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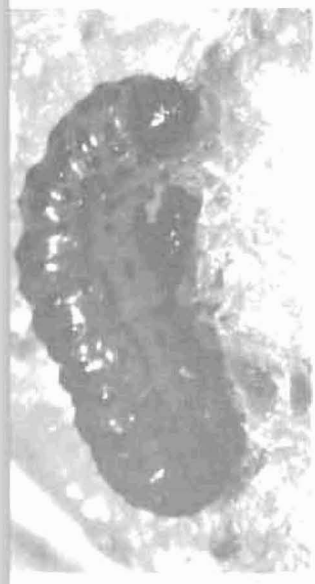

ENVIRONMENTAL LABORATORY

AQUATIC PLANT CONTROL
RESEARCH PROGRAM

TECHNICAL REPORT A-88-1

FIELD EVALUATION OF MICROBIOLOGICAL CONTROL AGENTS ON EURASIAN WATERMILFOIL

by

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US ARMY ENGINEER WATERWAYS EXPERIMENT STATION

February 1988
Final Report

Approved For Public Release; Distribution Unlimited

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

Under Contract No. DACW39-80-C-0029
(APCRP Work Unit 32202)

Monitored by Environmental Laboratory
US Army Engineer Waterways Experiment Station
PO Box 631, Vicksburg, Mississippi 39180-0631

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SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 0704-0188

1a. REPORT SECURITY CLASSIFICATION Unclassified	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION / AVAILABILITY OF REPORT Approved for public release; distribution unlimited.
2b. DECLASSIFICATION / DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S)	5. MONITORING ORGANIZATION REPORT NUMBER(S) Technical Report A-88-1
6a. NAME OF PERFORMING ORGANIZATION University of Massachusetts Dept. of Environmental Sciences 6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION USAEWES Environmental Laboratory
6c. ADDRESS (City, State, and ZIP Code) Amherst, MA 01003	7b. ADDRESS (City, State, and ZIP Code) PO Box 631 Vicksburg, MS 39180-0631
8a. NAME OF FUNDING / SPONSORING ORGANIZATION US Army Corps of Engineers 8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER Contract No. DACW39-80-C-0029
8c. ADDRESS (City, State, and ZIP Code) Washington, DC 20314-1000	10. SOURCE OF FUNDING NUMBERS PROGRAM ELEMENT NO. / PROJECT NO. / TASK NO. / WORK UNIT ACCESSION NO. APCRP 32202

11. TITLE (Include Security Classification)
Field Evaluation of Microbiological Control Agents on Eurasian Watermilfoil

12. PERSONAL AUTHOR(S)
Gunner, Haim B.; Limpa-Amara, Yuthana; Weilerstein, Philip J.

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Final report	FROM _____ TO _____	February 1988	39

16. SUPPLEMENTARY NOTATION
Available from National Technical Information Service, 5285 Port Royal Road, Springfield, VA 22161.

17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	Aquatic plants Aquatic weed control Biological control Microorganisms

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

Control of *Myriophyllum spicatum* L., as previously reported, was based on the multiple effects achieved by the fungus *Mycoleptodiscus terrestris* and selected bacterial isolates with strong pectinolytic abilities and the capacity to exercise a hormonelike stress effect on the plants. Enriched media were adopted for growth of the fungus and bacterial inoculants. This reduced incubation time to 72 hr for the fungus and to 48 hr for the bacterium.

These changes in inoculum growth procedures were reflected in the subsequent change in the pattern of *M. spicatum* decline after exposure to the control agents: from the appearance of isolated necrotic areas to generalized systemic decline and the ultimate disintegration of the plant. The most rapid and devastating response to the organisms grown in these new media was observed in the laboratory when the inoculated plants were maintained in jars. This reflected the sustained high concentration of the inoculum and isolation from environmental effects.

(Continued)

20. DISTRIBUTION / AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION
☒ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT. ☐ DTIC USERS	Unclassified
22a. NAME OF RESPONSIBLE INDIVIDUAL	22b. TELEPHONE (Include Area Code) / 22c. OFFICE SYMBOL

19. ABSTRACT (Continued).

In pool experiments, the most significant result obtained was the restriction in biomass after treatment with *M. terrestris*, either alone or in combination with the bacterium Isolate P-8. These data were further validated by the appearance of the treated plants: greater tissue decay, separation of stem from root, and scarcity of new tips. In the pool setting, the application of the bacterium alone again resulted in increased internodal length. There was also a slight delay in the growth cycle as evidenced by the appearance of the plant and the number of growing tips. However, there was no significant difference in biomass between untreated controls and bacteria-treated pools. Thus, bacterial action must be considered capable of enhancing fungal lethality, but not of exercising a lethal effect by itself. This is supported by the biomass data in which joint fungal-bacterial treatment resulted in the greatest decline in biomass.

The most encouraging dimension of this work was the recent extension to a field setting, in Stockbridge Bowl in western Massachusetts, and the demonstration that an explicit decline in *M. spicatum* could be achieved by the application of these organisms. In the course of the field experiments, unequivocal results were obtained that show:

a. Where sustained contact between microbial inoculum and plant was achieved, plant kill followed.

b. Symptoms of kill were systemic; leaves and stem tissue appeared bleached and chlorotic.

c. Bleached tissue subsequently decomposed and disintegrated.

That biomass figures did not appear to reflect these changes may be adduced to be a consequence of the difficulties in sequestering plants within the designated quadrant areas and the subsequent uncertainty in harvesting. At this time there may have been a loss of decayed material from the quadrant or the intrusion of new material rooted outside the treated area.

In general there was a significant drop in biomass in the 12 weeks of the experiment in all quadrants (treated and control), which reflected seasonal decline. This seasonal change may also have served to mask any biomass difference that could have resulted from treatment with the microbial control agents.

One may hypothesize that the pectinolytic organisms are primary decomposers, releasing nutrients and, by pitting the plant surface, providing additional ecosites for plant-associated microorganisms. This, in turn, is reflected by an increase in the numbers of these populations and the evidence of their action.

The results of these initial field tests on the control of *M. spicatum* by the application of plant-derived microorganisms provide grounds to pursue the development of this approach as a practical control strategy.

The results reported herein represent the findings of our most recent work (1984-1985) and are part of an ongoing series of studies, initiated in 1980, on the microbiological control of Eurasian watermilfoil. Beginning with laboratory studies and proceeding to pool-scale trials, the current work includes our first attempt to control Eurasian watermilfoil in a natural setting. The results provide an encouraging perspective for this microbial, ecosystem-compatible technique for the control of the aquatic weed.

PREFACE

The research reported herein was sponsored by the Office, Chief of Engineers (OCE), US Army, through the Aquatic Plant Control Research Program (APCRP), Work Unit 32202. Technical Monitor for OCE was Mr. E. Carl Brown. The APCRP is managed by the US Army Engineer Waterways Experiment Station (WES). Mr. Edwin Theriot, WES, served as Project Officer on behalf of APCRP. The study was conducted under the general supervision of Dr. Hanley K. Smith, Chief, Wetlands and Terrestrial Habitat Group; Dr. C. J. Kirby, Chief, Environmental Resources Division; and Dr. John Harrison, Chief, Environmental Laboratory, WES. Mr. J. Lewis Decell was Program Manager of the APCRP.

This report was prepared by Dr. Haim B. Gunner and Messrs. Yuthana Limpa-Amara and Philip J. Weilerstein of the Department of Environmental Sciences, University of Massachusetts, Amherst. Assistance in the laboratory and in the field was provided by Messrs. Beryl Bouchard, John Tumino, Seth Frisbee, and Eugene Davidov and Ms. Sara Berger of the University of Massachusetts. Dr. Gunner was Principal Investigator. The report was edited by Ms. Jessica S. Ruff of the WES Information Products Division.

Commander and Director of WES was COL Dwayne G. Lee, CE. Technical Director was Dr. Robert W. Whalin.

This report should be cited as follows:

Gunner, Haim B., Limpa-Amara, Yuthana, and Weilerstein, Philip J. 1988. "Field Evaluation of Microbiological Control Agents on Eurasian Watermilfoil," Technical Report A-88-1, prepared by the University of Massachusetts, Amherst, for the US Army Engineer Waterways Experiment Station, Vicksburg, Miss.

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PART I: INTRODUCTION

Background and Review of Previous Work

1. The introduction of Eurasian watermilfoil, *Myriophyllum spicatum* L., to the waters of North America in the late 19th century has resulted in its emergence as a significant nuisance aquatic plant, making appropriate control measures an urgent necessity (Hayslip and Zettler 1973; Aiken, Newroth, and Wile 1979).

2. Initially, chemical herbicides such as 2,4-D were reported to be an effective means of management even when used at low concentrations (Aiken, Newroth, and Wile 1978; Killgore 1984). However, mounting evidence of the toxicity of chemical herbicides and the danger they pose to other living systems has resulted in increasing restrictions on their use, particularly in sensitive water bodies (Amundsen and Brenkert 1978).

3. Mechanical control methods such as winter drawdown, bottom coverings, bottom tillage, and weed harvesting are also used, but application limitations and economic considerations restrict their use to specific situations (Bates, Burns, and Webb 1986; Newroth 1986).

4. Management methods based on the manipulation of biological systems have shown great potential advantages over other methods (Schuytema 1977). A variety of organisms have been tested for use as biocontrol agents against *M. spicatum*. A snail, *Pomacea australis*, and the manatee, *Trichechus manatus*, were reported to be potent control candidates (Blackburn, Sutton, and Taylor 1971), but no further report has been given of their use (Amundsen and Brenkert 1978). Among 25 insects found to be associated with *M. spicatum*, *Paraponyx stratiotata* was the only one with sufficient host specificity to be considered for use as a biological control (Spencer and Lebic 1974). Two species of fish, *Tilapia zillii* and the grass carp or white amur (*Ctenopharyngodon idella*), have been used for aquatic weed management (Blackburn, Sutton, and Taylor 1971; Amundsen and Brenkert 1978). The inability of *T. zillii* to survive low temperatures and the potential for the upset of ecological

balances by the aggressive grass carp are significant drawbacks that have restricted the use of herbivorous fish as biological control agents (Blackburn, Sutton, and Taylor 1971; Bates, Burns, and Webb 1986).

5. Plant pathogenic organisms have also been tested for their ability to infect *M. spicatum* (Hayslip and Zettler 1973). Two fungi, *Fusarium sporotrichoides* and *Acremonium curvulum*, were reported to attack *M. spicatum* (Andrews and Hecht 1981; Patlak 1982; Andrews, Hecht, and Bashirian 1982). Although *F. sporotrichoides* can cause localized symptoms and *A. curvulum* can kill the plant under specific environmental conditions that increase plant susceptibility (Patlak 1982), the limitations on the effectiveness of these organisms suggest that further study of the interactions of the plant, its microflora, and environmental conditions is necessary.

6. Work conducted in the laboratory at the University of Massachusetts has taken an ecosystems approach to the development of microbial control agents in which the emphasis is on organisms naturally present in the plant environment. Microflora native to the *M. spicatum* phyllosphere have been screened for the production of enzymes destructive to selected plant components, cellulose and pectin, as well as their ability to generally stress the plant either alone or in concert. The contention that this search among plant-associated flora can identify organisms which, after appropriate growth procedures and inoculation back onto the plant, are capable of bringing about its decline and death has been confirmed (Gunner 1983). A series of experiments in the laboratory has resulted in the selection of two promising microbial control agents, a fungus, *Mycoleptodiscus terrestris* (Gerdemann) Ostazeski, and a pectinolytic bacterium, Isolate P-8, for further investigation (Gunner 1983, 1984). Studies of plant-microbe interactions have demonstrated that the selected species occupy an econiche determined by the competitive advantage provided by their resistance to inhibitory substances released by the plant (Gunner 1984).

Purpose and Scope

7. The objectives of the work reported herein were to:

   a. Extend the scope of tests on the effectiveness of this control strategy from the jar, aquarium, and pool-scale studies previously reported (Gunner 1983, 1984) to a natural lake setting.

b. Further examine microbial interactions at the plant-microbe interface.

c. Initiate testing of the specificity of the selected microbial agents by application to a variety of other aquatic and terrestrial plants.

PART II: DESCRIPTION OF STUDIES

Materials

Experimental plant material

8. *Myriophyllum spicatum* L. from Stockbridge Bowl, Stockbridge, Mass., was used in all experiments. For laboratory experiments, healthy tips were cut from plants in the field and brought back in lake water. Vegetative tips were planted as described below and allowed to form roots prior to the initiation of experiments.

Microbial cultures

9. Fungal. *Mycoleptodiscus terrestris* (Gerdemann) Ostazeski (Ostazeski 1967), a cellulolytic organism originally isolated from necrotic areas on samples of *M. spicatum* as previously described (Gunner 1983), was used throughout these studies.

10. Bacterial. Isolate P-8, a pectinolytic gram-negative rod isolated from *M. spicatum*, was selected because of its resistance to plant secretions inhibitory to various other bacteria, its ability to coexist with *M. terrestris*, and its capacity to produce a giberellinlike effect on this plant (Gunner 1983, 1984).

Preparation of Inocula

11. Fungal inocula were propagated in potato dextrose salts broth (PDSB) (Appendix A) unless otherwise noted. Small-scale batch cultures were grown at 28° C on a rotary shaker at 125 rpm for 96 to 120 hr in Ehrlenmeyer flasks. Larger batch cultures were grown in a New Brunswick Scientific Microferm 24-ℓ fermenter at approximately 28° C for 96 to 128 hr at an agitation speed of 250 rpm. All fungal inocula consisted of mycelium grown as described above and subsequently blended at low speed in a Waring 4-ℓ blender for 1 min prior to inoculation. Inoculum viability was tested by plating samples onto potato dextrose agar (PDA) (BBL Company, Dickeysville, Md.) and measuring unit dry weight at the time of inoculation. Inocula were stored, when necessary, at 5° C in darkness. Inocula prepared from separate batches were pooled prior to blending.

12. Bacterial inocula were grown in trypticase soy broth (BBL Company) for all studies described below. Large batch cultures were grown for 48 hr at room temperature in 18-ℓ carboys aerated with sterile air. Bacterial inocula were prepared by mixing xanthan gum (Sigma Chemical Company, St. Louis, Mo.) at 1-percent weight per volume (w/v) with whole cultures to aid in bacterial adhesion to plant surfaces. Counts of viable bacteria were obtained by plating inocula onto trypticase soy agar (TSA) (BBL Company) and pectin agar (PA) (Appendix A) at the time of inoculation. When necessary, inocula were stored at 5° C in darkness.

Cell Counts

13. Microbial populations were enumerated by serial dilution of samples. Fungal counts were made on PDA and Martin's agar (MA) (Martin 1950) after 72 hr of incubation. Total bacterial counts were made on TSA while pectinolytic organisms were enumerated on PA. Bacterial counts were made after 36 to 48 hr of incubation at 28° C in darkness.

Visual Evaluation of Plant Condition

14. The condition of experimental plant samples was evaluated visually and rated on a scale of 0 to 3, with the higher numbers indicating a greater degree of change in the following characteristics:

a. Internode elongation.
b. Discoloration.
c. Turgidity.
d. Change in leaf structure.
e. New growth.
f. Amount of algae on plant surface.

The total number of points accrued in categories A-D and F, quantifying plant decline, was weighed against the total from category E, indicating recovery of the plant sample. This allowed computation of a score describing the condition of the plants in the sample under observation. Observations of each sample were made for the above characteristics by two independent observers.

Jar Experiment

15. Experiments were conducted in 960-ml canning jars to confirm previous results, test the effects of changes in media formulation, and provide a basis for experimental procedures prior to expansion to a larger scale. Five 10-cm tips of *M. spicatum* with roots were planted in 4 to 5 cm of a 3:1 mixture of sterile sand and pond soil in a plastic cup and lowered into a jar filled with distilled water. Plants in jars were maintained in an environmental chamber at 15° C on an 8:16 hr light:dark cycle.

16. Five millitres of each inoculum, prepared as described, was pipetted onto the surface of the plants in each jar. Data were collected by visual evaluation of samples at weekly intervals.

Pool Experiment

17. Twelve 1.6-m^2 pools were planted with healthy *M. spicatum* tips. Thirty-six plants were placed in a 3:1 sand:pool soil mixture in 0.09-m^2 seedling trays that were 6 cm in height. The trays were then placed in the semicircular half-pools at a depth of 65 cm. The plants were allowed to root for 2 weeks prior to inoculation. The pools were filled with tap water and aerated vigorously until inoculation. The water was maintained at the same level throughout the experiment by the addition of fresh tap water as needed.

18. Prior to inoculation, the 12 pools were randomly assigned to four groups:

a. Control, no treatment.

b. Treated with bacterium P-8 in xanthan carrier.

c. Treated with fungus *M. terrestris*.

d. Treated with fungus *M. terrestris* and bacterium P-8 in xanthan carrier.

19. Each of the inoculum formulations described above was dispensed into the appropriate pool under low pressure through a spray manifold constructed of 1.3-cm polyvinyl chloride (PVC) pipe with eight 2-mm apertures. Pools treated with fungus received 500 ml of *M. terrestris* solution containing 2.6×10^4 colony forming units (CFU)/ml; pools treated with bacteria received 1.0 ℓ of inoculum containing 1.25×10^7 cells/ml in 1-percent xanthan. Pools receiving both treatments were treated with the bacterial inoculum first.

Throughout the experiment, measurements were made of microbial populations, plant biomass, and plant condition.

20. Microbial populations were enumerated both on the plant surfaces and in the water column for each treatment. Plant samples were randomly selected from designated trays, and water samples were taken from each pool on a weekly basis for enumeration of microbial populations. Visual evaluation of plant conditions in each treatment was done at weekly intervals. Biomass data were collected weekly by the random harvest of five plants from each pool and at the termination of the experiment by measurement of total biomass in each pool.

Specificity Testing

21. Preliminary screening for pathogenicity of *M. terrestris* and bacterium P-8 toward nontarget aquatic and terrestrial plants was carried out before initiating the field experiments.

22. Four aquatic plants commonly found in New England lakes and ponds, *Vallisneria*, *Sagittaria*, *Elodea*, and *Lemna* (obtained from Connecticut Valley Biological Supply Company, Southampton, Mass.), were grown in aquaria and inoculated with 400 ml per aquarium of a solution of 1.0×10^7 CFU/ml of bacterium P-8 in 1-percent w/v xanthan gum and 200 ml per aquarium of a solution of 1.0×10^4 CFU/ml *M. terrestris* grown in PDSB. Plants were observed for 5 weeks after inoculation for pathological symptoms.

23. Five common terrestrial plants were selected for screening of *M. terrestris*. Rye, wheat, vetch, pea, and bean plants were exposed to *M. terrestris* grown in PDSB by covering the plant surface with a suspension of blended mycelium. Plants were grown to maturity and observed for evidence of pathogenicity.

Field Experiment

24. A field experiment was conducted at Stockbridge Bowl in the town of Stockbridge in the Berkshire region of western Massachusetts. The Bowl is a hardwater lake infested with *M. spicatum* in all areas less than 7 m in depth. The town operates a mechanical harvester during the summer to remove *M. spicatum* growth. An area 2.0 to 2.5 m in depth on the eastern side of the

lake was chosen as an experimental site, and arrangements were made to prevent harvesting in the proximity of this 5,000-m^2 site.

25. Eighteen 0.5-m^2 plots were marked with frames constructed of PVC tubing. Plots were randomly divided into two groups:

a. Control, no treatment.

b. Treated with fungus *M. terrestris* and bacterium P-8 in xanthan carrier.

26. Inocula were prepared as previously described and transported to the field site in 45-ℓ carboys packed in ice. Plots were inoculated first with bacterium P-8 in xanthan and then with *M. terrestris* with a spray manifold constructed of PVC tubing with sixteen 2-mm apertures. Inocula were delivered from a reservoir under 1.7 kg/cm^2 of pressure under the surface of the water in each treated plot. Treated plots were inoculated twice, once at the beginning of the experiment and again 8 weeks later. The first inoculum consisted of 10 ℓ of bacterium P-8 in 1-percent xanthan at a concentration of 7.7×10^7 CFU/ml and 4.0 ℓ of *M. terrestris* at a concentration of 1.35×10^4 CFU/ml. The second inoculum consisted of 10 ℓ of bacterium P-8 in 1-percent xanthan at a concentration of 3.2×10^8 CFU/ml and 6.0 ℓ of fungus *M. terrestris* at a concentration of 1.2×10^4 CFU/ml.

27. Measurements of microbial populations and visual evaluation of plant condition were carried out weekly. Biomass and chlorophyll content were measured for plant material in the plots at the termination of the experiment.

28. Microbial populations were measured on plant surfaces of samples taken from a designated plot for each treatment. Water grab samples were taken from all plots in each treatment and pooled prior to enumeration. Visual evaluations of plant condition were made for each plot as previously described.

29. Biomass was determined for plant material in all plots by cutting stems at the sediment level and bringing the plant material to the surface in wire baskets. Live and dead tissues were separated on the basis of differences in color and physical condition for separate measurement. For the purpose of this study, dead shoots were defined as those that were black and brown and had no green leaves emerging from them. All material was washed in tap water to remove marl; dead material was then dried for 72 hr at 105° C in tared weighing screens. Live material was analyzed for chlorophyll content as described below, after which its dry weight was determined.

30. Chlorophyll measurement was conducted according to a modification of the method of Burnison (1980) using an acetone-dimethyl sulfoxide (DMSO) extraction procedure. A food processor was used to chop live plant material into uniform segments 1 to 3 mm in length. Ten 1-g (wet weight) samples from the material from each plot were weighed out and placed into 20 ml of solvent mixture containing 60 parts 90-percent acetone to 40 parts DMSO. Samples were left at room temperature for 72 hr after which 6 ml was removed by pipette and clarified by centrifugation for 20 min at 500× gravity. One millilitre of clarified extract was then diluted in 4 ml of fresh solvent, and the absorbance at 664 nm (A664) was read in a Bausch and Lomb Spec 21 spectrophotometer. Samples were then acidified with concentrated hydrochloric acid to 0.012 normal, and the A664 was read again. Chlorophyll and pheopigment values were then calculated according to the method of Parsons and Strickland (1972) and expressed as micrograms per milligram dry weight of live plant tissue.

Statistical Methods

31. Statistical analysis of data was performed using the F-test for the determination of overall variance and the t-test to determine if significant differences existed between treatments. Analysis was performed by computer application of BMDP statistical programs (BMDP Statistical Software, Los Angeles, Calif.). The level of significance was determined within a 95-percent confidence interval ($p \leq 0.05$).

PART III: RESULTS AND DISCUSSION

Results

Jar experiment

32. As shown in Figure 1, *M. spicatum* responded to inoculation with bacterium P-8 and the fungal inoculant *M. terrestris*. In conformity with previous observations (Gunner 1984), the pectinolytic bacteria, in addition to pectinolysis, stimulated a hormonelike effect that was expressed in extended internodal distance. This clearly stressed the plant but was not sufficient, by itself, to bring about plant death. Inoculation with *M. terrestris* alone did, however, suffice to induce death. There was no significant increase in response when plants were inoculated with both the bacterium and the fungus. The data in Figure 2 represent averages of visual evaluations. The deviation observed between the control and bacterium P-8 reflects primarily internodal elongation generated by the bacterium. The significant decline of the plant after treatment with *M. terrestris* either alone or with bacterium P-8 confirms that the fungus *M. terrestris* is the essential agent in inducing plant decline.

Pool experiment

33. Photographs of randomly selected representative plants, one from each of the treatments, are shown in Figures 3-6. At the termination of the experiment, control pools 5, 10, and 11 (Figure 3) yielded plants that were generally representative of prevailing seasonal conditions, i.e., the period of decline, when new tips are produced while old stems decay to allow release of this new growth. Root structures remain intact, presaging regrowth of the plant in the new season.

34. Figure 4 shows the response of plants from pools 6, 7, and 12 that were inoculated with only the pectinolytic bacterium P-8. Consistent with the jar experiments is the internodal elongation of stems. What is further apparent is the delay of seasonal decline observed in the control pools. This is expressed by a reduction in the stem necrosis, greater amounts of green tissues with proportionately fewer tips.

35. The response of *M. spicatum* to the application of the fungus is shown in Figure 5 (pools 1, 4, and 9). Here the direct impact of the fungal attack can be seen in the widespread occurrence of necrosis and the lack of

a. Uninoculated control

b. Inoculated with bacterium P-8

c. Inoculated with *M. terrestris*

d. Inoculated with bacterium P-8 and *M. terrestris*

Figure 1. Representative samples of *M. spicatum* 4 weeks after inoculation in jar experiment

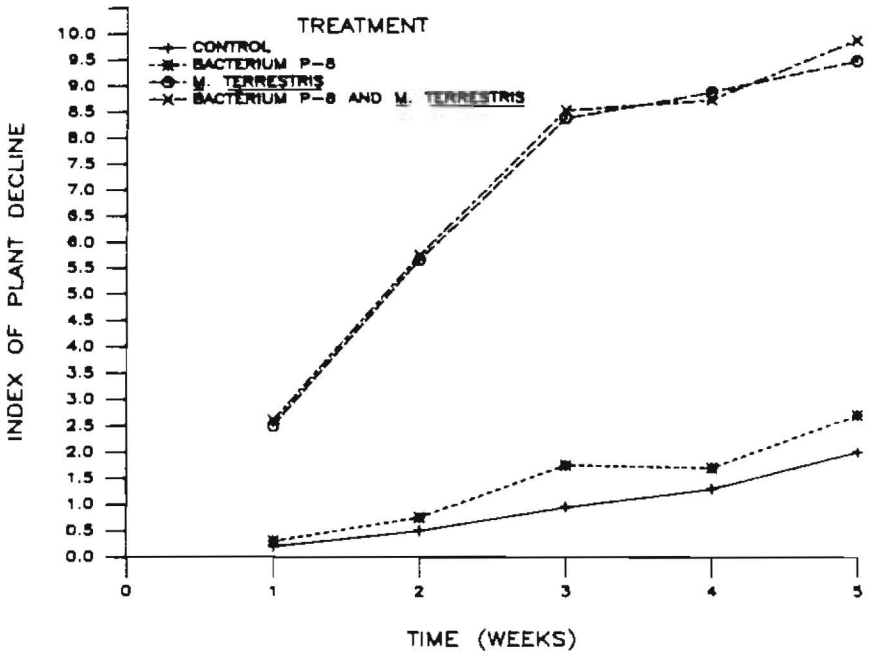

Figure 2. Visual evaluation of condition of *M. spicatum* in jars after microbial treatments. Index represents averages of observations of 10 replicates

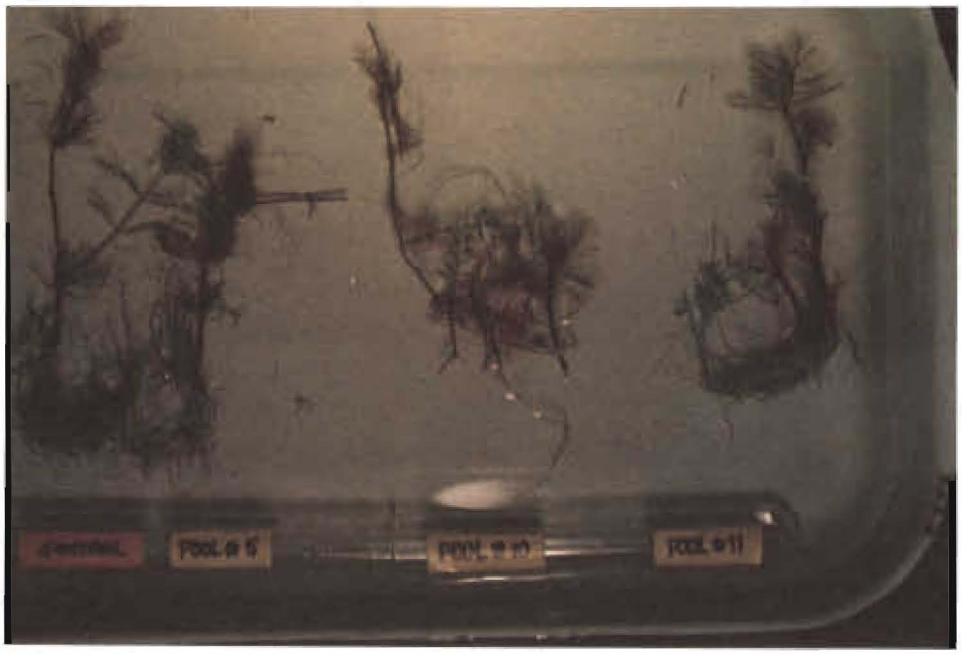

Figure 3. Representative plants from control pools after 9 weeks. Note green tips and substantial root development

Figure 4. *M. spicatum* 9 weeks after inoculation with bacterium P-8. Note internodal elongation and normal root development

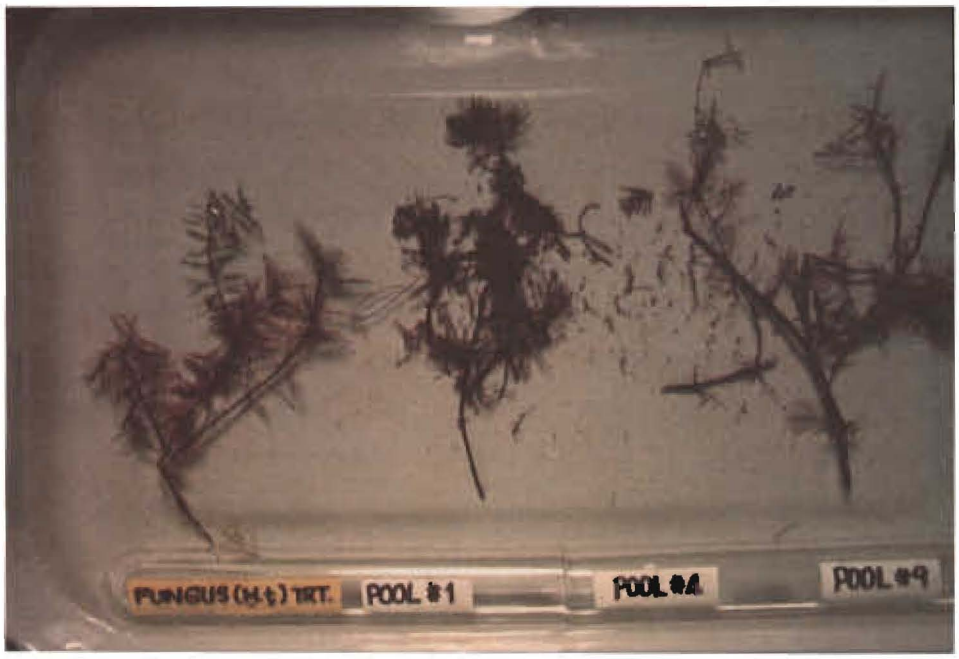

Figure 5. *M. spicatum* 9 weeks after inoculation with *M. terrestris*. Note virtual absence of green tips, generalized decay of plant tissue, and lack of healthy root structure

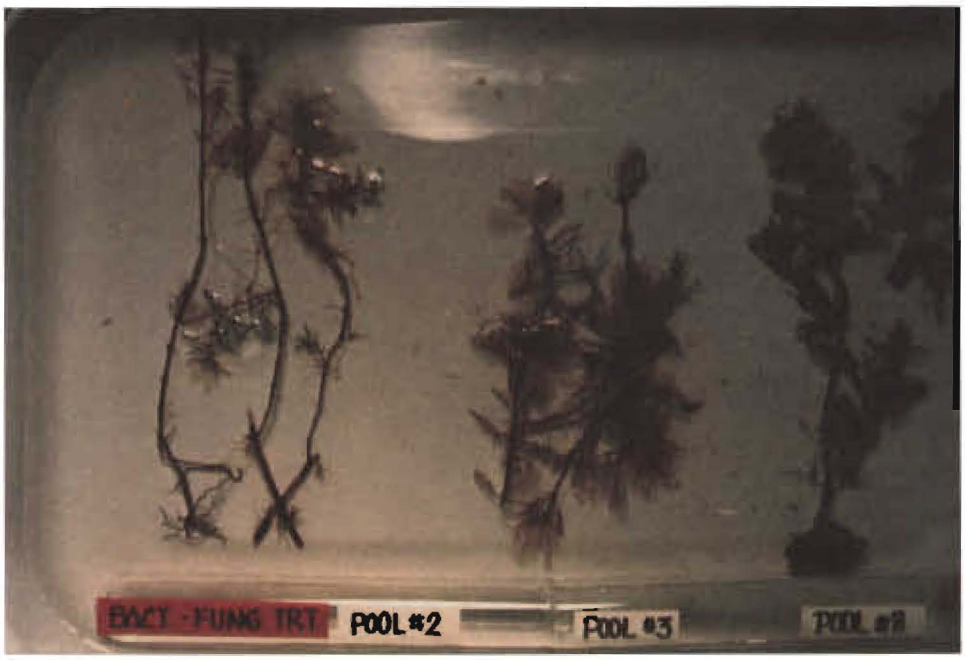

Figure 6. *M. spicatum* 9 weeks after inoculation with both both bacterium P-8 and *M. terrestris*. Note occurrence of internodal elongation, virtual absence of green tissue, extensive decay of plant, with lack of healthy root structure (sample at far left)

new tips or green material. Root tissue could not be recovered due to its severance from the decayed stems at the time of harvest.

36. The effects of treatment with both the bacterium and the fungus are shown in Figure 6 (pools 2, 3, and 8). Again, stem elongation is evident; there is a virtual absence of new growth and severance of roots at plant harvest.

37. In Figure 7 are shown the results of visual evaluations of changes in the appearance of pool-grown plants. Under these conditions, the emergence of extensive epiphytic algal populations in all groups hastened the decline of control plants and minimized the difference observed until the plants were harvested. Although the visual evaluations show a general decline in disease index, as evidenced by new tip growth, these data do not reflect the significantly reduced biomass in pools treated with fungus alone or fungus with bacterium P-8.

38. Analysis of biomass data (Table 1) shows that the biomass harvested from the control pools was significantly greater than that harvested from pools treated with the fungus alone or with the fungus and bacterium together.

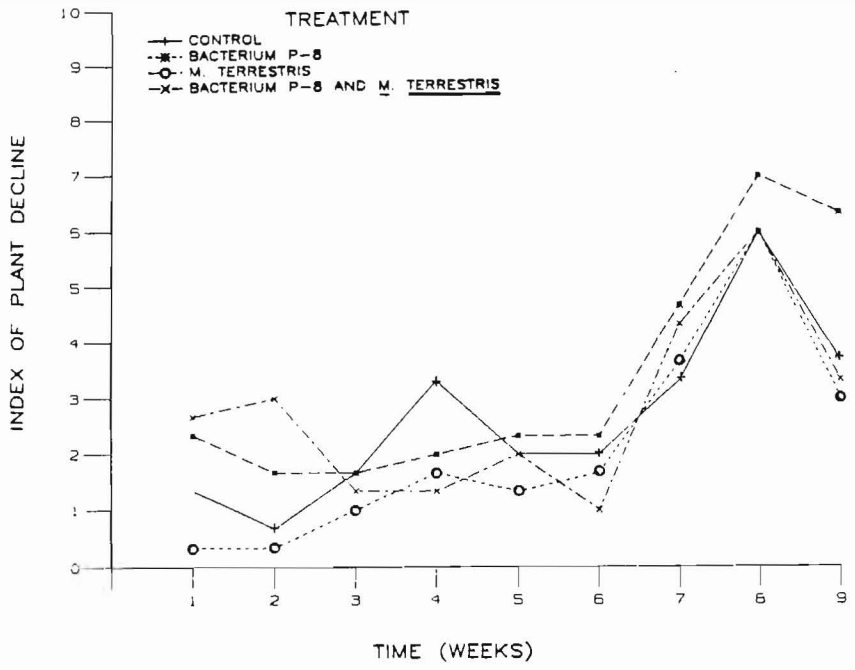

Figure 7. Visual evaluation of condition of *M. spicatum* in pools after microbial treatments. Index represents averages of three replicates

Table 1

Biomass of *M. spicatum* in Pools Treated with *M. terrestris* and Bacterium P-8

Treatment	Biomass Mean Value*	p Value**			
		Control	Bacterium	Fungus	Fungus and Bacterium
Control	2.5733	1.0000			
Bacterium	2.3621	0.3787	1.000		
Fungus	1.9429	0.0044	0.0592	1.000	
Fungus and bacterium	1.7358	0.0044	0.0081	0.2077	1.000

* Mean value of biomass (grams dry weight per tray) obtained by averaging three sets of replicates from each treatment. Values represent average weight of plants from 24 trays (8 trays/replicate) containing 36 plants each.

** p values obtained from ANOVA f values. (p values ≤ 0.05 are significant at a confidence level (CL) of 95 percent; p values ≤ 0.01 are highly significant, CL = 99 percent.)

Treatment with the bacterium alone did not result in any significant loss of biomass with respect to the control pools. The presence of the bacterium, even when added to the fungal inoculum, did not result in any significant loss of biomass in comparison to the treatment with the fungus alone. Comparison of the biomass from the groups treated with fungus and with the combination of bacterium and fungus showed no significant difference between the two treatments.

39. As shown in Figures 8 and 9, the total numbers of microorganisms isolated from plant surfaces or isolated from the water profile remain constant in their relationship throughout the various treatments. Inoculation did not result in any observable shift in microbial numbers, although an overall increase in colonization of the plant surface emerged as the experiment progressed. Total numbers in the water column were approximately three orders of magnitude lower than on the plant surface.

40. Examination of data on population changes induced by the addition of the fungal and bacterial inoculum reveals that the addition of these organisms did not result in a residual increase either on the plant surface or in the adjacent water profile. Numbers in the water column were lower than those on the plant surface and showed a greater variability.

Specificity tests

41. As noted in Table 2, no evidence of infection was obtained from the variety of aquatic and terrestrial plants inoculated with *M. terrestris*.

Field experiment

42. Comparison of Figures 10 and 11 illustrates the response of *M. spicatum* in Stockbridge Bowl to the application of the microbial agents *M. terrestris* and bacterium P-8. The condition of the treated plants 2 weeks after the second inoculation is shown in Figure 10. Leaves are necrotic, stems are bleached, and no green tips are observed. There are extensive areas of necrosis and bleaching of stems, and very little new growth is seen. Growth in an untreated plot is shown in Figure 11. The plants are green, there is little necrosis, and healthy growing tips are plentiful.

43. Biomass and chlorophyll determinations (Table 3) made at the termination of the experiment did not show significant differences between treated and untreated quadrants.

44. Analysis of microbial population dynamics on plant surfaces and in the adjacent water profile revealed that increases in microbial numbers were

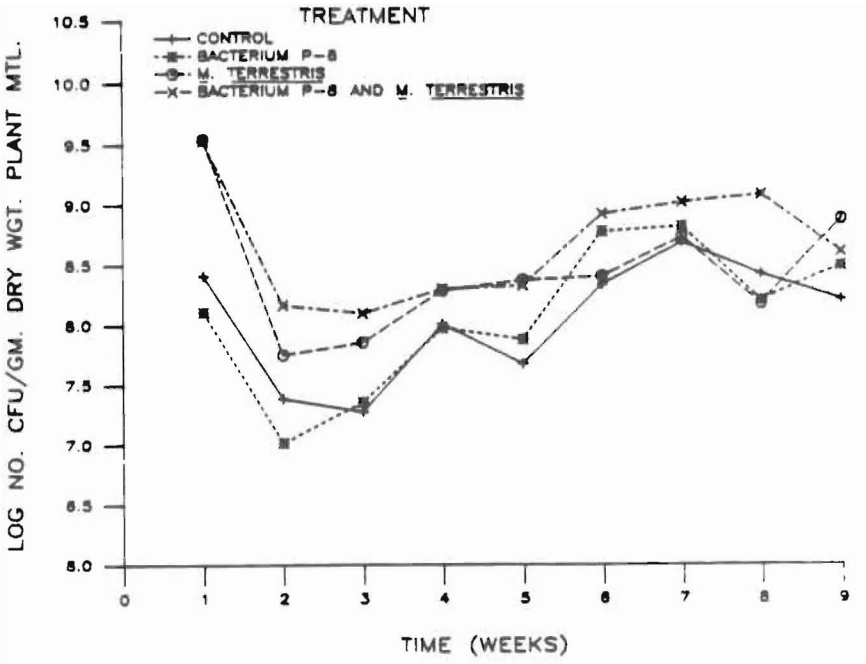

Figure 8. Microbial populations defined by recovery on TSA medium from control (untreated) and treated (inoculated) *M. spicatum* surfaces in pool experiment

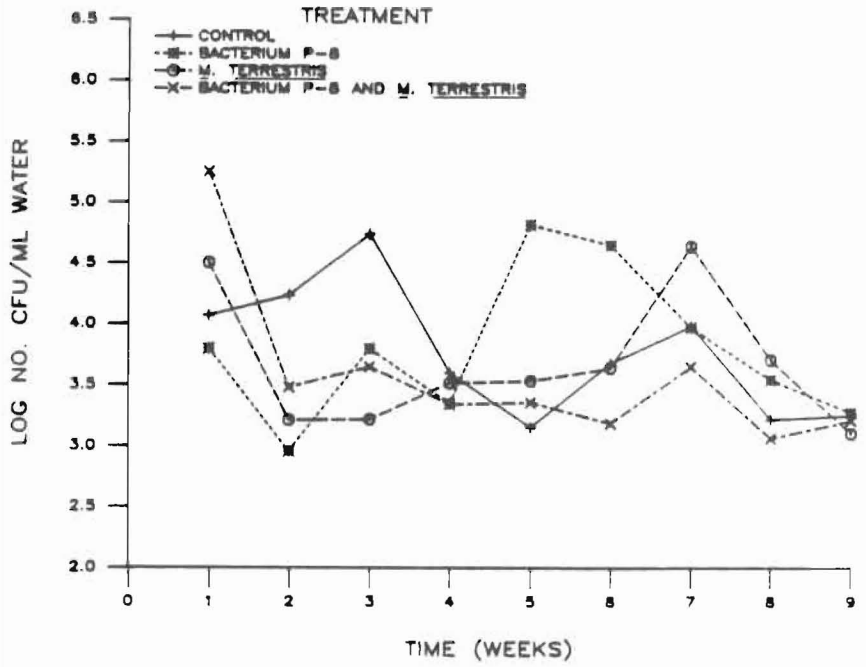

Figure 9. Microbial populations defined by recovery on TSA medium from control (untreated) and treated (inoculated) water profiles in pools containing *M. spicatum* in pool experiment

Table 2

Results of Host Range Specificity Tests of *M. terrestris* on Selected Aquatic and Terrestrial Plants

Common Name	Scientific Name	Visible Symptoms*
Aquatic plants		
Corkscrew eelgrass	*Vallisneria* sp.	-
Duckweed	*Lemna minor*	-
Elodea	*Elodea (Anacharis)*	-
Grass leaf	*Sagittaria* sp.	-
Terrestrial plants		
Green bean	*Phaseolus vulgaris*	-
Pea	*Pisum sativum* L.	-
Vetch	*Vicia* sp.	-
Wheat	*Triticum sativum*	-

* Observations were classified as: "+" (symptoms of infection observed) or "-" (no visible symptoms of infection).

Figure 10. Treated quadrant 2 weeks after second inoculation (the 10th week) showing extensive deterioration of tissue and loss of pigmentation

Figure 11. Uninoculated control 9 weeks after the initiation of field experiment

Table 3

Biomass and Pheopigment Data from Field Experiment

Treatment	Date of Harvest	Mean Biomass/Plot*			Mean Pheopigment**	
		Total	Dead	Live	Chlorophyll	Pheopigment
Control	9 July	322.9	--	--	--	--
Control	15 Oct	92.5	30.0	62.5	2.01	1.18
Treated	15 Oct	91.9	40.9	51.0	2.13	1.16

* Expressed in grams dry weight of plant material.
** Expressed in milligrams per gram dry weight of live tissue.

generally transient (Figures 12 and 13). Increases in numbers reflected the introduction of inoculum and were only briefly sustained. As the plant declined, there was a sustained peak of microbial populations. However, visual evaluation of the plant response to inoculation (Figure 14) showed that observed plant decline was coincident with maximum microbial numbers on the plant surface. It should be noted that the maximum decline followed reinoculation of the plant with fungus and bacterium. Figures 15 and 16, in which are shown the counts of pectinolytic bacteria on plant surfaces and in the water profile, reflect the greater stability of numbers on plant surfaces. Although numbers were significantly greater subsequent to inoculation, they declined to a baseline level not significantly higher than in the controls.

45. Fungal numbers (Figures 17 and 18) remained relatively stable on plant surfaces after inoculation but declined sharply in the water column to almost undetectable levels. However, counts of both fungal and pectinolytic bacterial populations on the plant surface rose sharply after the second inoculation before declining to near control levels.

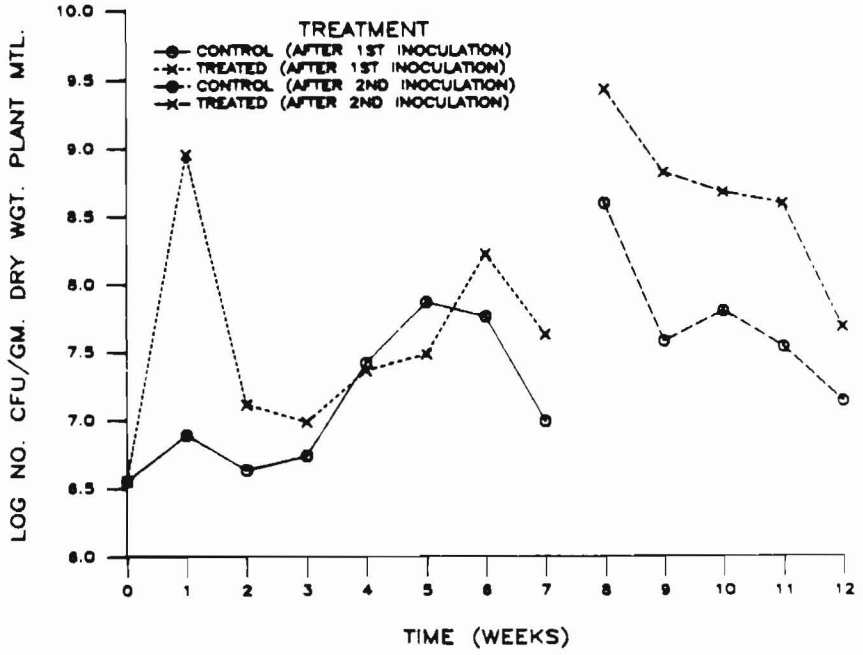

Figure 12. Microbial populations defined by recovery on TSA medium from control (untreated) and treated (inoculated) *M. spicatum* surfaces in field experiment

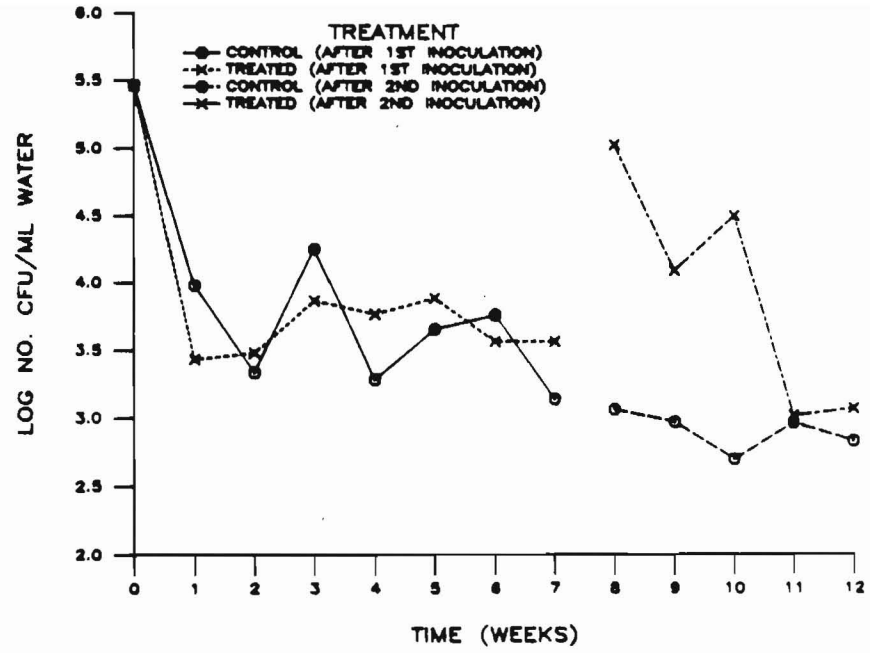

Figure 13. Microbial populations defined by recovery on TSA medium from control (untreated) and treated (inoculated) water profiles in plots containing *M. spicatum* in field experiment

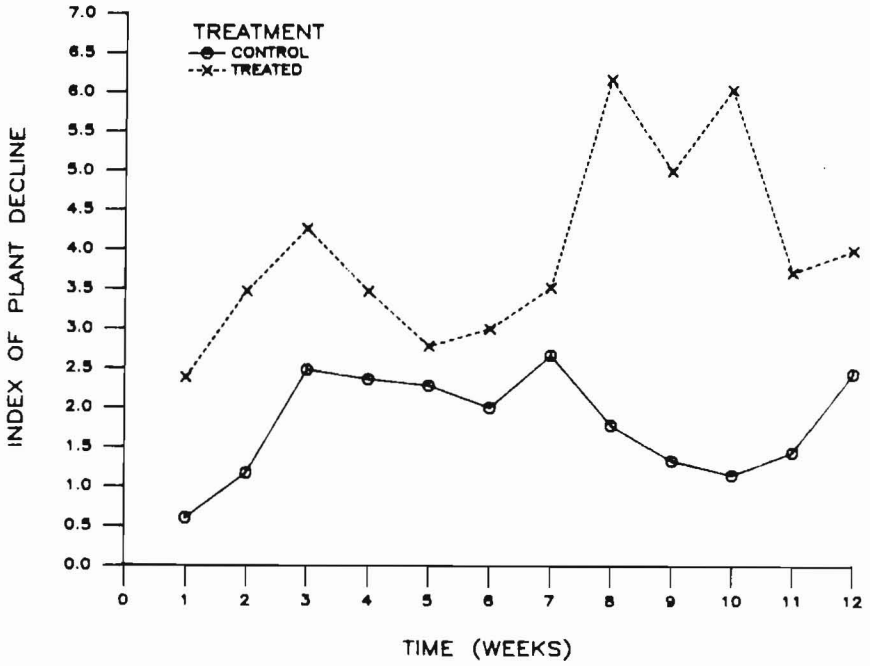

Figure 14. Visual evaluation of condition of *M. spicatum* in field experiment from control (untreated) and treated (with *M. terrestris* and bacterium P-8) plots. Index represents averages of observations from nine replicates

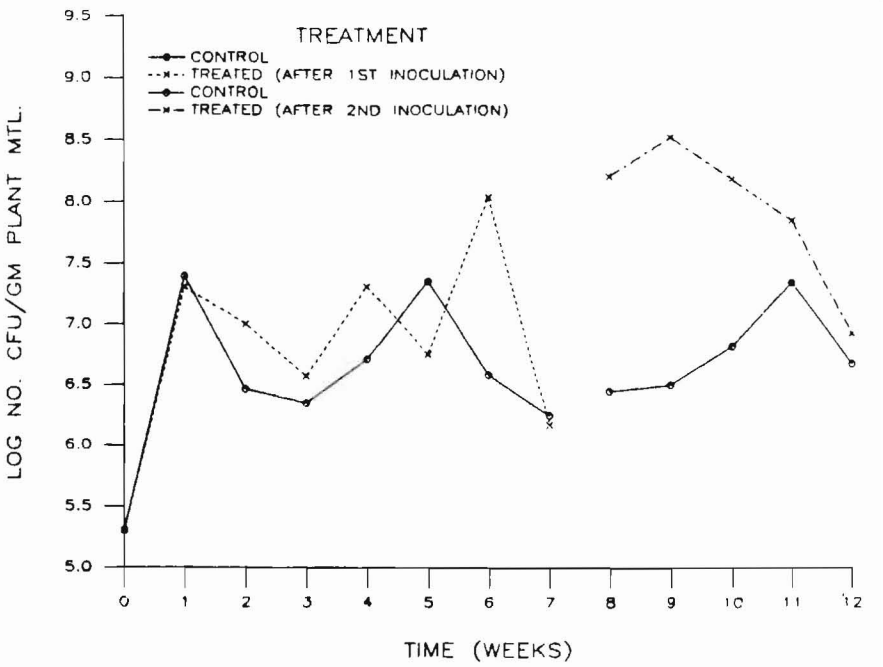

Figure 15. Populations of pectinolytic bacteria defined by recovery on PA medium from control (untreated) and treated (inoculated) *M. spicatum* surfaces in field experiment

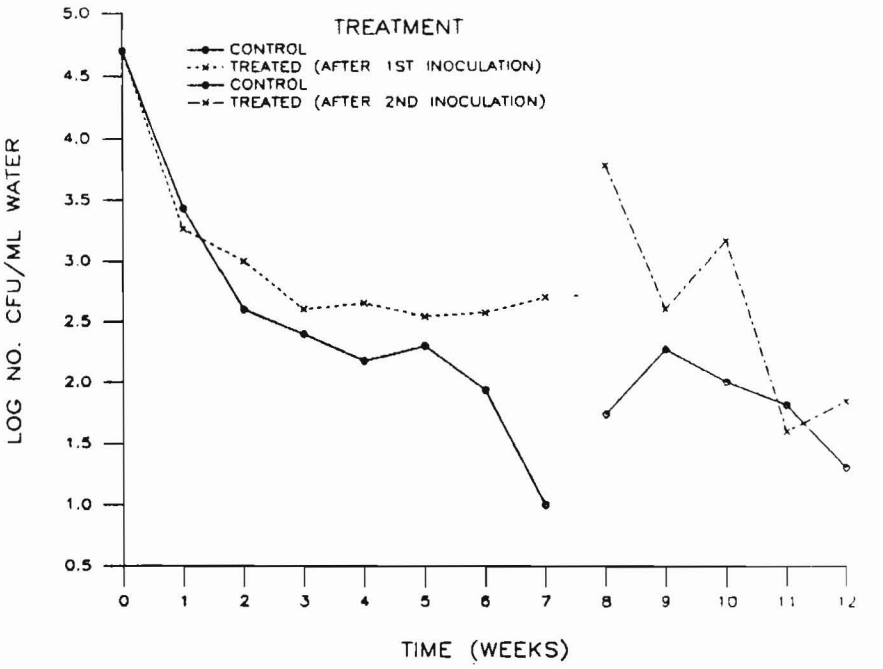

Figure 16. Populations of pectinolytic bacteria defined by recovery on PA medium from control (untreated) and treated (inoculated) water profiles in plots containing *M. spicatum* in field experiment

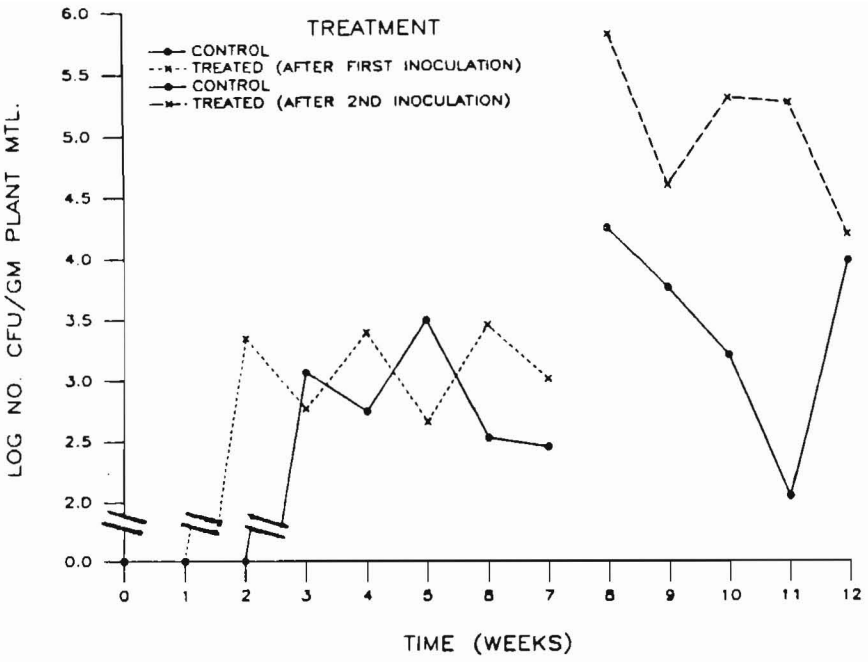

Figure 17. Fungal populations defined by recovery on MA medium from control (untreated) and treated (inoculated) *M. spicatum* surfaces in field experiment

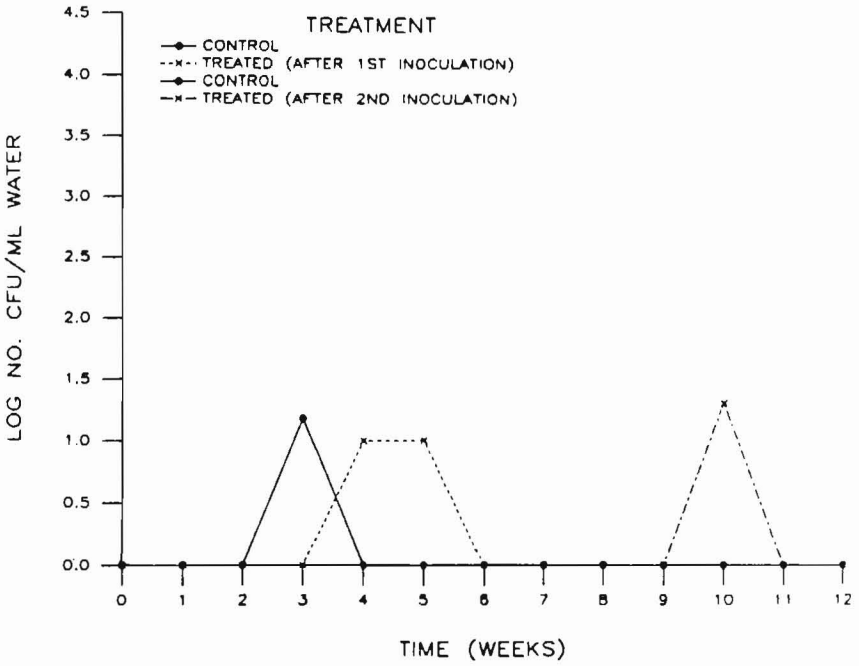

Figure 18. Fungal populations defined by recovery on MA medium from control (untreated) and treated (inoculated) water profiles in plots containing *M. spicatum* in field experiment

Discussion

46. Control of *M. spicatum*, as previously reported (Gunner 1983, 1984), was based on multiple effects achieved by the fungus *M. terrestris* and selected bacterial isolates with strong pectinolytic abilities and the capacity to exercise a hormonelike stress effect on the plants. This bacterial effect was manifested in significant internodal elongation and appeared to predispose the plant to subsequent assault by *M. terrestris*. In our previous studies, the fungus and bacterium were grown, respectively, on a cellulase-inducing medium or a medium induced for pectinase production. Due to the lengthy incubation required for growth and induction of enzymes (14 days for the fungus and 5 days for bacterium), a change was made to media that would maximize cell yield in a minimum time. As a result of the increases in the volume of inoculum required for field studies, fermentation techniques were adopted that involved enriched media with the elimination of the enzyme-induction process. This reduced incubation time to 72 hr for the fungus and 48 hr for the bacterium. To test whether *M. terrestris* and bacterium P-8 would continue to be active against *M. spicatum*, jar and pool-scale tests were repeated with organisms grown under the revised protocol.

47. The difference in inoculum procedures resulted in changes in the pattern of *M. spicatum* decline after exposure to the control agents. Previous plant decline associated with the application of *M. terrestris* (Gunner 1983, 1984) had been marked by the appearance of necrotic areas. In the results reported here, application of cultures grown in the new media caused symptoms of systemic plant decline: browning of plant tissue, collapse of stems, and ultimate disintegration of the plants. It is noteworthy, however, that bacterium P-8 continued to produce a hormonelike response in *M. spicatum*.

48. The most rapid response to the organisms grown under the new protocol was in the laboratory when plant tips were maintained in jars. Although effects identical to previous results were obtained with bacterium P-8 grown under the new regimen, *M. terrestris* with or without the bacterium exercised an enhanced ability to bring about plant decline, where previously the action of the bacterium isolate P-8 had been required. It may be speculated that this enhanced impact is a result of the addition of vitamins and mineral salts to the growth medium. That the most rapid and dramatic results were obtained

in the jar experiments can be attributed to the sustained high concentration of the inoculum and the isolation of the plants from environmental pressures.

49. In the pool experiments, the most significant result obtained was the restriction in biomass after treatment with *M. terrestris* either alone or in combination with bacterium P-8. These data were further validated by the appearance of the treated plants after their removal from the pools: greater tissue decay, separation of stem from root, and scarcity of new tips. In a sense this may be seen as the acceleration of the cycle of growth and decline. From an ecosystems point of view, this weakening of *M. spicatum* could be seen as a portal for the reentry of competing populations in a natural setting.

50. In the pool setting, the application of the bacterium alone again resulted in increased internodal length. There was also a slight delay in the growth cycle, as evidenced by the appearance of the plant and the number of growing tips. However, there was no significant difference in biomass between untreated controls and bacteria-treated pools. Thus, bacterial action must be considered capable of enhancing fungal action, but not of exercising a lethal effect by itself. This is supported by the biomass data in which joint fungal-bacterial treatment resulted in the greatest decline in biomass. However, the limitations of the pool environment in replicating the natural setting must not be overlooked. The shallow depth of the pool makes more drastic the effects of temperature change and light penetration (Barko, Adams, and Clesceri 1986). In particular, the intrusion of epiphytic algae obscured visual evaluation of the plants in situ, and a more accurate representation of their condition was obtained when plants were removed from the pool and observed individually.

51. The dynamics of fungal and bacterial population numbers in both the pool and field settings show a consistent relationship between communities in the water column and those associated with the plant. Peak populations observed subsequent to inoculation on the plant are followed by similar rises in the water profile. Most important is that these inocula do not become dominant members of the microbial ecosystem, whose number remains relatively constant. The perturbation in the numbers of pectinolytic organisms could reflect the interaction of these organisms with environmental factors such as the availability of substrate, the release of inhibitory and stimulatory factors from the plant, and predatory pressures.

52. In the field setting, a clearly sustained rise in the microbial populations subsequent to the second inoculation was observed. This rise suggests that the plants, by releasing additional substrates or by having available more ecosites, generated enhanced colonization opportunities. This may reflect the effects of the first inoculation as well as seasonal changes in plant condition.

53. Significantly, peak levels of microbial populations (total, fungal, and pectinolytic) coincide with an increase in the index of plant decline obtained by visual evaluation of the plants in situ. One may speculate that the coincidence of increased numbers and the visual evidence of plant decline encompasses a number of events, including:

a. The pectinolytic organisms may be seen as primary decomposers, releasing nutrients by attacking plant components. They may also provide additional ecosites for microbial populations.

b. Bacterial attachment generates plant stress, which serves as a predisposing element for successful fungal assault on the plant.

54. At the termination of the field studies, data on the total chlorophyll content and biomass of plant material from the harvest of all plots did not show significant differences between treated and control plots. In the case of the chlorophyll measurement, the technique, which is adapted from one used to measure chlorophyll in newly emerged shoots,* may be overly sensitive to interference from epiphytic algal populations on mature stems and leaves.

55. That biomass figures do not reflect changes in treated populations can also be attributed to the difficulties in sequestering decayed plant material within the designated quadrant areas and preventing the intrusion of new growth from outside the treated area during the course of the experiment.

56. In general, there was a drop of 71.4 percent in biomass in all the quadrants (treated and control) over the course of the experiment which reflected seasonal decline. This seasonal change, from dense early summer growth to the sparser stands of early autumn, may also have served to mask biomass differences resulting from treatment with the microbial agents.

57. Worthy of note was the shortening of the elapsed period between the appearance of effects and the second inoculation. It could be conjectured

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* Personal Communication, 1986, D. Scott Painter, National Water Research Institute, Environmental Canada, Burlington, Ontario, Canada.

that the plant response is more immediate because of its own incipient decline or that the initial inoculation had enhanced plant susceptibility. In any event, this would suggest that expanded application procedures should take advantage of the cumulative effect of serial applications as well as critical aspects of the *M. spicatum* growth cycle which render it more vulnerable.

58. In preliminary specificity testing, *M. terrestris* was active only against *M. spicatum* from which it had been originally isolated. In trials conducted with a number of commonly found aquatic plants and economically significant terrestrial plants, no evidence of infection was observed. More extensive tests of specificity of infection are being conducted.

PART IV: CONCLUSIONS AND RECOMMENDATIONS

59. Extension of our studies through jar, pool, and field studies has provided a consistent demonstration that an explicit decline of *M. spicatum* could be achieved by the application of microorganisms originally isolated from the plant and selected for their ability to degrade specific plant tissues. In the course of these experiments, unequivocal results were obtained that show:

a. Where sustained contact between microbial inoculum and plant was achieved, plant kill followed.

b. Symptoms of kill were systemic; leaves and stem tissue appeared bleached and chlorotic.

c. Bleached tissue subsequently decomposed and disintegrated.

60. Thus, it appears that the application of *M. terrestris* and bacterium P-8 clearly affected the plant and can bring about its decline. To proceed to the use of these organisms in a practical control strategy requires resolution of the following:

a. Elucidation of the mechanisms of pathogenesis. To enzyme and hormonelike effects, systemic effects appear to have been added; regimens for successful field application remain contingent on the detailed understanding of these multiple actions.

b. Elaboration of an appropriate field test methodology. To provide an unequivocal measurement of plant status after treatment in light of the influence of growth conditions, the plant physiological status and water quality parameters must be clearly defined as components of the test environment.

c. Development of an effective field applications and delivery methodology. To provide an effective basis for the successful implementation of the ecosystem approach to the control of *M. spicatum*, applications and delivery methods must be refined and the appropriate application equipment developed.

d. Establishment of host range. Comprehensive specificity trials must be conducted to test the cultivar of *M. terrestris* used in these studies for pathogenicity to all plants reported as susceptible to this fungus.

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APPENDIX A: MEDIA FORMULATIONS

1. Potato dextrose salts broth

Ingredients	Amount/liter of Medium
Potato starch	10.0 g
Dextrose	10.0 g
Yeast extract	2.0 g
Mineral salt cofactors	
$MgSO_4 \cdot 7H_2O$	200.0 mg
$CaCl_2 \cdot 2H_2O$	5.0 mg
$CoCl_2 \cdot 6H_2O$	6.0 mg
$ZnSO_4 \cdot 2H_2O$	5.0 mg
Na_2MoO_4	1.5 mg
$FeCl_2 \cdot 6H_2O$	1.7 mg
$CuSO_4 \cdot 5H_2O$	0.4 mg
$MnCl_2 \cdot 4H_2O$	3.7 mg
H_3BO_3	5.6 mg

2. Trypticase soy broth

Ingredients	Amount/liter of Medium
Trypticase peptone	17.0 g
Phytone	3.0 g
NaCl	5.0 g
K_2HPO_4	2.5 g
Dextrose	2.5 g

3. Pectin agar

Ingredients	Amount/liter of Medium
1.0 N NaOH	9.0 ml
10% $CaCl_2 \cdot 2H_2O$	6.0 ml
Yeast extract	5.0 g
Casamino acid	1.0 g
Agar	30.0 g
Sodium polypectate	20.0 g