US Army Corps of Engineers

ENVIRONMENTAL LABORATORY

AQUATIC PLANT CONTROL RESEARCH PROGRAM

TECHNICAL REPORT A-90-3

HOST SPECIFICITY OF MICROBIAL FLORA FROM EURASIAN WATERMILFOIL

by

John H. Andrews, Robin F. Harris
Craig S. Smith, Tara Chand

College of Agricultural and Life Sciences
University of Wisconsin-Madison
Madison, Wisconsin 53706

June 1990
Final Report

Prepared for DEPARTMENT OF THE ARMY
US Army Corps of Engineers
Washington, DC 20314-1000
Under APCRP Work Unit 32388
Monitored by Environmental Laboratory
US Army Engineer Waterways Experiment Station
3909 Halls Ferry Road, Vicksburg, Mississippi 39180-6199

Destroy this report when no longer needed. Do not return it to the originator.

The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorized documents.

The contents of this report are not to be used for advertising, publication, or promotional purposes. Citation of trade names does not constitute an official endorsement or approval of the use of such commercial products.

Unclassified
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-90-3
6a. NAME OF PERFORMING ORGANIZATION (6b. OFFICE SYMBOL (If applicable)) See reverse.	7a. NAME OF MONITORING ORGANIZATION USAEWES Environmental Laboratory
6c. ADDRESS (City, State, and ZIP Code) See reverse.	7b. ADDRESS (City, State, and ZIP Code) 3909 Halls Ferry Road Vicksburg, MS 39180-6199
8a. NAME OF FUNDING/SPONSORING ORGANIZATION US Army Corps of Engineers (8b. OFFICE SYMBOL (If applicable))	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER
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 32388

11. TITLE (Include Security Classification)
Host Specificity of Microbial Flora from Eurasian Watermilfoil

12. PERSONAL AUTHOR(S)
Andrews, John H.; Harris, Robin F.; Smith, Craig S.; Chand, Tara

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Final report	FROM _____ TO _____	June 1990	35

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

17. COSATI CODES		
FIELD	GROUP	SUB-GROUP

18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
Aquatic plants
Eurasian watermilfoil
Fungi
Host specificity
Microflora
Myriophyllum spicatum

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

A laboratory assay was developed to evaluate microbial colonization of Eurasian watermilfoil (*Myriophyllum spicatum*). The assay consisted of a 24-hr association phase of plants with inoculum, followed by two microbial growth cycles of 3 days each. The system was used to evaluate the colonization potential of the fungi *Colletotrichum gloeosporioides*, *Acremonium curvulum*, *Cladosporium herbarum*, *Aureobasidium pullulans*, *Paecilomyces* sp., and an unidentified sterile, septate fungus.

The fungi differed in the extent to which they attached to watermilfoil and in their ability to grow in association with it. There were relatively few significant differences among the tightly attached fungal populations after 24 hr, but growth of the better colonizers led to a greater number of significant differences after 4 and 7 days.

(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

6. NAME AND ADDRESS OF PERFORMING ORGANIZATION (Continued).

University of Wisconsin-Madison
College of Agricultural and Life Sciences
Madison, WI 53706

19. ABSTRACT (Continued).

The milfoil pathogen *C. gloeosporioides*, and *A. curvulum*, a fungus commonly found as an epiphyte on watermilfoil, were the two best colonizers. In host range tests, they were found to be nonspecific to milfoil. *Aureobasidium pullulans* was the only fungus that consistently failed to establish an increasing population on milfoil.

PREFACE

The research reported herein was sponsored by the Headquarters, US Army Corps of Engineers (HQUSACE), through the Aquatic Plant Control Research Program (APCRP), Work Unit 32388, and was conducted under Contract No. DACW39-86-K-002 to Dr. John H. Andrews of the University of Wisconsin, Madison, WI. Technical Monitor for HQUSACE was Mr. James W. Wolcott.

The APCRP is managed by the US Army Engineer Waterways Experiment Station (WES), under the Environmental Resources Research and Assistance Programs, Mr. J. Lewis Decell, Manager.

The study was conducted under the direct supervision of Dr. Alfred F. Cofrancesco and under the general supervision of Mr. Edwin A. Theriot, Chief, Aquatic Habitat Group; Dr. Conrad J. Kirby, Chief, Environmental Resources Division; and Dr. John Harrison, Chief, Environmental Laboratory, WES.

This report was prepared by Drs. John H. Andrews, Robin F. Harris, Craig S. Smith, and Tara Chand of the College of Agricultural and Life Sciences, University of Wisconsin-Madison. Assistance in the laboratory and in the field was provided by Mr. Simon Slade, Ms. Susan Adams, Mr. Erik V. Nordheim, Ms. Ann Buenzli, and Mr. Russell Spear of the University of Wisconsin-Madison. The report was edited by Ms. Jessica S. Ruff of the WES Information Technology Laboratory.

Commander and Director of WES was COL Larry B. Fulton, EN. Technical Director was Dr. Robert W. Whalin.

This report should be cited as follows:

Andrews, John H., Harris, Robin F., Smith, Craig S., and Chand, Tara. 1990. "Host Specificity of Microbial Flora from Eurasian Watermilfoil," Technical Report A-90-3, US Army Engineer Waterways Experiment Station, Vicksburg, MS.

CONTENTS

	Page
PREFACE	1
PART I: INTRODUCTION	3
Background	3
Sampling Procedures	4
The Assay System	5
PART II: ANALYTICAL RESULTS	13
Epiphytic Colonization	13
Endophytic Colonization	14
Host Specificity	15
PART III: CONCLUSIONS AND INTERPRETATION OF FINDINGS	16
REFERENCES	19
TABLES 1-6	
FIGURES 1-6	

HOST SPECIFICITY OF MICROBIAL FLORA FROM EURASIAN WATERMILFOIL

PART I: INTRODUCTION

Background

1. Eurasian watermilfoil (*Myriophyllum spicatum* L.), a submersed aquatic angiosperm, is an aggressive invader that is currently causing severe problems in many regions of the United States due to its high reproductive capacity and extraordinary competitive ability. The main problems that arise from disruption of the aquatic plant community are impeded water flow, interference with navigation or recreation, clogging of water intake structures, and the deleterious effects of decaying plant parts on water quality. Conventional control methods, including mechanical and chemical approaches, have been expensive and only partially successful. An alternative is to develop microbiological control strategies.

2. If indigenous or engineered microorganisms are to be used to control milfoil, there must be reasonable assurance that they do not pose a hazard to other plants. The objective of this study was to develop a protocol to screen for fungi or bacteria specific to milfoil. Specificity denotes the ability to grow exclusively on or in milfoil, that is, to colonize the plant. To the extent that the research required microorganisms for testing, some fieldwork was necessary to isolate prospective microbes. However, the emphasis was on developing a screening mechanism that would discriminate colonists from noncolonists, rather than searching for a microbe with specific features such as selective colonizing ability.

3. Study hypotheses were that microbes isolated from milfoil (or elsewhere) segregate by growth potential into colonists and noncolonists and that, when tested on a plant host range, milfoil colonists would be either selective or nonselective for milfoil. The premise for the research, which remains to be verified, is that membership of the above categories remains constant between laboratory and field trials, i.e., the assay is valid and has predictive value for events in a natural ecosystem.

4. At seven dates in 1986 and 1987, *M. spicatum* growing at various locations in Lake Wingra, Wisconsin (Table 1), was sampled for associated microbial populations. Likewise, on three and two occasions, respectively, samples were taken from coontail (*Ceratophyllum demersum*) and wild celery (*Vallisneria americana*) (Table 1).

5. In brief, the sampling protocol was as follows. Only healthy tissue was used. Each milfoil sample consisted of two 10-cm pieces from terminal 30-cm shoots. Samples approximately equivalent in wet weight to those from milfoil were taken from coontail (lateral or terminal segments) and from wild celery (whole mature leaves).

6. After overnight rinsing in flowing cold tap water, duplicate samples were transferred to 8-oz (0.2-dm^3) jars containing 100 ml of sterile water plus 0.01 percent Tween-80 and were then washed by shaking at 150 rpm for 10 min at about 25° C. Each pair of jars was then split into a nonsurface sterilized (NS) or surface sterilized (SS) regimen. Plant portions from the former were washed manually by swirling (four times, 1 min each) in 150 ml of sterile distilled water. Those from the latter were sterilized for 5 min in 100 ml of 15-percent hydrogen peroxide (H_2O_2) and rinsed in sterile water as above. The NS and SS samples were chopped aseptically, suspended in 25 to 30 ml of cold sterile buffer Tween (SBT) (0.005 percent Tween-80 in 0.005 M phosphate buffer, pH 7.0), and either ground for 10 min at 6,000 rpm with an Omni-Mixer (Model 17105, OCI Instruments, Waterbury, CT) or macerated in 20 to 25 ml of cold SBT using a 40-ml glass tissue homogenizer. No differences were recorded in the viable fungal or bacterial colony forming units (CFU) resulting from the two homogenization procedures.

7. Fungal populations were estimated by dilution plating homogenates on potato glucose agar (Tuite 1969) amended with 250 mg/ℓ chloramphenicol (PGA/ch). Plates were incubated at 25° ± 3° C and examined periodically for 10 to 15 days. Following enumeration of all colonies, subcultures were made from visually distinct colonies and identified by standard taxonomic criteria (e.g., Ellis 1971, Sutton 1980). The predominant fungi isolated are summarized in Table 2.

8. Bacterial populations were estimated from a dilution series in SBT of the homogenates planted on dilute nutrient broth agar (DNB) (0.8 g nutrient broth, 16 g Bacto agar per liter), by conventional dilution plating methods

(SS plants) or by the plate-dilution frequency technique (Harris and Sommers 1968) (NS plants). Plates were incubated at 25° ± 3° C and examined periodically for 21 days. Following enumeration of the total CFU, colonies were categorized visually (Smibert and Krieg 1981). One or more representatives from each set of colony types was streaked onto casein-peptone-starch (CPS) medium (Jones 1970), purified if necessary, and stored on CPS slants at 5° C. The isolates were assayed as follows: gram stain by the Burke method (Doetsch 1981), motility (Drake and Montie 1988), oxidase production (Kovacs 1956), oxidation/fermentation of glucose (Hugh and Leifson 1953), and endospore staining by the Schaeffer-Fulton method (Doetsch 1981). According to the results of these biochemical tests, the bacterial isolates were placed into groups, using a simplified scheme of Ramsay (1977) (see Figure 1). These results are summarized in Table 3.

The Assay System

Plant cultures

9. *Myriophyllum spicatum* plants were derived from the strain originally cultured by Gerloff (1975). Cultures of plant stem segments were maintained in a dilute mineral salts medium, which was derived from a modified Gerloff's medium (Andrews 1980), by adding sodium bicarbonate and potassium bicarbonate to produce a bicarbonate concentration of 4.4 mM/ℓ and by increasing the concentrations of calcium and magnesium to 1.2 and 1.0 mM/ℓ, respectively. Stock and experimental cultures were grown at approximately 25° C and were illuminated by fluorescent lamps, which provided 300 to 400 $\mu E/m^2/sec^2$ for 15 hr/day. The plant cultures originally contained a fungus, *Acremonium curvulum*, which was eliminated by exposing plants to thiabendazole (Andrews, Hecht, and Bashirian 1982). Although free of fungi, cultured plants supported large bacterial populations. Bacteria associated with plants used for colonization experiments were monitored routinely by plating plant macerates on DNB agar. The total bacterial population was found to range from 2 million to 60 million CFU/cm of plant.

10. Plants were transferred to fresh culture medium 2 to 3 weeks prior to the start of each colonization experiment to ensure that they were healthy and actively growing. Colonization experiments were conducted with unrooted, terminal stem segments that were cut to a uniform 4.0-cm length at the

beginning of each experiment. During 7-day experiments, the plants typically increased in length by approximately 1 cm.

11. *Myriophyllum farwellii*, *Elodea canadensis*, and *Potamogeton pectinatus*, isolated from Wisconsin lakes, were included in the host specificity portion of the assay. The latter two species were grown as described above for *M. spicatum*. Since *M. farwellii* does not grow well in carbonate-buffered media, it was cultured in Gerloff's medium without added bicarbonate. Otherwise, the environmental conditions were the same as for M. spicatum.

Microorganisms

12. Six fungi were used to test the assay protocol because, of the microbes isolated, they were feasible to track on the nonaxenic plants and they represented organisms likely to differ in the degree and nature of their interaction with *M. spicatum*. *Colletotrichum gloeosporioides* (Penz). Sacc. is a weakly virulent pathogen of *M. spicatum*. It was isolated from blackened stem lesions on *M. spicatum* growing in a small pond within the University of Wisconsin Arboretum in Madison (Smith et al. 1988). *Acremonium curvulum* W. Gams is a frequent associate of *M. spicatum* (Andrews, Hecht, and Bashirian 1982) and was isolated from the plant culture prior to treating it with thiabendazole (see paragraph 9). *Cladosporium herbarum* (Pers.) Link, *Aureobasidium pullulans* (DeBary) Arnaud (a yeastlike fungus), *Paecilomyces* sp., and an unidentified sterile, septate fungus were isolated from healthy *M. spicatum* plants collected from Lake Wingra, Wisconsin, as described earlier. Experimental cultures were initiated from storage cultures of *A. curvulum* and *C. gloeosporioides* maintained on silica gel crystals (Perkins 1962). The other species of fungi were isolated just prior to the start of these experiments. Between experiments, all of the fungi were grown on PGA/ch.

13. Inoculum for colonization experiments consisted of a suspension of freshly harvested conidia in sterile distilled water for all fungi except *Aureobasidium* and the sterile fungus. *Colletotrichum* and *Acremonium* conidia were produced by growing the fungi for 4 days in a 20-percent liquid V8 juice medium. *Cladosporium*, *Aureobasidium*, and *Paecilomyces* were grown on PGA/ch. *Cladosporium* and *Paecilomyces* were grown for 10 to 12 days, whereas *Aureobasidium* was grown for 3 to 4 days. Spores were harvested by washing them from the agar surface with SBT. Mycelia were removed by pouring cultures through a double layer of sterile cheesecloth. Propagule suspensions of all but the sterile fungus were counted using a hemacytometer, and their density was adjusted with distilled water to produce a final concentration of

approximately 10^3 viable propagules per milliliter. An inoculum density of 10^5/ml also was used for colonization experiments with *C. gloeosporioides*. Inoculum of the sterile fungus was a suspension of mycelial fragments prepared by macerating (in a glass tissue homogenizer) disks cut from just behind the advancing edge of a 4- to 5-day-old colony growing on PGA/ch.

14. In a preliminary experiment, it was found that homogenizing five 8-mm disks from a colony of this age in 25 ml of SBT yielded approximately 5×10^4 CFU/ml. This relationship was used to estimate the amount of homogenate necessary to produce an inoculum containing approximately 10^3 viable CFU/ml. In all cases, the actual density of viable propagules in the inoculum was checked by dilution plating samples on PGA/ch. Viable counts were generally 85 to 95 percent of the microscopic counts for *Colletotrichum*, *Acremonium*, and *Paecilomyces*, and 60 to 70 percent for *Cladosporium*.

15. Viable populations of fungi were assayed by spreading 0.1 to 0.5 ml of propagule suspension, plant growth medium, or plant macerate onto triplicate PGA/ch plates and counting the number of colonies that appeared after incubation for 3 to 5 days at room temperature. When necessary, samples were serially diluted with SBT, and plates with 10 to 100 colonies were selected for counting.

Fungal colonization of plants

16. Fungal colonization was assessed by inoculating plants with one of the test fungi, followed by one or more cycles of washing the plants and then incubating them in fresh plant growth medium. The initial series of experiments lasted 7 days and consisted of three phases: a 1-day association phase and two 3-day growth phases. Between phases and at the end of the experiment, the plants were washed and the fungal population in the culture was partitioned into the following somewhat arbitrarily defined components: those in the medium (not attached), those removed by washing (loosely attached), those remaining on the plant after washing (tightly attached), and those surviving surface sterilization (endophytic). Figure 2 describes the assay protocol for a single replicate of a colonization experiment.

17. In the first phase (the association phase), nine shoot segments were immersed in 300 ml of inoculum suspension (described above) for 24 hr to allow fungi to attach to the plants and become established. At the end of the association phase, samples of the inoculum suspension were dilution plated to determine the number of viable fungal CFU remaining in the medium (i.e., not attached to the plants).

18. One plant was removed, and its endophytic fungal population was estimated by surface sterilizing the intact plant for 5 min in 15-percent H_2O_2, macerating the plant, and dilution plating portions of the macerate. Each 4- to 6-cm plant was macerated by chopping it coarsely with a sterile scalpel, suspending the pieces in 50 ml cold SBT, and grinding in a chilled blender cup for 10 min at 6,000 rpm with an Omni-Mixer.

19. The eight remaining plants were washed to remove loosely attached fungal CFU by shaking the plants in 150 ml SBT for 10 min on a rotary shaker at 200 rpm, followed by rinsing briefly in three changes of sterile distilled water. The first three washes were combined, and fungal propagules in the pooled washes and in the fourth wash solution were enumerated by dilution plating.

20. The amount of fungal carryover into the next experimental phase in the surface film was estimated by assuming, based on measurements of the weight of water blotted from plants, that 0.7 ml of the fourth wash was transferred with each plant. After washing, two plants were removed for sampling. One was preserved in formalin-acetic acid-alcohol (FAA) for microscopic examination; the other was macerated, and the macerate plated as described above to determine the number of tightly attached epiphytic CFU. Measurements of tightly attached fungal populations were corrected by subtracting the estimated carryover and any endophytic CFU from the number of CFU in the macerate.

21. The six plants remaining at the end of the association phase were transferred to the second phase, the first growth phase (days 2-4), in which plants were placed in aerated culture jars containing 250 ml of plant growth medium and were incubated for 3 days under the standard conditions described above. At the end of the growth period, plants were washed and sampled; the medium was sampled exactly as at the end of the association phase. The three remaining plants were then transferred to the final phase, the second growth phase (days 5-7), and monitored exactly as in the first. A fresh culture container and growth medium were used.

22. When an endophytic fungal population was detected by the plating assay, FAA-preserved plants were examined for internal hyphae with a light microscope. Plants colonized by *Acremonium* were also examined, since this species has been reported to grow endophytically within *M. spicatum* (Andrews, Hecht, and Bashirian 1982). Prior to examination, plant pieces were cleared in a boiling, saturated aqueous solution of chloral hydrate and stained overnight with a mixture of aniline blue and trypan blue (0.025 percent each).

Stained specimens were rinsed briefly in distilled water, placed into saturated chloral hydrate at room temperature for 1 to 2 hr, and then mounted in lactophenol for observation.

23. Based on the results of the 7-day colonization experiments, a good colonist, *Colletotrichum*, and a poor one, the unidentified sterile fungus, were selected for a 28-day experiment to investigate the influence on colonization of a longer growth period. In this experiment, plants were washed after exposure to the initial inoculum but were not washed between growth periods. Washing was omitted because the previous results demonstrated that tightly and loosely attached components of the fungal populations were highly correlated; thus, the effort involved in separating them was not yielding useful information. Four replicate cultures of eight plants each were exposed first to an inoculum of 10^3 CFU/ml of the two fungi for 24 hr, washed, and transferred to plant growth medium as in the experiments described above. A single plant from each culture was then removed and used to determine the initial plant-associated fungal population using the standard maceration and plating techniques. Inoculation was followed by four growth periods, one lasting 6 days and three lasting 7 days each. At the end of each growth period, the plants were transferred (without washing) to fresh growth medium. At the end of the first, second, and fourth growth periods, two plants were removed and the endophytic and plant-associated components of the population were measured as described above.

Experimental design and analysis

24. To evaluate aspects of variability in colonization, 7-day colonization experiments were conducted and analyzed in two parts. First, colonization by each fungus was assessed in duplicate plant cultures. One or two fungi were examined concurrently, using plants taken from a single group of replicate stock cultures. The results from this series of experiments were used to quantify the amount of culture-to-culture variability in colonization so that the minimum detectable change in population size could be estimated. To quantitatively compare fungal colonization, all six fungi were then evaluated simultaneously on plants from the same stock culture, with one replicate per fungus. This comparison was conducted three times, each with newly cultured plants and freshly produced fungal inoculum.

25. The tightly attached component of fungal populations was emphasized in the analysis because it is the component most likely to interact with the host. In the initial colonization experiments used to determine the minimum

detectable change in population size, the percent standard deviation of tightly attached fungal populations from replicate plant cultures was always 31 percent or less (average, 14 percent). At least significant difference calculation indicated that fungal populations at two times would have to differ by approximately a factor of 2 in order for the difference to be detectable at $P = 0.05$. All other analyses of 7-day experiments considered only the results from the three experiments in which all six fungi were assessed simultaneously.

26. The amount of growth or decline in fungal numbers was calculated for each fungus in each colonization experiment by dividing the 7-day tightly attached fungal population by that at 1 day. Cultures in which the 7-day tightly attached fungal population was 0.5 to 2.0 times that at 1 day were considered to exhibit no change, while increases greater than 2.0 times and decreases to less than 0.5 times the 1-day value were considered to be detectable increases and decreases, respectively.

27. Sizes of the tightly attached populations of fungal species, measured at the end of each phase of the 7-day experiments, were compared using analysis of variance (ANOVA). Data from the three experiments were first logarithmically transformed to stabilize the variance, and a two-way ANOVA (with fungi and experiments as factors) was performed. Logarithmic transformation did not produce a homogeneous variance when results from the *Colletotrichum* 10^5 CFU/ml initial inoculum treatment were included, so this treatment was excluded from the analysis. The fungus × experiment interaction was used as an error term after a graphical analysis revealed that a pronounced interaction was not evident. Mean fungal population sizes were then compared using Duncan's Multiple Range Test at $P = 0.05$.

Host specificity

28. The host specificity of the two best colonizers of milfoil, *C. gloeosporioides* and *A. curvulum*, was evaluated using a simplified colonization assay and four potential host species, *M. spicatum*, *M. farwellii*, *E. canadensis*, and *P. pectinatus*. *Myriophyllum farwellii* was chosen because it is a species closely related to *M. spicatum*; *E. canadensis* and *P. pectinatus* were included because they frequently occur with milfoil. Experimental plants of *M. spicatum*, *M. farwellii*, and *E. canadensis* were 4.0-cm terminal shoot segments. Entire plants of *P. pectinatus* approximately 25 cm in length (rhizome to leaf tips) were used for experimental cultures. Cultures of the plants were grown as described above.

29. Colonization was measured using a simplified version of the colonization assay described above, in which each replicate experimental plant culture initially contained four plants. The simplified colonization assay for a single replicate used to examine host specificity is illustrated in Figure 3. Plants were cut to 4 cm, washed with sterile, distilled water, and placed in 200 ml of growth medium in a 250-ml jar equipped with an aerator and a vent. At the beginning of the assay, sufficient spore suspension was added to the culture to produce 10^3 viable spores/milliliter, and the culture contents were mixed by swirling. Plants were exposed to the inoculum for 24 hr, during which time cultures were kept under standard growth conditions and aerated by bubbling sterile air through them at about 100 cc/min. After 24 hr, plants were washed by shaking them four times for 5 min each at 200 rpm on a rotary shaker, each time in 100 ml of fresh SBT.

30. After washing, one plant was removed, and the population of fungi on it (the "initial plant-associated" population) was measured by maceration and plating as described above. The remaining three plants were placed in 200 ml of fresh growth medium and incubated under standard conditions, with aeration, for 6 days. At the end of the experiment, one plant was fixed in FAA for microscopy; the plant-associated fungal population of the second and the endophytic fungal population of the third were determined. The washing prior to maceration that had been used to separate tightly and loosely attached epiphytic CFU in early colonization experiments was omitted because the two fractions were highly correlated and separating them did not yield additional useful information.

31. The host specificity of *A. curvulum* was evaluated in three experiments using the above assay: an initial trial with one culture per plant species, and two trials in which each plant species was evaluated in duplicate. *Colletotrichum gloeosporioides* was evaluated twice, each time with duplicate cultures of all plant species. The relative change in the plant-associated fungal population in a single replicate plant culture (i.e., the 7-day plant-associated CFU divided by the 1-day plant-associated CFU) was used as a measure of fungal colonization. For each fungus, colonization measurements for the four plant species were analyzed using one-way ANOVA. Mean colonization values for the plant species were compared using Duncan's Multiple Range Test at $P = 0.05$.

PART II: ANALYTICAL RESULTS

Epiphytic Colonization

32. The dynamics of tightly attached fungal populations during the 7-day assay varied considerably among the fungi (Table 4). *Colletotrichum* and *Acremonium* were the best colonizers; their populations consistently increased during each experiment. The other fungi were noticeably poorer colonizers and, except for *Cladosporium*, exhibited less consistent population dynamics. Ranked in order of decreasing colonization, they were *Paecilomyces* sp. and the sterile fungus, followed by *Cladosporium* and *Aureobasidium*. *Aureobasidium* was usually a noncolonist, as it declined during two of three experiments. Inoculation with 10^5 propagules of *Colletotrichum* per milliliter led to larger tightly attached populations of the fungus, but the larger populations did not increase as consistently or as rapidly as those resulting from inoculation with 10^3/ml.

33. Few differences among the fungi were noted after 1 day, but growth of the better colonizers led to an increased number of differences after 4 and 7 days (Table 5). At the end of the 1-day association phase, there were two distinguishable fungal groups: a high one containing *Colletotrichum*, *Acremonium*, and the sterile fungus, and a low one containing the other three fungi. Differences between tightly attached fungal populations increased by the end of the first growth phase. The ordering of fungi did not change over time, but there was a greater number of significant differences between fungi than at the end of the association period. The 7-day rankings were very similar to those at 4 days, except that *Paecilomyces*, which had only a small tightly attached population initially, had grown enough by day 7 to replace *Cladosporium*, which had begun with a higher population density but did not increase during the experiment. Inoculation with 10^5 CFU/ml of *Colletotrichum* led to a tightly attached population that was much higher at all times than that produced by any other treatment.

34. Nonattached and loosely attached fungal populations were removed between experimental phases of the 7-day assay by transfer and washing, but these populations rebounded during the growth periods to an extent that varied among fungi. Figure 4 illustrates the dynamics during a typical experiment of loosely attached and nonattached populations of *Colletotrichum*, a fungus for

which the recovery of these fractions was particularly large, and *Cladosporium*, a fungus which rebounded less. The nonattached fungal component was quantitatively important only for *Colletotrichum* and *Acremonium*. Propagules in the medium at the end of the growth periods averaged 122 and 140 percent of the tightly attached populations for *Colletotrichum* and *Acremonium*, respectively. Hyphae of these two fungi growing on the plant surface were frequently observed to be sporulating, and presumably most of the propagules released into the medium were conidia. Loosely attached fungal populations at the end of the experiment averaged 32 percent of the tightly attached populations for all fungi and were highly correlated with tightly attached populations measured concurrently ($r^2 = 0.73$, $P < 0.01$).

35. Since loosely and tightly attached fungal populations were correlated, the observed ranking of fungi did not depend on the somewhat arbitrary separation of the attached fungal populations into these components. If the test procedure had omitted washing of the plants and had included measuring the total epiphytic fungal population (tightly attached + loosely attached), a quantity would have been measured that was highly correlated ($r^2 = 0.87$, $P < 0.01$) with the tightly attached population.

36. In the 28-day colonization assay, populations of *Colletotrichum* and the sterile fungus behaved much as they had in the 7-day assay (i.e., *Colletotrichum* remained a good colonizer, and the sterile fungus, a weak one). The plant-associated population of *Colletotrichum* increased throughout the 28 days of the assay, while that of the sterile fungus increased somewhat during the first 14 days of the assay and declined slightly during the second half (data not shown).

Endophytic Colonization

37. *Colletotrichum* was the only fungus that was detected in macerates of surface-sterilized plants. Endophytic CFU were not detected at the end of the association period, but appeared by 4 days and were present at 7 days (Figure 5) and at 14 and 28 days in the longer experiment (data not shown). Plants inoculated with 10^5 conidia/milliliter developed endophytic populations that were consistently higher than those inoculated with 10^3 conidia/milliliter, but the difference was not appreciable. Microscopic examination of *M. spicatum* stems and leaves colonized by *Colletotrichum* revealed

intracellular hyphae within the epidermis. *Acremonium* did not survive this surface-sterilization regimen, and endophytic hyphae were not found during microscopic examination.

Host Specificity

38. Both *A. curvulum* and *C. gloeosporioides* preferentially colonized *P. pectinatus* (Figure 6). Although there was considerable variability in the amount of growth on this host, for both fungi the degree of epiphytic growth was significantly higher than on either species of *Myriophyllum*. Neither fungus exhibited very much host specificity of endophytic colonization (Table 6). Endophytic CFU of *C. gloeosporioides* were frequently detected in all of the plant hosts except *M. spicatum*. In contrast, endophytic CFU of *A. curvulum* were rarely detected and were found only in *M. farwellii* and *P. pectinatus*. The number of endophytic CFU detected was always small, never exceeding 250 CFU/plant.

PART III: CONCLUSIONS AND INTERPRETATION OF FINDINGS

39. The term colonization is subject to various interpretations, and differences in colonization patterns can be expected to occur based on minor variations in bioassay conditions such as the precise physiological state of the host plants. Nevertheless, the assay protocol described herein proved capable of discriminating "colonists" from "noncolonists" of milfoil and of determining host specificity of the colonists. It is proposed that evidence for a detectable increase in the microbial population in 80 percent of the replicate experiments with milfoil as the host plant is sufficient to conclude that significant colonization of milfoil occurs. Such results would justify proceeding with the second phase, specificity screening. Conversely, if non-milfoil plants become colonized in more than 20 percent of the replicate experiments in this latter phase, it would be concluded that the candidate microbe is nonspecific. Operationally, such determinations should probably be made based on five replicate experiments.

40. Successful microbial colonists are defined by their ability to maintain a constant or increasing plant-associated population in which some or all of the members are metabolically active. Under the conditions of the colonization experiments, the fungi examined varied considerably in their ability to colonize milfoil, but all except *Aureobasidium pullulans* colonized the plant to some extent. Pathogenicity, per se, was not an overriding determinant of epiphytic colonization ability, since *Colletotrichum gloeosporioides*, the only pathogen of milfoil we included, was not a demonstrably better colonist than *Acremonium curvulum*.

41. When the two best colonizers, *A. curvulum* and *C. gloeosporioides*, were tested on a host range, neither was found to be specific to milfoil. Because the degree of colonization of the various hosts was quite variable, considerably more replication would be necessary to precisely define the host preferences of these fungi. Given the goals of this study, the additional effort required to evaluate host preferences of fungal species is not justified. It is recommended that a candidate fungus or bacterium be eliminated from further consideration once it has been determined that the microbe does not preferentially colonize milfoil.

42. Interpretation of the results of colonization experiments can be complicated by the ability of fungi to persist for relatively long periods in

an inactive state. A constant microbial population size may either reflect a balance between birth and death in an active population or the survival and retention of dormant propagules. In these experiments, the former explanation must apply, since quantitatively significant numbers of loosely attached CFU were washed out at the end of each phase of the experiment. Thus, the population of a fungus such as *Cladosporium*, which maintained a constant tightly attached population size throughout both 3-day growth periods, must have included metabolically active members.

43. As in other investigations (e.g., James, Suslow, and Steinback 1985), this study found that attachment is a rather poor predictor of longer term microbial colonization. Attachment was fairly uniform, as shown by the few significant differences between 1-day tightly attached fungal populations. Backwards extrapolation of 1- to 4-day growth rates can account for only a small part of the observed variation, and differences among 1-day fungal populations must therefore primarily reflect differential attachment. In contrast, differential growth led to a much greater number of significant differences after 4 and 7 days.

44. The failure of attachment as a predictor of growth is not unexpected, since microbial traits that promote attachment are likely to be distinct from those that favor growth. Microbial attachment is often a rather nonspecific response to the physical characteristics of the surface (Leben and Whitmoyer 1979, Fletcher 1980, Young and Kauss 1984). Actively growing bacteria and fungi produce extracellular polysaccharides (Martin and Adams 1956, Fletcher and Floodgate 1973), and some fungal spores store mucilage that is elaborated upon hydration (Hamer et al. 1988). The presence of such adhesive materials and other cell-surface characteristics are the microbial features most likely to influence attachment. In contrast, longer term colonization depends on the ability to grow, which is determined largely by the match between the metabolic requirements of the microorganism and the types and quantities of available nutrients.

45. The duration of the assay had little effect on the evaluation of fungal colonization ability, at least for the fungi and time range examined. Shortening the assay to less than 4 days would allow little time for fungal growth or decline and would make it difficult to detect changes in microbial populations. Lengthening the assay to 28 days did not alter the characterization of *Colletotrichum* as a good colonizer of *M. spicatum* or the

unidentified sterile fungus as a poor one. Since the population of the sterile fungus declined between 14 and 28 days, possibly it would have disappeared if an even longer growth period had been provided; however, an increase in the length of the assay to more than 28 days would alter the conditions under which colonization is measured. *Myriophyllum spicatum* leaves are relatively short-lived, and leaf senescence will cause measurements from longer assays to include saprophytic or opportunistic colonization of unhealthy leaves.

46. Colonization of plant surfaces in nature proceeds under conditions quite different from those of the laboratory assay. Perhaps the most significant difference is the relative simplicity of the indigenous microbial community on culture-grown plants. The effects of a more complex indigenous community on colonization are not known and cannot readily be predicted. Some members of a complex community may be competitors of the colonist and may reduce colonization. Others may facilitate colonization, by producing a biofilm to which other organisms more-or-less selectively attach (Mitchell 1984), by providing a source of organic material for colonists, or by some other mechanism. Despite these problems, laboratory colonization studies provide valuable information by identifying the extent to which patterns of host specificity in nature reflect variations in the ability of microorganisms to colonize different hosts. This study demonstrated that fungi differ in their ability to colonize *M. spicatum* plants, even when the hosts lack epifauna and flora other than a simplified bacterial community. Whatever the outcome in a more complex environment, it will likely reflect such basic differences in colonization ability.

REFERENCES

Andrews, J. H. 1980. "Plant Pathogens as Agents for Biological and Integrated Control of Aquatic Plants," Technical Report WIS WRC 80-01, University of Wisconsin Water Resources Center, Madison, WI.

Andrews, J. H., Hecht, E. P., and Bashirian, S. 1982. "Association Between the Fungus *Acremonium curvulum* and Eurasian Water Milfoil, *Myriophyllum spicatum*," Canadian Journal of Botany, Vol 60, pp 1216-1221.

Doetsch, R. N. 1981. "Determinative Methods of Light Microscopy," Manual of Methods for General Bacteriology, P. Gerhardt, ed., American Society of Microbiology, Washington, DC, pp 21-33.

Drake, D., and Montie, T. C. 1988. "Flagella, Motility and Invasive Virulence of *Pseudomonas aeruginosa*," Journal of General Microbiology, Vol 134, pp 43-52.

Ellis, M. B. 1971. Dematiaceous Hyphomycetes, Commonwealth Mycological Institute, Kew, England.

Fletcher, M. 1980. "The Question of Passive Versus Active Attachment Mechanisms in Non-specific Bacterial Adhesion," Microbial Adhesion to Surfaces, R. C. W. Berkeley et al., eds., Horwood, Chichester, England, pp 197-210.

Fletcher, M., and Floodgate, G. D. 1973. "An Electron-Microscope Demonstration of an Acidic Polysaccharide Involved in the Adhesion of a Marine Bacterium to Solid Surfaces," Journal of General Microbiology, Vol 74, pp 325-334.

Gerloff, G. C. 1975. "Nutritional Ecology of Nuisance Aquatic Plants," Ecological Research Series No. EPA-660/3-75-027, US Environmental Protection Agency.

Hamer, J. E., Howard, R. J., Chumley, F. G., and Valent, B. 1988. "A Mechanism for Surface Attachment in Spores of a Plant Pathogenic Fungus," Science, Vol 239, pp 288-290.

Harris, R. F., and Sommers, L. E. 1968. "Plate-Dilution Frequency Technique for Assay of Microbial Ecology," Applied Microbiology, Vol 16, pp 330-334.

Hugh, R., and Leifson, E. 1953. "The Taxonomic Significance of Fermentative Versus Oxidative Metabolism of Carbohydrates by Various Gram-Negative Bacteria," Journal of Bacteriology, Vol 66, pp 24-26.

James, D. W., Jr., Suslow, T. V., and Steinback, K. E. 1985. "Relationship Between Rapid, Firm Adhesion and Long-term Colonization of Roots by Bacteria, Applied Environmental Microbiology, Vol 50, pp 392-397.

Jones, J. G. 1970. "Studies of Freshwater Bacteria: Effect of Medium Composition and Method of Estimates of Bacterial Population," Journal of Applied Bacteriology, Vol 33, pp 679-686.

Kovacs, N. 1956. "Identification of *Pseudomonas pyocyanea* by the Oxidase Reaction," Nature, Vol 178, p 703.

Leben, C., and Whitmoyer, R. E. 1979. "Adherence of Bacteria to Leaves," Canadian Journal of Microbiology, Vol 25, pp 896-901.

Martin, S. M., and Adams, G. A. 1956. "A Survey of Fungal Polysaccharides," Canadian Journal of Microbiology, Vol 2, pp 715-721.

Mitchell, R. 1984. "Colonization by Higher Organisms," Microbial Adhesion and Aggregation, K. C. Marshall, ed., Life Sciences Research Report No. 31, Springer-Verlag, Berlin.

Perkins, D. D. 1962. "Preservation of *Neurospora* Stock Cultures with Anhydrous Silica Gel," Canadian Journal of Microbiology, Vol 8, pp 591-594.

Ramsay, A. J. 1977. "Aerobic Heterotrophic Bacteria Isolated from Water, Mud, and Macrophytes of Lake Grasmere, New Zealand," New Zealand Journal of Marine/Freshwater Research, Vol 11, pp 541-557.

Smibert, R. M., and Krieg, N. R. 1981. "General Characterization," Manual of Methods for General Bacteriology, P. Gerhardt, ed., American Society of Microbiology, Washington, DC, pp 409-443.

Smith, C. S., Slade, S. J., Andrews, J. H., and Harris, R. F. 1988. "Pathogenicity of the Fungus, *Colletotrichum gloeosporioides* (Penz.) Sacc., to Eurasian Watermilfoil (*Myriophyllum spicatum* L.)," Aquatic Botany (in press).

Sutton, B. C. 1980. The Coelomycetes, Fungi Imperfecti with *Pyonidia*, *Acervuli* and *Stomata*, Commonwealth Mycological Institute, Kew, England.

Tuite, J. 1969. Plant Pathological Methods--Fungi and Bacteria, Burgess Publishing, Minneapolis, MN.

Young, D. H., and Kauss, H. 1984. "Adhesion of *Colletotrichum lindemuthianum* Spores to *Phaseolus vulgaris* Hypocotyls and to Polystyrene," Applied Environmental Microbiology, Vol 47, pp 616-619.

Table 1

Total Microbial Colony Forming Units* from Milfoil and Associated Plants in Lake Wingra, Wisconsin

Plant	Date	Location	Nonsurface-Sterilized (NS)		Surface-Sterilized			
			Fungi	Bacteria, thousands	Fungi		Bacteria	
			CFU/g	CFU/g	CFU/g	Percent NS	Thousands CFU/g	Percent NS
Myriophyllum spicatum	Oct 1986	North 1	NR**	3,300	NR	34	37	1
	Mar 1987	North 1	750	6,900	<60	5	0.63	0.01
		West 1	460	7,400	300	3	24	0.3
	Apr 1987	North 1	1,200	11,000	<4	NE†	0.93	0.01
		West 1	<25	1,000	<4	NE	0.48	0.05
	Jun 1987	North 1	1,600	960	<4	NE	1.2	0.1
		2	1,100	590	<4	NE	0.63	0.1
		West 1	870	1,500	<4	NE	0.37	0.02
		2	<500	2,900	<4	NE	1.5	0.05
	Jul 1987	North 1	55	540	17	31	0.65	0.12
	Aug 1987	North 1	430	7,700	42	10	31	0.4
	Oct 1987	North 1	770	1,100	57	7	26	2
Ceratophyllum demersum	Jul 1987	North 1	95	3,000	<4	NE	9.3	0.3
	Aug 1987	North 1	730	980	12	2	0.64	0.07
	Oct 1987	North 1	1,400	1,900	56	4	8	0.4
Vallisneria americana	Jul 1987	North 1	10	460	10	100	<0.02	NE
	Aug 1987	North 1	16	460	5	31	<0.02	NE

* Expressed as colony forming units per gram fresh weight plant material.

** NR = not reported.

† NE = not estimable because of the below detection limit restriction.

Table 2

Predominant Fungi Isolated from Milfoil and Associated Plants in Lake Wingra, Wisconsin (North 1 Location)*

Fungus	Myriophyllum spicatum						Ceratophyllum demersum			Vallisneria americana	
	Oct 1986	Mar 1987	Apr 1987	Jul 1987	Aug 1987	Oct 1987	Jul 1987	Aug 1987	Oct 1987	Jul 1987	Aug 1987
Acremonium spp.	-	++	++	++	+	-	+	-	++	-	-
Alternaria alternata	-	-	-	-	-	-	+	+	++	-	-
Arthrinium phaeospermum	-	-	-	-	-	++	-	-	-	-	-
Ascochyta spp.	-	-	-	-	-	-	-	+	-	-	-
Aspergillus spp.	-	-	-	-	+	-	-	+	-	-	-
Beauveria spp.	-	-	-	-	-	-	-	+	-	-	-
Chaetomella spp.	-	-	-	-	+	-	-	+	-	-	-
Choanephora spp.	-	-	-	-	-	-	-	++	-	-	-
Cladosporium spp.	++	++	++	++	++	++	+	++	++	-	+
Curvularia spp.	-	-	-	-	+	-	-	+	-	-	-
Cytospora spp.	-	-	++	-	++	-	-	-	-	-	-
Fusarium spp.	-	-	++	+	++	-	++	++	-	-	-
Microsphaeropsis olivacea	++	-	-	-	-	++	-	-	++	-	-
Microsphaeropsis spp.	-	++	++	-	++	-	+	++	++	-	-
Mucor spp.	-	-	-	-	-	-	-	-	++	-	-
Myrothecium spp.	-	-	-	-	+	-	-	+	-	-	-
Paecilomyces spp.	-	-	-	-	+	-	-	+	-	-	-
Penicillium spp	-	-	-	-	++	++	+	++	++	-	-
Pestolotia spp.	-	-	-	-	-	-	-	+	-	-	-
Phoma eupyrena	-	-	-	-	-	-	+	+	++	-	+
Phoma leveillei	-	-	-	-	+	-	-	-	-	-	-
Phoma lingam	-	-	-	-	++	-	-	+	-	+	-
Phoma medicaginis	-	-	-	-	-	-	-	+	-	-	-
Phoma spp.	-	++	++	-	+	++	-	-	++	-	-
Pseudorobillardia spp.	-	-	-	-	+	-	-	-	-	-	-
Stemphylium spp.	-	-	-	-	+	-	-	-	-	-	-
Trichoderma spp.	-	++	++	-	+	++	-	++	++	-	-
Volutina spp.	-	-	-	-	+	-	-	-	-	-	-
Unidentified fungi	++	++	-	+	++	++	+	++	++	-	-
Yeasts	-	-	-	-	+	++	+	-	++	+	-

* Codes are as follows: ++, fungus was present at >25 CFU/g fresh plant weight; +, fungus was present at 4 to 25 CFU/g fresh plant weight; and -, fungus was not isolated (<25 and <4 CFU/g fresh plant weight up to April 1987 and after July 1987, respectively).

Table 3

Characteristics of the Predominant Aerobic Heterotrophic Bacteria

Associated with Milfoil from Lake Wingra, Wisconsin

Code Name	Colony Properties Used for Enumeration*: Color	Approx. Size mm	Morphology**: 1	2	3	4	5	6	7	Relative Amount (% CFU)	Cell and Biochemical Properties of the Representative Isolate: Gram Stain	Shape	Endospores	Motile	Biochemical Properties: Ox/Ferm†	Oxidase	Presumptive Genus of the Representative Isolates††
July 1987 (73 million bacterial CFU/cm)																	
BF87-7-1	Colorless	4	T	C	F	U	G	V	N	37	-	Rods (medium)	ND‡	+	NR	+	Pseudomonas/Alcaligenes
BF87-7-8	Colorless	<1.0	T	C	F	E	S	V	N	37	-	Rods (short,stout)	ND	+	F	-	Enterobacter
BF87-7-4!	Dull white	1	O	C	P	E	S	V	N	8	-	Rods (medium)	ND	-	NR	+	Moraxella
BF87-7-5!	Bluish white	1	O	C	P	E	S	V	N	6	-	Rods (medium)	ND	-	NR	+	Moraxella
BF87-7-9	Cream	2.5	O	C	R	E	S	V	N	6	-	Rods (medium)	ND	+	F	+	Vibrio/Aeromonas
BF87-7-7	Red	1	O	C	R	E	S	V	N	2	-	Rods (medium)	ND	+	NR	+	Motile Flavobacterium
BF87-7-2	Yellow	2.0	T	C	F	U	S	V	N	2	-	Rods (medium)	ND	+	NR	+	Xanthomonas
BF87-7-3	Cream	1.5	O	C	C	E	S	V	N	2	+	Cocci (small)	-	-	NR	-	Micrococcus
BF87-7-6	Yellow	<1.0	O	C	R	E	D	V	N	1	+	Cocci (small)	-	-	NR	-	Micrococcus
August 1987 (1,000 million bacterial CFU/cm)																	
BF87-8-2	Colorless	4.0	T	C	F	O	S	V	N	33	-	Rods (medium)	ND	-	NR	-	Acinetobacter
BF87-8-4	Cream	3.0	O	C	C	E	S	V	N	25	-	Rods (short)	ND	-	NR	-	Acinetobacter
BF87-8-3	Dull cream	2.5	T	C	R	U	S	V	N	19	-	Rods (medium)	ND	+	F	+	Vibrio/Aeromonas
BF87-8-1	Light peach	10	T	C	F	U	D	D	Y	11	-	Rods (medium)	ND	+	NR	+	Cytophaga
BF87-8-6	Milky white	<1.0	O	C	C	E	S	V	N	8	-	Rods (short,stout)	ND	-	NR	-	Acinetobacter
BF87-8-7	Colorless	<1.0	T	C	C	E	S	V	N	2	-	Rods (short)	ND	+	F	-	Enterobacter
BF87-8-5	Yellow	2.0	O	C	C	E	S	V	N	1	-	Rods (medium)	ND	+	NR	+	Xanthomonas
October 1987 (73 million bacterial CFU/cm)																	
BF87-10-1	Yellow	3	T	C	F	E	S	V	N	41	-	Rods (medium)	ND	-	NR	+	Flavobacterium
BF87-10-2	Colorless	4.5	T	I	F	L	S	V	N	18	-	Rods (medium)	ND	-	NR	-	Acinetobacter
BF87-10-5	Light amber	1.0	T	C	C	E	S	V	N	18	-	Rods (medium)	ND	-	NR	-	Acinetobacter
BF87-10-3	Reddish	1.5	T	C	C	E	S	V	N	11	-	Rods (medium)	ND	+	NR	+	Motile Flavobacterium
BF87-10-6!	Colorless	3.0	T	P	F	U	S	V	N	7	-	Rods (medium)	ND	-	NR	+	Moraxella
BF87-10-4	Colorless	2.0	T	I	F	L	S	V	N	5	-	Rods (medium)	ND	-	NR	-	Acinetobacter

* Properties after 21-day incubation on the enumeration medium, DNB (10% normal strength nutrient agar).

** Colony morphology key: 1. Opacity: T = transparent; O = opaque, 2. Form: P = punctiform; C = circular; I = irregular; 3. Elevation: F = flat; R = raised; C = convex; P = pulvinate; 4. Margin: E = entire; U = undulate; L = lobate; O = erose; 5. Smoothness: S = shiny; G = granular; D = dull; 6. Texture: V = viscous; D = dry; 7. Spreading nature: N = no; Y = yes. (Spreading colonies generally showed the following combination of properties: large diameter; irregular form; flat elevation; undulate/lobate margin; granular/dull smoothness.)

† Oxidation/fermentation key: F = fermentative (acid response within 7 days under aerobic and anaerobic conditions); NR = no response (no acid response within 7 days under either aerobic or anaerobic conditions).

†† Classification assignments based solely on scheme in Figure 1.

‡ ND = not determined (for gram-negative bacteria).

Table 4

Relative Change in Tightly Attached Populations of Fungi on *M. spicatum* for Three Experiments

Fungus	Change in Fungal Population (Fold)* by Experiment 1	2	3	Interpretation** 1	2	3
	Initial Inoculum = 10^3 CFU/ml					
Colletotrichum	2.4	7.2	8.4	+	+	+
Acremonium	9.6	2.6	3.2	+	+	+
Paecilomyces	6.5	5.5	1.0	+	+	0
Sterile septate	6.4	3.4	1.1	+	+	0
Cladosporium	1.2	0.9	1.4	0	0	0
Aureobasidium	1.4	0.2	0.1	0	-	-
	Initial Inoculum = 10^5 CFU/ml					
Colletotrichum	4.1	2.2	1.6	+	+	0

* Each value is the change that occurred in the fungal population in a single plant culture. Values are the day 7 population size divided by the day 1 value.

** Symbols are defined as follows: +, detectable increase; -, detectable decrease; and 0, no detectable change.

Table 5

Tightly Attached Fungal Populations on M. spicatum After Three Phases of Colonization Assay

Fungus	Fungal Numbers,* 10^3 CFU/plant		
	Association Phase	Growth Phase 1	Growth Phase 2
	Initial Inoculum = 10^3 CFU/ml		
Colletotrichum	3.1^{A}	7.1^{A}	13.1^{A}
Acremonium	2.8^{A}	8.4^{A}	19.8^{A}
Sterile septate	1.7^{A}	2.8^{AB}	7.7^{AB}
Cladosporium	0.8^{B}	0.9^{BC}	0.9^{C}
Paecilomyces	0.6^{B}	0.9^{C}	3.0^{BC}
Aureobasidium	0.4^{B}	0.2^{D}	0.2^{D}
	Initial Inoculum = 10^5 CFU/ml		
Colletotrichum	50.0	66.1	117.3

* Mean of three experiments. Within a column, values with the same letter were not significantly different at P = 0.05 in a Duncan's Multiple Range Test.

Table 6

Fraction of Replicates in Which Endophytic CFU Were Detected in Plant Species

Plant Species	Fraction of Replicates Having Endophytic CFU*	
	C. gloeosporioides	*A. curvulum*
M. spicatum	0/4	0/5
M. farwellii	4/4	1/5
E. canadensis	3/4	0/5
P. pectinatus	3/4	2/5

* Number of replicates in which endophytic CFU were detected at end of host-specificity experiments/total number of replicates examined.

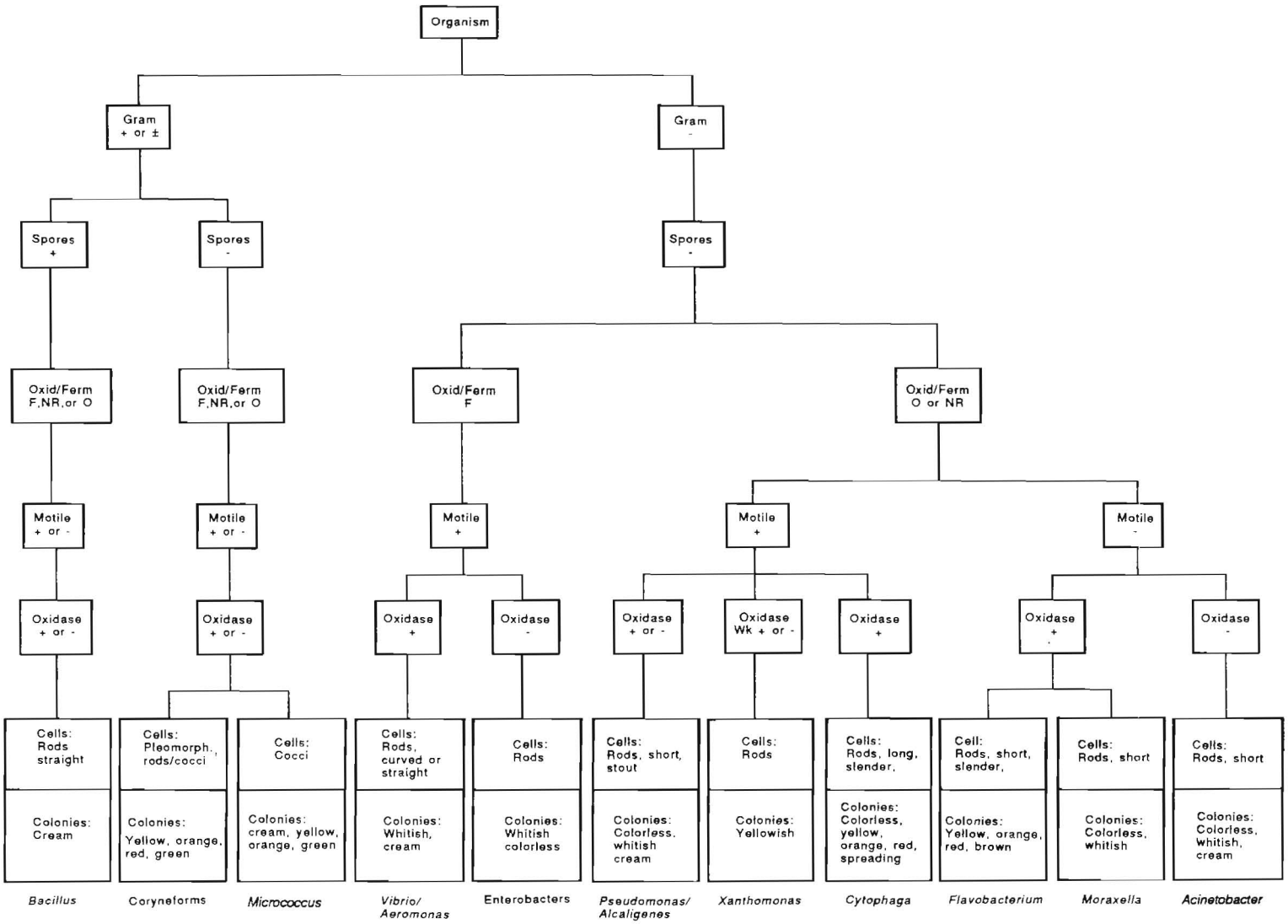

Figure 1. Classification scheme for aerobic heterotrophic bacteria commonly associated with aquatic plants, based on classical, routinely measurable, taxonomic properties (see Table 3 for definition of codes)

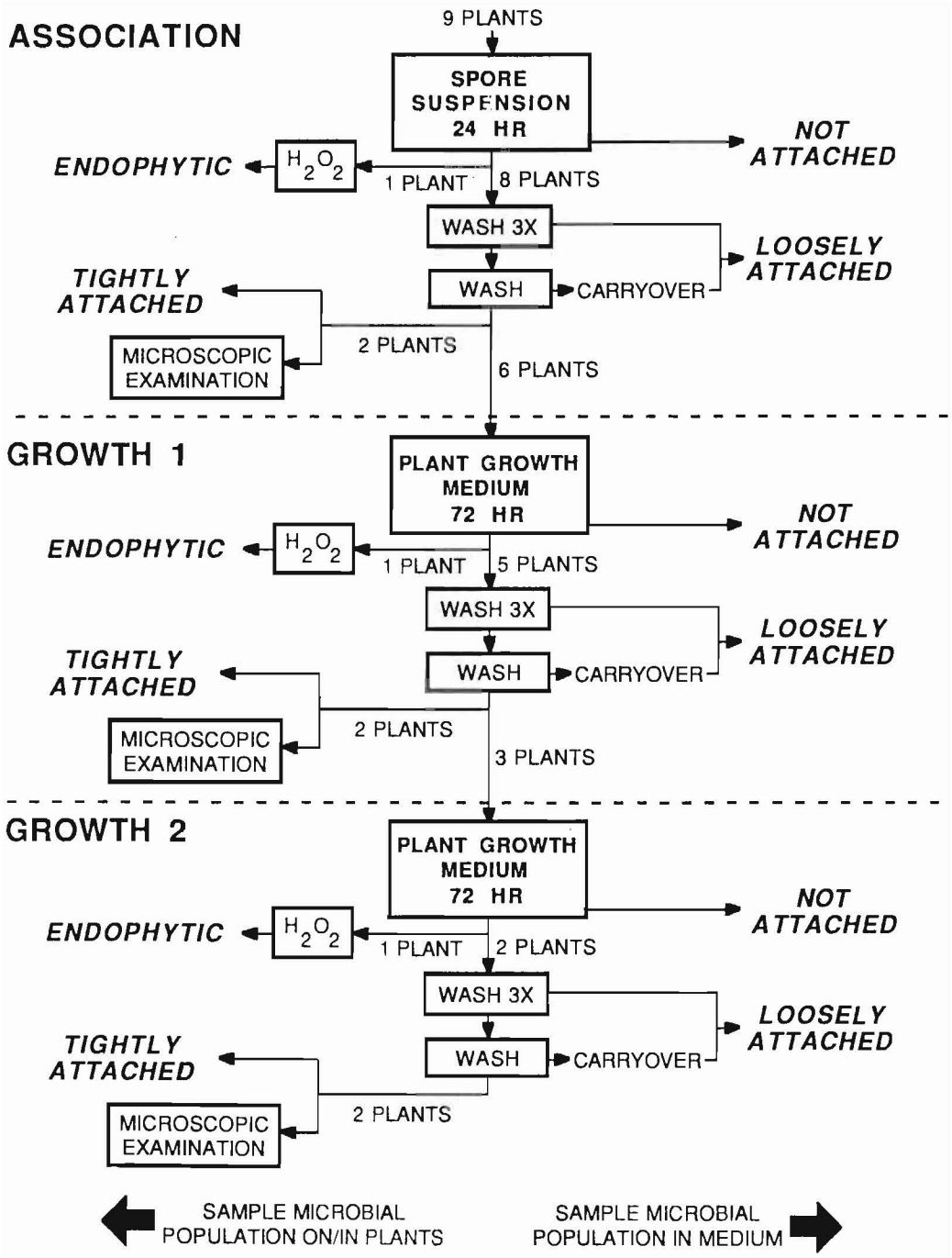

Figure 2. Experimental protocol for a single replicate in the standard milfoil colonization experiment

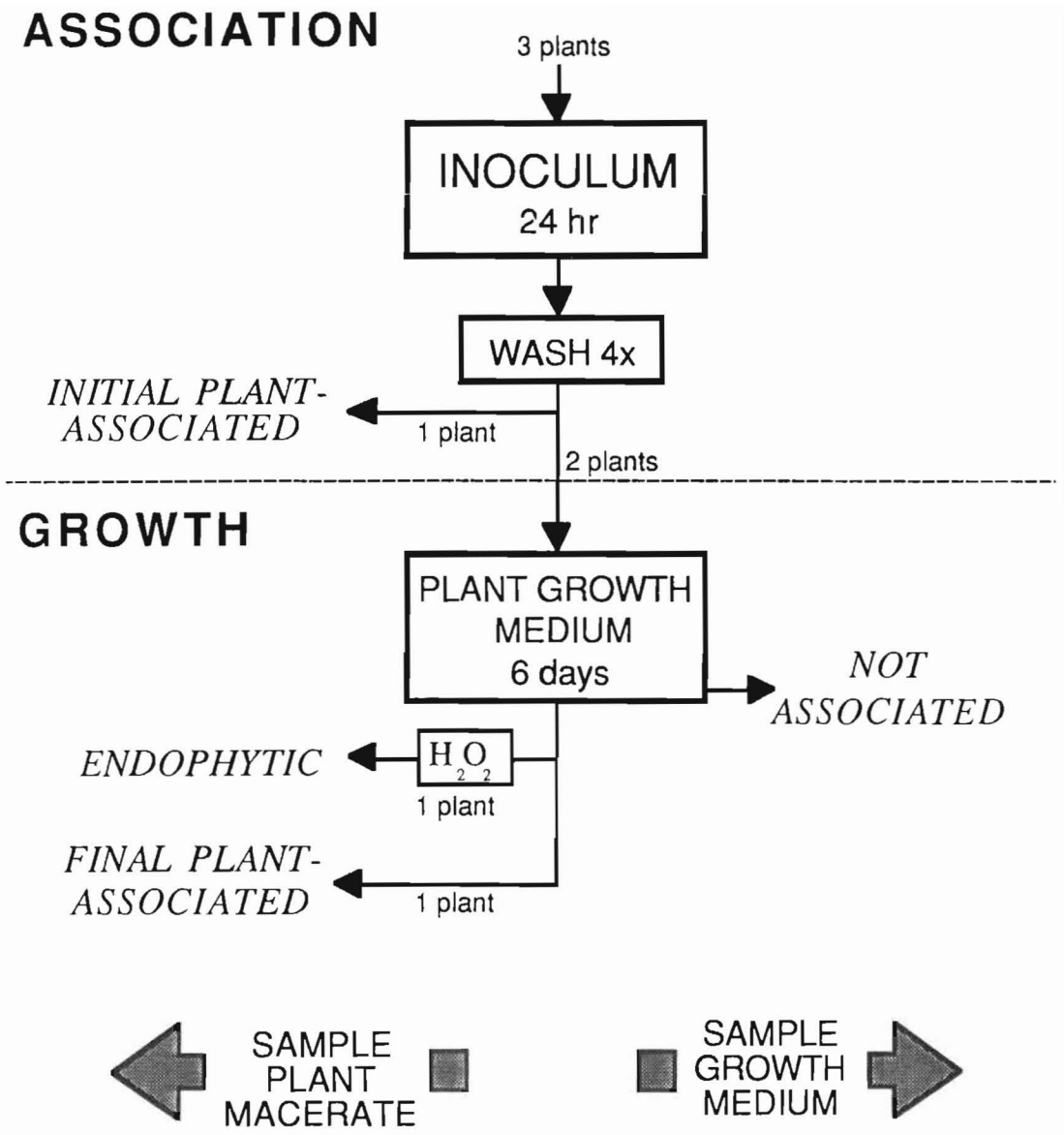

Figure 3. Experimental protocol for one replicate of the simplified colonization assay used to examine host specificity

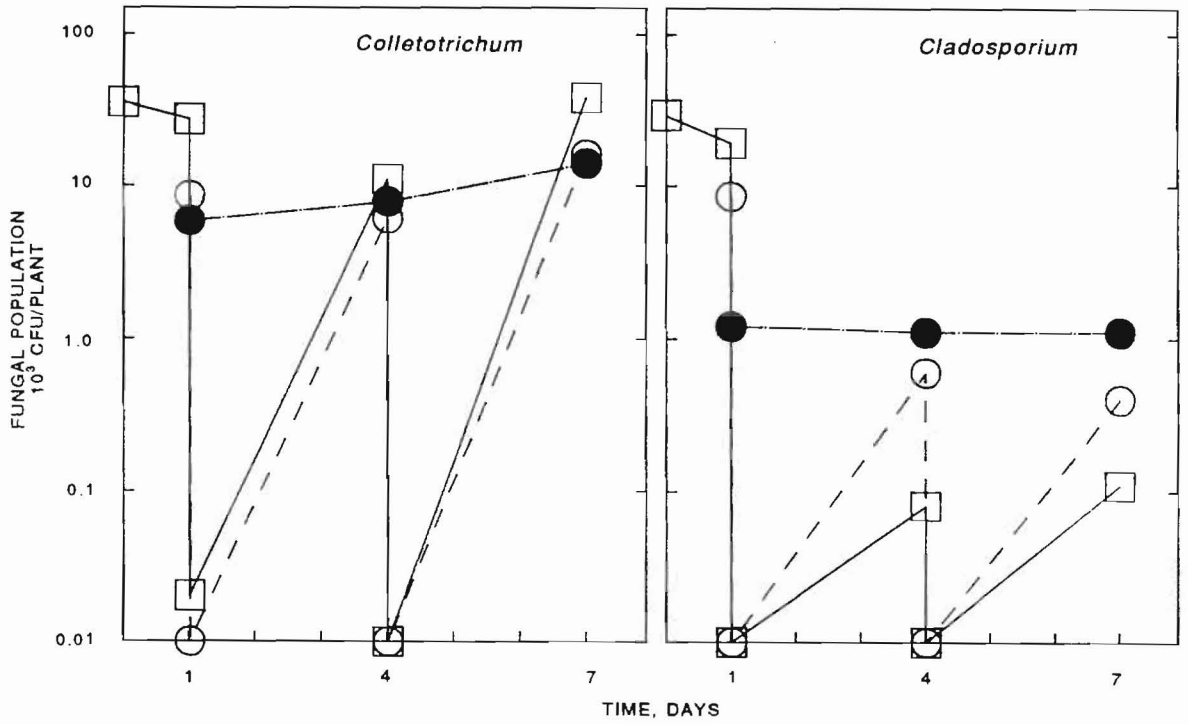

Figure 4. Dynamics of the nonattached (□---□), loosely attached (o---o), and tightly attached (●-·-●) components of *C. gloeosporioides* and *C. herbarum* populations during a typical colonization experiment

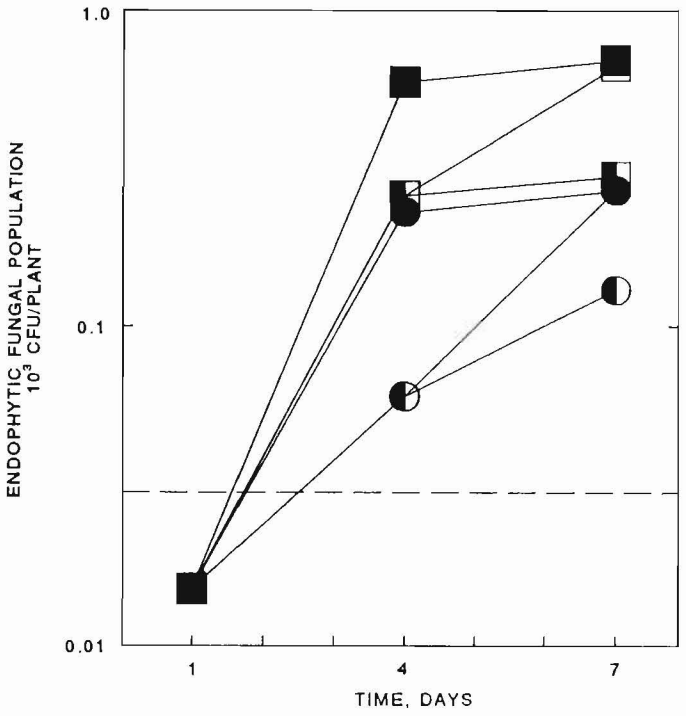

Figure 5. Endophytic colonization of *M. spicatum* by *C. gloeosporioides*. Initial inoculum: circles (10^3 CFU/ml), squares (10^5 CFU/ml). The shading patterns of the symbols indicate the results of individual experiments. The dashed line is the detection limit

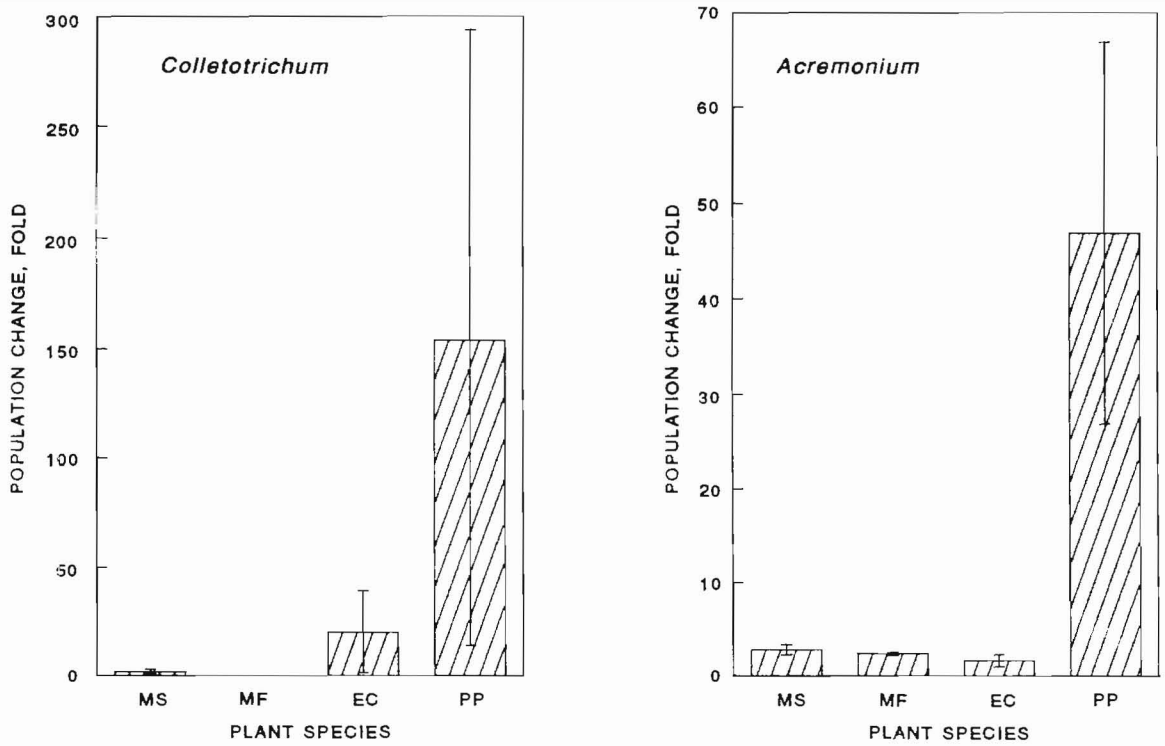

Figure 6. Colonization of *M. spicatum* (MS), *M. farwellii* (MF), *E. canadensis* (EC), and *P. pectinatus* (PP) by *C. gloeosporioides* and *A. curvulum*. Each bar is the average ratio of the size of the 7-day plant-associated fungal population to that at 1 day for three to five replicate cultures. Bars indicate ±1 standard error of the mean