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EVALUATION OF FUNGI FOR BIO' CONTROL OF *HYDRILLA VER* (L.f.) ROYLE

by

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Fungal isolates collected from the microsphere of hydrilla were evaluated for their use as potential biological control agents of this weed. Test-tube and aquaria pathogenicity tests were conducted with each isolate alone and in combination. Fungal isolates were also tested in conjunction with commercially produced enzymes to determine if pathogenicity could be enhanced. Environmental parameters including pH, temperature, and light were evaluated for their effect on the growth and sporulation of 16 fungal isolates that exhibited an impact on hydrilla. Preliminary host range and acute fish toxicity tests were performed on the fungal isolate *Cladosporium cladosporioides* (number 224), which exhibited the greatest potential as a biological control agent of hydrilla.

Preliminary test-tube studies of individual isolates indicated that several fungi may impact hydrilla. Although *C. cladosporioides* did not impact hydrilla in the test-tube

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assay, it did impact this weed in an aquarium assay, with plants being killed within 2 weeks after inoculation. In the combination studies, isolates 170/249 and 170/59 significantly damaged hydrilla. In this same study, tests were also performed to determine if there was any inhibitory growth action between fungal combinations. No such activity was found to occur between the fungal isolates used.

Studies using commercial enzymes were performed in Petri dishes. Results indicated that enhanced biocontrol capabilities may be possible for fungi with the addition of pectinolytic enzymes.

The environmental parameters of pH, temperature, and light all affected growth of fungal biocontrol agents to varying degrees, with temperature exhibiting the most pronounced effect.

In preliminary host range studies, isolate 224 did not cause symptoms of disease on any of the test plant species, including hydrilla. In repeated experiments, isolate 224 was unable to impact hydrilla. This fungus apparently lost its pathogenic ability through continuous reculturing on artificial media. Isolate 224 did not cause any significant detrimental effects to test fish in acute toxicity tests.

Although none of the fungi in these studies could be considered a serious candidate for use as a biocontrol agent, other fungi have been found to cause disease of hydrilla.

Preface

This study was conducted by personnel of the US Army Engineer Waterways Experiment Station (WES) as part of the US Army Corps of Engineers Aquatic Plant Control Research Program (APCRP). Funds were provided by the Headquarters, US Army Corps of Engineers (HQUSACE), under Department of the Army Appropriation No. 96X3122, Construction General. Mr. E. Carl Brown, HQUSACE, was Technical Monitor.

The principal investigators for this work were Dr. Gary F. Joye and Mr. Edwin A. Theriot of the Aquatic Habitat Group (AHG), and Ms. Susan Hennington, Wetlands and Terrestrial Habitat Group (WTHG), Environmental Resources Division (ERD), Environmental Laboratory (EL). They were assisted by Mr. Stewart Kees of the AHG.

The work was conducted under the general supervision of Dr. John Harrison, Chief, EL, and Dr. Conrad J. Kirby, Chief, ERD, and under the direct supervision of Dr. Hanley K. Smith, Chief, WTHG, and Mr. Theriot, Chief, AHG. Mr. J. Lewis Decell was the Program Manager of the APCRP. This report was edited by Ms. Jessica S. Ruff of the WES Information Technology Laboratory.

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Contents

EVALUATION OF FUNGI FOR BIOLOGICAL CONTROL OF *HYDRILLA VERTICILLATA* (L.f.) ROYLE

Background

1. These studies were based on the theory that certain microorganisms, which exist in the natural microflora of hydrilla, have a latent ability to attack the plant when conditions favor the microbe and/or stress the plant. This research is similar to the biological control project conducted by Dr. Haim B. Gunner, University of Massachusetts at Amherst (Gunner 1983).

2. Previous research has identified 20 fungal isolates that produced enzymes lytic to hydrilla tissues; six of these enzymes caused damage to hydrilla in test-tube assays (Theriot and Pennington 1985). Isolate 224 (*Cladosporium cladosporioides*), which was the only isolate to produce pectinase enzyme, failed to cause disease of hydrilla in test-tube assays. Pectinase production may be a predisposing factor for the pathogenicity of many plant pathogens. Therefore, isolate 224 was included with the other six as primary candidates. These seven isolates were evaluated in aquaria assays. The isolates that showed potential in these assays will be tested in the field. Aquaria assays and plant culture techniques have been refined to evaluate candidates on plants growing in lake sediment (Pennington 1985).

3. To be effective as a biocontrol agent, a candidate organism must express pathogenicity, be host specific, thrive in conditions favored by the target weed species, and compete with other microflora for sites of attachment (Shrum 1982). The enzyme production of these candidates was enhanced so they would become stronger pathogens (Pennington 1985).

Objectives

4. The objectives of this report were to present results of studies conducted to (a) evaluate growth of the candidate microorganisms under different environmental conditions to determine how well they may inhabit an area where hydrilla may thrive, (b) determine the potential efficacy of isolates alone and in combination on hydrilla, (c) determine the efficacy of the best candidates on other selected aquatic species commonly growing in habitats

similar to those of hydrilla, and (d) determine the effects of the candidate fungi in combination with commercially produced pectinase enzymes that may have synergistic activity.

Materials and Methods

5. All organisms used in these studies were fungi collected from apparently healthy hydrilla foliage. In no case was any isolate observed causing disease of hydrilla in the field. This is not to say that the organism was not a potential pathogen. The pathogenic effects of one organism on another are often density dependent (Shrum 1982). Throughout these studies, large propagule counts were needed to produce an impact on hydrilla. The minimum level of inoculum has not been accurately defined. Therefore, a minimum inoculum count of at least 10^6 colony forming units (CFU) per millilitre was generally used. All data were analyzed using PC-SAS on an IBM-AT (SAS Institute, Inc. 1985).

Test-tube assays

6. Two test-tube assays were conducted using previously collected fungal isolates. The first test included isolates known to produce lytic enzymes (Pennington 1985). The second test included fungal isolates collected from hydrilla during summer 1987.

7. In the first test, eight fungal isolates were tested for efficacy on hydrilla by the method described by Pennington (1985). These isolates included the fungal species *Aspergillus awomori*, *Cephalosporium acremonium*, *Humicola* sp. with *Trichoderma* sp., *Fusarium moniliforme* var. *subglutinans*, *C. cladosporioides*, and *Fusarium roseum* var. *culmorum*. (The *F. roseum* was received from Dr. R. Charudattan of the University of Florida, Gainesville.) Test tubes (20 by 2.3 cm) were filled with 60 ml of nutrient solution (described in methods for the host-specificity test, paragraphs 22-23) amended with 3 mg/ℓ streptomycin. A 10-cm apical tip of hydrilla was inserted into each tube.

8. The fungi were grown separately in 100 ml of potato dextrose broth (PDB) in 250 Erlenmeyer flasks on a shake table at 150 rpm at 25° C. *Cladosporium cladosporioides* was grown in a V-8 Juice media as described in paragraph 23. Two millilitres of inoculum of each fungus was randomly placed in each hydrilla test tube. The hydrilla-inoculated test tubes were incubated

at 25° C with 12-hr light periods (Hotpack incubator, model 352620, Philadelphia, PA). The test continued for 6 weeks, after which time the remaining living biomass was weighed (in grams). All treatments were replicated five times.

9. The second test-tube assay was conducted in a manner similar to the first. Fifteen fungal isolates collected from hydrilla during 1987 (see Figure 1) were screened in this assay. These isolates were grown on solid media that consisted of potato dextrose agar (PDA) amended with 3 mg/ℓ streptomycin and 2 g yeast extract. A 1-cm plug was placed into each test tube and forced to the bottom of the tube (Rejmankova, Blackwell, and Culley 1986). All treatments were replicated 10 times. After 6 weeks, the remaining living biomass was weighed.

10. Data from both assays were subjected to analysis of variance (ANOVA) procedures, and mean comparisons between isolates and controls were made using Tukey's test (Steel and Torrie 1980).

Isolate growth studies

11. Two growth experiments were conducted to identify isolates that grow best under conditions common to hydrilla. For the first experiment, candidate isolates were grown at temperatures of 16°, 25°, and 30° C on PDA adjusted to pH 7 in Petri dishes (1.5 by 9 cm). Isolate inoculum, taken from stock culture, was placed in the center of each dish with a sterile loop. Growth diameter measurements were taken 5 and 10 days after inoculation. Isolates were also grown at 25° C on PDA adjusted to pH levels of 5, 7, and 9 to determine the optimum pH for growth.

12. In the second experiment, 16 of 21 fungal isolates known to produce lytic enzymes (Pennington 1985) (Table 1) were tested in Petri dishes to determine the effects of different temperature, pH, and light regimes on their growth and reproduction. This experiment was similar to the previous one except that the candidates were also grown under light and dark conditions, and the temperatures were 16°, 25°, and 35° C. The fungi were grown on PDA amended with streptomycin (3 mg/ℓ). The pH was adjusted to one of three levels (5, 7, and 9) with hydrochloric acid or potassium hydroxide.

13. After the fungal isolates were placed on the PDA, they were allowed to grow in a dark or lighted incubator (Freas, model 815 GCA, Precision Scientific for dark; Lab-line, model Imperial II, Lab-line instruments with light attachment, 40 lux, for lighted). The temperature was preset to 16°, 25°, or

35° C. All treatment combinations were replicated five times. Fungal isolates were allowed to grow to the edge of the Petri dish. Radial growth was measured (in millimetres) at 24-hr intervals. Growth of fungal cultures was statistically compared between isolates for each treatment through ANOVA. Mean comparisons were made using Duncan's New Multiple Range Test (Steel and Torrie 1980).

Isolate combination studies

14. Experiment 1. Isolates were grown in pairs on PDA in Petri dishes to determine compatibility. Two isolate inocula were centered on opposite sides of the same dish. A zone of inhibition (no growth) between isolates on the same dish signified incompatibility.

15. Compatible combinations of isolates were evaluated in test-tube assays using healthy sprigs of hydrilla. One millilitre each of the two isolates was inoculated in each test tube. The inoculum was prepared in the manner described by Pennington (1985). Each treatment was replicated 10 times.

16. A damage index (Pennington 1985) was given to each plant in the test tubes once a week for 6 weeks. The damage index was determined by arbitrarily assigning a number to a plant with a specific disease severity, as follows:

Index	Description
1	Vigorous, apparently 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, less than 50 percent of plant damaged.
4	Markedly chlorotic plants, exhibiting pronounced damage exceeding 50 percent of sprig.
5	Chlorotic to brown plants, brown stems and most leaves transparent and disintegrating, or dead plants.

Data were subjected to ANOVA procedures, and mean comparisons were made using Duncan's New Multiple Range Test (Steel and Torrie 1980).

17. Experiment 2. Sixteen lytic enzyme-producing (Pennington 1985) fungal isolates collected from hydrilla were tested for antibiotic activity

against one another. All possible paired combinations of the fungi were tested. Two fungi were placed opposite one another in 1.5- by 9-cm Petri dishes with 25 ml of PDA (pH 6.5) approximately 1 cm from the dish edge. Cultures were allowed to grow at ambient temperature under normal laboratory conditions. As the fungal colonies grew, they were monitored for any inhibitory activity. Fungal colony radii were measured at 24-hr intervals. All combinations were replicated five times. Data were presented as a positive or negative response, with a positive response being the presence of antibiotic activity.

Assay of pectinase enzymes and fungal isolates

18. Eight purified pectinase enzymes (seven lyases and one hydrolase) were reconstituted in 50-mM Tris buffer. The lyases were dissolved in Tris at pH 8.0 and the hydrolase in Tris at pH 5.5, to accommodate their respective pH ranges of optimum activity. The final concentration of the enzyme solution was 100 µg/ml, the recommended minimum.* Eight fungal isolates were also used. The final ratio of media to enzyme was 9 to 1 (i.e., 4.5 ml media:0.5 ml enzyme). The plant nutrient solution for the seven lyases was pH 5.0.

19. A 1-cm portion of the apical tip of hydrilla was excised and used as the plant component. A total of 72 enzyme treatments were prepared (64 treatments of enzyme plus isolate and 8 treatments of enzyme alone). All treatments were replicated four times. Plants were rated using the damage index previously described. Data were subjected to ANOVA procedures; Least Significant Difference (LSD) separation (Steel and Torrie 1980) was used for mean comparisons.

Aquaria assays

20. Assay 1. Aquaria assays of the seven primary candidates were conducted as recommended by Pennington (1985), using first-generation field-collected hydrilla plants and whole liquid inoculum of the isolate. Eight aquaria were set up, one for each treatment plus an untreated control. Nine 0.24-ℓ plastic cups containing lake sediment were planted with three sprigs of hydrilla and placed in each of the eight aquaria. Liquid inoculum was grown

* Personal Communication, 1987, Dr. Shauping Lei, Genetic Institute, Berkeley, CA.

for 4 weeks at ambient temperature in roux culture bottles containing 200 ml of V-8 Juice broth to obtain a mixture of both hyphae and spores. The inoculum was blended with a Waring blender, and 600 ml of inoculum was applied to each treated aquarium. The assay was run for 8 weeks. The remaining living biomass was weighed. Data were subjected to ANOVA, and mean comparisons were made using Tukey's test.

21. Assay 2. A study was conducted to test the effects of five fungal isolates that showed potential as biocontrol agents on the growth of hydrilla in test-tube assays. Species included in this assay were *A. awomori*, isolate 56; *F. roseum* var. *culmorum*, isolate 621P (donated by Charudattan, University of Florida); *C. cladosporioides*, isolate 224; and two unidentified isolates, isolate FHY18 and FHY20. Three hydrilla stem tips (13 cm) were planted in plastic cups (0.24 ℓ), filled with pond sediment, and covered with silica sand. Nine cups were placed in each of six aquaria. One aquarium was designated as the control. Environmental conditions were maintained as previously described. After the plants had grown to the top of the water column, whole inoculum of each isolate was poured into one of the six aquaria. For each isolate, a concentration of 10^6 CFU/ml was applied. The test was continued for 3 weeks. Biomass of each cup from each aquaria was weighed. Data were subjected to ANOVA procedures, and Tukey's test was performed to compare means (Steel and Torrie 1980).

Host specificity of *C. cladosporioides*

22. A preliminary host-specificity aquarium test was conducted under controlled conditions [light (10,000 footcandles (107,640 lux), 14-hr light period, and a constant temperature of 25° C) in an environmental chamber measuring 2.6 by 2.3 by 2.4 m]. Aquaria measured 30 by 30 by 75 cm. Each of the eight aquaria was filled with 48.5 ℓ of nutrient solution containing 48.5 ℓ of reverse osmosis (RO) water, $CaCl_2 \times 2H_2O$, 4.456 g; $MgSO_4$ (anhydrous), 1.635 g; $KHCO_3$, 0.747 g; and $NaHCO_3$, 2.84 g. Three 13-cm apical nonflowering sprigs of hydrilla (*Hydrilla verticillata*), Eurasian watermilfoil (*Myriophyllum spicatum*), elodea (*Elodea densa*), and coontail (*Ceratophyllum demersum*) were planted in one 0.24-ℓ plastic cup four-fifths filled with autoclaved (1 hr) Brown Lake sediment (US Army Engineer Waterways Experiment Station, Vicksburg, MS) and covered with 1 cm of washed silica sand. Each aquarium contained three cups of hydrilla and three cups each of two of the other species for a total of nine cups and three species per aquarium. The plants were allowed to

grow for 4 weeks before application of the fungal inoculum. Two aquaria were used for each plant species combination--one for control and one for treatment.

23. The inoculum consisted of the fungus and the culture media (whole inoculum). The formulated media was made of 200 ml of V-8 Juice, 3 g of $CaCO_2$, 5 g of sodium polypectate, 2 g of yeast extract, and 800 ml of distilled water. After 1 week in this media, the fungus had reached a concentration of 10^5 CFU/ml. For each treatment, 600 ml of whole inoculum plus 3×10^6 spores/ml was applied to four aquaria. Spores were grown by the open-tray method (Walker 1980). The test continued for 8 weeks. In order to detect any significant change in the growth of the plant in the presence of the fungi (even if no visual symptoms were evident), data were collected on total living biomass above and below ground, number of shoots from roots, number of branches off shoots, and maximum plant length. Data were subjected to a T-test (Steel and Torrie 1980).

Fish toxicity of *C. cladosporioides*

24. *Cladosporium cladosporioides* was tested for toxicity to the white amur. The triploid white amur, female *Ctenopharyngodon idella* × male *Hypophthalmichthys nobilis* (Ozark Catfisheries, Springfield, MO), was chosen as the test fish because it feeds on hydrilla foliage. Therefore, the fish would be in direct contact with the fungus both through ingestion and contact in the water. Ten aquaria measuring 30.5 by 21.5 by 15.2 cm were filled with 8 ℓ of RO water. Within each aquarium, 50 g of hydrilla foliage was emersed in the water. Ten white amur were placed in each aquarium. Five randomly assigned aquaria were inoculated with 150 ml containing fungal whole inoculum (10^5 CFU/ml) plus a spore concentration of 3×10^6/ml. the water was not filtered or changed after inoculation, since filtering may remove the toxic activity. The fish were observed daily for any toxic effect by the fungus. The data collected included pretest weight of total fish/aquarium, posttest fish weight, and number of dead fish. Changes in feeding habit were monitored, and an examination was made of the digestive tract and gills of five randomly sampled fish. The test continued for 4 weeks. Data were subjected to a paired T-test analysis comparing differences between treated fish and untreated fish (Steel and Torrie 1980). The test was conducted twice.

Results and Discussion

Test-tube assays

25. Assay 1. The ANOVA procedure showed a significant difference in efficacy between the treatments ($r^2 = 0.66$, $P > f = 0.0001$). Fungal isolates 56, 116, and 236 were significantly more effective than isolates 224 or 621P in reducing hydrilla biomass. However, the more effective isolates were not significantly better than the treatment with PDB (Figure 2).

26. Assay 2. The ANOVA procedure showed a significant difference in the effectiveness of the fungal isolates ($r^2 = 0.16$, $P > f = 0.04$). There was an expected lower coefficient of determination in this test than in the first test. The fungal isolates used in assay 2 were not evaluated for any particular mode of pathogenicity, such as the degradation of host tissue from the production of lytic enzymes. Thus, any isolate could express a wide range of efficacy to hydrilla. Several isolates were significantly more effective than others (Figure 3). Those isolates that showed potential were tested in greenhouse aquaria studies.

Isolate growth studies

27. Experiment 1. Results of the growth experiment demonstrated that 5 of the 16 isolates tested grew best at 25° C. The remainder preferred temperatures between 25° and 30° C. Mean water temperatures, 15 to 30 cm from the surface, in hydrilla mats of the southeastern United States often reach 25° C in late summer.

28. All fungal isolates grew moderately well at all three pH levels tested. One isolate grew best at pH 5, and 10 isolates preferred pH 7 or higher (Table 2). No significant difference in growth was noted for the remaining nine isolates. It was expected that the majority of the isolates would grow best at the higher pH levels, since they were all collected from healthy field hydrilla. Seven of the isolates that grew well at the higher pH levels preferred the lower temperatures (Table 2). Three of the seven were isolates that damaged hydrilla in test-tube assays--156, 161, and 170. Isolate 224 also preferred a pH range of 7 to 9 and grew best at 25° C.

29. Experiment 2. Results of the second growth experiment suggested that all of the isolates would grow under a wide range of temperatures, pH levels, with or without light. Eight isolates grew best at 25° C, eight grew best at 35° C, and none grew well at 16° C (Figure 4). The isolates grew

moderately well at all three pH levels (Figure 5). Generally, most isolates grew best at the 5 or 7 pH level, as was the case in the first experiment.

30. Little difference in growth of isolates was noted between the light and dark treatments (Figure 6). The ability of fungi to exist in both light and dark conditions further suggests a compatible relationship with hydrilla. Hydrilla has been reported to survive under a wide range of light intensities, from low to high penetration of sunlight (Bowes et al. 1977; Van, Haller, and Bowes 1978).

31. The isolates showed a wide range of growth rates over time. Ten of the 16 isolates grew significantly faster than the remaining 6 (Figures 7 and 8). Isolates 56 and 249, which grew quickly, were reported to cause disease of hydrilla (Pennington 1985). Isolate 224, which grew slowly, was also reported to cause disease of hydrilla (Theriot 1987).

32. Microbes can probably coexist with hydrilla, if they grow well at 25° C or lower, in neutral to alkaline pH levels, and under a wide range of light intensities. Results of the experiments described herein suggest that the pathogenic fungi tested may coexist with hydrilla and survive under a wide range of environmental conditions.

Isolate combination studies

33. All 20 isolates tested proved to be compatible with each other in experiment 1. In the second experiment, most isolates tested were compatible (Table 3). However, several isolate combinations exhibited weak inhibitory activity. This weak inhibition was exhibited as a zone of aggregating mycelium at the point of contact between the two isolates, which eventually grew together. The slowing of growth over the media may simply be competition for nutrients. Toxic antibiotic affects are usually more distinguishable, exhibiting distinct lines of inhibition.

34. Test-tube assays were conducted on all combinations to determine the efficacy on hydrilla sprigs. Three weeks after treatments were applied to hydrilla test-tube assays, two combinations exhibited significantly greater damage than the control: 249/170 and 170/59. Mean disease index values for the two combinations were also significantly greater than the individual isolates. These results suggest a synergistic effect.

Assay of pectinase
enzyme and fungal isolates

35. Crude pectinase lyase (PL-C) was the most efficacious enzyme on hydrilla. The combinations of the enzyme PL 74% and fungal isolate 56 caused a significantly higher disease rating than any other used alone. Other enzyme/fungal combinations that showed a significant degree of pathogenicity include PL-B/249 and PL-C (pure 100%)/244 (Table 4). These results suggest that the addition of purified pectinase enzyme to opportunistic microorganisms may provide a portal of entry for infection of hydrilla. These results also suggest that if the enzymatic activity of weak fungal pathogens could be increased, their pathogenicity may be increased. This could be accomplished through various means, including media modifications and/or the genetic engineering of fungi to produce specific enzymes.* However, the specificity of such organisms must be determined, and this would require extensive laboratory and greenhouse testing to meet the stringent requirements of the US Environmental Protection Agency prior to field testing.

Aquaria assays

36. Assay 1. Two weeks after the aquarium test was initiated, isolate 224 had completely destroyed hydrilla (Figure 9). Plant tissues disintegrated and settled to the bottom of the aquarium. Plant decomposition was followed by an algal bloom. The rapid decline and decomposition of the tissues indicated possible toxin and/or enzyme effects.

37. Isolates 244, 56, and 249 achieved maximum shoot length significantly greater than the control, but none exhibited significant increases in aboveground biomass. Isolates 244, 56, and 249 elicited a plant growth hormone reaction, suggestive of gibberellin production. Gibberellin causes cell elongation in plants, which would explain the increase in length without increase in biomass (Raven, Evert, and Curtis 1981). No disease symptoms were observed in any aquarium other than the one treated with 244.

38. Assay 2. None of the test isolates impacted hydrilla plants. No significant difference was noted between the control and treatments. Even isolate 224, which had severely impacted hydrilla in a previous experiment, did not cause disease of hydrilla. Since these organisms were not impacting

* Personal Communication, 1988, Dr. Olen Yoder, Cornell University, Ithaca, NY.

hydrilla initially in the field, they may have been effective only in the test-tube assay because of a limited nutrient source or some other unknown factor. A scaleup of several orders of magnitude may have rendered the isolate useless as far as being efficacious to hydrilla. However, in the case of 244, pathogenicity of organisms may be lost through repeated transfer of cultures. Changes in pathogenicity of organisms such as *C. cladosporioides* are usually genetic. This being the case, the isolates of 224 in the culture collection have been recultured to the point that they have become laboratory artifacts. Use of a new cryofreezer should reduce the chance of such changes occurring in stock cultures. Ultrafreezing of cultures reduces genetic mutations, and many fragments may be taken from a single isolate (Dhingra and Sinclair 1985). A survey in the area where 224 was originally collected will be conducted to reisolate the organism.

Host specificity of *C. cladosporioides*

39. None of the test species was diseased by *C. cladosporioides*, including hydrilla. This may be explained by evidence that long-term storage of pathogenic organisms results in loss of virulence, although their viability is maintained (Hawksworth 1984). This change is generally a genetic mutation, and the gene(s) for virulence may be lost. Occasionally, virulence may be regained through modification of the growth media. However, this has not been the case with *C. cladosporioides*. Reisolation of the field strain is necessary.

Fish toxicity of *C. cladosporioides*

40. The white amur were not affected by the presence of *C. cladosporioides*. Ninety percent or more of the hydrilla was consumed within 10 days. Fish in the treated aquaria were observed ingesting the fungus as well as the hydrilla foliage. Their feces were of a much darker color than the untreated fish. Both fungal hyphae and hydrilla tissue were removed from their digestive tracts. No fungal material was removed from the gills. No significant differences in mortality or weight loss were noted between treatments. Results of this test suggest that exudates from this fungus would not pose a threat to fish behavior or mortality. The fact that this organism is known to exist in endogenous waters would indicate cohabitation with a variety of fish species with no adverse effects.

Conclusions and Future Research

Conclusions

41. Conclusions of this study are:

a. Several fungal isolates were able to grow well at the temperature and pH levels tested, whether grown in the presence or absence of light.

b. Fungal isolate 224 was originally capable of attacking and destroying apparently healthy hydrilla growing in pond sediment. However, continual reculturing of isolate 224 resulted in loss of its pathogenicity.

c. All paired combinations of the lytic enzyme-producing isolates were compatible.

d. Two combinations of isolates significantly damaged hydrilla in test-tube assays: 249/270 and 170/59.

e. The use of fungi in combination with certain enzymes (PL 74%/fungal isolate 56, PL-B/fungal isolate 249, and PL-C (pure 100%)/244) produced significant disease symptoms on hydrilla in Petri dishes.

f. Isolate 224 did not impact coontail, Eurasian watermilfoil, egeria, eelweed, or hydrilla in host-specificity tests.

g. Isolate 224 was not toxic to triploid grass carp.

Future research

42. Several fungal isolates now in culture will be tested in the greenhouse, and titer tests will be conducted to determine the minimum CFU/ml required for effective control of hydrilla. A survey will continue in search for plant pathogens of hydrilla, and special effort will be made to reisolate a pathogenic form of 224. For a candidate organism to be an effective biocontrol agent, it must express pathogenicity, be host specific, thrive in conditions favored by the target weed species, and compete with other microflora for sites of attachment. These basic criteria should be used to determine whether a microorganism could be used for biological control of hydrilla.

References

Bowes, G., Van, T. K., Gerrard, L. A., and Haller, W. T. 1977. "Adaptation to Low Light Levels by Hydrilla," Journal of Aquatic Plant Management, Vol 15, pp 32-35.

Dhingra, O. D., and Sinclair, J. B. 1985. Basic Plant Pathology Methods, CRC Press, Boca Raton, FL.

Gunner, H. B. 1983. "Microbiological Control of Eurasian Watermilfoil," Miscellaneous Paper A-83-4, US Army Engineer Waterways Experiment Station, Vicksburg, MS.

Hawksworth, D. L. 1984. "Fungi in Culture," Nature, Vol 310, p 18.

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, MS.

Raven, P. H., Evert, R. F., and Curtis, H. 1981. Biology of Plants, Worth Publishers, New York.

Rejmankova, E., Blackwell, M., and Culley, D. D. 1986. "Dynamics of Fungal Infection in Duckweeds (Lemnaceae)," Veroff. Geobot. Inst. ETH, Stiftung Rubel, Zurich, Vol 87, pp 178-189.

SAS Institute, Inc. 1985. SAS User's Guide: Statistics, Version 5, Cary, NC.

Shrum, R. D. 1982. "Creating Epiphytotics," Biological Control of Weeds Using Plant Pathogens, R. Charudattan and H. L. Walker, eds., John Wiley and Sons, New York, pp 113-136.

Steel, R. G. D., and Torrie, J. H. 1980. Principles and Procedures of Statistics, McGraw-Hill, New York.

Theriot, E. A. 1987. "Microbial Control of *Hydrilla verticillata*," Proceedings, 21st Annual Meeting, Aquatic Plant Control Research Program, Miscellaneous Paper A-87-2, US Army Engineer Waterways Experiment Station, Vicksburg, MS.

Theriot, E. A., and Pennington, J. C. 1985. "Microbiological Control of Hydrilla with Lytic Enzyme-Producing Microorganisms," Proceedings, 19th Annual Meeting, Aquatic Plant Control Research Program, Miscellaneous Paper A-85-4, US Army Engineer Waterways Experiment Station, Vicksburg, MS.

Van, T. K., Haller, W. T., and Bowes, G. 1978. "Some Aspects of the Competitive Biology of Hydrilla," Proceedings, EWRS 5th Symposium on Aquatic Weeds, Amsterdam, The Netherlands, pp 117-125.

Walker, H. Lynn. 1980. "Production of Spores for Field Studies," Agricultural Research (Southern Region), Science and Education Administration, US Department of Agriculture, New Orleans, LA.

Table 1

Fungal Isolates Used in Hydrilla Efficacy Studies

(Modified from Pennington 1985)

Isolate Number	Scientific Name
	Cellulase Positive
49	*Penicillium* sp.
56	*Aspergillus awomori* Nakazawa
57	*Aspergillus* sp.
59	*Cephalosporium acremonium* Corda
101*	
111	*Cephalosporium acremonium* Corda
116*	
156	*Humicola* sp. with *Trichoderma* sp.
157	*Aspergillus luchuensis* Invi
161	*Humicola* 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
	Undetermined Enzyme Activity
621P†	*Fusarium roseum* var. *culmorum* (Lk. and Fr.) Synd. and Hans.

* Unidentified.

** Not previously described.

† Donated by Dr. R. Charudattan, University of Florida, Gainesville.

Table 2

Optimum Growth Temperature and pH Values for Lytic Enzyme-Producing Fungal Isolates Examined in Isolate Growth Experiment 1

Isolate No.	Temperature °C	pH
49	25	5-9
56*	30	9
57	25-30	5-9
59	25	7-9
101	25-30	9
111	30	5-9
116	25-30	5-9
156*	25-30	7
157	30	9
161*	25-30	7
162	25-30	5
170*	25-30	7
224**	25	7-9
236*	25	5-9
237	25-30	5-9
240	30	5-9
242	25	7-9
244	25-30	5-9
249*	25-30	5-9
250	30	9

* Isolates that diseased hydrilla in test-tube assays.
** Isolate 224 was the only pectinase-producing fungus.

Table 3

Fungal Isolate Compatibility

Isolate No.	49	56	57	101	116	157	224	236	237	240	242	244	249	250	621P
49	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
56		+	+	+	+	+	-	-	+	+	+	+	+	+	+
57			+	+	+	+	+	+	+	+	+	+	+	+	+
101				+	+	+	+	+	+	+	+	+	+	+	+
116					+	-	+	-	+	+	+	+	+	+	+
157						+	-	+	+	+	+	+	+	+	+
224							+	+	+	+	+	+	-	+	+
236								+	+	+	+	+	+	+	+
237									+	+	+	+	+	+	+
240										+	+	+	+	+	+
242											+	+	+	+	+
244												+	+	+	+
249													+	+	+
250														+	+
621P															+

Note: * = compatibility; - = noncompatibility.

Table 4

Results of Enzyme/Fungal Combination Assay

Treatment*	Fungal Isolate	Mean Disease Index**
PL 74%	56	3.84 a
PL-B	249	3.56 ab
PL-C (pure 100%)	244	3.31 b
PL-C (crude 100%)		2.84 c
PL 74%		2.18 d
PL 344		2.15 d
Cooper's pectinase		2.06 d
Hydrolase		1.43 e
PLA		1.31 e
Control		1.21 e
PL-B		1.12 e
PL-C (pure 100%)		1.03 e

Note: P = 0.05, LSD = 0.50.

* Enzymes were furnished by Genetic Institute, Berkeley, CA.

** Means followed by the same letter are not significantly different. (Disease index rating 1 = little or no disease; 5 = plant diseased to point that death is certain.)

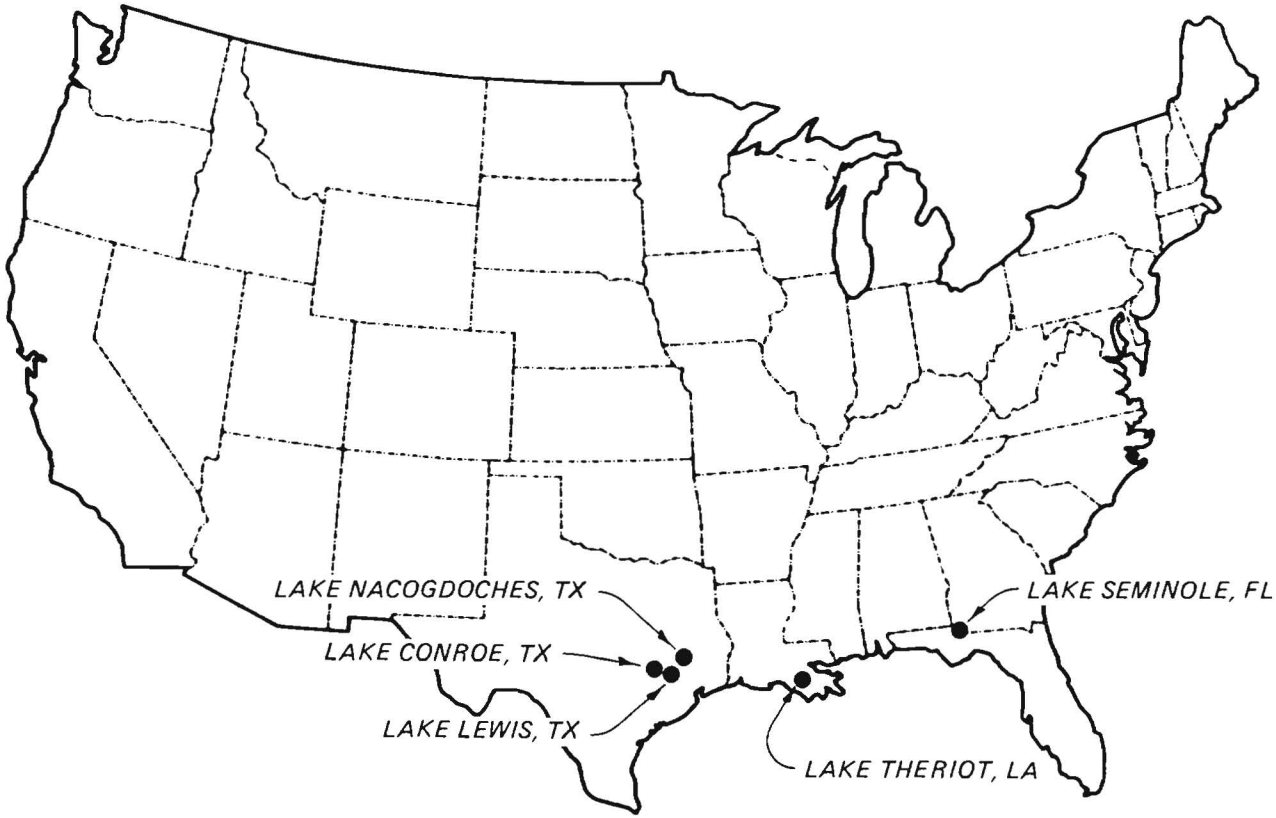

Figure 1. Locations of microbial isolate collections made during 1987

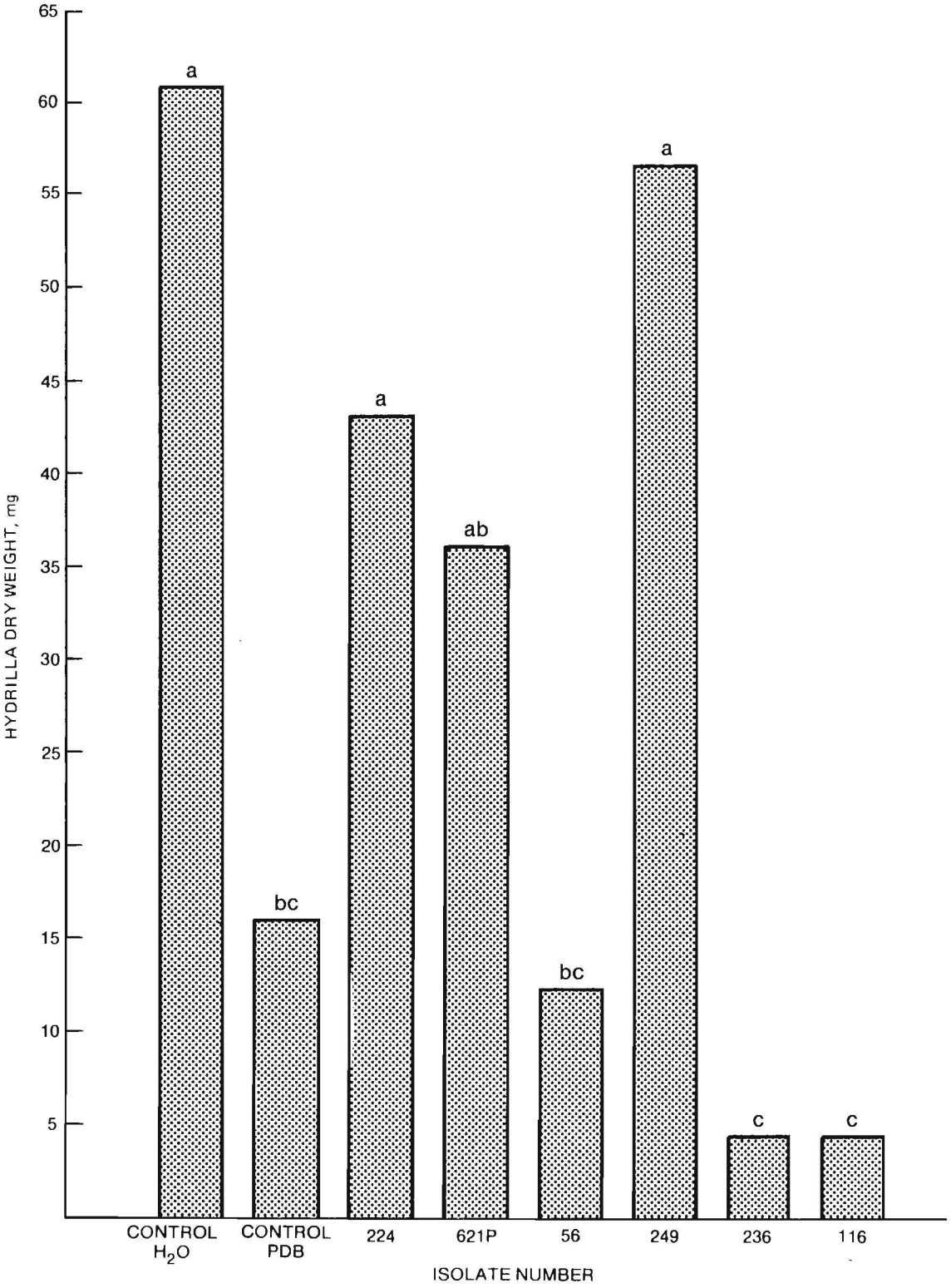

Figure 2. Comparative efficacy of lytic enzyme-producing fungal isolates on hydrilla, based on Tukey's test ($P < 0.05$). Bars with the same letter are not significantly different

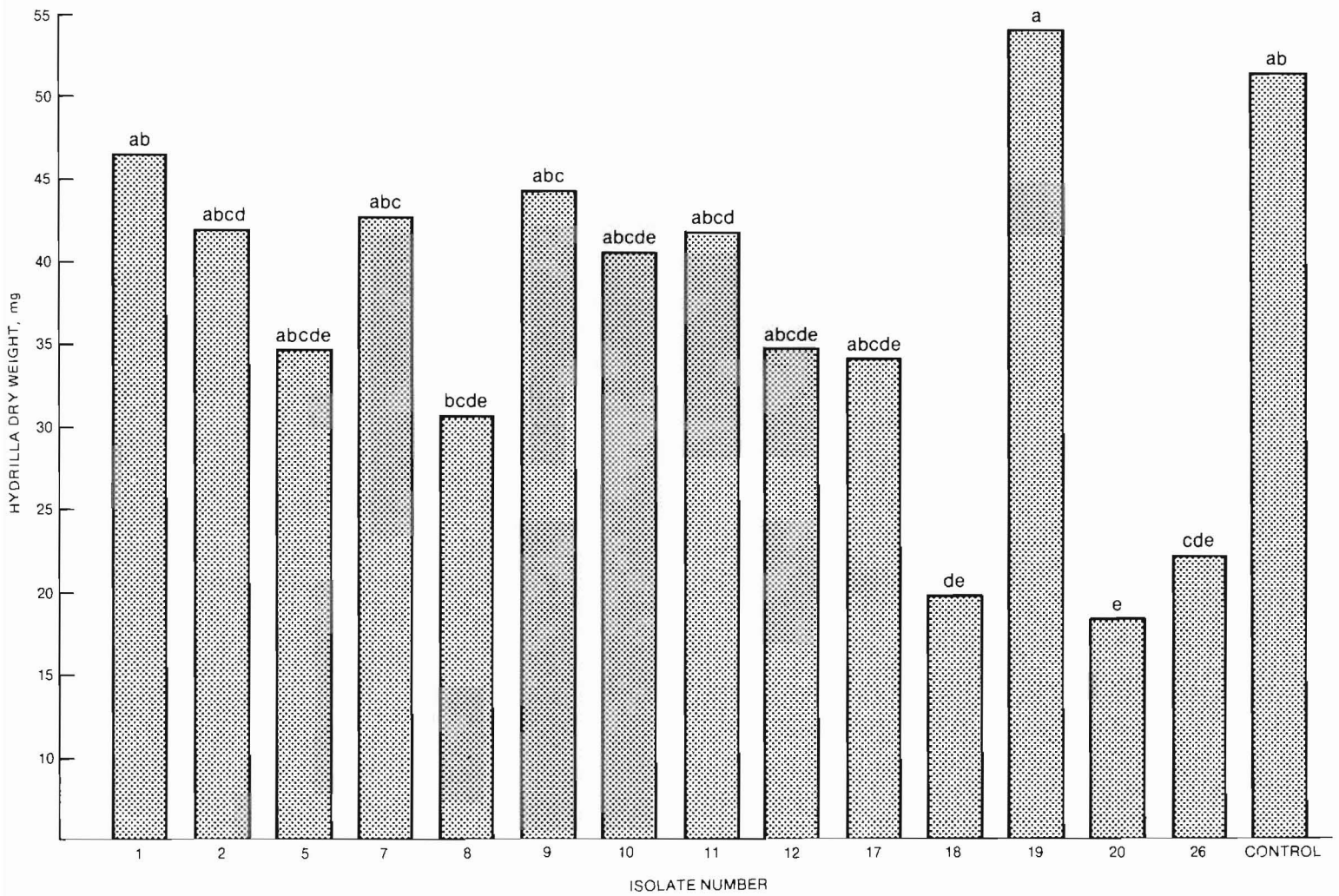

Figure 3. Comparative efficacy of fungal isolates collected from hydrilla during the 1987 growing season, based on Tukey's test ($P < 0.05$). Bars with the same letter are not significantly different

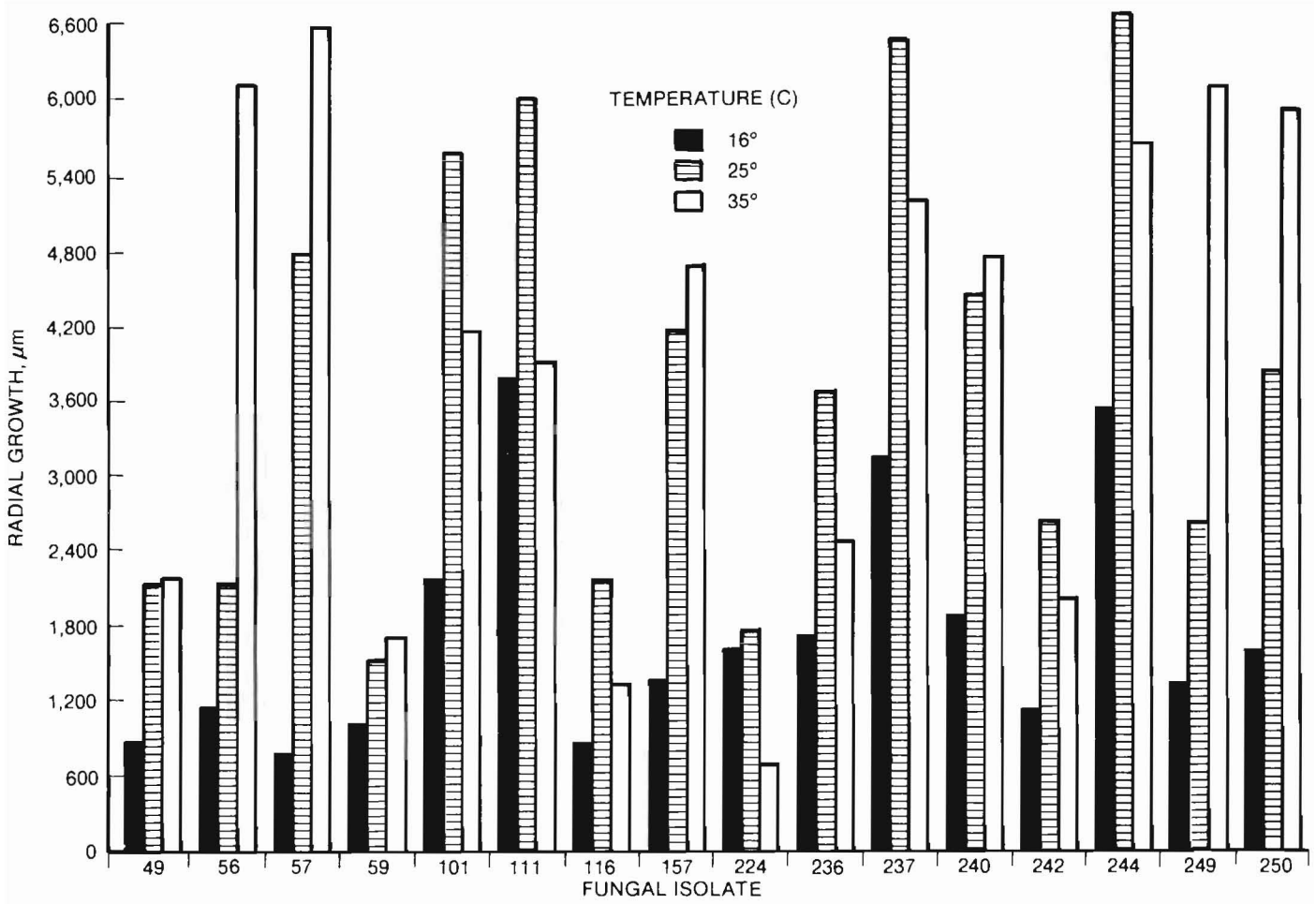

Figure 4. Comparative effects of temperature on fungal isolate growth

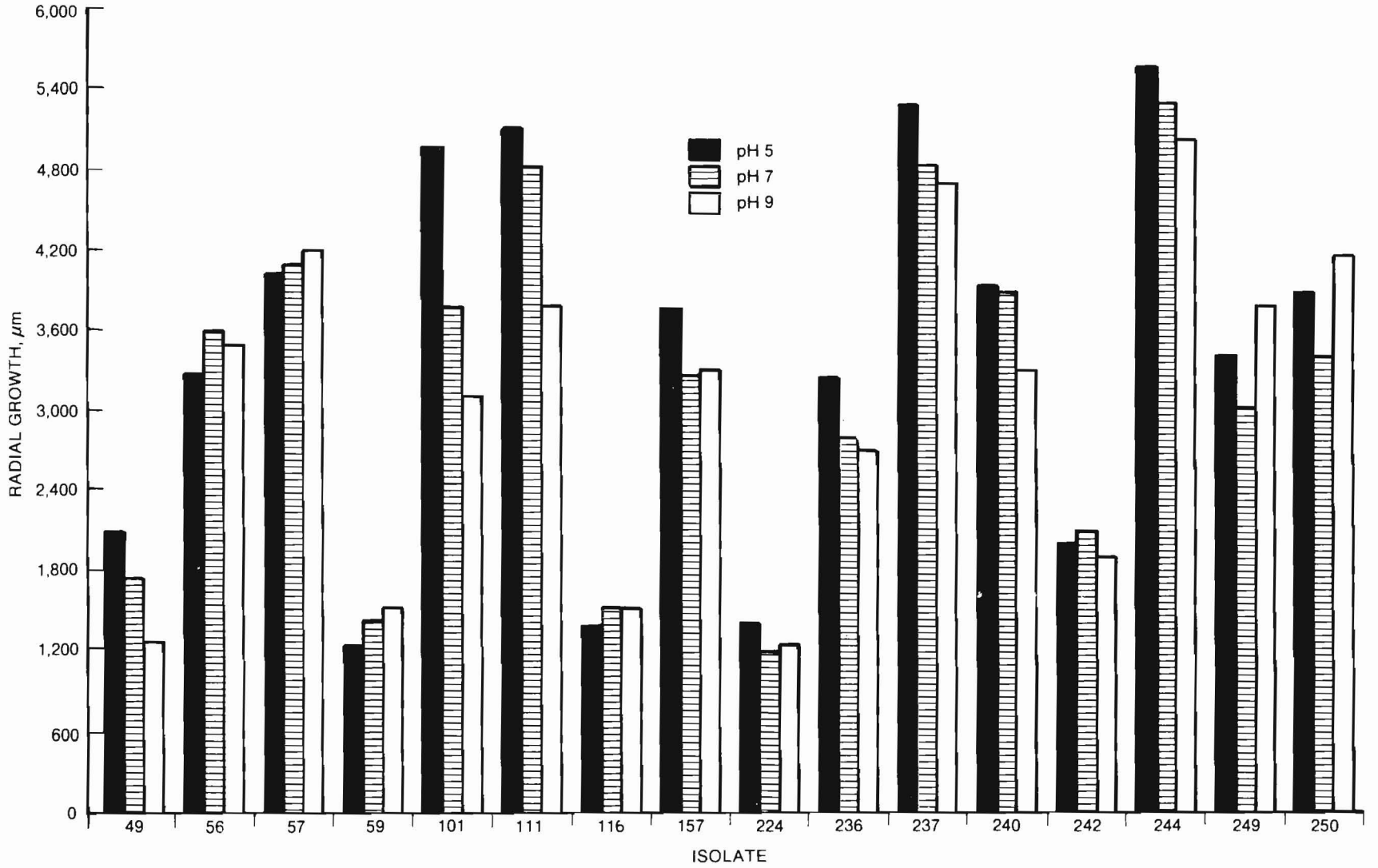

Figure 5. Comparative effects of pH on fungal isolate growth

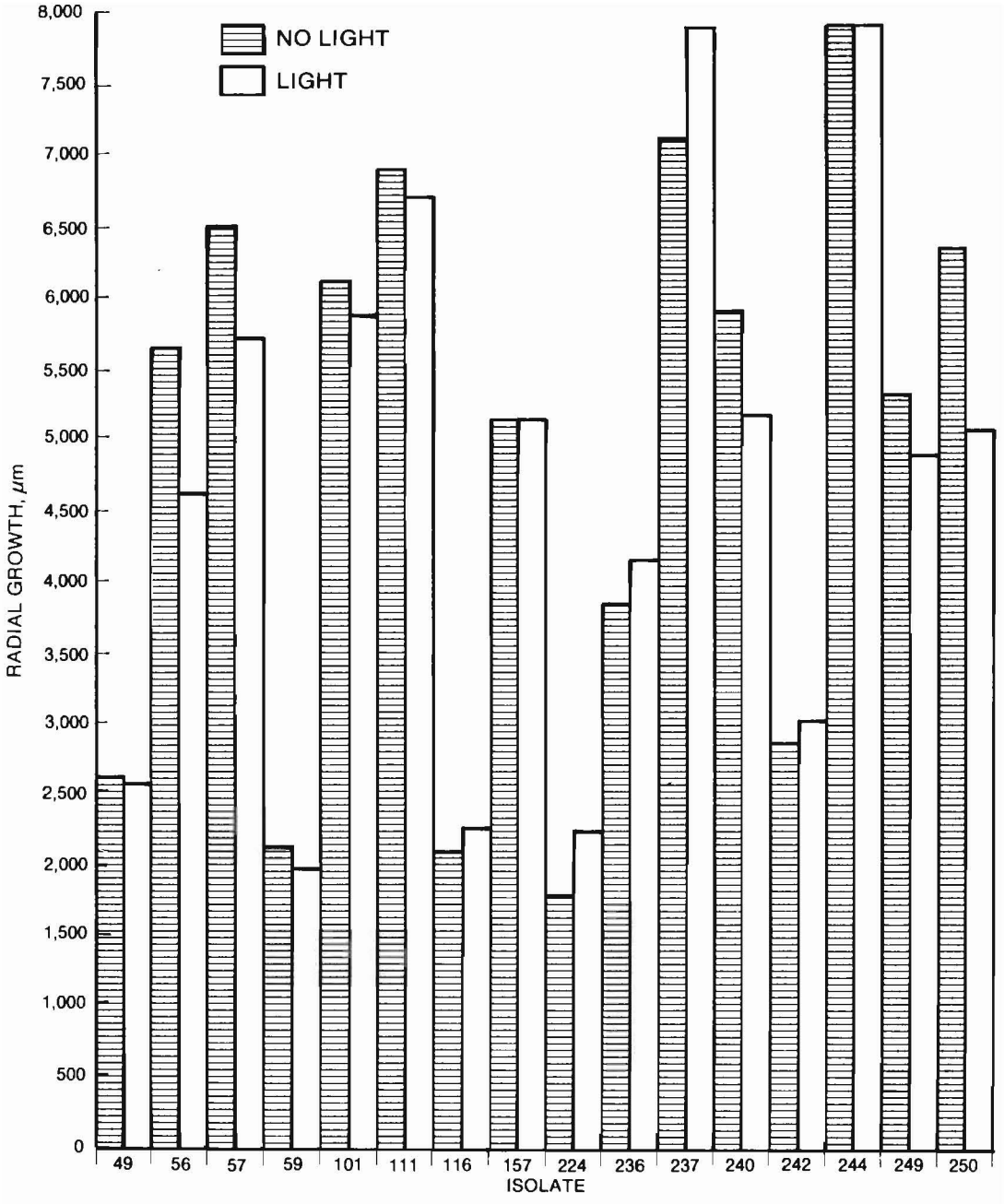

Figure 6. Comparative effects of light on fungal isolate growth

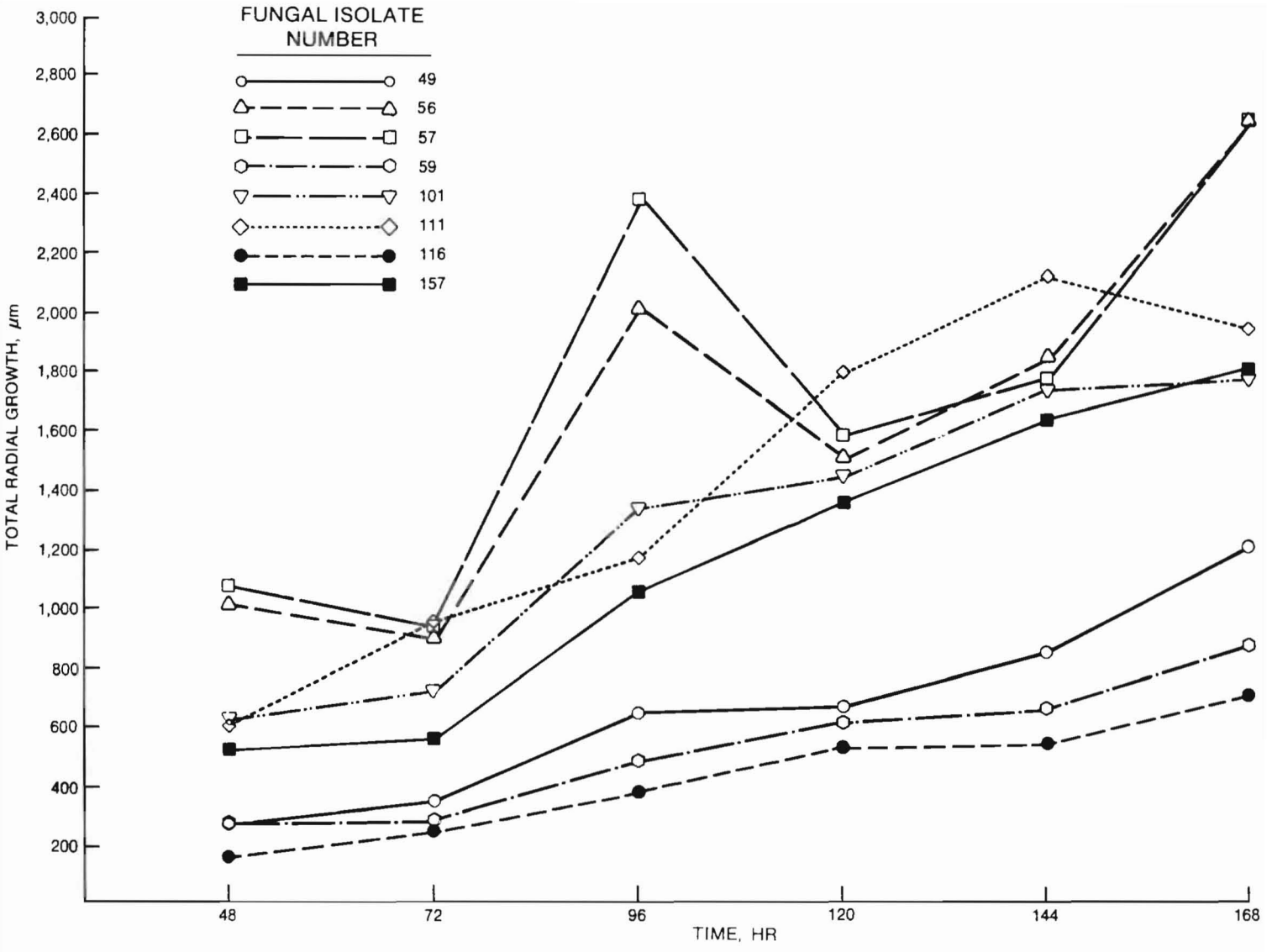

Figure 7. Mean radial growth of fungal isolates 49-157 over time

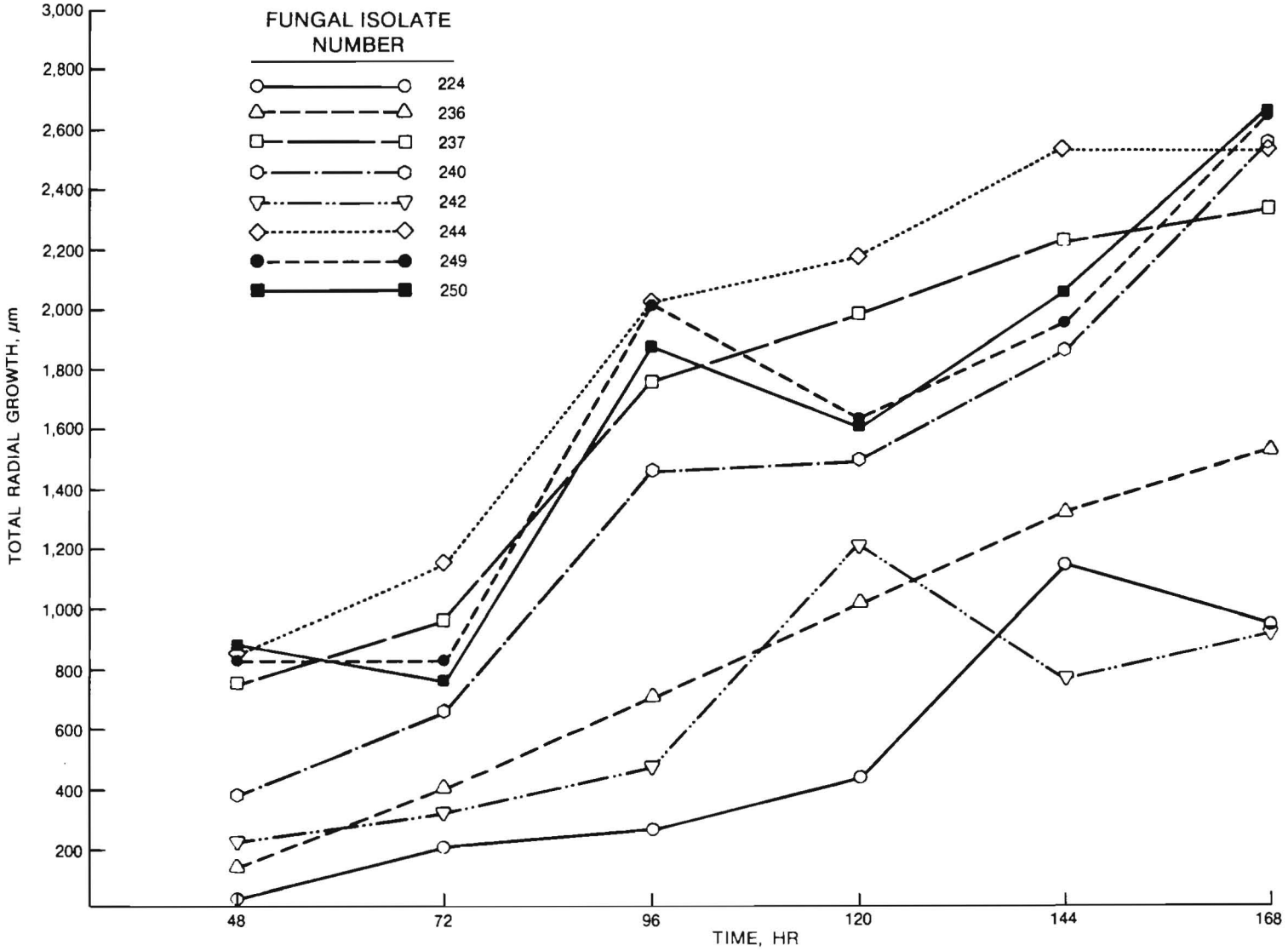

Figure 8. Mean radial growth of fungal isolates 224-250 over time

a. Control

b. Isolate 224 treatment

Figure 9. Efficacy of isolate 224 (*C. cladosporioides*) on hydrilla growing in aquaria, 2 weeks after inoculation