically susceptible American elm was selected. Two other American elm selections were used, Delaware #2 and #8630, classified as intermediate-resistant and resistant, respectively (Schreiber, unpublished) based on their response to inoculation trials with the pathogen, *C. ulmi.*

Callus initiation and culture

Callus cultures were initiated from young leaves obtained from greenhouse stock plants. The first, fully expanded young leaf below the shoot tip was routinely used. Leaves were surface sterilized in a 0.5% commercial sodium hypochlorite solution for 10 min, followed by 4 rinses in sterile, distilled water. Leaf segments including a portion of the midvein were plated adaxial side up on a Murashige and Skoog (MS) medium [16] supplemented with 200 mg/l casein hydrolysate, 10% (v/v) coconut milk (processed from fresh coconuts deproteinated by heat coagulation), 8 μM 6-benzylaminopurine, 0.5 μM 2,4-dichlorophenoxyacetic acid, 3% (w/v) sucrose, and 0.7% (w/v) Difco Bacto agar (pH 5.7). Cultures were incubated in the dark at 23 ± 2°C for 1—2 months. Proliferating callus cultures were then routinely transferred every 4 weeks to fresh half strength MS media containing the same supplements.

Pathogen culture

The fungal isolates of *C. ulmi* used were PMP1, isolated from a diseased American elm in Delaware, OH; PG442, a non-aggressive isolate obtained from Tomar, Portugal and H961, an aggressive isolate obtained from Quebec, Canada. Axenic cultures of the fungal isolates were maintained on potato dextrose agar medium [17] at 23°C in the dark, and were routinely transferred to fresh medium every 2 weeks.

Culture filtrates of C. ulmi

Culture filtrates of *C. ulmi* isolates were prepared from liquid shake cultures. Modified Salemink's medium [18] (100 ml) in 250 ml Bellco Delong flasks was autoclaved at 121°C for 15 min. The fungal isolates used to inoculate the sterile medium were obtained from 4-day-old fungal stock cultures. Each flask of sterile medium (total of 1 l for each isolate) was inoculated with a 5 × 5 mm plug of agar containing the fungus. Liquid cultures were incubated at 23 ± 2°C in the dark on a gyratory shaker (125 rev./min) for 8 days. Uninoculated fungal culture medium was similarly handled. The liquid cultures were then centrifuged for 30 min at 1200 × *g* to remove spores and mycelia. Supernatant fractions from the same isolates were pooled. The pH of the filtrates were adjusted to 5.7 with 0.1 N NaOH, and the filtrates were filter sterilized through a 0.45 μm Nalgene filter unit.

Callus testing

Culture filtrate was added to MS medium to give concentrations of 1, 5, 10, 20 and 50% filtrate and a final volume of half strength MS medium. The test media were dispensed into 60 × 20 mm sterile, Petri plates. Control plates contained either the uninoculated fungal culture medium or sterile, distilled-deionized water at the same concentrations.

Calli (0.5 g pieces) of each elm selection were placed on the surface of the test media and incubated in the dark at 23°C for 8 weeks. All callus samples were preweighed under sterile conditions. Fresh weights were obtained from 10 plates per elm selection per treatment. The experiments were conducted in triplicate. The 2,3,5-triphenyltetrazolium chloride (TTC) assay [19] was used as a viability assay for callus

exposed to filtrates. The data were analyzed using analysis of variance and linear regression.

Assay with stem cuttings

Five 15-cm long stem cuttings (with 4–6 leaves) from each of 10 greenhouse stock plants per elm selection were removed from the stock plants and placed in a beaker of water. Cuttings were prepared for the test solutions by making a second cut, at an angle, while the stems were immersed in autoclaved (degassed) water to avoid the possibility of air-embolisms in the xylem vessels. The cuttings were then placed in flasks containing 100 ml of filter-sterilized uninoculated fungal culture medium or culture filtrates, at full strength or diluted 50% with distilled water and kept at a temperature of 25 °C with 16 h day length (60–80 μE m^{-2} s^{-1}). Foliar symptoms were rated after 48 h, on a scale of 0–3, where 0 = green leaves, no wilting; 1 = general wilting; 2 = wilting and necrosis of leaf margins; 3 = wilting, necrosis, and leaf curl.

Results

Effect of culture filtrates on callus

The differences in growth response among the susceptible, the intermediate-resistant, and the resistant American elm calli exposed to the culture filtrates of the three pathogenic isolates of *C. ulmi* were quite apparent. The culture filtrates of all isolates of *C. ulmi* tested suppressed callus growth of the susceptible elm as the concentration of culture filtrate increased (Table I).

Table I. Effect of culture filtrates of *C. ulmi* on growth of callus from elm genotypes with differing susceptibility to Dutch elm disease. Mean fresh weights (g) ± S.E. for 30 observations.

% v/v	Pathogenic isolates of *C. ulmi*			Control (uninoculated)
	PMP1	PG442	H961	
Susceptible				
0	7.37 ± 0.33	6.89 ± 0.15	6.96 ± 0.12	4.38 ± 0.06
1	6.98 ± 0.55	6.40 ± 0.08	5.88 ± 0.09	4.98 ± 0.23
5	6.25 ± 0.58	6.61 ± 0.11	5.61 ± 0.23	6.25 ± 0.20
10	4.30 ± 0.20	3.87 ± 0.10	3.92 ± 0.09	6.20 ± 0.24
20	4.28 ± 0.31	3.46 ± 0.09	3.77 ± 0.14	7.28 ± 0.37
50	3.28 ± 0.42	3.23 ± 0.06	3.10 ± 0.05	6.78 ± 0.62
SIG.[a]	**	**	**	**
Intermediate				
0	6.62 ± 0.39	6.61 ± 0.16	6.77 ± 0.08	7.25 ± 0.13
1	6.79 ± 0.69	6.59 ± 0.09	6.94 ± 0.11	6.14 ± 0.20
5	6.35 ± 0.22	6.02 ± 0.13	6.97 ± 0.12	7.80 ± 0.80
10	6.87 ± 0.30	6.40 ± 0.07	6.58 ± 0.09	7.12 ± 0.63
20	6.62 ± 0.30	5.90 ± 0.13	6.69 ± 0.07	8.33 ± 0.79
50	6.73 ± 0.58	6.74 ± 0.05	6.04 ± 0.14	6.14 ± 0.27
SIG.	NS	NS	**	NS
Resistant				
0	10.63 ± 0.21	9.23 ± 0.05	10.28 ± 0.18	8.33 ± 0.32
1	10.18 ± 0.36	9.07 ± 0.08	10.39 ± 0.18	12.00 ± 0.21
5	10.38 ± 0.64	9.10 ± 0.12	10.25 ± 0.14	13.21 ± 0.39
10	11.64 ± 0.88	9.46 ± 0.11	10.16 ± 0.13	12.29 ± 0.56
20	10.93 ± 0.73	9.31 ± 0.08	10.41 ± 0.16	15.86 ± 0.32
50	10.35 ± 0.26	9.57 ± 0.08	10.25 ± 0.21	13.81 ± 0.55
SIG	NS	**	NS	**

[a] Linear regression analyses; non-significant (NS) $P > 0.20$ or significant (**) $P < 0.001$.

Table II. Effect of *C. ulmi* isolate-PMP1 culture filtrate on cell viability (TTC assay) of susceptible, intermediate-resistant, and resistant American elms. Mean $A_{485\ nm}$ of triplicate samples per treatment. Numbers in parenthesis are percent viable cells.

% v/v	Susceptible	Intermediate	Resistant
0	0.72 (100)	0.72 (100)	0.64 (100)
1	0.68 (94)	0.68 (98)	0.62 (97)
5	0.64 (89)	0.72 (100)	0.62 (97)
10	0.44 (61)	0.68 (94)	0.66 (100)
20	0.42 (58)	0.70 (100)	0.62 (97)
50	0.20 (27)	0.72 (100)	0.64 (100)

Growth was approximately 50% inhibited when the filtrates were added as 50% of the total medium volume for all isolates. This was seen against a growth stimulation as a result of the addition of uninoculated culture filtrate. This may have masked some of the effects of the fungal culture filtrate. Growth inhibition was less severe with the 1 or 5% culture filtrate concentration; however, callus growth was also reduced significantly when 10 or 20% filtrate was added. The culture filtrate of the aggressive isolate, H961 significantly reduced callus fresh weight at all concentrations. Both the non-aggressive PG442 and the PMP1 isolates significantly reduced callus growth at 10, 20 and 50% dilutions. No significant differences were observed between these levels.

The callus fresh weights of the intermediate-resistant elm were not discernably different when grown on media containing filtrate of PMP1 or PG442 (Table I). Callus growth decreased significantly only when the filtrate of the aggressive isolate H961 was incorporated into the medium at the 50% concentration. The uninoculated filtrate also enhanced callus fresh weight of this elm, especially at the 20% level.

The callus fresh weights of the resistant elm were not significantly decreased as a result of the culture filtrate in the medium (Table I). The fresh weights did, however, significantly increase in the presence of isolate PG442. The resistant elm callus growth increased significantly at all levels of uninoculated fungal filtrate in the callus medium, with the greatest increase at the 20% dilution.

A decrease in cell viability (as measured with the tetrazolium assay) of the susceptible elm was observed when callus cultures were exposed to the culture filtrate of an isolate of *C. ulmi* (Table II). As the concentration of culture filtrate was increased in the culture medium, a

Table III. Effect of exposure of elm stem cuttings to culture filtrates after 48 h. Rating scale: 0, green leaves, no wilt; 1, general wilting; 2, wilting and necrosis of leaf margins; 3, wilting, necrosis, and leaf curl; based on average of 10 cuttings per elm selection. DW, sterile, distilled water; UFM, uninoculated fungal culture medium; CF, culture filtrate.

Elm selection	DW	UFM/DW (1 : 1)	UFM	CF/DW (1 : 1)	CF
Susceptible	0	0	0	2	3
Intermediate	0	0	0	1	1
Resistant	0	0	0	0	0

significant decrease in cell viability was noted. Calli from the resistant and intermediate-resistant elms maintained normal cell viability.

Assay with stem cuttings

Cuttings of the susceptible elm had Dutch elm disease, wilt-like symptoms 48 h after taking up culture filtrate (Table III). Leaves of the filtrate-treated cuttings wilted and then developed a necrosis that usually began at the margins of the leaves. Leaves often curled inward as they became necrotic and desiccated. Severe symptoms developed on the susceptible cuttings after 4 days in full strength culture filtrate. Cuttings from the intermediate-resistant elm had some leaf chlorosis and wilting. However, the symptoms were not as severe as on the susceptible elm after a 4 day exposure to the filtrate. Cuttings from the resistant elm had no disease symptoms when exposed to the fungal filtrate.

Discussion

We demonstrated that a reduction in callus growth of a susceptible American elm occurs when the callus culture medium is amended with a cell-free culture filtrate of an isolate of *C. ulmi*. This decrease in callus fresh weight was the result of the presence of toxic metabolites in the culture filtrate. Resistant and susceptible elms were differentially distinguished using this in vitro callus screening system. The intermediate-resistant genotype was less discernible, except where the aggressive isolate was used at the highest concentration. The correlation between callus reactions and cut stem assay reactions indicates that callus testing is feasible for screening *Ulmus* germplasm for Dutch elm disease resistance. This screening procedure may also be useful in performing cellular selection for tolerance to the putative toxin.

After repeated attempts to obtain differentiation from the elm callus by media manipulation, growth regulator concentrations, environmental factors, etc., we have been unable thus far, to regenerate plants from callus derived from adult phase tissue. To date, no one has successfully propagated the American elm through tissue culture, using material from mature trees. Regeneration has been achieved using juvenile tissue [20–23]. Additional research on the factors controlling organogenesis is necessary to aid in subsequent regeneration of plants from calli resistant to the culture filtrate of *C. ulmi*, and to determine whether resistance observed in vitro can be maintained after subsequent transfer of regenerated plants to field conditions.

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