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Effects of Metabolic Products of Cellulose-Utilizing Organisms on Hydrilla

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ABSTRACT

Organic-rich sediments from lakes where growth of hydrilla (*Hydrilla verticillata* (L.f.) Royle) appeared to be inhibited were extracted previously, and the extracts were shown to inhibit growth of hydrilla cultured under laboratory conditions. The same sediments were used to isolate organisms that may utilize cellulosic material with production of metabolic products that inhibit growth of hydrilla. A cellobiose-based medium was used, and metabolic products were isolated by filtering the medium and autoclaving the filtrate. A 3-day growth period produced the maximum yield of hydrilla-inhibiting material, as measured by changes in fresh weight of hydrilla and by changes in chlorophyll content. High performance liquid chromatograms for the metabolic product and for the hydrilla-inhibiting extract provided an indication of the similarity of the inhibitors.

Key words: inhibitors, fungi, bacteria, cellobiose, sediment, cellulose degradation.

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INTRODUCTION

Dooris and Martin (1980) suggested that the hydrilla-inhibiting organic material(s) found in certain lakes may be a precursor or an intermediate in lignin synthesis and is the result of microbial degradation of lignified tissue. Though the lakes seem to be unique because of associated cypress (*Taxodium distichum*), aqueous extracts of the trees (bark or leaves) did not inhibit hydrilla growth.

The presence of this hydrilla inhibitor was determined in various natural waters using HPLC (high performance liquid chromatography). The intensity of a diagnostic peak (*i.e.*, one characteristic of the inhibitor) in the HPLC was directly related to the relative concentration of the inhibitor, and was inversely related to the relative abundance of hydrilla (Martin *et al.* 1986).

Additional studies provided more information about the hydrilla inhibitor(s). Rate studies indicated the inhibitor suppressed photosynthesis and increased respiration rates (Barltrop and Martin 1983, 1984). In addition, evidence was obtained that indicated the inhibitor served as a sensitizer for singlet oxygen. Finally, the effect of the inhibitor on the ultrastructure of hydrilla was examined through the use of

electron microscopy (Dooris *et al.* 1988). This study revealed that the inhibitor caused an increase in starch accumulation and a distortion of chloroplasts.

The production of the inhibitor by organisms acting on organic substrate has remained unstudied until recently. The present study was concerned with production of hydrilla inhibitors in the laboratory. The strategy involved inducing growth of microorganisms present in lake sediments (Lake Starvation and White Trout Lake, Hillsborough County, Florida) by providing a suitable organic substrate (a cellobiose-based medium).

MATERIALS AND METHODS

Hydrilla samples were obtained from a retention pond located northeast of Fowler Avenue and Bruce B. Downs Boulevard, north of University Square Mall in Tampa. Other samples were obtained from the Hillsborough River under Morris Bridge west of I-75 and from an area south of the 40th Street underpass. Hydrilla samples were rinsed, cleaned of debris in tap water, then stored in Floridan aquifer well water in aquaria with lighting provided by cool white fluorescent lamps (12 hr light: 12 hr dark); 80 μmol/m^2/sec at 23C.

Lake sediments were taken from stored samples whose source has been described previously (Dooris and Martin 1980, Martin *et al.* 1986, Dooris *et al.* 1988). The White Trout Lake sample had been stored dry at room temperature for several years in a sealed container; Lake Starvation samples had been stored in a freezer at -17C.

Hydrilla inhibitor preparations were made as follows: Sediment (5.0 g dry weight basis) was added to each of eight 250-ml Erlenmeyer flasks containing sterilized growth medium, containing (g/L): cellobiose (Sigma), 10; ammonium sulfate, 0.5; $MgSO_4 \cdot 7\ H_2O$, 0.2; calcium chloride, 0.1; yeast extract (Difco), 0.5; distilled water (to 1 liter). A control culture was prepared using the same ingredients, except dextrose (10.0 g), an alternative carbon source, was substituted for the cellobiose. Flasks (with gauze or foam stoppers) were placed in a gyrorotary shaker bath (New Brunswick Scientific, model G76, 60 rpm) at 27C for a known period, typically 10 days, by which time all bubbling had ceased. The pH was monitored daily with a pH meter (Chemtrix, Type 40 E). After 10 days pH was constant at 3.0.

The extracts from 3-, 6-, and 10-day cultures were collected by filtering through Whatman #1 filter paper, then the filtrates were autoclaved at 120C for 20 min. [The filter paper was saved and stored in a sealed container for further culturing of the spores on growth media.]

The procedure of Dooris and Martin (1980) was used for bioassays. Specifically, 1.0-g hydrilla sprigs were weighed and placed into 500-ml Erlenmeyer flasks containing varying volumes of filtrate duplicate samples (0 to 400 ml) and diluted with sterilized 10% Hoagland's medium (Steward and Elliston 1974) until the flasks were filled. The flasks were sealed with rubber stoppers, inverted, and exposed to 40-W cool white fluorescent lights on both sides (12 hr light: 12 hr dark) with an intensity of 80 μEinsteins/m^2/sec for 10 days, and then the fresh weight was again determined, and the percent change was calculated.

Bioassays were also done by measuring the change in chlorophyll content. Samples of hydrilla were treated as before, but at the end of the bioassay, the sprigs were collected from each flask, and homogenized in a Waring blender. Chlorophylls were extracted into 80% (v/v) aqueous acetone, and the absorbance was measured at 647 and 664 nm, using a Shimadzu recording spectrophotometer. Chlorophyll *a* and *b* and total chlorophyll were calculated using standard equations (Combes *et al.* 1985).

HPLC chromatograms were obtained using a Beckman (Altex) model 110 liquid chromatograph equipped with model 160 solvent programmer, and a LKB (model 2238) multiwavelength detector set at 254 nm for these experiments. A Dupont preparative scale Zorbax™ (21.2 x 350 mm) column was used, and in all analyses a linear gradient was used during a 20-min analysis starting with 60:40 water-methanol, and ending with 100% water. Prior to injection, the sample was passed through a C-18 Bond-elut® cartridge (Analytichem International) by centrifuging for 3 to 5 min on a clinical centrifuge. Inorganic and total carbon were measured using a Beckman model 915 carbon analyzer.

RESULTS AND DISCUSSION

The extracts obtained from culturing the soil samples had certain characteristics in common, regardless of the replicate or source. The pH of the medium decreased from an initial value of about 6.0 to a minimum (pH 2.9 to 3.5). The extract was malodorous.

Additions of filtrates from the extract were deleterious to the hydrilla: within 72 hr, tissue became chlorotic and soft. Roots disintegrated and detached from the plant. Turions wilted and became flaccid. In contrast, control samples in 10% Hoaglands at pH 7, had a fresh-weight increase of 10 to 20% in a 10-day period, and the sprigs were green. Other controls were prepared. One cellobiose control consisted of the initial medium pH 6.0 (without sediment) with the pH adjusted to 3.0. The change in fresh weight was 11.0 ± 4.1% (mean ± SE) for 10 to 100 ml of filtrate (Table 1).

Another cellobiose control was used to check the effect of acidity. The extract from the cellobiose culture (pH 3.0) was neutralized with 0.25 *M* NaOH prior to dilution with Hoagland's solution (final pH 6.7) and compared with the

TABLE 1. SUMMARY OF CONTROL SYSTEMS TESTING THE EFFECT OF pH AND MEDIA ON CHANGES IN THE FRESH WEIGHT OF HYDRILLA OVER A 10-DAY PERIOD.

Sample	N	% change[1]
10% Hoagland's at different initial pH		
pH 6.6	10	11.4 ± 2.3
pH 6.6	10	12.0 ± 2.8
pH 5.7	20	9.0 ± 1.3
Celloboise extract, pH 3.0 adjusted to pH 7.0, different volumes of extract (10 to 100 ml) diluted to 500 ml with 10% Hoaglands with final pH 6.7	10	12.1 ± 2.7
Celloboise control, without sediment, initial pH 6.0, adjusted to pH 3.0, 10 to 100 ml diluted to 500 ml with 10% Hoaglands	10	11.0 ± 4.1

[1]Mean ± S.E.

result of not acidifying when 10 to 100 ml of filtered culture media were added. No significant differences were noted (see Table 1) indicating the cellobiose was not being degraded at pH 3.0 to produce hydrilla inhibitors.

A third cellobiose control (20 ml) consisted of the medium left uncovered in the laboratory for a 10-day period, then added to 10% Hoaglands and bioassayed. The mean change in fresh weight was 18 ± 9%, consistent with controls, *i.e.*, 10% Hoaglands at pH 7 (21 ± 8.3%).

Experiments were conducted to check for the effectiveness of related media in which dextrose was substituted for cellobiose. The extract (after 10 days) was malodorous and had a pH of 3.5. Twenty milliliters of extract was diluted to 500 ml with 10% Hoaglands to give a final pH of 5.7 and the effect on hydrilla was bioassayed. The control system (10% Hoaglands adjusted to pH of 5.7) had a growth increase of 9.0 ± 1.3% over a 10-day period, whereas the test fresh weight decreased 6.2 ± 1.1% (N=20). Each test flask, however, showed an overgrowth of microbes, and most of the stoppers were pushed open by gas bubbles. For better comparison, 20 ml of dextrose medium was added to 10% Hoagland's and the mixture (pH 6.6) was bioassayed. Both control (dextrose medium) and test (dextrose medium plus inhibitor) experienced excessive organism growth, and there was no statistically significant difference (Student's t-test) between the two systems.

The effect of re-culturing sediment samples was investigated. A previously used sample was autoclaved, then cultured, and after 10 days, the filtrate was collected. No unusual odor was noted, and addition of 20 ml of extract did not cause notable decrease in growth of hydrilla. Apparently the organisms were not airborne nor an adventitious contaminant, *i.e.*, the organisms were isolated from the sediment.

The results of 10-day incubation in cellobiose medium yielded extracts that consistently inhibited hydrilla. Extracts from three different cultures were bioassayed, and the volume of extract needed to produce zero growth or loss of biomass was 16 ± 3 ml. Possibly subsequent experience might lead to a lowering of the value of extract to produce the zero-growth effect in the bioassays. One possibility for improvement was length of culturing.

Sediments were cultured for 0, 3, 6, and 10 days, and 20 ml samples of each were used for the bioassay (10 replicates) with the 20-ml extract diluted to 500 ml with 10% Hoaglands of final pH 7. The mean percent changes in fresh weight were -40 ± 7 (3 days), -16 ± 3.5 (6 days), and -26 ± 7 (10 days) and -21 ± 7 (10 days). These results suggest that optimum yield of hydrilla inhibitor occurred at an early stage, e.g., after 3 days and that the subsequent growth inhibition was about constant within experimental error. The bioassay behavior of the three extracts (three versus 6- and 10-day incubation) was different. While chlorosis was not observed for the 3- and 6-day incubation extracts, damage was severe: leaves had disintegrated, and it was necessary to collect the sample on cheesecloth. Disintegration was also observed for 6-day extracts. Chlorosis and disintegration were observed with 10-day extracts.

Two different types of bioassays were run. Typically, change in fresh weight was studied, but for one bioassay, the change in total chlorophyll content was also studied in parallel with change in fresh weight for doubly replicated samples as a function of volume of 10-day extract (Figure 1). The agreement between the results for total chlorophyll and change in fresh weight was generally good, and supports the validity of using change in fresh weight as a means of characterizing inhibition.

However, there is a complicating factor involved in this particular bioassay: the pH was not adjusted, and the pH of solutions containing more than 30 ml of extract were less than 5.0. The effect of pH probably becomes significant only at pH of 3.5 or less (Trent *et al.* 1978, Table 1). In previous bioassays, the pH of the final solution of Hoaglands was adjusted so that the pH with extract was 6.7.

The research strategy was formulated from a general hypothesis of Dooris and Martin (1980) that a chemical inhibitor detected in water and sediment from lakes that do not support prolific growth of hydrilla is derived from microbial degradation of cellulose material. This material may derive from bald cypress, but past experience (Martin *et al.* 1986) has indicated that other sources of carbon may be involved. It was hypothesized that fungi might be the primary organisms responsible for the degradation.

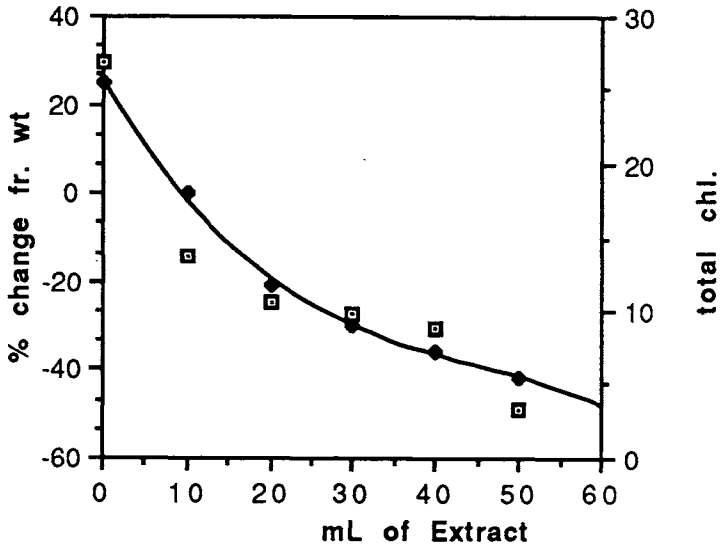

Figure 1. Percent change in fresh weight of hydrilla (closed diamonds) and total chlorophyll content (squares) as a function of extract. Total chlorophyll content was calculated from mean absorbance of chlorophyll *a* and *b*.

Previously collected sediment samples from White Trout Lake and Lake Starvation were used to inoculate media, and several considerations were involved. Cellobiose was selected because it is a smaller unit of cellulose, and it was believed that the chemical inhibitor was derived from degradation of the cellulose/lignin material. Also, the culture medium, pH = 6.0, was reported (Booth 1971) to be highly selective for fungi. The pH was selected because lakes that tend to be rich in tannins and acidic do not support rampant growth of hydrilla. In addition, a pH of 6.0 would inhibit the overpopulation of competing organisms.

Cultures were allowed a growth period of about 10 to 14 days. At the end of this time, gas evolution was at a minimum or could not be detected. The pH had reached a minimum and remained constant at 2.5 to 3.0. Presumably at this pH, competitive organisms that might initially be able to utilize cellobiose media would be eliminated. In retrospect, it appears that a shorter period of incubation (perhaps 3 to 4 days) might have the advantage of greater activity.

Previous experiments indicated that a pH of 3.5 did not affect the growth of hydrilla (Trent *et al.* 1978), but there was still concern that the low pH of the extract could be adversely affecting the growth of hydrilla in the assays. Four controls were tested: 10% Hoagland's at different initial pH, varying amounts of cellobiose extract (day 1) with initial pH 3.0 added to Hoaglands (with final pH adjusted to pH 6.7) (Table 1). We conclude from these results that neither pH, nor the volume of cellobiose medium used, affected the change in fresh weight of hydrilla under assay conditions.

Assays of extract activity indicated several features about the production of the hydrilla-inhibiting chemicals. The volume of inhibitor to reduce growth to 0% was consistent (16 ± 3 ml) for a 10-day incubation period. Probably maximum activity was reached during the first few days, and similar results were obtained whether monitoring change in fresh weight or change in total chlorophyll content (Figure 1). The pattern of behavior was consistent with that observed previously, i.e., development of significant chlorosis.

While attention was focused on a medium for fungi development, we did not neglect the possibility of other organisms. The effectiveness of dextrose utilization was compared with that of cellobiose. A control dextrose medium was evaluated, and the control sample (medium only, no sediment) clearly showed an inhibition as well as an overpopulation of organisms attached to the hydrilla sprigs. This experiment reaffirmed that opportunistic organisms living on hydrilla can become detrimental to its survival (Mansell and Silver 1974). Possibly the growth inhibitor studied here affects the change of phenolic substances produced by the plant (*cf.* Woodward *et al.* 1974).

We are not the first to be concerned about deleterious organisms on hydrilla. Charudattan (1973) has been concerned with the pathogenicity of fungi and bacteria for years. More recently, Joye and Cofrancesco (1991) described the isolation of about 200 fungal and 27 bacterial organisms from hydrilla. From their isolates, Joye and Cofrancesco (1991) determined that *Macrophomina phaseolina* isolate was detrimental to hydrilla and duck lettuce *Ottelia alissmoides* (L.) Pers.

We believe that the organisms that we are examining act in a different manner, i.e., they act on a cellulose substrate and produce an inhibitory substance that occurs in locations where there is no hydrilla.

The present study is linked to previous studies (Dooris and Martin 1980, Martin *et al.* 1986, Dooris *et al.* 1988) in a common search for the significance and identity of a naturally occurring hydrilla inhibitor. The similarity of properties between the previously studied hydrilla inhibitor and the material produced in this study has been described. One other significant similarity exists: similarity of HPLC chromatograms. For cellobiose extract, the HPLC peak appeared at 69 ± 3% of the programmed run versus 65% for the Lake White Trout extract. While the absolute location is different from previous results, the injection loop used in the HPLC unit was also different, 100 µl vs 20 µl.

Given the convenience of the method of production of hydrilla inhibitor by culturing, it should be much easier to seek out the chemical identity of the hydrilla inhibitor(s).

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