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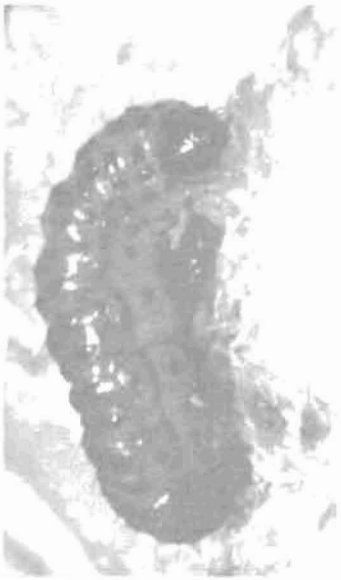

ENVIRONMENTAL LABORATORY

AQUATIC PLANT CONTROL RESEARCH PROGRAM

TECHNICAL REPORT A-85-3

BIOLOGICAL CONTROL OF *HYDRILLA VERTICILLATA* (L.f.) ROYLE WITH LYTIC ENZYME-PRODUCING MICROORGANISMS

by

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September 1985
Final Report

Prepared for

DEPARTMENT OF THE ARMY
US Army Corps of Engineers
Washington, DC 20314-1000

Unclassified

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE

READ INSTRUCTIONS BEFORE COMPLETING FORM

1. REPORT NUMBER: Technical Report A-85-3
2. GOVT ACCESSION NO.:
3. RECIPIENT'S CATALOG NUMBER:

4. TITLE (and Subtitle): BIOLOGICAL CONTROL OF *HYDRILLA VERTICILLATA* (L.f.) ROYLE WITH LYTIC ENZYME-PRODUCING MICROORGANISMS

5. TYPE OF REPORT & PERIOD COVERED: Final report

6. PERFORMING ORG. REPORT NUMBER:

7. AUTHOR(s): Judith C. Pennington

8. CONTRACT OR GRANT NUMBER(s):

9. PERFORMING ORGANIZATION NAME AND ADDRESS:
US Army Engineer Waterways Experiment Station
Environmental Laboratory
PO Box 631, Vicksburg, Mississippi 39180-0631

10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS:
Aquatic Plant Control Research Program

11. CONTROLLING OFFICE NAME AND ADDRESS:
DEPARTMENT OF THE ARMY
US Army Corps of Engineers
Washington, DC 20314-1000

12. REPORT DATE: September 1985

13. NUMBER OF PAGES: 41

14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office):

15. SECURITY CLASS. (of this report): Unclassified

15a. DECLASSIFICATION/DOWNGRADING SCHEDULE:

16. DISTRIBUTION STATEMENT (of this Report):
Approved for public release; distribution unlimited.

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report):

18. SUPPLEMENTARY NOTES:
Available from National Technical Information Service, 5285 Port Royal Road, Springfield, Virginia 22161.

19. KEY WORDS (Continue on reverse side if necessary and identify by block number):

Aquatic plant control	Lytic enzyme-producing microorganisms
Biocontrol	Mycoherbicides
Hydrilla	
Induced pathogenicity	

20. ABSTRACT (Continue on reverse side if necessary and identify by block number):

Bacteria and fungi isolated from the microsphere of hydrilla were screened for production of the lytic enzymes cellulase and pectinase. Lytic enzyme-positive isolates were successively subcultured on restrictive media in the laboratory to enhance enzyme production. Twenty-two of the enzyme-enhanced isolates were assayed using hydrilla sprigs in test tubes to determine their ability to infect the plant. Seven cellulase-producing fungi

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20. ABSTRACT (Continued).

produced significantly greater damage to hydrilla than exhibited by treated or untreated controls.

Six of these were assayed using hydrilla plants in 75.7-ℓ aquaria. None of the fungi significantly damaged hydrilla in the aquaria. Inoculum for the aquarium assay consisted of filtered organisms, whereas whole inoculum including nutrient growth medium (PDB) and accumulated exogenous metabolites (e.g. cellulase) was used in the preliminary test tube assay. This difference in inoculum for the two assays and the negative results of the aquarium assay suggested that the isolates required nutrient or metabolite supplements in the inoculum to inflict damage on hydrilla. The preliminary test tube assay was conducted on hydrilla from a field site, whereas the aquarium assay was conducted on hydrilla grown in the greenhouse. To examine the requirement for nutrients or metabolites and to compare resistance of field and greenhouse hydrilla, two additional test tube assays were conducted. The assays were designed to compare effects of whole inoculum, filtered organisms, and filtrate (i.e. accumulated metabolites and nutrients). One assay used field plants; the other used greenhouse plants. Results showed that whole inoculum produced significantly greater damage than filtered organisms and that nutrients must be present for the organisms to impact hydrilla. Results of the two assays also showed that greenhouse plants were more resistant to attack than field plants.

PREFACE

This report describes a laboratory study to develop microbiological agents for the control of hydrilla by enhancing lytic enzyme production of isolates from the microsphere of the plant.

Funding for this study was provided by the Office, Chief of Engineers (OCE), under appropriation number 96X3122, Construction General, to the Aquatic Plant Control Research Program (APCRP), US Army Engineer Waterways Experiment Station (WES), Vicksburg, Mississippi. The OCE Technical Monitor of the APCRP was Mr. E. Carl Brown.

The study was conducted by Mrs. Judith C. Pennington of the Wetland and Terrestrial Habitat Group (WTHG), Environmental Resources Division (ERD), Environmental Laboratory (EL), WES.

Team Leaders for the Biocontrol Team during the study were Mr. Edwin A. Theriot and Dr. Dana R. Sanders, Sr. The study was conducted under the direct supervision of Dr. Hanley K. Smith, WTHG, and under the general supervision of Dr. Conrad J. Kirby, Jr., Chief, ERD, and Dr. John Harrison, Chief, EL. Manager of the Aquatic Plant Control Research Program was Mr. J. Lewis Decell. The report was edited by Ms. Jamie W. Leach of the WES Publications and Graphic Arts Division.

During the preparation of this report COL Tilford C. Creel, CE, and COL Robert C. Lee, CE, were Commanders and Directors of WES and Mr. Fred R. Brown was Technical Director. At the time of publication, COL Allen F. Grum, USA, was Director of WES and Dr. Robert W. Whalin was Technical Director.

This report should be cited as follows:

Pennington, J. C. 1985. "Biological Control of *Hydrilla verticillata* (L.f.) Royle with Lytic Enzyme-Producing Microorganisms," Technical Report A-85-3, US Army Engineer Waterways Experiment Station, Vicksburg, Mississippi.

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CONVERSION FACTORS, NON-SI TO METRIC (SI)
UNITS OF MEASUREMENT

Non-SI units of measurement used in this report can be converted to metric (SI) units as follows:

Multiply	By	To Obtain
acres	4046.873	square metres
feet	0.3048	metres
miles (U.S. statute)	1.609347	kilometres

BIOLOGICAL CONTROL OF *HYDRILLA VERTICILLATA* (L.f.) ROYLE WITH LYTIC ENZYME-PRODUCING MICROORGANISMS

PART I: INTRODUCTION

Background

1. Hydrilla [*Hydrilla verticillata* (L.f.) Royle] is a submersed aquatic macrophyte introduced into the United States from Asia or Africa. Like many other introduced species, in the absence of natural enemies or native inhibitors, hydrilla reproduced so rapidly that it became a problem in temperate waterways of the southeastern United States and California. Its abundant masses impede water flow, restrict navigation, and interfere with recreational activities such as swimming and fishing.

2. Hydrilla has not yet reached its potential distribution in the United States. Its presence as far north as Iowa and Delaware has been confirmed. This demonstrates that it is capable of survival in colder climates than it now inhabits. In the past 25 years, it has spread to 11 states and the District of Columbia. All Gulf and Atlantic Coast states south of Delaware, except Virginia and Mississippi, have confirmed populations (Haller 1982).

3. The rapid proliferation of hydrilla is attributable to vegetative reproduction from stem fragments, tubers, and turions (winter buds produced in leaf axils). Tubers and turions enable the species to survive cold temperatures and drought. Tubers and turions also provide a means of regeneration if the vegetative mat is eliminated. Although hydrilla is capable of sexual reproduction by seeds, male flowers have been identified rarely in the United States, and reproduction from seeds is not yet a problem (Tarver et al. 1978).

4. Hydrilla exhibits tolerance to a wide range of ecological conditions. It occurs in water that is clear to very turbid, oligotrophic to highly eutrophic, and shallow to more than 15 m deep. It can tolerate alkaline to slightly acidic conditions and moderate salinity. Its low light requirement enables it to successfully compete with other submersed aquatic plants (Tarver et al. 1978).

Approach

5. The conventional approach to biological control of any exotic species has been to introduce a natural enemy imported from the country of origin. However, no natural enemies of hydrilla have yet been imported that are promising biocontrol candidates. Therefore, a less conventional approach was pursued in this study. The research began with a search among the endogenous microflora of the plant for saprophytes that break down plant tissues during normal seasonal senescence. Some investigators (Blotnick et al. 1980) believe that aquatic macrophytes such as hydrilla possess a unique complement of microorganisms that function in the decay process. These microorganisms produce enzymes capable of lysing specific plant components such as cellulose and pectin. If such microorganisms can be induced to increase their production of lytic enzymes, they may become capable of attacking plant tissues at any time during the growing season. Several unseasonal declines of hydrilla have been observed in which only saprophytic microorganisms were isolated.

6. Enhancement of lytic enzyme production was performed in the laboratory by successively subculturing isolates on media containing cellulose or pectin as the sole carbon source. These microorganisms were then reintroduced to hydrilla and several were capable of attacking and destroying the plant. Advantages of utilizing such induced pathogens over conventional approaches are: (a) elimination of costly overseas searches for natural enemies; (b) preclusion of the need for long quarantine trials required for imported organisms; (c) production of pathogens derived from, and therefore already acclimated to, the host environment; and (d) reduction of environmental risks by producing pathogens that are likely to be host-specific.

Purpose and Objectives

7. The purpose of this study was to develop a biocontrol agent capable of reducing the growth and reproduction of hydrilla to acceptable levels. Specific objectives were as follows:

a. Isolate endogenous microorganisms from hydrilla collected in several states.

b. Select candidate microorganisms by testing isolates for production of cellulase and pectinase.

c. Taxonomically characterize candidates.

d. Enhance production of lytic enzymes by successively subculturing candidates on restrictive media.

e. Demonstrate efficacy of candidate microorganisms on hydrilla.

f. Determine whether isolates require nutrients and accumulated metabolites in the inoculum.

g. Compare resistance to isolates by hydrilla grown in the field to hydrilla grown in the greenhouse.

PART II: MATERIALS AND METHODS

Sampling of Hydrilla

8. To provide a broad complement of isolates, hydrilla was collected from four locations across its southern range. The locations were Imperial Valley, California; Lake Conroe, Texas; Lake Theriot, Louisiana; and Lake Trafford, Florida. All available portions of the plant were sampled, including roots, tubers, turions, stems, and leaves.

9. Eight sites in the Imperial Valley were sampled, one during spring 1981 and the other seven during fall 1981. The spring samples were collected from Reese's Pond, a 2-acre* pond located 5 miles north of Brawley, California. Hydrilla was confined to the fringe of the pond following an unexplained decline. The seven sites sampled in the fall were: (a) stressed and unstressed plants in the Calexico International Golf Course Lake, a 1-acre lake heavily infested with hydrilla; (b) three sites in lateral three of Wormwood Canal, a 1-mile, moderately infested canal off the All-American Canal; (c) one heavily infested site in the All-American Canal located 1 mile west of Wisteria check, or gate; and (d) one site at J. M. Sheldon Reservoir, a 56-acre temporary re-regulating reservoir averaging 5 to 6 ft in depth and containing only sparse hydrilla growth because of previous chemical control efforts. Four sites were sampled on Lake Conroe, Texas, a 21,000-acre reservoir with an average depth of 20.5 ft. The lake supported dense hydrilla infestations along its fringe and in the many shallow coves. Two sites were sampled in spring 1981 near League Line Marina and two sites were sampled southwest on Twin Shores, all in the southeastern portion of the Lake. Two sites were sampled in summer 1981 in a cove near Lake Conroe Hills and two sites were sampled between Walden on Lake Conroe and Cape Conroe. Four sites in Lake Lewis, a small lake very near and connected to Lake Conroe by streams, were also sampled in summer 1981 because hydrilla in the lake had become necrotic and was dropping out. Lake Lewis averages less than 11 ft in depth and had supported a dense hydrilla infestation. The Walden/Cape Conroe sites

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* A table of factors for converting non-SI units to metric (SI) units of measurement is presented on page 3.

were resampled in fall 1982. Four sites were sampled in a half-mile reach of a feeder canal located about 1 mile north of Lake Theriot, Louisiana. The canal supported a moderate to sparse fringe of hydrilla. Samples were collected randomly in this area in spring 1981 and fall 1982. Lake Trafford, Florida, a small lake of 100 to 200 acres, was sampled twice in spring 1981 and once in summer 1982. Lake Trafford was heavily infested with hydrilla. Each quadrant of the lake was sampled once and two additional stressed areas were sampled in summer 1982.

Isolation of Fungi and Bacteria

10. Plant material from each location was separated into the following plant parts: meristematic stem tips, stems, leaves, roots, and, when found, turions and tubers. Plant parts from the same location were combined, rinsed thoroughly through three portions of sterile distilled water, and ground in a manual tissue grinder. The well-mixed slurry was streaked for isolation on three plates of potato dextrose agar (PDA) per tissue type for isolation of fungi and on three plates on nutrient agar (NA) per tissue type for isolation of bacteria.

Lytic Enzyme Screening

Cellulase production

11. All isolates were screened for cellulase production by several methods. The most rapid method consisted of a tube containing 2 ml of mineral salts agar overlaid with 0.5 ml of cellulose-azure* agar (Smith 1977). As the cellulose was utilized by the microorganism, the azure (a blue dye) was uncoupled and diffused into the clear basal layer (Figure 1). The intensity of blue color in the basal layer was a semiquantitative indication of the amount of cellulose utilized by the microorganism. Positive results were obvious in 1 or 2 days. Another test for cellulase production consisted of test tubes containing 2 ml of mineral salts medium and 1- by 14-cm filter paper strips (Whatman No. 1) (Rodina 1972). Isolates utilizing the filter paper strips

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* Calbiochem, 10933 N. Torney Pines Rd., LaJolla, Calif.

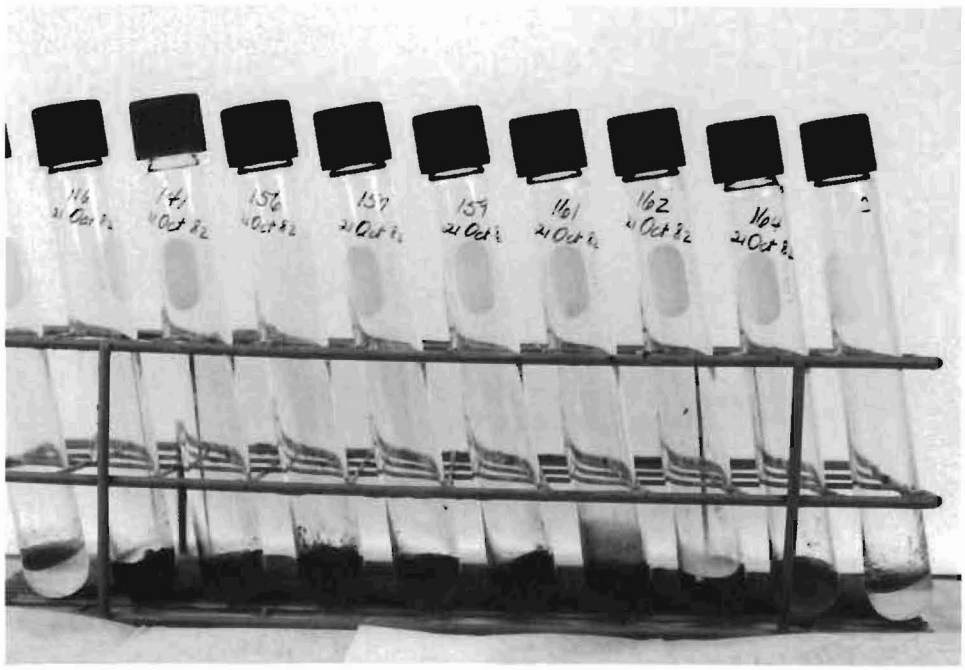

Figure 1. Cellulose-azure screening test for cellulase production. Microorganisms capable of utilizing cellulose uncouple the azure (a blue dye) allowing it to diffuse into the clear basal layer. The intensity of blue color in the basal layer is a semiquantitative indication of the amount of cellulose utilized. Each end tube is a negative control

(cellulose) eventually severed the strip at the air/liquid interface and digested the strip below the medium surface (Figure 2). Cellulase positive isolates were successively subcultured on medium in which cellulose was the sole carbon source (cellulose agar plates) through a minimum of ten transfers to enhance enzyme production. More elaborate quantification of cellulase production was considered to be beyond the scope of the present study, but could become necessary if results of this study indicate that cellulase-enhanced isolates are capable of damaging hydrilla.

Pectinase production

12. All isolates were also screened for pectinase production by streaking on pectin medium (Skermann 1967). Pectin medium consisted of a petri dish containing a basal layer of nutrient agar with 0.50-percent $CaCl_2$ added and a thin (5-ml) overlay of citrus pectin* solution dried to a solid film.

* Sodium Polypectate, Exchange Brand, Sunkist Growers, 720 East Sunkist St., Ontario, Calif.

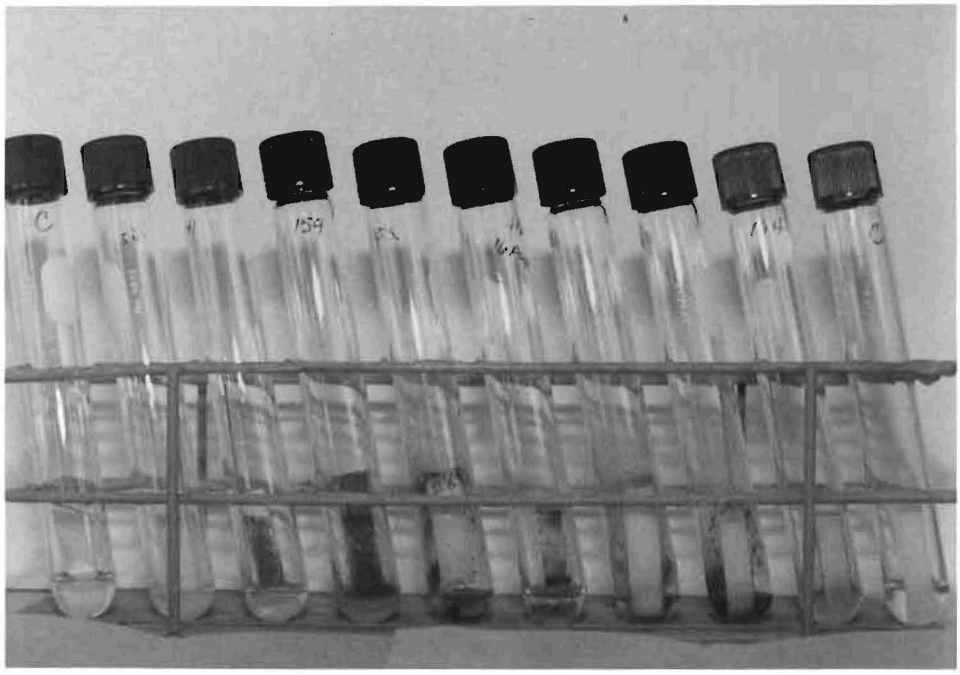

Figure 2. Screening test for cellulase production using mineral salts medium containing filter paper strips. Positive isolates digested the strip, eventually severing it at the air/liquid interface (third through sixth tubes from left). Each end tube is a negative control

Positive enzyme production was indicated by pitting of the pectin layer around bacterial colonies or fungal mycelia (Figure 3). Pectinase production was enhanced by successively subculturing (a minimum of ten times) colonies or mycelia producing the widest zones of indentation in the pectin medium.

Identification of Isolates

13. Limited chemical and microscopic characterization of all bacterial isolates and presumptive identification of all fungal isolates were conducted. Table 1 lists microscopic examinations and chemical tests conducted with bacterial isolates. Fungal isolates were examined on Sabouraud's dextrose agar and V-8 Juice agar for gross mycelial characteristics. Slide cultures were prepared for examination of spores and other fine structures. All fungal isolates were sent to Dr. Tim Schubert, Florida Department of Agricultural and Consumer Services, University of Florida, for more comprehensive taxonomic characterization.

Figure 3. Pectin medium showing pitting caused by utilization of the pectin by bacterial colonies

Preliminary Hydrilla Test Tube Assay

Testing

14. Twenty-two lytic enzyme-producing isolates were tested: twenty cellulase positive fungi, and two pectinase positive isolates (i.e. one bacterium and one fungus).

15. Fungal inocula were prepared by introducing each isolate into a 125-ml erlenmeyer flask containing 50 ml of sterile potato dextrose broth (PDB). Cultures were incubated for 3 days on an intermittent shaker at ambient temperature with alternating dark to low light intensities. Mycelia were broken apart and mixed with an Omni-mixer for 5 min. The resulting slurry was stirred mechanically while being dispensed for testing.

16. Bacterial inocula were prepared by inoculating 10 ml of nutrient broth (NB) with each candidate and incubating at 25°C for 24 hr. Cultures were mixed on a vortex mixer prior to dispensing.

17. Inoculum levels were quantified by serial dilution followed by plate counts.

18. Sprigs from healthy field populations of hydrilla were placed in 200- by 25-mm test tubes containing 50 ml of sterile macronutrient solution deficient in nitrogen and phosphorus to inhibit algal growth. Hydrilla sprigs 11 cm long were selected to avoid branching, lateral budding, and axillary root development. Only healthy sprigs with a strong apical meristem were selected. Sprigs were rinsed carefully three times in sterile distilled water before introduction to tubes. Tubes were covered with cotton plugs followed by loose metal caps and placed in an environmental chamber maintained at 25°C with a 12- to 12-hr light to dark cycle. Plants were thus maintained for 4 days prior to inoculation.

19. Each hydrilla tube culture received 1 ml of inoculum (viabilities presented in Table 2). Two treated controls were necessary to test the possible effects of culture media on the plants. Since fungal isolates were culture in PDB, a PDB-treated control was tested. Bacterial isolates were grown in NB, which necessitated an NB-treated control. Untreated controls received sterile distilled water. Each treatment was replicated seven times.

20. Damage index values were defined prior to test initiation as follows:

Index	Description
1	Vigorous, healthy plants with normally spaced internodes and no evidence of disease or damage.
2	Faintly chlorotic plants only slightly paler than healthy sprigs and exhibiting few or no damaged spots.
3	Chlorotic plants, or plants showing less than 50 percent damage.
4	Markedly chlorotic plants, or plants exhibiting pronounced damage exceeding 50 percent of sprig.
5	Chlorotic to brown plants, plants with brown stems and most leaves transparent and disintegrating, or obviously dead plants.

21. A damage index value was assigned to each sprig prior to inoculation and every week for 6 weeks after inoculation. Any axillary shoots or roots were counted weekly and their condition noted. At the end of the study, one composite index value was assigned to all axillary shoots in each tube and subjective notes were made describing the likelihood of their regeneration.

Other qualitative notes included appearance of algae and of inoculum mycelia or colonies.

22. All test sprigs developing a damage index value of 4 or 5 were sampled for recovery of the isolate. Approximately 2 cm of the lower stem, including a single node when possible, were excised aseptically, sterilized by immersing for 1 min in 1 percent NaHClO, and rinsed with sterile distilled water three times. The tissue was ground and the resulting slurry was streaked for isolation on two PDA plates (fungi) and two NA plates (bacteria). At the same time, fungal isolates were taken from stock cultures and streaked in a similar manner so that parallel development could be closely observed. Mycelia or colonies suspected of being the tested isolate were transferred to slant culture for later taxonomic confirmation.

Data analysis

23. Statistical analyses were conducted using the procedures available with Statistical Analysis System (SAS) (SAS Institute, Inc. 1982). For each sampling period, the damage-index data were evaluated by a one-way analysis of variance (ANOVA) as a test of differences between treatment means. When a statistical difference was shown, Duncan's Multiple Range Test was used to determine which treatments (isolates) had mean damage index values significantly higher than the corresponding controls (PDB or NB).

Hydrilla Aquarium Assay

Selection and testing

24. Healthy, third generation greenhouse hydrilla sprigs were selected, rinsed gently but thoroughly in tap water, and planted in 250-ml beakers containing one part sterile lake sediment mixed with one part sand. Three unbranched sprigs 15 cm in length were planted in each beaker. Eight beakers were placed in each of eight 75.7-ℓ aquaria (30.5 cm by 30.5 cm by 76.2 cm). Aquaria were filled to a depth of 56 cm (56.8 ℓ) with a 1:1 mixture of unfiltered tap water and tap water that had been filtered through activated carbon and three ion exchange filters (Figure 4). Plant growth lights suspended 60 cm above the aquaria were programmed for 10 hr light/14 hr dark, and each aquarium was aerated with a small air pump. Sides of the aquaria were wrapped

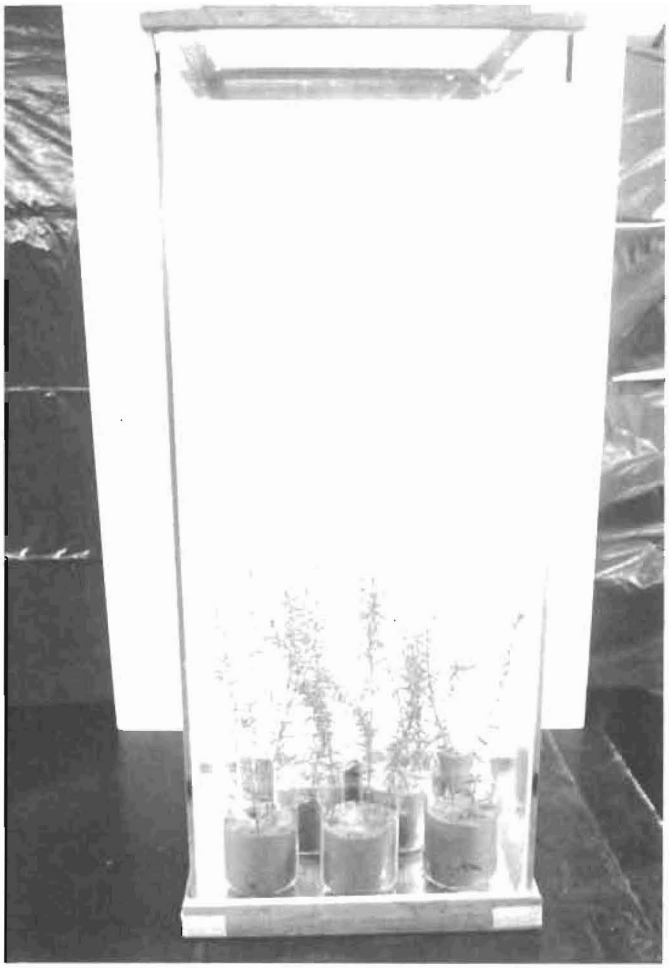

Figure 4. Hydrilla aquarium assay unit showing uninoculated hydrilla sprigs planted in beakers

with black plastic to reduce lateral light that could stimulate algal growth on aquaria walls. Plants were allowed 10 days to become established.

25. Six candidate fungi were selected based on results of the preliminary hydrilla test tube assay (paragraph 40). Each candidate was inoculated into six 500-ml erlenmeyer flasks, each containing 250 ml of sterile PDB. Cultures were allowed to grow for 10 days, after which all cultures of the same candidate were combined and blended for 2 to 3 min in a Waring blender. The slurry was filtered in a Buchner funnel under vacuum to remove PDB because, in a previous aquarium test, inoculation with the same nutrient levels caused a sufficiently rapid proliferation of the candidates to disrupt data collection. The mycelia were resuspended in 1 ℓ of sterile distilled water and stirred mechanically while a 1-ml aliquot was extracted for serial

dilution. Four dilutions (10^5, 10^6, 10^7, and 10^8) were inoculated onto PDA plates (three replicates per dilution) for quantification of inoculum. Inoculum was stored at 8°C for 48 hr. Colonies were counted and inocula were diluted to be consistent with levels applied in the preliminary hydrilla test tube assay (Table 2). Treatments and controls were randomly assigned to aquaria. To confirm inoculum levels, serial dilutions and plate counts were repeated at the same time that aquaria were inoculated.

26. A single damage index value was assigned to all plants in each beaker as a composite before inoculation and each week after inoculation for 6 weeks. Mean plant height per beaker was estimated weekly. Qualitative notes were made on development of axillary shoots and roots, water clarity, visible signs of mycelial development, specific infection foci, algal growth in aquaria and on plants, and general plant vigor.

27. At the end of the test, plants from each aquarium were sampled and cultured for recovery of the inoculated candidate. Dry weights of treated plants were determined and compared to dry weights of untreated controls.

Data analysis

28. Application of statistical analysis was unnecessary since damage index values remained the same on test plants throughout the aquarium assay. However, mean dry weights of treated and control plants were compared using a t-test.

Filtered Inoculum Assays

Assay I: greenhouse plants

29. A test tube assay was conducted using the six best isolates from the preliminary hydrilla test tube assay (i.e. 56, 156, 161, 170, 236, and 249). Fungal inoculum was prepared by introducing each isolate into two 500-ml erlenmeyer flasks each containing 100 ml of sterile PDB. Cultures were incubated for 6 days. Mycelia were broken apart and mixed with an Omni-mixer for 5 min. One inoculum flask for each isolate was filtered through Whatman No. 1 filter paper on a Buchner funnel under mild suction. The residue was resuspended in 100 ml of sterile distilled water. The filtrate was centrifuged to remove any visible residues.

30. Whole (unfiltered) inoculum, inoculum filtrate, and resuspended filtered organisms were quantified by serial dilution followed by plate counts.

31. Hydrilla from greenhouse cultures were selected and placed in test tubes as described in paragraph 18, except that 50 ml of sterile distilled water was used rather than macronutrient solution. The hydrilla sprigs were typical of greenhouse culture, i.e. having thin stems and leaves with a generally pale, fragile appearance.

32. Each hydrilla tube culture received 1 ml of inoculum at levels shown in Table 3. Untreated controls received 1 ml of sterile distilled water and treated controls received 1 ml of PDB. Each treatment and control was replicated seven times.

33. Damage index values were assigned as described in paragraph 20. Separate data were not taken from axillary shoots.

34. Hydrilla sprigs were cultured as described (paragraph 22) except that tissues were not ground, but plated directly onto a single PDA plate. Cultures were not subjected to exact taxonomic verification.

Assay II: field plants

35. A hydrilla test tube assay identical to Assay I was conducted using hydrilla from the field. These plants had thick stems and leaves, were dark green, and generally coarse and robust in appearance. Test procedures, including inoculum preparation, were the same as described in paragraphs 29-34.

Data analysis

36. Differences among treatment means and means of treatment types (i.e. whole inoculum, filtered organisms, etc.) were determined using DUNCAN option to the PROC GLM procedure (SAS Institute, Inc. 1982).

PART III: RESULTS AND DISCUSSION

Results

Isolates

37. Two hundred sixteen of the microorganisms isolated from hydrilla were screened for cellulase and pectinase production.* Only 26 percent of the isolates were fungi, but they comprised the larger group of lytic enzyme producers. Twenty fungal isolates were cellulase positive and one was pectinase positive. Only one of 139 bacterial isolates was pectinase positive and none were cellulase positive. Forty-eight percent of the lytic enzyme-producing isolates were collected in fall, 28 percent in spring, and 24 percent in summer. Table 4 shows the number of isolates by site including those that were lytic enzyme positive. Although no attempt was made to analyze isolates by sites, the six best isolates were from the following locations:

Isolate No.	Location
56	Imperial Valley, California
156	Lake Theriot, Louisiana
161	Lake Theriot, Louisiana
170	Lake Theriot, Louisiana
236	Lake Conroe, Texas
249	Lake Conroe, Texas

Isolate 56 was taken from Reese's Pond, where an unexplained decline of hydrilla had occurred. However, no relationship between the decline and this isolate has been established. None of the remaining five isolates were collected from sites exhibiting unusual conditions. The Lake Theriot sites exhibited only fringe growth of hydrilla. Exact conditions at the Lake Conroe sites, from which isolates 236 and 249 were collected, are not known.

38. Table 5 lists 32 fungal isolates examined by Dr. Timothy S. Schubert, Florida Department of Agriculture and Consumer Services, Division of Plant Industry, Gainesville, Fla. All but one cellulase positive fungus and twelve cellulase negative fungi are listed. One cellulase positive fungus

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* Two hundred fifty-two microorganisms were isolated from hydrilla and given numbers. However, thirty-six of these were lost before screening for lytic enzymes could be performed.

was lost in shipping. The 12 cellulase negative fungi identified were representative of all cellulase negative fungi. A comprehensive, definitive identification of all lytic enzyme negative isolates was not warranted.

39. Table 6 lists bacterial genera associated with hydrilla, including the number of isolates of each genus. Identifications are presumptive because of the limited number of tests conducted (Table 1). More definitive identifications were not warranted since only bacterial isolate 39 (*Erwinia herbicola*) was lytic enzyme (pectinase) positive.

Preliminary hydrilla test tube assay

40. Five fungal isolates (56, 249, 156, 170, 161) exhibited damage index values significantly greater than PDB-treated controls 1 week after inoculation (Table 7). Mean damage index values for two of these (56, 156) remained significantly greater than controls throughout the 6-week study period (Figures 5-7). Two weeks after treatment, damage index values of eight isolates (56, 170, 249, 161, 156, 236, 116, 244) were significantly greater than controls. Damage index values continued to increase for each of these, but values for PDB-treated controls also exhibited a rapid increase in the third and fourth weeks. All isolates producing significantly greater damage index values than PDB-treated controls more than once during the test were selected for the aquarium assay. These included isolates 56, 156, 161, 170, 236, and 249. All lytic enzyme-producing isolates were maintained in stock culture for possible examination after efficacy studies with most promising candidates were completed.

41. Damage index values for bacterial isolate 39 showed no significant difference from NB-treated controls at any time and was excluded from further study. Damage index values for the pectinase positive fungal isolate (224) showed no significant difference from PDB-treated controls at any time and was also excluded from further study.

42. Untreated control damage index reached a mean of 2.29 at 6 weeks, with all of the increase occurring the fifth and sixth weeks. There were no significant differences between untreated and NB-treated controls at any time during the study. However, PDB-treated controls exhibited significantly greater damage index values than untreated controls at the third, fourth, and fifth weeks.

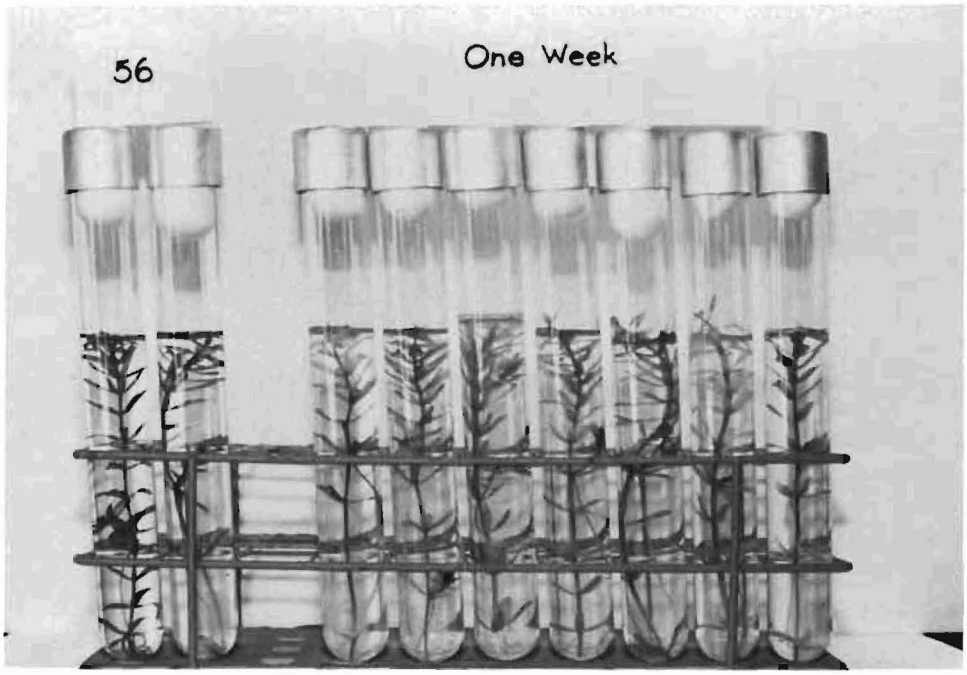

Figure 5. Hydrilla test tube assay for isolate 56 after 1 week. Note browning of treated sprigs as compared to untreated control (far left) and PDB-treated control (second from left)

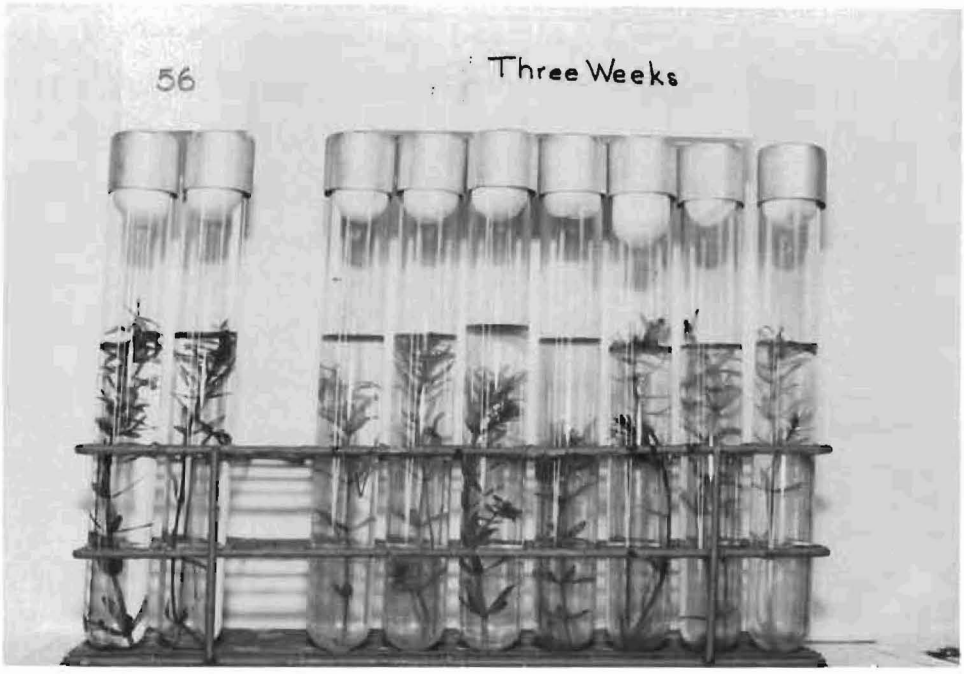

Figure 6. Hydrilla test tube assay for isolate 56 after 3 weeks. Note breakdown of plant sprigs in the first, third, and fourth treated tubes

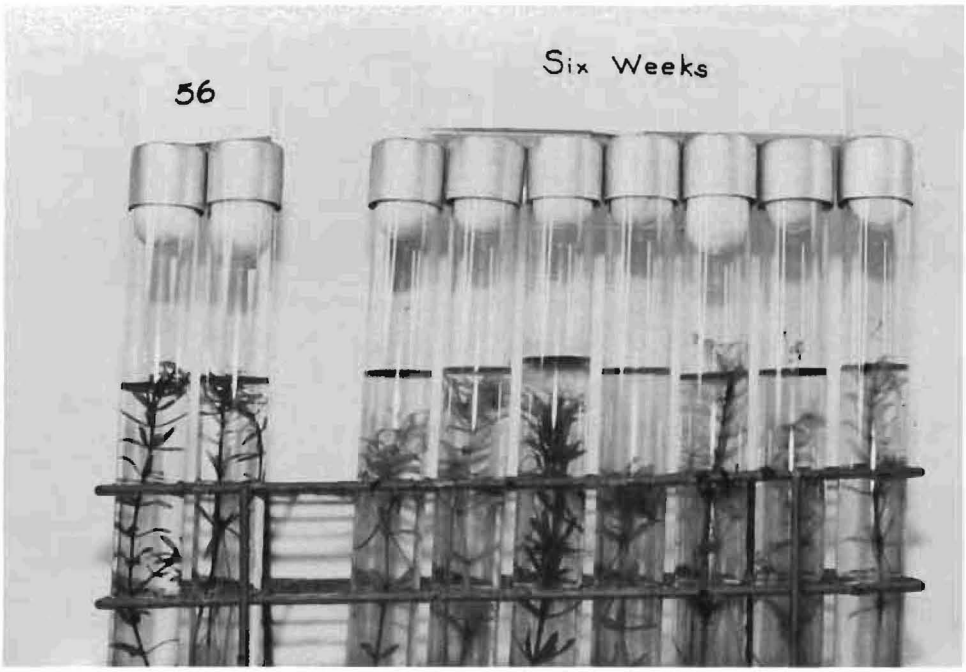

Figure 7. Hydrilla test tube assay for isolate 56 after 6 weeks. Note decline of axillary shoot in the third treated tube and slight browning of PDB-treated control (second tube)

43. All but four isolates exhibited damage index values of 4 in at least one replicate during the study. At least one of these tubes for each isolate was cultured to recover the original inoculum. The originally inoculated isolates were recovered in 75 percent of the cultured tubes, most in pure culture. All successful recoveries occurred on or before the fourth week of testing.

44. Five isolates (56, 249, 236, 170, 161) exhibited significantly greater composite damage index values on axillary shoots than treated and untreated controls after 6 weeks (Table 8). All of these isolates were among those producing significant damage to the primary shoot at some time during the study.

45. Algal contamination was not a problem on hydrilla sprigs in the preliminary test tube assay. Blue-green and filamentous green algae began to appear the second week of the study in about 14 percent of the tubes, but were not extensive. Only 21 percent of the tubes had algal growth at any time during the study and less than 5 percent showed moderate growth.

Hydrilla aquarium assay

46. Colony counts conducted at the time of inoculum preparation and immediately prior to inoculation 48 hr later indicated that four of the six isolates decreased in viability and two remained the same. However, viability at the time of inoculation compared favorably with the viability of isolates at the time of inoculation in the preliminary test tube assay (Table 3).

47. Damage index values for control and all test aquaria remained unchanged throughout the 6-week study period. All beakers of plants were assigned an initial damage index value of 1 prior to inoculation and this value remained constant throughout the study. Mean plant height increased steadily at 2.6 cm per week in both controls and treatments. Development rate of axillary shoots and roots was consistent for controls and treatments. No signs of active fungal growth were noted except in one aquarium (isolate 236). At 1 week, a brown coating completely enveloped sprigs in the affected aquarium, weighting some down slightly. However, hydrilla sprigs showed no signs of damage. New growth developing by the second week was free of mycelia, and by the fourth week only brown mycelial debris and turbid water remained. The aquarium had returned to normal by the fifth week. Attempts to culture the brown material on PDA and NA failed and only amorphous debris was observed upon microscopic examination. No noticeable algal growth occurred in any aquarium, and plants remained vigorous and free of damage throughout the 6-week study.

48. At the end of the study, plants from each aquarium were sampled and cultured for possible recovery of isolates, but none were found.

49. Dry weights of treated plants were compared with dry weights of controls at the end of the study. Aquaria treated with isolates 56 and 249 exhibited mean dry weights significantly greater than the control at the 0.05 level of significance. No other treatments exhibited any difference from controls.

Filtered Inoculum

Assay I: greenhouse plants

50. Mean damage index values as indicated by Duncan's Multiple Range Test by treatment types (Table 9) showed that whole inoculum produced significantly greater damage to hydrilla than was produced by filtrate, filtered organisms, or untreated controls. Whole inoculum produced significantly

greater damage than was produced by PDB-treated controls in all except the sixth week.

51. There were no significant differences between mean damage index values of filtrates and filtered organisms except in the last 2 weeks. In the fifth and sixth weeks means of filtrates were significantly greater than means of filtered organisms. PDB-treated controls produced significantly greater mean damage index values than filtrates except in the first 2 weeks. There were never any significant differences between mean damage index values of filtrates and of untreated controls.

52. Filtered organisms were never significantly different from untreated controls. PDB-treated controls produced significantly greater mean damage index values than filtered organisms except in the first 2 weeks when there were no significant differences.

53. The PDB-treated controls produced significantly greater mean damage index values than untreated controls only after the second week.

54. Duncan's Multiple Range Test treatment means for Assay I are presented in Appendix A.

55. The inoculated organisms were recovered from 79 percent (n = 28) of the cultured hydrilla sprigs that received whole inoculum, from none (n = 4) of those that received filtered organisms, and from one (n = 7) that received filtrate.

Filtered Inoculum
Assay II: field plants

56. Duncan's Multiple Range Test by treatment types (Table 9) showed that mean damage index values for whole inoculum were significantly greater than means for filtered organisms, PDB-treated controls, or untreated controls. Mean damage index values of whole inoculum were never significantly different from those of filtrates.

57. Mean damage index values of filtrates were significantly different from those of filtered organisms except in the first 2 weeks. Mean damage index values of filtrates oscillated from no significant differences to significantly greater than means of PDB-treated controls (Table 9). Filtrates produced significantly greater means than untreated controls after the first week.

58. Mean damage index values of filtered organisms were never significantly different from PDB-treated controls, but were significantly greater than untreated controls after the first week.

59. The PDB-treated controls produced significantly greater mean damage index values than untreated controls after the second week.

60. Duncan's Multiple Range Test of mean damage index values for filtered inoculum Assay II are shown in Appendix A.

61. The inoculated organisms were recovered from 40 percent (n = 40) of cultured hydrilla sprigs that received whole inoculum, from 42 percent (n = 19) of those that had received filtered organisms, and from 30 percent (n = 33) of those that had received filtrate.

Discussion

Preliminary hydrilla test tube assay

62. The fact that mean damage index values for untreated controls did not increase until the fifth week and was only 2.29 at the end of the study indicates that the test sprigs were capable of surviving with only limited decline through the study period. The significant increase in mean damage index values of PDB-treated controls beginning the third week probably reflects simulation by increased nutrient availability to the endogenous microflora associated with control plants. The reason that NB-treated controls failed to produce a similar response may be related to differences between the composition of the two media and may also reflect a preference of the endogenous microflora for PDB.

63. The 75-percent inoculum recovery rate, and the frequency with which pure cultures were obtained, strongly suggested that the inoculated isolates not only maintained viability during the study, but also caused the damage to sprigs.

64. Of the 22 lytic enzyme-producing isolates, 5 inflicted rapid and severe damage to hydrilla in the preliminary test tube assay. This demonstrated that endogenous saprophytes from the natural microsphere of the plant could be induced through enhancement of lytic enzyme production to exert a

significant impact on hydrilla. The fact that 47 percent of the lytic enzyme-producing isolates were collected in the fall strongly suggests that these saprophytes contribute to the natural seasonal decline of hydrilla.

65. The significant and sustained damage induced by the tested isolates to both axillary shoots as well as primary shoots indicated that fungal isolates could produce permanent damage from which hydrilla would be incapable of recovering.

Hydrilla aquarium assay

66. The six isolates producing the greatest impacts on hydrilla in the preliminary test tube assay did not impact hydrilla in the aquarium assay. Examination of differences between the two assays suggested five factors that could have been responsible for the ineffectiveness of candidates in the aquarium assay: (a) inoculum nutrient levels, (b) sources of hydrilla, (c) methods of inoculum preparation, (d) aeration of aquaria, and (e) test scale. These factors are discussed in the following paragraphs.

67. Nutrient level. High nutrient levels in the water column have been shown to enhance the effectiveness of lytic enzyme-producing isolates that stress Eurasian watermilfoil (Gunner 1983), presumably by sustaining the microorganisms and/or stimulating increases in their numbers until they are able to attack the plant. The mechanisms involved have not yet been elucidated. No effort was made to remove nutrient media (PDB or NB) from inoculum used in the preliminary test tube assay. However, nutrients (PDB) were removed from inoculum for the aquarium assay because a previous test at the time nutrient concentration had produced a rapid explosion of microbial growth so extensive that it would have precluded data collection. It is possible that a threshold level of nutrients must exist before the microorganisms can impact hydrilla.

68. Hydrilla source. Hydrilla plants for the preliminary test tube assay were collected from a healthy field population. They were thick stemmed, dark green, and robust in appearance. In contrast, plants for the aquarium assay were third generation greenhouse plants, grown in nutrient deficient water, and screened by shade cloth to control algal growth. These plants had thin stems, were pale, and were more fragile in appearance than those used in the preliminary test tube assay. Even though apparently less vigorous, the third generation greenhouse plants may have been more disease resistant than

first generation plants, because resistant may have been selected by culturing of disease-free, undamaged sprigs through several generations. Strong plant resistance could account for the ineffectiveness of candidates in the aquarium assay.

69. Another factor relating to the use of greenhouse plants that have been subcultured through several generations is that some pathogens and saprophytes have been shown to produce their effects synergistically.* Greenhouse hydrilla cultures are typically maintained in the actively growing (log) phase, precluding the opportunity for natural senescence processes to commence. Such culturing practices could produce a tremendous reduction in the natural saprophytic microflora associated with hydrilla. If a synergistic effect between natural saprophytes and the inoculated candidates were a factor in the success of isolates in the preliminary test tube assay, it is likely that a similar effect was impossible in the aquarium assay.

70. Inoculum preparation. Removal of the nutrient medium (PDB) in which the candidates were grown required more extensive manipulation of the inoculum for the aquarium assay than for the preliminary test tube assay. Virulence of the candidates may have been reduced by subjecting them to filtration under mild vacuum and holding for 48 hr in water, even though viability of the isolates on culture medium (PDA) was not significantly different. Filtration of inoculum could have simultaneously removed any accumulated lytic enzymes from the culture. It is possible that the very early impacts on hydrilla sprigs seen in the preliminary test tube assay were the result of high levels of lytic enzymes already secreted into the inoculum media.

71. Aeration. The aquaria were aerated to prevent stagnation of the water and to maintain uniform temperature and oxygen concentrations. The constant water currents may have prevented fixation of the fungal candidates to hydrilla long enough to initiate infection.

72. Test scale. Each increase in test scale affords the plants a greater advantage than the preceding scale by removing some stresses. In the preliminary test tube assay, the cut end of sprigs may have provided a port of entry that was unavailable to the fungi in the aquarium assay. It is

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* Personal Communication, Dr. H. B. Gunner, September 1982, Department of Environmental Studies, University of Massachusetts, Amherst, Mass.

frequently necessary to abrade or puncture plant tissue before weak pathogens can inflict damage (Andrews and Hecht 1981; Gunner 1983).

73. Lytic enzyme systems of test isolates may have been insufficient to impact healthy, actively growing aquarium plants, even though they did impact hydrilla sprigs in a smaller, more confined system.

Filtered inoculum assays

74. Results of assays with both greenhouse and field plants showed that whole inoculum was significantly more damaging to hydrilla sprigs than filtered organisms. Moreover, filtered organisms never produced damage significantly greater than untreated controls in Assay I. In Assay II this was not true, but mean damage index values for filtered organisms were consistently close to values for untreated controls. These results suggest that the organisms require nutrients or metabolites to inflict damage on hydrilla. Results of Assay I suggest that nutrients are very important since PDB treatment produced significantly greater damage after the second week than all other treatments except whole inoculum. In Assay II, PDB was less damaging compared to whole inoculum; however, filtrate treatments produced consistently high means in Assay II that were also significantly greater than all other treatments in the third and fifth weeks (except whole inoculum from which filtrate was never significantly different). These tests provide additional evidence that the organisms require nutrients and, perhaps, accumulated metabolites (cellulase) to damage hydrilla. These results are also consistent with results of the hydrilla aquarium assay in which filtered organisms alone produced no damage to hydrilla (paragraph 47).

75. Statistical comparisons of results between the two filtered inoculum assays could not be made because inoculum levels were not identical. However, examination of the data suggested that the greenhouse plants of Assay I were more resistant to attack by filtered organisms than the field plants of Assay II. In Assay I, filtered organisms never produced mean damage index values that were significantly different from untreated controls. In Assay II, filtered organisms produced significantly greater mean damage index values than untreated controls after the first week (Table 9). Mean inoculum levels for whole inoculum and for filtered organisms were higher in Assay II than in Assay I, but there was greater difference between means of whole inoculum levels for the two assays (Δ = 2000x) than between means of filtered

organism inoculum levels ($\dot{\Delta}$ = 100x) (Table 3). The significant damage exerted by whole inoculum implied that sufficient numbers of organisms were present even at the lower inoculum levels and that test results must be explained by some other mechanism than differences in inoculum levels. Nutrient levels (paragraph 67) and hydrilla resistance as determined by plant source (paragraph 68) may account for differences between effects of filtered organisms in the two assays.

76. The fact that whole inoculum produced significantly greater damage index values than filtered organisms lends support to the possibility that the filtration process impacts the organisms.

PART IV: CONCLUSIONS AND RECOMMENDATIONS

Conclusions

77. Conclusions of this study were:

a. Two hundred sixteen isolates from hydrilla collected in California, Texas, Louisiana, and Florida were screened for lytic enzyme production.

b. Twenty fungal isolates were cellulase positive. No bacterial isolates were cellulase positive.

c. Only one fungal and one bacterial isolate were found to be pectinase positive.

d. Fourteen cellulase positive fungi, the pectinase positive fungus, and nine cellulase and pectinase negative fungi were identified to genus (Table 5).

e. One hundred twelve bacterial isolates were identified to genus, including the pectinase positive isolate (Table 6).

f. All lytic enzyme-producing isolates were successively subcultured to enhance lytic enzyme production.

g. Six enzyme-enhanced isolates proved to be efficacious on hydrilla sprigs in test tubes by effecting a rapid and dramatic decline of the plants.

h. Filtered inocula of the six isolates producing decline of hydrilla in the preliminary test tube assay did not impact hydrilla in the aquarium assay.

i. The lytic enzyme-producing isolates need nutrients, and perhaps accumulated metabolites (cellulase) to damage hydrilla.

j. Hydrilla from greenhouse cultures are more resistant to filtered organisms than hydrilla from healthy field cultures.

Recommendations

78. It is recommended that the level of nutrients necessary to enable organisms to damage hydrilla be determined, practical means of providing the required nutrient levels be investigated, and effects of these nutrient levels on the ecosystem be evaluated. To this end, it is suggested that a dry formulation of the isolates be produced in the laboratory using a method developed by Walker and Connick (1983) and tested at the aquarium level for efficacy, effects on endogenous microflora, and effects on general water quality. The

formulation should incorporate nutrients and provide other desirable inoculum characteristics that would facilitate aquarium level and larger scale testing (e.g. consistent viability, sinkability, extended shelf life, and general ease in application and storage).

79. Concurrent with nutrient level testing, a small test tube assay should be conducted to determine effects of the filtration process on isolates. The assay would compare results of hydrilla treatment with filtered organisms to which filtrate had been resupplied with results of treatment with whole (unfiltered) inoculum.

80. It is also recommended that the cellulase production of isolates be quantified and the induction of lytic enzymes be confirmed.

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Table 1

Tests Used to Determine Morphological and Chemical Characteristics of Bacterial Isolates

Cell morphology

- Shape: rod, coccus, spiril
- Length and width: μm
- Endospores: present or absent
- Capsule: present or absent
- Gram stain reaction: positive or negative
- Motility: motile or nonmotile

Colony characteristics

- Margin: entire or undulant
- Elevation: flat or convex
- Surface: smooth, wrinkled, or granular
- Opacity: opaque, translucent, or transparent
- Consistency: mucoid or butyrous
- Pigment: color

Biochemical tests

- API strip
  - ONPG (O-nitrophenyl-β-d-galactoside)
  - ADH (Arginine dihydrolase)
  - LDC (Lysine decarboxylase)
  - ODC (Ornithine decarboxylase)
  - TDA (Tryptophane deaminase)
  - VP (Voges-Proskauer test)
  - Gelatin
  - Glucose
  - Mannitol
  - Inositol
  - Sorbitol
  - Rhamnose
  - Sucrose
  - Melibiose
  - Amygdalin
  - Arabinose
  - Oxidase
  - Nitrate reduction
  - Catalase
- O_2 requirement: aerobic, microaerophilic, facultative, or anaerobic
- Oxidative/fermentative
- MacConkey agar growth
- Cellulase
- Pectinase

Table 2

Inoculum Viability in Preliminary Hydrilla Test Tube Assay

Isolate	CFU*/ml	Isolate	CFU/ml
39**	5×10^6	161	1×10^5
49	1×10^5	162	8×10^6
56	2×10^5	170	1×10^5
57	3×10^5	224	1×10^3
59	8×10^6	236	3×10^6
60	6×10^7	237	1×10^5
101	5×10^6	240	1×10^4
111	3×10^6	242	4×10^4
116	1×10^5	244	1×10^4
156	3×10^5	248	5×10^4
157	1×10^6	249	4×10^5

* Colony forming units.
** Bacterial isolate. All others are fungi.

Table 3

Inoculum Viabilities (CFU/ml)*

Isolate No.	Preliminary Test Tube Assay	Aquarium Assay	Filtered Inoculum			
			Assay I		Assay II	
			Whole**	Organisms†	Whole	Organisms
56	2×10^5	9×10^6	5×10^4	9×10^5	1×10^5	5×10^4
156	1×10^5	8×10^5	6×10^3	6×10^3	6×10^4	1×10^5
161	1×10^5	3×10^4	8×10^2	9×10^3	8×10^6	1×10^6
170	3×10^6	5×10^5	7×10^3	2×10^4	3×10^4	8×10^4
236	4×10^5	4×10^6	4×10^6	8×10^5	2×10^6	4×10^6
249	3×10^5	1×10^5	1×10^5	4×10^5	7×10^6	5×10^5
$\bar{X}$			7×10^5	4×10^5	2×10^7	1×10^6

* Counts given are for quantification conducted at the time of inoculation.
** Whole (unfiltered) inoculum.
† Filtered organisms.

Table 4

Numbers of Bacterial and Fungal Isolates by Site

Site	Total Isolates*			Cellulase Positive		Pectinase Positive	
	F	B	L	F	B	F	B
Imperial Valley	28	50	9	3	0	0	1
Lake Trafford	3	21	4	3	0	0	0
Lake Theriot	11	27	3	6	0	0	0
Lake Conroe	13	41	6	8	0	1	0
Totals	55	139	22	20	0	1	1
		216		20		2	

* These three columns list the total number of fungal isolates (F), bacterial isolates (B), and lost isolates (L) that did not survive long enough to designate with certainty as fungal or bacterial.

Table 5

Fungal Isolates

Isolate Number	Name
	Cellulase Positive
49	*Penicillium* sp.
56	*Aspergillus awomori* Nakazawa
57	*Aspergillus* sp.
59	*Cephalosporium acremonium* Corda
101	*
111	*Cephalosporium acremonium* Corda
116	* with Chlamydospores
156	*Humicola* sp. with *Trichoderma* sp.
157	*Aspergillus luchuensis* Invi
161	*Humicola* sp. with *Trichoderma* sp.
162	*Cephalosporium acremonium* Corda
170	*
236	*Fusarium moniliforme* Sheldon var. *subglutinans* Wr. & Reink
237	*Rhizoctonia* sp.** with *Trichoderma* sp.
238	*
240	*
242	*
244	*
249	*Aspergillus awomori* Nakazawa
250	*Aspergillus awomori* Nakazawa
	Pectinase Positive
224	*Cladosporium cladosporioides* (Fresen.) de Vries
	Cellulase/Pectinase Negative
20	*Aspergillus* sp.
21	*
28	*Cylindrocarpon* sp.
31	*Rhizoctonia* sp. (with sclerotia, not same sp. as 237)**
32	*Rhizopus stolonifer* (Ehrenb. ex Link) Lind.
45	*Cladosporium cladosporioides* (Fresen.) de Vries
159	*Aspergillus luchuensis* Inui
163	*Aspergillus luchuensis* Inui
164	*Aspergillus luchuensis* Inui
245	*
246	*

* Unidentified.

** Not previously described.

Table 6

Genera of Bacteria Associated with Hydrilla

Isolate	Number of Isolates
Pseudomonas	33
Pseudomonas (fluroescent)	5
Acinetobacter	27
Aeromonas	14
Flavobacterium	12
Bacillus	5
Enterobacter	3
Alcaligenes	2
Flexibacter	2
*Erwinia**	1
Myconostoc	1
Rhodopseudomonas	1
Serratia	1
Caulobacter	1
Agrobacterium	1
Hafnia	1
Klebsiella	1
Sporocytophaga	1
Unidentified**	19
Lost†	8

* Pectinase positive.
** Isolates that could not be keyed to genera with test performed.
† Isolates lost while holding cultures.

Table 7

Mean Damage Index Values for Preliminary Hydrilla Test Tube Assay*

1st Week		2nd Week		3rd Week		4th Week		5th Week		6th Week	
Isolate No.	$\bar{X}$**	Isolate No.	$\bar{X}$	Isolate No.	$\bar{X}$	Isolate No.	$\bar{X}$	Isolate No.	$\bar{X}$	Isolate No.	$\bar{X}$
56	3.14^{a}	56	3.71^{a}	56	3.86^{a}	56	4.57^{a}	56	4.71^{a}	56	4.86^{a}
249	2.71^{ab}	170	3.14^{ab}	170	3.71^{ab}	156	4.29^{ab}	156	4.43^{ab}	156	4.57^{ab}
156	2.43^{bc}	249	3.14^{ab}	156	3.43^{abc}	170	4.00^{abc}	249	4.29^{ab}	170	4.57^{ab}
170	2.43^{bc}	161	2.86^{bc}	161	3.43^{abc}	249	4.00^{abc}	170	4.14^{abc}	249	4.29^{abc}
161	2.30^{bcd}	156	2.71^{bcd}	236	3.29^{abc}	161	3.86^{abc}	161	4.00^{abcd}	236	4.14^{abcd}
116	2.14^{bcde}	236	2.71^{bcd}	249	3.29^{abc}	236	3.86^{abc}	236	4.00^{abcd}	161	4.00^{abcde}
236	2.14^{bcde}	116	2.57^{bcde}	244	2.86^{bcd}	244	3.29^{bcd}	244	3.57^{abcde}	244	3.71^{bcdef}
244	2.00^{cdef}	244	2.43^{cdef}	116	2.57^{cde}	PDB	3.00^{cde}	PDB	3.00^{cdefg}	116	3.43^{bcdefg}
PDB†	1.43^{efgh}	PDB	1.57^{ghij}	PDB	2.14^{def}	116	3.00^{cde}	116	3.00^{cdefg}	PDB	3.29^{cdefg}
NB††	1.00^{h}	NB	1.00^{j}	NB	1.14^{gh}	NB	1.29^{fg}	NB	1.57^{hi}	Unt	2.29^{ghij}
Unt‡	1.00^{h}	Unt	1.00^{j}	Unt	1.00^{h}	Unt	1.00^{g}	Unt	1.43^{hi}	NB	1.71^{ij}

* Data presented include only isolates producing significantly (according to Duncan's Multiple Range Test) greater damage index values than controls at some time during the study.

** $\bar{X}$ = Mean damage index values (n = 7). Means in the same column followed by the same superscript are not significantly different ($P < 0.05$).

† Potato dextrose broth-treated controls.

†† Nutrient broth-treated controls.

‡ Untreated controls.

___ Isolates appearing above solid line exhibited significantly greater mean damage index values than untreated controls.

-- Isolates appearing above dashed line exhibited significantly greater mean damage index values than PDB-treated controls. Dashed line is consonant with solid line in 2nd week.

Table 8

Mean Damage Index Values of Axillary Shoots in Preliminary Hydrilla Test Tube Assay*

Isolate No.	Means
56	4.43^{a}
249	4.00^{ab}
236	3.57^{b}
170	2.43^{c}
161	2.14^{cd}
156	1.86^{cde}
PDB**	1.14^{e}
Unt†	1.00^{e}

* Means represent composite damage index values assigned at the sixth week to axillary shoots (n = 7). Data presented include only the six most damaging isolates from the preliminary hydrilla test tube assay. Means followed by the same superscript are not significantly different ($P < 0.05$) according to Duncan's Multiple Range Test. Isolates above line produced mean damage index values significantly greater than PDB-treated and untreated controls.

** Potato dextrose broth-treated controls.

† Untreated controls.

Table 9

Mean Damage Index Values for Hydrilla by Treatment Types

1st Week		2nd Week		3rd Week		4th Week		5th Week		6th Week	
Treatment Type*	$\bar{X}$**	Treatment Type	$\bar{X}$	Treatment Type	$\bar{X}$	Treatment Type	$\bar{X}$	Treatment Type	$\bar{X}$	Treatment Type	$\bar{X}$
				Filtered Inoculum Assay I: Greenhouse Plants							
W	1.81^{a}	W	2.79^{a}	W	3.05^{a}	W	3.43^{a}	W	3.71^{a}	PDB	4.00^{a}
F	1.10^{b}	PDB	1.71^{b}	PDB	2.29^{b}	PDB	2.71^{b}	PDB	3.29^{b}	W	3.93^{a}
A	1.02^{b}	F	1.48^{b}	F	1.60^{c}	F	1.79^{c}	F	2.14^{b}	F	2.48^{b}
PDB	1.00^{b}	Unt	1.14^{b}	Unt	1.14^{c}	Unt	1.29^{c}	Unt	1.43^{bc}	Unt	1.71^{bc}
Unt	1.00^{b}	A	1.05^{b}	A	1.10^{c}	A	1.17^{c}	A	1.29^{c}	A	1.62^{c}
				Filtered Inoculum Assay II: Field Plants							
W	1.98^{a}	W	3.19a	W	3.88^{a}	W	4.31^{a}	W	4.45^{a}	W	4.62^{a}
F	1.60^{ab}	F	2.64^{ab}	F	3.38^{a}	F	3.69^{ab}	F	3.90^{a}	F	4.00^{ab}
A	1.29^{b}	A	1.88^{bc}	PDB	2.29^{b}	PDB	2.86^{bc}	PDB	3.00^{b}	PDB	3.29^{bc}
PDB	1.14^{b}	PDB	1.71^{cd}	A	2.17^{b}	A	2.45^{c}	A	2.62^{b}	A	2.76^{c}
Unt	1.00^{b}	Unt	1.00^{d}	Unt	1.00^{c}	Unt	1.14^{d}	Unt	1.29^{c}	Unt	1.29^{d}

* Key to treatment types:
W = Whole inoculum
F = Filtrate
A = Filtered organisms
PDB = PDB-treated controls
Unt = Untreated controls.

** $\bar{X}$ = mean damage index values of all treatments of the same type. Means in the same column followed by the same superscript are not significantly different ($P < 0.05$) according to Duncan's Multiple Range Test.

APPENDIX A: TREATMENT MEANS

Table A1

Mean Damage Index Values for Filtered Inoculum Assay I: Greenhouse Plants

1st Week		2nd Week		3rd Week		4th Week		5th Week		6th Week	
Isolate No.	$\bar{X}$**	Isolate No.	$\bar{X}$	Isolate No.	$\bar{X}$	Isolate No.	$\bar{X}$	Isolate No.	$\bar{X}$	Isolate No.	$\bar{X}$
249	2.71^{a}	249	4.00^{a}	249	4.00^{a}	249	4.86^{a}	249	5.00^{a}	249	5.00^{a}
56	2.43^{a}	56	3.71^{ab}	56	3.71^{ab}	56	4.00^{b}	56	4.29^{ab}	56	4.29^{ab}
161	1.71^{b}	161	3.14^{bc}	161	3.14^{bc}	161	3.71^{bc}	161	3.86^{bc}	PDB	4.00^{ab}
156	1.57^{bc}	156	2.86^{cd}	156	2.86^{cd}	156	3.14^{cd}	156	3.43^{bcd}	161	4.00^{ab}
236	1.43^{bcd}	170	2.29^{bcde}	170	2.29^{de}	PDB	2.71^{de}	PDB	3.29^{bcd}	156	3.71^{bc}
161F	1.29^{bcd}	161F	2.00^{cdef}	236	2.29^{de}	170	2.57^{def}	170	3.00^{cde}	236	3.43^{bcd}
249F	1.14^{cd}	236	1.86^{defg}	PDB	2.29^{de}	161F	2.43^{def}	161F	3.00^{cde}	161F	3.29^{bcd}
170F	1.14^{cd}	249F	1.71^{efg}	161F	2.14^{de}	236	2.29^{defg}	170F	2.86^{cde}	170	3.14^{bcd}
156A	1.14^{cd}	PDB	1.71^{efg}	170F	1.71^{ef}	170F	2.00^{efgh}	236	2.71^{de}	170F	3.14^{bcd}
161A	1.00^{d}	170F	1.43^{efg}	249F	1.71^{ef}	249F	1.86^{efghi}	249F	2.14^{ef}	156F	2.71^{cde}
170A	1.00^{d}	156F	1.43^{efg}	156F	1.57^{ef}	156F	1.71^{fghi}	156F	2.00^{ef}	156A	2.57^{cdef}
170	1.00^{d}	236F	1.14^{fg}	56F	1.29^{f}	236F	1.43^{ghi}	236F	1.57^{f}	249F	2.29^{defg}
236A	1.00^{d}	Unt	1.14^{fg}	161A	1.14^{f}	156A	1.43^{ghi}	156A	1.57^{f}	236F	2.00^{efg}
236F	1.00^{d}	56F	1.14^{fg}	Unt	1.14^{f}	Unt	1.29^{hi}	Unt	1.43^{f}	Unt	1.71^{efg}
156F	1.00^{d}	249A	1.14^{fg}	236F	1.14^{f}	56F	1.29^{hi}	56F	1.29^{f}	249A	1.71^{efg}
249A	1.00^{d}	156A	1.14^{fg}	249A	1.14^{f}	161A	1.14^{hi}	161A	1.29^{f}	170A	1.57^{efg}
Unt	1.00^{d}	161A	1.00^{g}	236A	1.14^{f}	249A	1.14^{hi}	249A	1.29^{f}	56F	1.43^{fg}
PDB	1.00^{d}	170A	1.00^{g}	156A	1.14^{f}	170A	1.14^{hi}	170A	1.29^{f}	161A	1.43^{fg}
56A	1.00^{d}	56A	1.00^{g}	56A	1.00^{f}	236A	1.14^{hi}	56A	1.14^{f}	236A	1.29^{g}
56F	1.00^{d}	236A	1.00^{g}	170A	1.00^{f}	56A	1.00^{i}	236A	1.14^{f}	56A	1.14^{g}

* Isolate numbers followed by F indicate treatment with filtrate of that isolate; those followed by A indicate treatment with filtered organisms; and those followed by no letter indicate treatment with whole inoculum. PDB indicates controls treated with potato dextrose broth and Unt indicates untreated controls.

** Mean damage index values (n = 7). Means in the same column followed by the same superscript are not significantly different ($P < 0.05$) according to Duncan's Multiple Range Test.

__ Isolates appearing above solid line exhibited significantly greater mean damage index values than untreated controls.

-- Isolates appearing above dashed line exhibited significantly greater mean damage index values than PDB-treated controls. Dashed lines are consonant with solid lines in 1st and 6th weeks.

Table A2

Mean Damage Index Values for Filtered Inoculum Assay II: Field Plants

1st Week		2nd Week		3rd Week		4th Week		5th Week		6th Week	
Isolate No.*	$\bar{X}$**	Isolate No.	$\bar{X}$	Isolate No.	$\bar{X}$	Isolate No.	$\bar{X}$	Isolate No.	$\bar{X}$	Isolate No.	$\bar{X}$
56	2.86^{a}	56	4.00^{a}	56	4.71^{a}	56	4.86^{a}	56	4.86^{a}	56	5.00^{a}
161	2.43^{ab}	161F	3.43^{ab}	161F	4.43^{ab}	156	4.57^{ab}	156	4.86^{a}	156	4.86^{ab}
161F	2.14^{abc}	56F	3.43^{ab}	56F	4.14^{abc}	56F	4.43^{ab}	236	4.57^{ab}	236	4.86^{ab}
56F	2.14^{abc}	156	3.29^{abc}	156	4.00^{abcd}	161F	4.43^{ab}	161	4.57^{ab}	161F	4.71^{abc}
156	2.14^{abc}	161	3.14^{abcd}	161	3.86^{abcde}	161	4.29^{ab}	161F	4.43^{ab}	161	4.71^{abc}
170	1.86^{bcd}	236	3.14^{abcd}	170	3.71^{abcdef}	236	4.29^{ab}	236F	4.43^{ab}	236F	4.57^{abc}
170F	1.71^{bcd}	249	2.86^{abcde}	236	$3.57^{abcdefg}$	170	4.00^{abc}	56F	4.43^{ab}	56F	4.43^{abcd}
161A	1.57^{bcd}	156F	2.71^{bcde}	249	$3.43^{abcdefg}$	236F	3.86^{abc}	170	4.00^{abc}	170	4.29^{abcde}
156A	1.57^{bcd}	170	2.71^{bcde}	156F	3.29^{bcdefg}	249	3.86^{abc}	156F	4.00^{abc}	249	4.00^{abcde}
236	1.43^{cd}	161A	2.57^{bcdef}	156A	3.29^{bcdefg}	156A	3.71^{abc}	249	3.86^{abc}	236A	4.00^{abcde}
236A	1.29^{cd}	156A	2.57^{bcdef}	236F	$3.14^{bcdefgh}$	156F	3.57^{bc}	156A	3.71^{abc}	156F	4.00^{abcde}
156F	1.29^{cd}	249F	2.14^{bcdefg}	161A	3.00^{cdefgh}	161A	3.43^{bc}	161A	3.57^{bc}	156A	3.71^{bcde}
249F	1.29^{cd}	170F	2.14^{bcdefg}	170F	2.71^{defghi}	170F	3.00^{cd}	236A	3.57^{bc}	161A	3.57^{cde}
249A	1.14^{d}	236F	2.00^{cdefg}	249F	2.57^{efghi}	236A	3.00^{cd}	249F	3.14^{cd}	PDB	3.29^{de}
PDB	1.14^{d}	236F	2.00^{cdefg}	236A	2.43^{fghi}	249F	2.86^{cd}	PDB	3.00^{cd}	249F	3.14^{ef}
56A	1.14^{d}	249A	1.86^{defg}	PDB	2.29^{ghi}	PDB	2.86^{cd}	170F	3.00^{cd}	170F	3.14^{ef}
249	1.14^{d}	PDB	1.71^{efg}	249A	1.86^{hij}	249A	2.00^{d}	249A	2.00^{de}	249A	2.14^{fg}
236F	1.00^{d}	56A	1.29^{fg}	56A	1.43^{ij}	56A	1.57^{e}	56A	1.71^{e}	56A	1.86^{g}
170A	1.00^{d}	170A	1.00^{g}	170A	1.00^{j}	Unt	1.14^{e}	Unt	1.29^{e}	170A	1.29^{g}
Unt	1.00^{d}	Unt	1.00^{g}	Unt	1.00^{j}	170A	1.00^{e}	170A	1.14^{e}	Unt	1.29^{g}

* Isolate numbers followed by F indicate treatment with filtrate of that isolate; those followed by A indicate treatment with filtered organisms; and those followed by no letter indicate treatment with whole inoculum. PDB indicates PDB-treated controls and Unt indicates untreated controls.

** Mean damage index values (n = 7). Means in the same column followed by the same superscript are not significantly different ($P < 0.05$) according to Duncan's Multiple Range Test.

__ Isolates appearing above solid line exhibited significantly greater mean damage index values than untreated controls.

-- Isolates appearing above dashed line exhibited significantly greater mean damage index values than PDB-treated controls. Dashed line is consonant with solid line in the first week.