Azolla filiculoides Nitrogenase Activity Decrease Induced by Inoculation with Chlamydomonas sp.†

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Experiments were conducted to determine the influence of Chlamydomonas sp. on nitrogen fixation (C2H2 → C2H4) in Azolla filiculoides and on the nitrogen fixation and growth of free-living Anabaena azollae 2B organisms. Inoculation of azolla medium with Chlamydomonas sp. was associated with decreased nitrogenase activity in A. filiculoides and with increases in the density of a fungal population identified as Acremonium sp. Subsequent inoculation of azolla medium with this fungus was also accompanied by a significant decrease in nitrogenase activity of A. filiculoides. However, the extent of depression of nitrogenase activity was significantly higher when azolla medium was inoculated with Chlamydomonas sp. than when it was inoculated with Acremonium sp. Inoculation of nitrogen-free Stanier medium with either Acremonium sp. or Chlamydomonas sp. did not adversely affect the growth or nitrogenase activity of free-living A. azollae. Decreased nitrogenase activity in A. filiculoides is apparently related to the adverse influence of the green alga and the fungus on the macro symbiont. The mechanisms that might be involved are discussed.

Among the nitrogen-fixing plant-cyanobacterium associations that are currently known, the most frequently studied and perhaps the most important one is the Anabaena sp.-Azolla sp. association. Azolla sp. is a free-floating aquatic fern of worldwide occurrence whose usefulness as a source of nitrogen for lowland rice cultivation has long been recognized in parts of Asia, particularly in Vietnam and in the People's Republic of China (2).

The nitrogen fixation potentials of the Anabaena sp.-Azolla sp. association have been variously estimated, with some estimations placing it on an equal footing with the Rhizobium sp.-legume association (2, 7).

Like the Rhizobium sp.-legume association, the Anabaena sp.-Azolla sp. association is susceptible to restrictions imposed by biotic and abiotic factors. The most widely recognized biotic factors limiting the productivity of Azolla sp. are insects, diseases, and aquatic weeds (7, 14). Biotic factors that could limit the N2 fixation activity of the Azolla sp.-Anabaena sp. association are not, however, limited to the above categories. Field surveys of Azolla sp. and cyanobacterial populations in Hawaii (Habte, unpublished data) have indicated that populations of green and other algae that tend to antagonize Azolla sp. as well as cyanobacteria appear to be abundant in Azolla sp.-supporting habitats of Hawaii. Greenhouse and laboratory observations have also shown that failure to maintain and multiply healthy Azolla sp. cultures is often associated with the buildup of algal populations such as Chlamydomonas, Cladophora, Spirogyra, and Stigeoclonium species (Habte, unpublished data). These algal developments in turn associated with the colonization of fronds of Azolla sp. by fungi. The interference of the green alga Spirogyra sp. in the multiplication of Azolla sp. has also been recently reported by investigators in India (9). Quantitative information on the extent to which green algae influence nitrogen fixation activities of the Anabaena sp.-Azolla sp. association is nonexistent.

The objective of this investigation was to determine the influence of Chlamydomonas sp. on the nitrogenase activity of Azolla filiculoides and on the growth and nitrogenase activity of Anabaena azollae 2B.

MATERIALS AND METHODS

Cultures and media used. A. filiculoides was obtained from the collection of the Department of Agronomy and Soil Science, University of Hawaii. Tips of very young and healthy fronds were cut and surface sterilized as described by Watanabe et al. (14). The frond tips were then inoculated into 3-liter black polyethylene beakers containing sterile medium consisting of 154 mg of MgSO4·7H2O, 42 mg of CaCl2, 67 mg of K2SO4, 44 mg of KH2PO4, 13 mg of NaCl, 40 μg CoCl2·6H2O, 0.4 mg of CuSO4·5H2O, 0.56 mg of H2BO3, 3.6 mg of MnCl2·4H2O, 0.25 mg of Na2MoO4·2H2O, 2.2 mg of ZnSO4·7H2O, and 42 mg of iron sequestrene (10% Fe) per liter of distilled water. The pH of the medium was adjusted to 6.0 before autoclaving. Every other day, the fronds were transferred into fresh sterile medium to maintain the culture in a clean state. The beakers containing the culture were incubated at 26°C in a Biotronette Mark III environmental chamber (Labline Instruments Inc., Melrose Park, Ill.) maintained at maximum fluorescent light intensity.

Free-living Anabaena azollae 2B was obtained from J. W. Newton of the Northern Regional Research Center, U.S. Department of Agriculture, Peoria, Ill. The culture was maintained and cultivated in a sterile nitrogen-free modification of Stanier medium (10). Cultures were maintained at 26°C under subdued light in 124-ml Erlenmeyer flasks containing 50-ml portions of the medium. Cells were grown by inoculating 100-ml portions of the medium in 250-ml Erlenmeyer flasks and incubating the flasks at 26°C in the Biotronette environmental chamber at maximum fluorescent light intensity. The medium amended with 1.75 or 15.0 g of agar per liter was used for assessing the nitrogenase activity of Anabaena azollae or for enumerating its CFU by the standard plate count technique.

Chlamydomonas sp. was isolated from partially deteriorating fronds of A. filiculoides. The green algal cells were first allowed to multiply rapidly by inoculating pieces of the

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necrotic fronds into 250-ml Erlenmeyer flasks containing 100-ml portions of azolla medium amended with 0.25 g of KNO₃ per liter and incubating the flasks at 26°C in the Biotronette environmental chamber at maximum fluorescent light intensity. After 10 to 15 days of incubation, serial dilutions of the turbid suspension were prepared. The dilution suspensions were poured plated on N-containing azolla medium agar. Plates were incubated at 26°C 10 cm directly below a 40-W cool white fluorescent lamp for 10 to 15 days, after which time individual colonies were picked up, further purified by streak plating, and transferred into nitrogen-containing azolla medium for multiplication and identification. Purity was ascertained by the lack of turbidity in nutrient broth when it was inoculated with *Chlamydomonas* sp. organisms and incubated in the dark at 27°C. Pure cultures of *Chlamydomonas* sp. were maintained in nitrogen-containing azolla medium under subdued light. *Acremonium* sp. was isolated from necrotic azolla tissues and from azolla medium in which *A. filiculoides* was interacting with *Chlamydomonas* sp. Pieces of the fronds or 1.0 ml of the spent medium was transferred into azolla medium and vigorously vortexed for 1 min. The suspensions were serially diluted and then poured plated out on a modification of Vincent yeast-mannitol-agar medium (13). The medium had a pH of 6.0 and contained one-fourth of the normal amounts of mannitol and yeast extract per liter and 30 µg of streptomycin per ml. Plates were incubated in the dark at 26°C for 4 days, and single colonies were picked up and grown on modified yeast-mannitol-agar slants for further identification or maintenance at 4°C. The fungus was identified by using the diagnostic features outlined by Domsh et al. (3) and through the close consultation of M. Aragaki, Department of Plant Pathology, University of Hawaii. Cells were multiplied by inoculating spores and hyphae into 100-ml portions of modified yeast-mannitol broth in 250-ml Erlenmeyer flasks. The flasks were incubated on a rotary shaker (100 rpm) at 26°C in the dark for 4 days before cells were harvested.

Microbial cultures were harvested by centrifugation at 8,000 × g and washed twice in saline (4). This saline was also used for suspending pellets to the original volume and for preparing serial dilutions of samples. All media used in this study were sterilized by autoclaving at 121°C for 20 min at 15 lb/in². Heat inactivation of inocula was also achieved by autoclaving at 121°C for 10 min.

**Influence of Chlamydomonas sp. on *A. filiculoides***. Portions (200 ml) of sterile azolla medium in 500-ml Erlenmeyer flasks were inoculated with 0.15 g (fresh weight) of surface-sterilized *A. filiculoides* multiplied as described above. The flasks were also inoculated with 5.0 ml of a washed suspension of a 15-day-old *Chlamydomonas* sp. culture before and after heat inactivation. Flasks were incubated at 26°C in the Biotronette environmental chamber at maximum fluorescent light intensity. At regular intervals, the nitrogenase activity of the ferns was determined. For this purpose, the original flasks from each treatment were sealed with serum stoppers after the medium in the flask was brought up to its original volume. Ten percent of the air in the flasks was then replaced with C₂H₂, and the flasks were incubated under the same conditions used for growing the ferns. After 2 h of incubation the headspace was sampled for C₂H₂ and C₂H₄ content. The gasses were analyzed on a Perkin-Elmer Sigma 3B gas chromatograph equipped with a flame ionization detector and a 1-m column packed with a 2.0 g of phenylisocyanate-Porasil c (Applied Sciences Laboratories Inc., Gardena, Calif.). The acetylene reduction assay was determined on six replicates per treatment. Upon termina-

tion of the assay, the contents of the flasks were vigorously shaken by hand to release propagules of fungi and *Chlamydomonas* sp. into the medium. Portions (1 ml) of the medium were then withdrawn, and serial dilutions were prepared for determining the numbers of CFU of *Chlamydomonas* sp. and fungi. Fungal CFU were recovered on the modified yeast-mannitol-agar, whereas the CFU of *Chlamydomonas* sp. were recovered on N-containing azolla medium. The former plates were incubated at 26°C in the dark for 4 days, whereas the latter plates were incubated at 26°C 10 cm directly below a cool-white fluorescent light source for up to 15 days.

**Relative influence of Acremonium sp. or Chlamydomonas sp. on *A. filiculoides***. The relative influence of *Acremonium* sp. or *Chlamydomonas* sp. on the acetylene reduction activity of *A. filiculoides* was determined by employing procedures that are similar to those used for determining the influence of *Chlamydomonas* sp. on the fern. Portions (5.0 ml) (5.0 × 10⁵ CFU/ml) of a washed suspension of the 4-day-old fungus or 5.0 ml (4.9 × 10⁵ CFU/ml) of the washed suspension of a 15-day-old culture of *Chlamydomonas* sp. before and after heat inactivation was inoculated into flasks containing azolla medium and *A. filiculoides* plants. The flasks were incubated at 26°C in the Biotronette environmental chamber for 18 days before acetylene reduction assays were made. Acetylene reduction assays were made as described above, except in this case only the activity at day 18 was measured, and this activity was monitored at 0.5-h intervals for 2.5 h.

**Influence of Acremonium sp. or Chlamydomonas sp. on the nitrogenase activity of Anabaena azollae**. Portions (5 ml) of nitrogen-free sterile semisolid Stanier medium in 20-ml test tubes were inoculated with 0.2 ml (2.9 × 10⁴ CFU/ml) of a washed suspension of *Anabaena azollae* and 0.2 ml (5.2 × 10⁴ CFU/ml) of the washed suspension of a 4-day-old culture of *Acremonium* sp. or 0.2 ml (5.5 × 10⁵ CFU/ml) of a washed suspension of a 15-day-old culture of *Chlamydomonas* sp. Inoculation with the latter two microorganisms was done before or after heat inactivation. Tubes were incubated at 26°C in the Biotronette environmental chamber at maximum fluorescent light intensity for 10 days. The tubes were then sealed with serum stoppers, and the acetylene reduction activity of the cells was determined as described above.

**Interaction of Acremonium sp. with Chlamydomonas sp.** Portions (1 ml) of a washed suspension of a 4-day-old culture of *Acremonium* sp. with or without 1.0 ml of a washed suspension of a 15-day-old culture of *Chlamydomonas* sp. were inoculated into 50 ml of azolla medium amended with 0.06% nitrogen. Similar inoculations were also made into the medium after heat inactivation of the cells. Inocula were applied in triplicate. Flasks were incubated at 26°C in the Biotronette environmental chamber at maximum fluorescent light intensity. Samples (1 ml) were aseptically withdrawn from the flasks at regular intervals, and the numbers (CFU) of *Acremonium* sp. and *Chlamydomonas* sp. determined by the standard plate count technique as described before.

**Population changes of *A. azollae* interacting with Acremonium sp. or Chlamydomonas sp.** Portions (50 ml) of sterile nitrogen-free Stanier medium in 125-ml Erlenmeyer flasks were inoculated with 1.0 ml of a washed suspension of a 15-day-old culture of *Anabaena azollae*. Some of these flasks were also inoculated with 1.0 ml of a washed suspension of a 4-day-old culture of *Acremonium* sp. or 15-day-old culture of *Chlamydomonas* sp. before or after heat inactivation. The density of the interacting populations was regularly monitored by employing procedures and media already described.
RESULTS

Figure 1 represents a summary of the nitrogenase activity (C\textsubscript{2}H\textsubscript{2} \rightarrow C\textsubscript{2}H\textsubscript{4}) values obtained when \textit{A. filiculoides} was grown in uninoculated medium or in medium inoculated with \textit{Chlamydomonas} sp. The nitrogenase activity of \textit{A. filiculoides} in the inoculated medium, expressed as a proportion of that observed in the uninoculated medium, decreased with time. The values recorded on day 24 showed that the presence of \textit{Chlamydomonas} sp. was associated with the loss of as high as 65\% of the activity inherent in \textit{A. filiculoides} grown in the absence of the green alga.

The changes in the densities of \textit{Chlamydomonas} sp. and fungi associated with algal inoculation are illustrated in Fig. 2. The number of \textit{Chlamydomonas} sp. CFU in the uninoculated medium did not exceed 100/ml for up to 12 days. Beyond this point the population of the alga increased rapidly, attaining a maximum density of about 10\textsuperscript{6} CFU/ml. On the other hand, in medium inoculated with the alga the initial density of the alga was in the vicinity of 10\textsuperscript{4} cells/ml. This population increased slowly until day 18, after which a stable density of about 10\textsuperscript{6} cells per ml was maintained. The changes in fungal CFU closely followed those of the alga in both the uninoculated and inoculated (with alga) medium; there were about 10\textsuperscript{4} and 10\textsuperscript{6} fungal CFU per ml respectively, at the end of the experiment, and 90 to 95\% of the colonies of the fungi appearing on the recovery medium were identified as \textit{Acremonium} sp. These results demonstrate that the development of \textit{Acremonium} sp. in azolla medium was closely linked with the development of \textit{Chlamydomonas} sp. in the medium. To further verify this observation an experiment was conducted in which the two microbial populations were allowed to grow together or separately in a nitrogen-containing azolla medium (Table 1). \textit{Chlamydomonas} sp. clearly was capable of stimulating the growth of the fungus, whereas the growth of the alga did not appear to be influenced by the presence of \textit{Acremonium} sp.

Since inoculation of azolla medium with \textit{Chlamydomonas} sp. had led to depressed nitrogenase activity of the \textit{Anabaena} sp.-Azolla sp. association and since this decline in activity was associated with a buildup in the population of \textit{Acremonium} sp., a study was undertaken to determine the extent to which nitrogenase activity was influenced by each of these microorganisms. The results showed that both \textit{Acremonium} sp. and \textit{Chlamydomonas} sp. were capable of decreasing nitrogenase activity in \textit{A. filiculoides}, although the greatest decrease in activity was associated with the inoculation of \textit{Chlamydomonas} sp. (Fig. 3).

TABLE 1. Interaction of \textit{Chlamydomonas} sp. with \textit{Acremonium} sp. in nitrogen-amended azolla medium\textsuperscript{a}

<table>
<thead>
<tr>
<th>Day</th>
<th>CFU/ml, 10\textsuperscript{3}</th>
<th>\textit{Chlamydomonas} sp.</th>
<th>\textit{Acremonium} sp.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>\textit{Chlamydomonas} sp.</td>
<td>\textit{Acremonium} sp.</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.61 ± 0.08</td>
<td>0.63 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>0.73 ± 0.19</td>
<td>16 ± 4.9</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>0.80 ± 0.1</td>
<td>55 ± 24.8</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>1.2 ± 0.25</td>
<td>16 ± 6.0</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>0.9 ± 0.25</td>
<td>11 ± 3.9</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a} Values represent means of three replicates ± 95\% confidence intervals.
Experiments were also conducted to determine the influence of *Acremonium* sp. or *Chlamydomonas* sp. on the growth of *A. azollae*. Neither the presence of the green alga nor that of the fungus had significant influence on the growth of *A. azollae*, whereas the growth of the former two organisms was stimulated as a result of their association with the cyanobacterium (Tables 2 and 3). Likewise, the presence of *Chlamydomonas* sp. did not have significant influence on the nitrogenase activity of *A. azollae* (Fig. 4). On the other hand, the cyanobacterium had significantly higher nitrogenase activity in the presence of *Acremonium* sp. than in its absence (Fig. 4).

**DISCUSSION**

Since the growth of *Acremonium* sp. is closely related to the growth of *Chlamydomonas* sp., it appears that the alga influences nitrogenase activity in *A. filiculoides* not only by its deleterious interaction but also by stimulating the development of an antagonistic fungus. The direct relationship between the density of *Acremonium* sp. and *Chlamydomonas* sp. is explainable in terms of the release of algal extracellular products which are essential for the growth of *Acremonium* sp. This observation is consistent with the well-known role algal excretions play in the nutrition of associated organisms (1, 5).

The development of *Chlamydomonas* sp. and *Acremonium* sp. in azolla medium, despite the fact that the medium was inoculated with *A. filiculoides* started from surface-sterilized frond tips, would suggest that the microorganisms were capable of colonizing the fern internally. This observation is supported by microscopic examinations, which revealed that both *Acremonium* sp. and *Chlamydomonas* sp. were capable of colonizing the leaf cavities of *A. filiculoides* as well as the interior of its roots. Much of the deleterious effects caused by these microorganisms could be a result of the loss of dissolved nutrients as well as fixed nitrogen, for which they could compete effectively with *A. filiculoides* by virtue of their intimate contact with the fern, and their tendency, particularly the tendency of *Chlamydomonas* sp., to proliferate in it. Moreover, the possibility that

**TABLE 2. Growth of *A. azollae* in the presence or absence of *Chlamydomonas* sp.**

<table>
<thead>
<tr>
<th>Day</th>
<th>CFU/ml, 10^3</th>
<th><em>Chlamydomonas</em> sp.</th>
<th><em>A. azollae</em></th>
<th><em>Chlamydomonas</em> sp. + <em>A. azollae</em></th>
<th><em>Chlamydomonas</em> sp. - <em>A. azollae</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4.5 ± 0.5</td>
<td>4.7 ± 0.5</td>
<td>5.1 ± 1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>48 ± 12</td>
<td>64 ± 6</td>
<td>170 ± 58</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>210 ± 45</td>
<td>190 ± 69</td>
<td>150 ± 49</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>300 ± 99</td>
<td>273 ± 99</td>
<td>310 ± 75</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>260 ± 112</td>
<td>200 ± 72</td>
<td>160 ± 50</td>
<td></td>
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</tbody>
</table>

* Values represent means of three replicates ± 95% confidence intervals.
these microorganisms secrete substances that might be toxic to *A. filiculoides* cannot be ruled out. Many green algae, including those that parasitize land plants, are known to liberate such potent biologically active compounds as peroxidases, polyphenols, and antibiotics during active growth as well as in senescence (6, 8).

The decline in the nitrogenase activity of the *Anabaena* sp.-Azolla sp. association in the presence of *Acremonium* sp. or *Chlamydomonas* sp. or both cannot be explained in terms of antagonism of these microorganisms to *Anabaena azollae*, since neither the growth of the cyanobacterium nor its nitrogenase activity was adversely influenced by the presence of the green alga or the fungus in culture medium.

The ability of *Chlamydomonas* sp. to grow in nitrogen-free media in the presence of *A. filiculoides* or free-living *A. azollae* indicates that the green alga was deriving its nitrogen from fixed nitrogen excreted into the medium by *A. filiculoides* or *A. azollae*. In the course of their growth, cyanobacteria and *A. filiculoides* are known to liberate substantial quantities of combined nitrogen and other compounds essential for growth (7, 11, 12). It will be interesting to know to what extent nitrogenase activity would be enhanced if *A. filiculoides* could be grown free of the above antagonistic microorganisms. However, such an effort is likely to be frustrated by the extreme sensitivity of *A. filiculoides* to antimicrobial agents.

**ACKNOWLEDGMENTS**

I thank M. Aragaki for his assistance in the identification of the antagonistic fungus and I. Abbot for her confirmation of the identity of the green alga.

**LITERATURE CITED**