Colonization of a Submersed Aquatic Plant, Eurasian Water Milfoil (Myriophyllum spicatum), by Fungi under Controlled Conditions

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A laboratory assay to assess colonization of a submersed aquatic plant, Eurasian water milfoil (Myriophyllum spicatum), by fungi was developed and used to evaluate the colonization potential of Colletotrichum gloeosporioides, Acremonium curvatum, Cladosporium herbarum, Aureobasidium pullulans, and a Paecilomyces sp., and an unidentified sterile, septate fungus. Stem segments of plants were first immersed in suspensions of fungal propagules for 24 h and then washed to remove all but the tightly attached component of the population. Inoculation was followed by two growth cycles of 3 days each. At the start of each cycle, washed plants were transferred to a mineral salts medium to provide an opportunity for the attached fungal populations to grow. After each growth period, plants were again washed, and fungal populations in the medium (nonattached), loosely attached and tightly attached to the plant, and within the plant (endophytic) were assayed by dilution plating. The fungi differed in the extent to which they attached to water milfoil and in their ability to grow in association with it. There were relatively few significant differences among the tightly attached fungal populations after 24 h, but growth of the better colonizers led to a greater number of significant differences after 4 and 7 days. In addition, the better colonizers showed sustained regrowth of loosely and nonattached fungal propagules in the face of intermittent removal by washing. A milfoil pathogen, C. gloeosporioides, was the only endophytic colonizer; it was also among the best epiphytic colonizers but was not demonstrably better than A. curvatum, a fungus commonly found as an epiphyte on watermilfoil. The yeastlike hyphomycete Aureobasidium pullulans was the only fungus that consistently failed to establish an increasing population on the plant.

Plant-microbe interactions begin with the colonization of the plant surface; i.e., the microorganism must attach and begin to grow if it is to appreciably influence the growth of the plant or to prompt a defensive response. Colonization of plants by pathogens and nitrogen-fixing symbionts has been studied extensively, but much less is known about the colonization of plant surfaces by microorganisms that form other types of relationships. Whether or not epiphytic colonization occurs can have important implications for the outcome of the microbe-plant interaction. For example, disease incidence has been related to the size of epiphytic populations of plant pathogenic bacteria (27), and colonization by saprophytic fungi can provoke defensive reactions in the plant and reduce yield even in the absence of visible symptoms (31).

While epiphytic colonization of submersed plants by bacteria has attracted considerable attention (for a review, see reference 4), virtually nothing is known about their colonization by fungi. Aerial plant surfaces, except in the tropics (28), are viewed as generally being a hostile environment for microbial growth because of the limited availability of moisture (5, 8, 9). In contrast, leaves and stems of submersed plants would seem to be a good habitat for microbial growth; free water is always present, and the plants leak substantial quantities of dissolved organic matter (37). Indeed, the surfaces of submersed plants present vast potential for microbial colonization and are frequently covered by a dense growth of algae and other organisms (29).

Eurasian water milfoil (Myriophyllum spicatum L.) is a submersed plant that grows as a nuisance weed in lakes and streams across much of North America (1, 13). Identification of microbial colonists of water milfoil may lead to the discovery of organisms that can be used as part of a biological or integrated control strategy for the plant, since even organisms that alone have a minimal effect on the plant can contribute to integrated control strategies (33).

In this paper we present the results of laboratory studies of colonization of Eurasian water milfoil by six species of fungi. We describe the development and evaluation of a protocol for the evaluation of fungal colonization of M. spicatum and compare the relative colonization ability of the fungi. Operationally, we equate colonization with growth (including regrowth in the face of repeated plant washing) and equate growth with an increase in fungal CFU, whether these be spores, mycelia, or both. There is no unambiguous method for quantifying growth on solid substrates (21). The direct microscopic count procedure is tedious, relatively insensitive in practice, and because of highly variable microbe distributional patterns, difficult to analyze statistically (7, 16). Many of the physiological or biochemical methods are lengthy and require expensive equipment and experienced laboratory personnel. Most, such as the ATP method (15), respirometry (20), and fluorescein diacetate hydrolysis (34), do not discriminate among categories of microbes (e.g., bacteria versus fungi), and depending on the assay, results may be affected by the physiological state of the organism. Ergosterol measurements may be unsatisfactory due to high background interference or confusion with similar sterols in the plant (22). Growth-linked enzymes, such as laccase or esterases, are prone to being affected by substrate composition (22). Chitin analyses can vary independently of growth owing to the age of the fungus or presence of nonfungal

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hexosamines (22, 30), and they cannot be used for certain aquatic fungi, such as the oomycetes which have cellulose walls. Accordingly, our assessments are based on the conventional dilution plate count procedure applied to growth media (nonattached CFU), plant washings (loosely attached CFU), and macerated plant material (closely attached CFU). Water agar is a well-recognized approach for removing fungal spores from soil (6) and plant (26) systems supporting fungal colonization. Maceration of plant material followed by dilution plating is a relatively simple yet useful method to follow fungal population dynamics under specific conditions (24, 38).

MATERIALS AND METHODS

Plant cultures. *M. spicatum* plants for this study were from the strain originally cultured by Gerloff (12). Cultures of plant stem segments were maintained in a dilute mineral salts medium which was derived from a modified Gerloff medium (2) by adding NaHCO₃ and KHCO₃ to produce a bicarbonate concentration of 0.4 N by raising the concentrations of calcium and magnesium to 1.2 and 1.0 mM/liter, respectively. Stock and experimental cultures were grown at approximately 25°C and were illuminated by fluorescent lamps which provided 300 to 400 microeinstein/m² per s for 15 h/day. The plant cultures originally contained a fungus, *Acremonium curvulum*, which was eliminated by exposing plants to thiabendazole (3). 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 dilute nutrient agar (0.8 g nutrient broth per liter), and the total bacterial population was found to range from 2 × 10⁷ to 60 × 10⁶ CFU/cm² of plant.

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 which were cut to a uniform 4.0-cm length at the beginning of the experiment. During 7-day experiments the plants typically increased in length by approximately 1 cm.

Fungi. The six fungi were chosen for inclusion in this study because they represent organisms likely to differ in growth characteristics and in the degree and nature of their interaction with *M. spicatum*. *Colletotrichum gloeosporioides* (Penz.) Sacc. is a weakly virulent pathogen of *M. spicatum* which was isolated from blackened stem lesions on *M. spicatum* growing in a small pond within the University of Wisconsin Arboretum in Madison (32). *Achromonium curvulum* W. Gams is a frequent associate of *M. spicatum* (3) and was isolated from our plant culture prior to treating it with thiabendazole (see above). *Cladosporium herbarum* (Pers.) Link, *Aureobasidium pullulans* (De Bary) Arnaud (a yeastlike fungus), *Paecilomyces sp.*, and an unidentified sterile, septate fungus were isolated from healthy *M. spicatum* plants collected from Lake Wingra, Wis. The nonsporulating fungus (isolate MN-2U-12) was included so that the ability of the assay to detect mycelial growth could be checked. *Cladosporium herbarum* was isolated commonly from *Curvulum*, while the other three species were encountered infrequently (T. Chandy, unpublished data). Experimental cultures were initiated from storage cultures of *A. curvulum* and *C. gloeosporioides* maintained on silica gel crystals (25). The other species of fungi were isolated just prior to the start of these experiments. Between experiments all of the fungi were grown on potato glucose agar (PGA; 35) or PGA amended with 250 mg of chloramphenicol per liter (PGA/ch).

The inoculum for colonization experiments was a suspension of freshly harvested conidia in sterile distilled water for all the sporulating fungi. *Colletotrichum* and *Acremonium* conidia were produced by growing the fungi for 4 days in a 20% liquid V8 juice medium, and mycelium was removed by pouring cultures through a double layer of sterile cheesecloth. *Cladosporium herbarum*, *Aureobasidium pullulans*, and the *Paecilomyces* sp. were grown on PGA/ch. *Cladosporium herbarum* and *Paecilomyces* sp. were grown for 10 to 12 days, whereas *Aureobasidium pullulans* was grown for 3 to 4 days, and spores were harvested by washing them from the agar surface with sterile buffered Tween (SBT; 0.05 to 0.1% Tween 80 in 0.005 M pH 7.1 phosphate buffer). Propagule suspensions of all but the sterile fungus were counted by using a hemacytometer, and the density was adjusted with distilled water to produce a final concentration of approximately 10⁷ viable propagules per ml. An inoculum density of 10⁶/ml was also used for colonization experiments with *C. gloeosporioides*.

The 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. In a preliminary experiment we found that homogenizing five 8-mm disks from a colony of this age in 25 ml of SBT yielded approximately 5 × 10⁴ CFU/ml, and this relationship was used to estimate the amount of homogenate necessary to produce an inoculum containing approximately 10³ viable CFU/ml. In all cases the actual density of viable propagules in the inoculum was checked by dilution plating samples on PGA. Viable counts were generally 85 to 95% of the microscopic counts for *C. gloeosporioides*, *A. curvulum*, *Paecilomyces sp.*, and *Aureobasidium pullulans* and 60 to 70% for *Cladosporium herbarum*.

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 plates of PGA/ch. The number of colonies that appeared after incubation for 3 to 5 days at room temperature (19 to 21°C) were counted. When dilution was necessary, samples were serially diluted with SBT and plates with 10 to 100 colonies were selected for counting.

Fungal colonization of plants. Colonization was assessed by inoculating plants with one of the test fungi, followed by one or more cycles of washing the plants and transferring them into fresh plant growth medium and allowing time for the microorganisms to grow. Unless specified otherwise, each experiment lasted 7 days and was divided into three phases: a 1-day association phase and two 3-day growth phases. Between phases and at the end of the experiment, plants were washed and the fungal population in the culture was partitioned into the following, somewhat arbitrarily defined CFU components: those in the medium (nonattached), those removed by washing (loosely attached), those remaining on the plant after washing (tightly attached), and those surviving surface sterilization (endophytic). The assay protocol for a single replicate of a colonization experiment is summarized in Fig. 1. The maceration-dilution plating procedure used for determining tightly attached and endophytic CFU was analogous in principle to that of Visser and Parkinson (36), Kuter (18), and Pfender and Woottke (26).

In the first phase, the association phase, nine shoot segments were immersed in 300 ml of inoculum suspension (described above) for 24 h to allow fungi to attach to the

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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., nonattached to the plants. One plant was removed, and its endophytic fungal population was estimated by surface sterilizing the intact plant for 5 min in 15% H₂O₂, 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 scalpels, suspending the pieces in 50 ml of cold SBT, and grinding them in a chilled blender cup for 5 min at 6,000 rpm with an Omni-Mixer (OCI Instruments, Waterbury, Conn.). The eight remaining plants were washed to remove loosely attached fungal CFU by shaking plants in 150 ml of 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. 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 for microscopic examination, and the other was macerated, and the macerate was 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. The six plants remaining at the end of the association phase were transferred to the second phase, the first growth phase, in which plants were placed into aerated culture jars containing 250 ml of plant growth medium and incubated for 3 days under the standard conditions described above. At the end of the growth period, plants were washed and sampled and 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 to 7), and monitored exactly as in the first. A fresh culture container and growth medium were used.

When an endophytic fungal population was detected by the plating assay (only C. gloeosporioides of the fungi was evaluated), Formalin-acetic acid-alcohol-preserved plants were examined for internal hyphae with a light microscope. Plants colonized by A. curvulum were also examined microscopically, since this species has been reported to grow endophytically within M. spicatum (3), and plants colonized by the sterile fungus were observed to see if the fungus produced spores when growing in association with the plant. In addition, control, noninoculated plants were examined routinely to confirm the absence of internal hyphae. Prior to examination, plant pieces were cleared in a boiling saturated aqueous solution of chloral hydrate and were stained overnight with a mixture of aniline blue and trypan blue (0.025% each). Stained specimens were rinsed briefly in distilled water, placed into saturated chloral hydrate at room temperature for 1 to 2 h, and then mounted in lactophenol for observation.

In all, 10 experiments were run at different times with different fungi. C. gloeosporioides was included as an internal control in all experiments. The reproducibility data (see below) were based on experiments 1 to 4. Experiments 5 to 7 were the basis of Tables 1 and 2 and Fig. 2 and 3. Experiments 8 to 10 evaluated the effect on colonization trends of assay duration (7 versus 28 days) and of time (1 h versus 1 day) and cultural variables (distilled water versus plant growth media; air bubbling versus occasional stirring) during the association phase. The results of experiments 8 to 10 are not part of this paper and are noted only in passing in the Discussion to confirm trends in the earlier experiments.

**Experimental design and analysis.** To evaluate aspects of variability in colonization, standard 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, we then evaluated all six fungi 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 a freshly produced fungal inoculum.

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% or less (average, 14%). A 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
TABLE 1. Relative change in tightly attached populations of fungi on M. spicatum from day 1 to day 7 of the colonization assay for each of three experiments

<table>
<thead>
<tr>
<th>Fungus*</th>
<th>Change in fungal population (fold)* in exp</th>
<th>Interpretation*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colletotrichum gloeosporioides</td>
<td>2.4</td>
<td>7.2</td>
</tr>
<tr>
<td>Acronemum curvulum</td>
<td>9.3</td>
<td>6.5</td>
</tr>
<tr>
<td>Paecilomyces sp.</td>
<td>5.4</td>
<td>5.4</td>
</tr>
<tr>
<td>Sterile, septate</td>
<td>1.6</td>
<td>0.9</td>
</tr>
<tr>
<td>Cladosporium herbarum</td>
<td>1.4</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Colletotrichum gloeosporioides<sup>a</sup> 4.1 | 2.2 | 1.6 | + + 0 |

<sup>a</sup> Initial inoculum size was 10<sup>5</sup> CFU/ml unless noted otherwise.
<sup>b</sup> 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.
<sup>c</sup> Symbols for three experiments: +, detectable increase; -, detectable decrease; 0, no detectable change.
<sup>d</sup> Initial inoculum size, 10<sup>5</sup> CFU/ml.

only the results from experiments 5 to 7, in which all six fungi were assessed simultaneously.

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.

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. Data from the three experiments were first logarithmically transformed to stabilize the variance, and a two-way analysis of variance with fungi and experiments as factors was performed. Logarithmic transformation did not produce a homogeneous variance when results from the C. gloeosporioides 10<sup>5</sup> 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.

RESULTS

Epiphytic colonization. The dynamics of tightly attached fungal populations during the 7-day assay varied considerably among the fungi (Table 1). C. gloeosporioides and A. curvulum were the best colonizers; their populations consistently increased during each experiment. The other fungi were noticeably poorer colonizers and, except for Cladosporium herbarum, exhibited less consistent population dynamics. Ranked in order of decreasing colonization, they were the Paecilomyces sp. and the sterile fungus, followed by Cladosporium herbarum and then Aureobasidium pullulans. Aureobasidium pullulans was usually a noncolonist, as it declined during two of three experiments. Inoculation with 10<sup>5</sup> propagules of C. gloeosporioides per ml led to larger tightly attached populations of the fungus, but the larger populations did not increase as consistently or rapidly as those resulting from inoculation with 10<sup>7</sup>/ml.

There were few differences among fungi after 1 day, but growth of the better colonizers led to an increased number of differences after 4 and 7 days (Table 2). At the end of the 1-day association period, there were two distinguishable fungal colonization groups: a higher one containing C. gloeosporioides, A. curvulum, and the sterile fungus and a lower one containing the other three fungi. Differences between tightly attached fungal populations increased by the end of the first growth period. 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 the Paecilomyces sp., which had only a small tightly attached population initially, had grown enough by day 7 to switch rankings with Cladosporium herbarum, which had begun with a higher population density but did not increase during the experiment. Inoculation with 10<sup>5</sup> CFU of C. gloeosporioides per ml led to a tightly attached population that was much larger at all times than that produced by any other treatment.

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 2 illustrates the dynamics during a typical experiment of loosely attached and nonattached populations of C. gloeosporioides, a fungus for which the recovery of these

![FIG. 2. Dynamics of the nonattached (○), loosely attached (○), and tightly attached (●) components of C. gloeosporioides and Cladosporium herbarum populations during a typical colonization experiment. Plants were washed at 1 and 4 days; thus, nonattached and loosely attached population components plummet at those times and then rebound.](http://aem.asm.org/)

TABLE 2. Tightly attached populations of fungi on M. spicatum at the end of each of the three phases of the colonization assay

<table>
<thead>
<tr>
<th>Fungus&lt;sup&gt;a&lt;/sup&gt;</th>
<th>No.&lt;sup&gt;a&lt;/sup&gt; of tightly attached fungi (10&lt;sup&gt;5&lt;/sup&gt; CFU/plant) at end of phase:</th>
<th>Association</th>
<th>Growth 1</th>
<th>Growth 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colletotrichum gloeosporioides</td>
<td>3.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>7.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>13.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Acremonium curvulum</td>
<td>2.8&lt;sup&gt;e&lt;/sup&gt;</td>
<td>8.4&lt;sup&gt;e&lt;/sup&gt;</td>
<td>19.8&lt;sup&gt;e&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Sterile, septate</td>
<td>1.7&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.8&lt;sup&gt;c&lt;/sup&gt;</td>
<td>7.7&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Cladosporium herbarum</td>
<td>0.8&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.9&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.9&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Paecilomyces sp.</td>
<td>0.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Aureobasidium pullulans</td>
<td>0.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Colletotrichum gloeosporioides&lt;sup&gt;d&lt;/sup&gt;</td>
<td>50.0</td>
<td>66.1</td>
<td>117.3</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Initial inoculum size was 10<sup>5</sup> CFU/ml unless noted otherwise.
<sup>b</sup> Values are means of three experiments.
<sup>c</sup> Within a column, values with the same letter are not significantly different at P = 0.05 in Duncan’s multiple range test.
fractions was particularly large, and *Cladosporium herbarum*, a fungus which rebounded less.

The nonattached fungal component was quantitatively important only for *C. gloeosporioides* and *A. curvulum*. Propagules in the medium at the end of the growth periods averaged 120 and 140% of the tightly attached populations for *C. gloeosporioides* and *A. curvulum*, respectively. Hyphae of these two fungi growing on the plant surface were frequently observed to be sporulating, and microscopic checks indicated that most of the propagules released into the medium and present in the plant washes were conidia, as might be expected (6, 26). Loosely attached fungal populations at the end of the experiment averaged 32% of the tightly attached population for all fungi and were highly correlated with tightly attached populations measured concurrently ($r^2 = 0.73$, $P < 0.01$). Since final 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 we had omitted washing the plants and instead measured the total epiphytic fungal population (tightly attached plus loosely attached), a quantity would have been measured that was highly correlated ($r^2 = 0.87$, $P < 0.01$) with the tightly attached population.

Hyphae of the sterile fungus were observed sporadically on the leaf epidermis, but there was no evidence that the fungus invaded the plant, and no spores or sporulating structures were seen.

**Endophytic colonization.** *C. gloeosporioides* 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 (Fig. 3) and at 14 and 28 days in the longer experiments (data not shown). Plants inoculated with $10^5$ conidia per ml developed endophytic populations that were consistently higher than those inoculated with $10^4$ conidia per ml, but the difference was not appreciable. Microscopic examination of *M. spicatum* stems and leaves colonized by *C. gloeosporioides* revealed intracellular hyphae within the epidermis (Fig. 4). *A. curvulum* did not survive this surface sterilization regimen, and endophytic hyphae were not found during microscopic examination. Endophytic hyphae were also found in noninoculated, control plants.

**DISCUSSION**

On the basis of extensive experience with colonization experiments done at different times under the standard and modified (e.g., incubation up to 28 days with media replacement but no washing of the plants at 7 and 14 days) assay conditions, we believe that the fungal propagule dynamics shown by the assay provide a meaningful index of fungal colonization (and, with less certainty, lack of colonization). However, as noted in the introduction, quantifying plant colonization by fungi is severely complicated by lack of appropriate routine methods for determining forms, amounts, and distribution of fungal biomass. Enumeration of viable propagules is a compromise approach and has theoretical limitations. Foremost among these is that it does not discriminate between different kinds and sizes of propagules (spores versus mycelial fragments) and, accordingly, cannot be used as an absolute index of fungal biomass (nor can any other method without a conversion factor).

For our test fungi, it appears reasonable to assume from other work (6, 26) that viable fungal propagules in the plant growth medium (nonattached propagule category) and in washes of the plant (loosely attached propagules) reflect a mixture of spores and, to a lesser extent, fragments of mycelia. This assumption is supported experimentally by (i) confirmatory periodic microscopic examination of these propagule categories for different fungi and (ii) barely detectable viable counts in the nonattached and negligible counts in the loosely attached category for the sterile fungus (in the presence of readily measurable tightly attached counts). In contrast, reestablishment of high levels of nonattached and loosely attached propagules of *C. gloeosporioides* and *A. curvulum* following repeated removal of such propagules by washing is strong evidence for colonization.

Interpretation of the dynamics of the tightly attached category, obtained from plant macerates, is complicated in principle by the possibility of highly variable decreases in viable propagules caused by abrasion-induced death or, conversely, of increases by mechanical shearing of mycelial fragments. The magnitude of the effect could depend on growth phase or maceration conditions or both. A preliminary experiment on the effect of maceration time (1, 5, and 10 min) on viable propagule counts of the sterile fungus showed no appreciable effect of maceration time. In addition, (i) tightly attached propagule numbers were very consistent for replicates within a single run, (ii) tightly attached propagule numbers were similar over an extended time for a given fungus, (iii) the population dynamics of the fungi were...
generally similar in different experiments, and (iv) tightly attached and loosely attached counts (the latter being derived from washes that were not macerated) were highly correlated. In aggregate this evidence suggests that the CFU-based assay provides meaningful information on fungal colonization, despite the theoretical objections acknowledged earlier. While conclusions based on two or more distinct methods would be desirable, it is not clear which other approaches would be the most useful complement to the relatively sensitive CFU approach, nor is it clear that the vastly increased effort needed for inclusion of other methods would be merited in terms of correspondingly more reliable interpretations of fungal colonization trends.

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 our colonization experiments, the fungi examined varied considerably in the 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 C. gloeosporioides, the only pathogen of milfoil included, was not a demonstrably better colonist than A. curvulum.

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 reflect either a balance between birth and death in an active population or the survival and retention of dormant propagules. In our experiments the former explanation must apply, since (except for the sterile fungus) quantitatively significant numbers of nonattached and loosely attached fungal CFU were washed out at the end of each phase of the experiment. Thus, the population of a fungus such as Cladosporium herbarum, which maintained a constant tightly attached population size throughout both 3-day growth periods, must have included metabolically active members.

We found, as have others (17), that attachment (14, 19, 38) is a rather poor predictor of longer-term microbial colonization. Attachment was fairly uniform, as shown by the few significant differences among 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. The failure of attachment as a predictor of growth is not unexpected, since microbial traits which promote attachment (9–11, 39) are likely to be distinct from those which favor growth.

Of necessity, the appropriate time period for this assay was decided somewhat arbitrarily. Shortening it to less than 4 days would allow little time for fungal growth or decline and would make changes in microbial populations difficult to detect. Lengthening the assay in runs 8 to 11 to 28 days did not alter our relative ranking of the two representative fungi tested in those experiments, C. gloeosporioides and the unidentified sterile fungus. Assays lasting more than 28 days would be complicated by substantial growth of the plant, and conditions under which colonization is assessed would change. M. spicatum leaves are relatively short-lived, and longer assays would inevitably involve saprophytic or opportunistic colonization of unhealthy leaves. In overview, duration of the assay had little effect on the evaluation of fungal colonization ability, at least for the fungi and time range examined.

Colonization of plant surfaces in nature proceeds under conditions quite different from those of our laboratory assay. Perhaps the most significant difference is the relative simplicity of the microbial community on culture-grown plants. The effects of a more complex indigenous community on colonization are not known and cannot be predicted readily. Some members may compete with the colonist and reduce colonization. Others may facilitate colonization by producing a biofilm to which other organisms more or less selectively attach (23). 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. We demonstrated here 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.

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LITERATURE CITED


