INFLUENCE OF COCONUT MILK SOURCE ON HOST-PATHOGEN INTERACTIONS BETWEEN OPHIOSTOMA ULMI AND ULMUS SELECTIONS

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Abstract. This study compared the effects of two sources of coconut milk in modified Murashige and Skoog medium on growth of *Ophiostoma ulmi* on five selections of elm callus cultures. One medium (CMC) contained coconut milk from fresh coconuts; the other medium, (CMS), coconut milk obtained from Sigma Chemical Co. These studies showed that the coconut milk source affected fungal growth significantly, varying with elm selection and aggressiveness of the *Ophiostoma ulmi* isolate. An evaluation was made on production of secondary metabolites in callus cultures with each source of coconut milk. High performance liquid chromatography from *Ulmus americana* showed a higher level of those secondary metabolites using CMS medium when callus was from mature, DED-susceptible trees, but higher levels with CMC when callus was from DED-resistant trees.

Dutch elm disease (DED), is one of the most devastating tree diseases in the world. First reported in 1922, this rapidly fatal disease caused by the fungus *Ophiostoma ulmi*, has considerably damaged the North American population of American elm (*Ulmus americana*). Extensive research has been conducted to control DED to develop DED-resistant elms. Conventional techniques of selecting and screening elms for resistance to DED are extremely time consuming. New techniques may expedite the screening program and aid in the development of a DED-resistant American elm. Using tissue culture technology in studies on host-pathogen interactions, it has been shown that in several instances callus cultures act like in vivo systems (1,2,10,11,13,16,17,18). In other instances, the response has been less assuring (3,12,15). Previous studies show that temperature, inoculum concentration, growth regulators in the medium, and other environmental factors can affect fungal colonization of susceptible and resistant callus tissue (4,9,17). These factors can also influence correlations in host-pathogen interactions between callus and intact plants.

Holmes reported using coconut milk in Gautheret's medium to grow elm callus (7,14). Domir et al. (5) showed that coconut milk in the medium can influence the fungal growth on callus cultures generated from various elm selections. In other studies, biochemical profiles of extracts, obtained from callus tissue generated on media containing coconut milk from two sources, were different (T.L.Graham, personal communication). The present study was to see whether different sources of coconut milk would have different effects on fungal colonization and on the production of secondary metabolites by callus tissue from susceptible and resistant elm selections.

Materials and Methods

Callus cultures were initiated from leaf tissue of American elm (A), susceptible to DED; American 8630 (8630) and Delaware #2 (DEL2), two American elm selections resistant to DED; and Siberian elm (*U. pumila*)(S), an elm species resistant to DED. Stock plants of these selections were propagated in the greenhouse from softwood cuttings obtained from mature trees. Callus was also generated from 6-month-old American elm seedlings (JA). These cultures were initiated on full strength modified Murashige and Skoog (20) culture medium (MS). The details of this procedure have been described elsewhere (4). Two types of media were prepared, one (CMC) containing coconut milk obtained from the fresh coconuts and the other (CMS) with coconut milk received from Sigma Chemical Co. (St. Louis, MO). These cultures were routinely subcultured

every 6 weeks onto fresh half-strength modified MS medium. Cultures were maintained in the dark at 22±1°C.

Two strains of *O. ulmi*, one aggressive (PMP1) and one nonaggressive (TN), were grown 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 distilled water and diluted to 2.2 X 10^6 spores/ml.

Eleven days before inoculation with the DED fungus, callus pieces of A, 8630, DEL2, S and JA, approximately 15 mm in diameter, were transferred from stock cultures to fresh, half- strength modified MS medium. Ten replicates from each of the five callus types were placed on CMC and CMS. The callus pieces were inoculated and fungal growth was measured after 96 hours as described in earlier publications (4,5). Appropriate uninoculated controls were used as in earlier studies. This study was repeated six weeks later. The fungal growth response variable was fitted to a 2 X 2 X 5 X 2 factorial analysis-of- variance model. The four factors were media, isolate, selection, and run, respectively. Thirty contrasts were tested among the 40 factor-level means, i.e. three contrasts within each of the ten run X selection combinations:

1) The media main effect, i.e. the difference between CMC and CMS averaged over both isolates.
2) The isolate main effect, i.e. the difference between PMP1 and TN averaged over both media.
3) The media X isolate interaction, i.e. the difference between the media effect for PMP1 and the media effect for TN.

To prepare samples for the analysis of soluble aromatic metabolite by high performance liquid chromatography (HPLC), the inoculated callus pieces were divided into two parts: a) tissue within the area of fungal growth, and b) healthy tissue just beyond the fungal front. Uninoculated callus pieces were provided as controls. The HPLC analysis of soluble aromatic secondary metabolites was carried out using the method published by Graham (8) but with the following changes. Wet tissue was extracted with 0.2 ml of 80% ethanol per 0.1 g of tissue. The Waters HPLC system (Millipore Corporation, Marlborough, MA) consisting of two 510 pumps, a 710B autoinjector, a 490 detector, and an 840 data control workstation, was used for chromatography. A 20 µl (microliter) aliquot of each sample was injected onto a Nova Pak C18 8 mm x 10 cm Radial-Pak cartridge at a flow rate of 2.5 ml/min with output monitoring at 236 nm (nanometer). A linear gradient of acidified water, pH 3 with H_3PO_4, to acetonitrile (0-55% in 25 min) was used to resolve sample components.

Significant differences in levels of metabolites are reported when the three chromatograms representing a triplicate callus trial of a given condition are all higher in level or all lower in level than a similarly triplicate set of another trial, when overlayed.

Results

Results on growth of PMP1 and TN isolates on elm callus cultures generated on modified MS media, CMC and CMS, are summarized in Table I. In the first run, the source of coconut milk significantly affected the growth of *O. ulmi* isolates on callus cultures from all elm selections except JA. Fungal growth on A was faster on callus generated on CMC than on CMS, whereas, reverse was observed with 8630, DEL2, and S. In the second run the source of coconut milk did not differentially affect the fungal growth on callus tissue of S while fungal growth was faster on A, JA, and DEL2 and slower on 8630 when callus cultures were generated on CMC than on CMS.

Analysis of growth differences between PMP1 and TN are also presented in Table 1. In the first run, we observed that PMP1, an aggressive isolate, grew slower on calli from JA, DEL2, and S than nonaggressive isolate TN. No significant growth differences between isolates were observed on callus from A and 8630. When repeated six weeks later, data showed the fungal growth on A, JA, 8630, and S followed the pattern established in the first run. No significant growth difference between two isolates was observed on DEL2 callus.

The data from the first run showed significant media x isolate interaction only in selection A. Isolate PMP1 grew much faster than isolate TN

Table 1. Analysis of growth of two isolates O. ulmi on callus cultures generated from elm selections on media containing two types of coconut milk[a].

Coconut Milk	Isolate	Fungal growth (mm) in Run 1 Selections A	JA	8630	DEL2	S	Fungal growth (mm) in Run 2 Selections A	JA	8630	DEL2	S
CMC	PMP1	11.1	4.3	6.3	1.9	2.0	4.1	1.0	2.6	3.2	1.6
CMC	TN	9.3	7.0	5.0	4.4	6.0	6.2	6.5	3.2	5.3	4.9
CMS	PMP1	3.3	3.3	12.6	3.6	4.0	3.9	0.9	5.1	0.9	0.4
CMS	TN	7.5	6.0	10.8	6.9	7.7	1.4	2.0	4.7	1.1	4.0
Contrasts[b]											
Media main effect		4.8 (.000)	1.0 (.241)	-6.1 (.000)	-2.1 (.014)	-1.9 (.031)	2.5 (.003)	2.3 (.007)	-2.0 (.019)	3.3 (.000)	1.1 (.208)
Isolate main effect		-1.2 (.151)	-2.7 (.002)	1.6 (.070)	-2.9 (.001)	-3.9 (.000)	0.2 (.837)	-3.3 (.000)	-0.1 (.907)	-1.2 (.203)	3.5 (.000)
Media x isolate interaction		6.1 (.000)	0.0 (.999)	-0.5 (.769)	0.8 (.641)	-0.3 (.860)	-4.7 (.007)	-4.4 (.011)	-1.0 (.558)	-1.9 (.275)	0.3 (.883)

[a] Each number is the averages of ten replicates 96 hours after inoculation.
[b] The pair of values given for each contrast are the estimate of the contrast and, in parentheses, the P value corresponding to the hypothesis that the contrast equals zero.

on A callus maintained on CMC in comparison to callus maintained on CMS. Among the other four selections, no significant media x isolation interaction occurred. This indicates that the differences in growth due to media are consistent across isolates. The results from the second run indicated that interaction was significant in both A and JA. Nonaggressive isolate TN grew at a faster rate on callus cultures generated on CMC than on CMS, but PMP1 showed no significant difference in growth regardless of media.

The HPLC analysis was used to determine the relative levels of aromatic secondary metabolites expressed from the callus cultures. Comparing callus growth on two media, significant differences were observed for some, but not all, of the explant selections tested. For A, callus grown on CMS yielded higher chromatogram profiles than callus grown on CMC for both of the respective callus regions and fungal challenges tested (Figure 1). In dramatic contrast, however, the DEL2 selection yielded higher metabolite levels for callus grown on CMC than on CMS (Figure 2).

Results were mixed for JA and 8630 selections, with neither CMC nor CMS yielding consistently higher levels of metabolites under any respective condition tested in triplicate (data not shown). In the case of S, the comparisons were complicated, as the species of metabolites expressed were different for CMC and CMS (Figure 3).

The HPLC analysis did not show the effect of isolate differences to the same degree as the fungal growth assay. The fungal isolates used to challenge these callus growths showed no significant effect, with the exception of PMP1 on JA calli grown on CMS media, which yielded lower chromatogram profiles relative to TN and uninoculated controls.

The age of callus cultures significantly affected the expression profile. Chromatograms of extracts from A callus grown on CMS for 12 weeks were lower in level than those from 6-week ones and more closely resembled 6-week ones grown on CMC.

Discussion

We have investigated the possibility of using in vitro systems for early screening of susceptibility

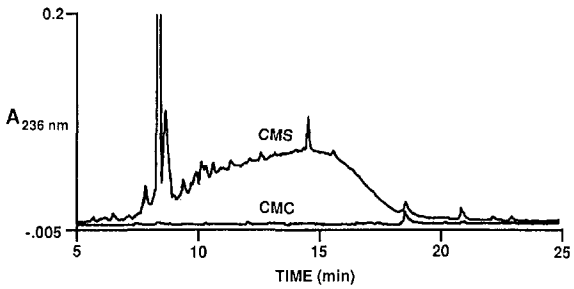

Figure 1. Overlayed HPLC profiles of metabolites extracted from callus derived from *U. americana* selection A. Top trace is from callus grown on CMS medium; bottom trace is from callus grown on CMC medium. Calli were inoculated with *O. ulmi* PMP1 and tissue distal to the fungal growth front was harvested for extraction.

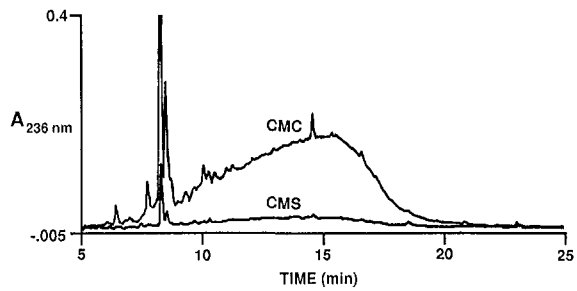

Figure 2. Overlayed HPLC profiles of metabolites extracted from callus derived from U. americana selection DEL2. Top trace is from callus grown on CMC medium; bottom trace is from callus grown on CMS medium. Calli were not inoculated with fungus (control group).

and resistance of elm selections to the DED fungus, *O. ulmi.* Several studies have shown definite correlation between in vivo and in vitro systems; while other reports show absence of such a correlation. Various factors such as media composition, temperature, inoculum level, phytohormones and tissue morphology can influence the host-pathogen interaction in vitro (5,9,11,19).

In an earlier study, we reported that the presence or absence of coconut milk can affect the growth of *O. ulmi* on callus cultures from different elm selections (5). This was also reported by Holmes (7). In the present study, we have shown that the source of coconut milk can also influence fungal growth. In addition, we now find that the

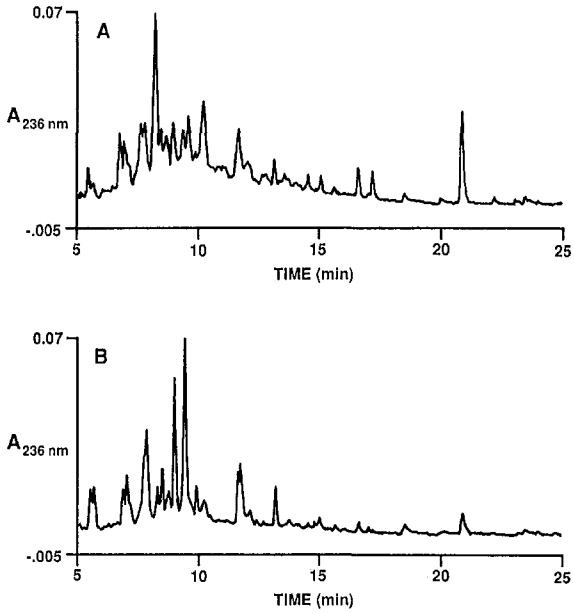

Figure 3. HPLC profiles of metabolites extracted from callus derived from U. pumila. A. Callus grown on CMC medium B. Callus grown on CMS medium. Callus pieces were inoculated with O. ulmi PMP1 and tissue distal to the fungal growth front was harvested for extraction.

secondary metabolite production by callus culture was also influenced by the type of coconut milk in the medium and by the elm selection.

In another previous study, we reported that the age of callus tissue can affect the growth response of fungus, depending upon the elm selection (6). That observation is confirmed in this paper. Such a relationship was further substantiated by our chromatographic analysis which showed that the profile of our 12-week-old callus differed from that of the 6-week-old ones. This difference suggests that over a period of time callus begins to change, probably due to somaclonal variation.

In vivo studies (21) have shown that young American elm seedlings are more resistant to O. ulmi than mature elm trees. However, fungal growth data using callus cultures from mature A trees and juvenile JA seedlings do not clearly show such a relationship.

Results from this and other studies (4,5,6) show that it may be difficult to use a laboratory glassware elm model system to predict responses

in the field. We found that environmental factors such as temperature, media constituents, and callus age can affect the fungal growth response and the secondary metabolite production. These factors, as well as others that are either unknown or difficult to control may significantly influence results obtained in a laboratory setting.

Importance to arborists

Selecting elms for resistance to DED is laborious and time consuming. This study and others are attempts to find out if by using tissue culture technology, we can speed up identification of resistant elms. Callus cultures, from susceptible and resistant elms, initiated on media with two different sources of coconut milk, were exposed to aggressive and non-aggressive isolates of *O. ulmi*. The results showed that source of coconut milk can affect growth of fungus. Thus it may be difficult to develop a laboratory system which corroborates the responses obtained in the field.

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Résumé. Cette étude comparait les effects de deux sources de lait de noix de coco sur la croissance de *Ophiostoma ulmi* sur cinq cultures différentes de tissu de cal d'orme. Un des medium (CMC) contenait du lait de noix de coco provenant de noix de coco fraîches, et l'autre médium (CMS) était additionné de lait de noix de coco provenant de la Sigma Chemical Co. Ces études démontrèrent que la source de lait de noix de coco affectant la croissance fongique était reliée, de façon significative, à la sélection d'orme et à l'agressivité de *Ophiostoma Ulmi* pris comme organisme isolé. Une évaluation était également réalisée sur la production de métabolites secondaires sur les cultures de tissus de cal en fonction de la source de lait de noix de coco.

Zusammenfassung. Diese Studie vergleicht die Auswirkungen von zwei Quellen von Korkusnußmilch auf das Wachstum von Ophiostoma ulmi auf fünf Selektionen von Ulmenkallus-gebilden. Ein Medium (CMC) enthielt Kokusnußmilch aus frischen Kokusnüßen und das andere Medium erhielt den Zusatz von Kokusnußmilch, die von der Sigma Chemical Co. geliefert wurde. Diese Studien zeigten, daß basierend auf der Ulmenselektion und der Agressivität des Ophiostoma-ulmi-Isolats die Quelle der Kokusnußmilch entscheidend für das Pilzwachstum ist. Basierend auf der Quelle der Kokusnußmilch wurde eine Bewertung der Produktion von sekundären Metaboliten der Kalluskulturen vorgenommen.