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Nitrogen Fixation (Acetylene Reduction) Associated with Duckweed (*Lemnaceae*) Mats

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Duckweed (*Lemnaceae*) mats in Texas and Florida were investigated, using the acetylene reduction assay, to determine whether nitrogen fixation occurred in these floating aquatic macrophyte communities. N_2-fixing microorganisms were enumerated by plating or most-probable-number techniques, using appropriate N-free media. Results of the investigations indicated that substantial N_2-fixation (C_2H_2) was associated with duckweed mats in Texas and Florida. Acetylene reduction values ranged from 1 to 18 μmol of C_2H_4 g (dry weight)$^{-1}$ day^{-1} for samples incubated aerobically in light. Dark N_2 fixation was always two- to fivefold lower. 3-(3,4-Dichlorophenyl)-1,1-dimethylurea (7 to 10 μM) reduced acetylene reduction to levels intermediate between light and dark incubation. Acetylene reduction was generally greatest for samples incubated anaerobically in the light. It was estimated that 15 to 20% of the N requirement of the duckweed could be supplied through biological nitrogen fixation. N_2-fixing heterotrophic bacteria (10^5 cells g [wet weight]$^{-1}$ and cyanobacteria (10^5 propagules g [wet weight]$^{-1}$ were associated with the duckweed mats. *Azotobacter* sp. was not detected in these investigations. One diazotrophic isolate was classified as *Klebsiella*.

Numerous investigators have reported the occurrence of free-living nitrogen-fixing microorganisms in association with aquatic macrophytes. These associations include both freshwater and marine plants, such as sea grasses (1, 12), floating marine macroalgae (5), salt marsh vegetation (9, 19), mangrove vegetation (22), rice grown in waterlogged soils (20), and floating freshwater macrophytes (4, 17; T. P. Duong, Ph.D. thesis, Michigan State University, East Lansing, 1972). In these associations the diazotrophs occur as epiphytes on or in leaves, shoots, and roots of the macrophyte "host." Many of these reports indicate that significant quantities of combined nitrogen are made available to the macrophyte as a result of the activities of the nitrogen-fixing constituents in these associative symbioses.

Duckweeds are small floating plants (members of the *Lemnaceae*) which occur throughout the world and frequently develop extensive mats on ponds and lakes. These mats may range from a few millimeters to several centimeters in thickness, and substantial biomass production is associated with the mats. Productivity values as high as 7,100 kg ha^{-1} year^{-1} have been reported (6).

The purpose of this study was to investigate the occurrence and extent of biological nitrogen fixation associated with duckweed mats in Texas and Florida, using the acetylene reduction (AR) assay, and to characterize N_2-fixing populations involved in the association.

MATERIALS AND METHODS

Plant materials. Plant material for these studies was collected from ponds in College Station, Tex., and Gainesville, Fla., containing duckweed mats in various stages of development, i.e., mat thickness (1 to 2 cm) and area of cover. A total of six different ponds were investigated. In some cases the mats were as thick as 2 cm, probably due to piling by wind action. Materials collected in midsummer (June) consisted primarily of *Lemna* sp., whereas those collected later (September to November) contained *Wolffia* sp. in addition to *Lemna* sp.

The mats were sampled in a random manner over a 1-m^2 area. Plant material was placed in plastic pails containing pond water, returned to the laboratory, and processed for AR assays.

Incubation conditions. In preparation for AR (C_2H_2) assays, samples of duckweed were washed several times with distilled water and collected on nylon mesh (100 μm) with suction. The material was pressed gently to remove excess water. Unwashed samples were also subjected to C_2H_2 reduction assays. Replicate samples (5 to 10 g, fresh weight) from washed and unwashed batches were placed in screw-cap Erlenmeyer flasks (125 ml) or 0.5-pint (240-ml) wide-mouth screw-top jars containing 20 or 30 ml of distilled water. The jars and flasks were fitted with serum stoppers for gas sampling.

Samples were incubated in the dark by wrapping in aluminum foil, whereas most lighted samples were held in constant light (135-W, CW, VHO fluorescent tubes; 420 μE m^{-2} s^{-1}) unless otherwise stated. Samples incubated anaerobically were flushed thoroughly with argon before gassing with C_2H_2. All experiments were conducted at 25 to 30°C.

Effects of DCMU on C_2H_2 reduction. 3-(3,4-Dichlorophenyl)-1,1-dimethylurea (DCMU) was used to inhibit nitrogen-fixing organisms relying on photosystem II and to inhibit photosynthesis by the duckweed plants. To determine the effects of DCMU on AR associated with duckweed mats, plant materials were incubated in distilled water containing DCMU (7 to 10 μM). Plant materials exposed to DCMU were assayed under aerobic and anaerobic conditions in the light and in the dark.

CO_2 enrichment. To determine the effects of elevated levels of CO_2 on C_2H_2 reduction associated with mat samples, six replicate samples were flushed with air containing 5% CO_2 (Matheson Co.) and six were flushed with normal air (0.03% CO_2). All samples were incubated in the light as described above, with the exception that a 14-h light period followed by a 10-h dark period were imposed.

C_2H_2 reduction assays. All samples treated as described above were incubated in the presence of 0.11 atm of C_2H_2. This was accomplished by replacing 11% of the enclosed gas phase (air or argon) with C_2H_2 (Matheson Co.). All treatments were assayed with three replicate samples. Data are presented as the means of three replicates.

Ethylene (C_2H_4) production was determined by gas chromatography, using a hydrogen flame ionization detector and a stainless-steel column (0.3-cm outside diameter by 182 cm) containing Porapak N. Peak heights were compared with those from samples of known concentrations of ethylene.

Microbial enumeration. Enumeration of nitrogen-fixing bacteria and cyanobacteria associated with homogenized plants was accomplished on a limited number of samples by most-probable-number techniques, using the appropriate N-free media. Heterotrophic bacteria were enumerated by using the medium of Okon et al. (11) modified to contain glucose as the carbon and energy source in soft-agar deeps. N_2-fixing algae were estimated by using BG-11 medium of Stanier et al. (17). Tubes were capped with serum stoppers, gassed with 0.11 atm of C_2H_2, and incubated for 24 h before analysis for ethylene production. The tubes giving positive AR indicated the presence of diazotrophs.

Isolation of *Azotobacter* sp. from homogenized plant tissues was attempted with an alkaline N-free medium (13) incubated aerobically. These estimates were conducted by the dilution spread plate technique.

Duckweed biomass. The biomass of a mat containing only *Lemna minor* was determined on one occasion (June 1981), using material from a stock pond located in a cattle pasture in College Station, Tex. The mat completely covered the pond and was approximately 1.5 to 2 cm thick. Biomass was estimated by taking nine samples with a 0.11-m^2 quadrat dropped randomly onto the mat. All plant material in the quadrat was collected and weighed after expressing excess water from the sample bags. Dry weights were determined after drying to a constant weight at 80°C.

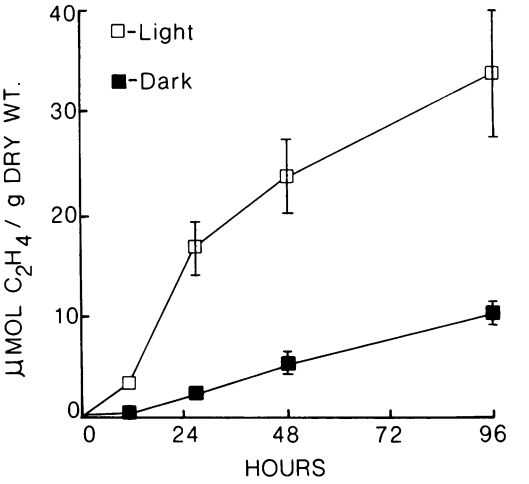

FIG. 1. AR by duckweed mat samples collected in Gainesville, Fla. (June 1977). This particular set of samples exhibited the greatest activity observed in these studies.

RESULTS

The results of the initial AR assay on washed duckweed samples from Gainesville, Fla., are shown in Fig. 1. Substantial rates of C_2H_2 reduction (AR) were associated with this washed plant material, and samples incubated in the light reduced acetylene at considerably greater rates than samples incubated in the dark. The temperature inside light and dark containers did not differ by more than 1 or 2°C. It was noteworthy that C_2H_2 reduction commenced without a lengthy lag period and was nearly linear for at least a 24-h incubation period.

C_2H_2 reduction rates of washed and unwashed duckweed mat samples were further compared under light or darkness with or without DCMU (Fig. 2). There was little difference in the rates of AR between washed and unwashed mat samples except on one occasion (Fig. 3), when washing reduced the activity of light-incubated samples about 42%. Activity was generally reduced only slightly by washing, indicating that diazotrophic microorganisms were well attached to the small macrophytes. C_2H_2 reduction of light-incubated samples was approximately fivefold greater than that of dark-incubated samples. DCMU suppressed but did not eliminate AR. The DCMU concentration (7 to 10 μM) used in these experiments should have been sufficient to inhibit photosynthesis by any algae in the system as well as by the duckweed plants. That AR was not completely eliminated by DCMU and the rates exceeded those of dark-incubated samples strongly suggest that some phototrophic AR was not suppressed by the compound. It is also probable that the cyanobacteria continued to fix

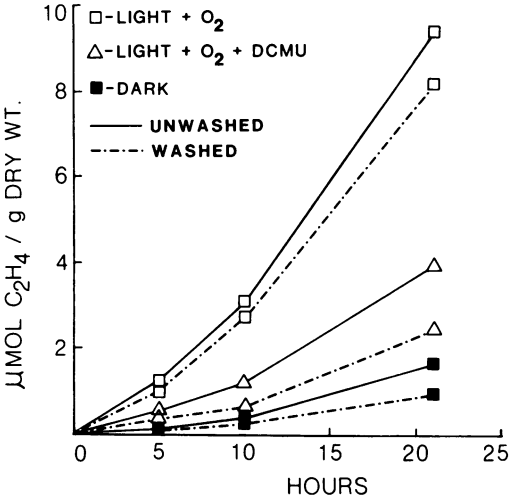

FIG. 2. Effects of washing (five times in distilled water) and addition of DCMU on C_2H_2 reduction by duckweed mat samples from Gainesville, Fla. (June 1977).

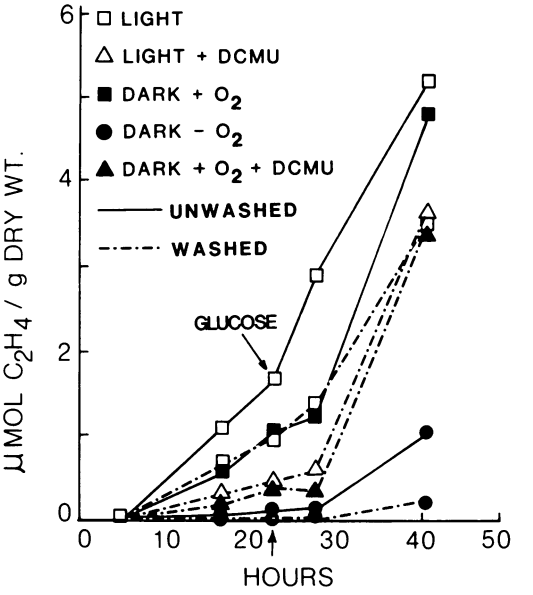

FIG. 3. AR by duckweed samples collected from a second site in Gainesville, Fla. (September 1977). Note the stimulation of nitrogenase (C_2H_2) activity by glucose (1.0 ml of 1 M solution added to all treatments) in both the light and dark samples containing DCMU and incubated aerobically.

nitrogen heterotrophically. Also, photosynthate production by the duckweed and subsequent use by diazotrophs may not have been eliminated. This phenomenon was observed in all subsequent assays in which DCMU was used.

The effect of DCMU on AR by dark-incubated samples was determined as part of several separate experiments from both the Florida and Texas sampling sites. Although Fig. 3 and 4 are for Florida samples only, similar results were observed with mat samples from Texas sites (data not shown). Data shown in Fig. 4 (and additional data not shown) generally indicate that DCMU did not appreciably modify the low rates of AR observed for dark-incubated samples (1 and 1.2 nmol g^{-1} h^{-1} with and without DCMU, respectively). One exception to this is shown in Fig. 3, where DCMU apparently caused a twofold reduction of AR by dark-incubated samples. This reduction did not occur in any other experiment. Also shown in Fig. 3 and 4 is the effect which glucose amendment (1 ml of a 1 M solution; final concentration, 10 mg ml^{-1}) exerted on samples after 24 h of incubation. Glucose stimulates AR under all incubation conditions. However, dark anaerobic samples consistently showed the lowest increases in AR. Also, glucose stimulates a diazotrophic population in light-incubated samples suppressed by DCMU.

Duckweed mat samples collected in College Station, Tex., 1 year after the Florida samples were assayed exhibited quite similar patterns of AR (Fig. 5 and 6). Light-incubated samples were generally at least twice as active as dark-incubated samples. Samples incubated in the light under an argon atmosphere were usually most active in AR (Fig. 5 and 6). As with the Florida samples, DCMU reduced AR to a level intermediate between light and dark samples but did not eliminate it. On one occasion (July 1981) samples of *Azolla* were subjected to the same type of treatments as duckweed samples. C_2H_2 reduction by the *Azolla* was greatest for light anaerobic conditions (40 μmol g [dry weight]$^{-1}$ day^{-1}), and DCMU caused a fivefold decrease in activity (whether incubation was aerobic or anaerobic). C_2H_2 reduction in the dark (97 nmol g [dry weight]$^{-1}$ day^{-1}) was 76-fold lower than in the light (7,400 nmol g [dry weight]$^{-1}$ day^{-1}) and 15-fold lower than samples incubated in light with DCMU (1,452 nmol g [dry weight]$^{-1}$ day^{-1}). This suggests that the algal symbiont is not fully inhibited by the DCMU or that it functions heterotrophically on stored reserves. Failure of DCMU to fully suppress AR in duckweed mats is probably due at least in part to similar phenomena. It appears that the patterns of AR activity as demonstrated for both Florida (three sites) and Texas (three sites) samples result from colonization of the mats by similar physiological groups of microorganisms.

Enhancing the CO_2 concentration in the flasks had no effect on AR (data not shown). There were no significant differences (0.05% level)

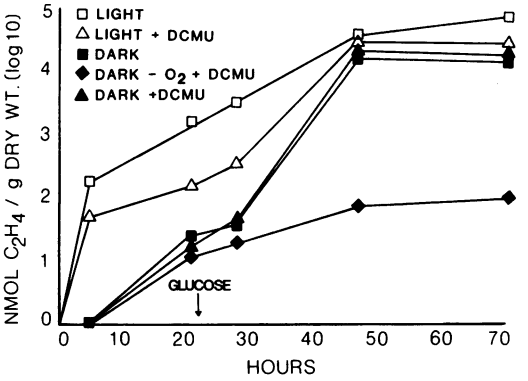

FIG. 4. AR associated with duckweed mat samples in late September 1977. Note the approximate 10-fold decrease due to DCMU and the low activity of all samples incubated in the dark before glucose addition. Glucose was added to all treatments after 24 h of incubation (arrow). Note that C_2H_4 production is expressed as $\log_{10}$ nanomoles of C_2H_4 per gram (dry weight). Samples were collected in Gainesville, Fla.

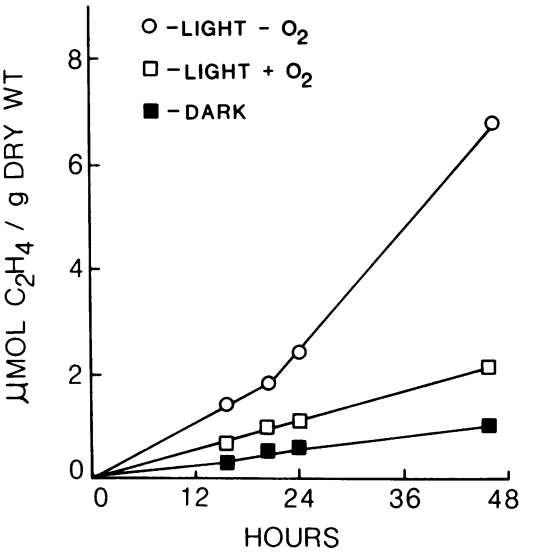

FIG. 5. AR associated with duckweed collected at College Station, Tex. (March 1979). Note the enhanced nitrogenase (C_2H_2) activity of samples incubated in the light under an argon atmosphere.

between the means of six replicates each of CO_2-enriched (5%) and nonenriched (0.03%) samples. In all of these experiments, ambient pond water as well as the wash water was assayed with the plant materials. These samples routinely produced negligible ethylene in comparison to the mat samples.

Attempts to obtain *Azotobacter* sp. with the alkaline nitrogen-free medium were unsuccessful. Organisms characteristic of *Azotobacter* were not recovered with the medium, although other unclassified aerobic diazotrophs were encountered.

By using the most-probable-number technique with soft-agar glucose deeps, populations of aerobic (and probably facultative) nitrogen-fixing bacteria were estimated to be approximately 10^5 diazotrophs g of fresh plant material (roots and fronds)$^{-1}$. Similar populations of cyanobacteria (10^5 propagules g [wet weight]$^{-1}$) were estimated. Both heterocystous and non-heterocystous forms were observed in tubes exhibiting AR. No attempts were made to assure that the tubes were free of bacteria. Though not illustrated here, numerous cyanobacteria were observed attached to the small roots of *L. minor* viewed by fluorescence microscopy.

No attempts have been made at rigorous classification of the diazotrophic bacteria responsible for the AR. One isolate from Florida has been classified as a member of the genus *Klebsiella*.

The result of the biomass collection from the pasture stock pond indicated that there was 2.99 kg (wet weight) m^{-2} (178 g [dry weight] m^{-2}). This was equivalent to a standing biomass of 3 × 10^4 kg (wet weight) ha^{-1} (13.5 tons [wet weight] acre^{-1}).

DISCUSSION

The results of these investigations indicated that substantial rates of AR were associated with duckweed mats in Florida and Texas. This activity was observed throughout the summer months when the mats were present in various thicknesses and amounts of cover. As a general observation, it appears that AR was greater when the mats were denser (thick). The observed AR is a product of the entire microbial community associated with the macrophytes in the mat. The major part of the activity was due to phototrophic diazotrophs which are sensitive to DCMU (i.e., possess photosystem II). The inhibition by DCMU would eliminate light-driven nitrogenase activity by epiphytic cyanobacteria and would probably reduce algal excretion products which might serve as energy sources for the heterotrophic diazotrophs. The AR activity which remained after treatment with DCMU was possibly due to diazotrophic members of the *Rhodospirillaceae* in the epiphytic microbial community since they are reported to be relatively insensitive to DCMU (7). It may also have been due in part to the heterotrophic population utilizing carbon substrates leaked by the duckweed plants and in part to "dark" heterotrophic N_2 fixation by cyanobacteria. Although evidence has been presented for the presence of

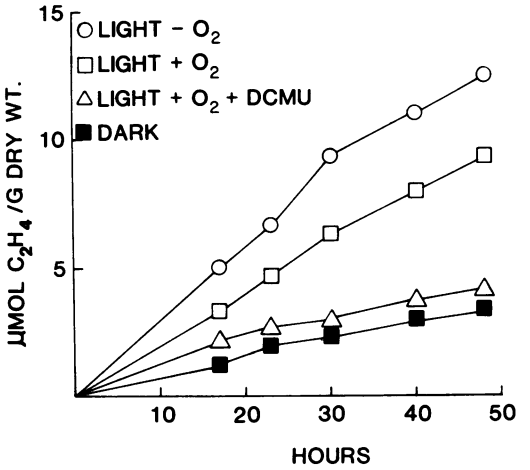

FIG. 6. AR associated with Texas duckweed samples (April 1980). Note that DCMU caused a partial reduction in nitrogenase (C_2H_2) activity as it did with Florida duckweeds.

N_2-fixing cyanobacteria and heterotrophic N_2-fixing bacteria, there is no direct information regarding non-cyanobacterial phototrophs, the role of which remains to be clarified.

Dark nitrogen fixation (C_2H_2) observed in these studies was most likely due to dark N_2 fixation by cyanobacteria and the heterotrophic utilization of macrophyte and algal excretion products as well as carbon liberated from senescent plants. Duckweed mats contain plants in all stages of development and senescence.

The duckweed mat is a community ideally suited to supporting a variety of nitrogen-fixing microbial groups. Morris and Barker (10) have shown that during peak daylight hours duckweed mats may become supersaturated with oxygen and, in the absence of light, they quickly reach very low oxygen tensions. These reduced O_2 tensions within the mats would provide favorable conditions for facultative diazotrophs and would perhaps enhance the nitrogen-fixing efficiency of the aerobic populations.

The enhanced activity exhibited by mat samples incubated anaerobically in the light might represent an expression of maximal community activity. Under these conditions one would expect that most of the organisms in the community would exhibit some nitrogenase activity. Photosynthetic oxygen evolution will meet some of the aerobic demand, whereas the reduced O_2 tension would allow activity by facultative and anaerobic organisms, including non-cyanobacterial phototrophs. These environmental conditions are not inconceivable in dense mats of duckweed where the bottom of the mat is subject to shading.

It has been reported that plants of *L. minor* are colonized by microorganisms (2, 8; J. Rho and M. Taylor, Abstr. Annu. Meet. Am. Soc. Microbiol. 1981, N43, p. 180). The studies reported herein indicated that N_2-fixing bacteria and cyanobacteria are part of the microflora attached to roots and fronds of *L. minor* (21; D. A. Zuberer, Abstr. Annu. Meet. Am. Soc. Microbiol. 1981, N36, p. 179). Further studies on the nature of this colonization will be reported in a subsequent paper. The results of AR assays and some microscopic observations are in agreement with earlier data reported by Duong (Ph.D. thesis, 1972). He reported that AR associated with duckweeds in Michigan was due primarily to epiphytic blue-green algae. The significance of N_2 fixation to the productivity of these communities is difficult to assess; however, in nutrient-limited aquatic habitats it no doubt contributes to the growth of the community. However, the high reproductive potential (doubling time of 2 to 4 days) of *Lemna* could not be achieved solely on the input from biological nitrogen fixation.

Sutton and Ornes (18) reported a daily growth rate of approximately 2.0 g (dry weight) m^{-2} day^{-1} for duckweed growing in pond water held in containers. The nitrogen content of duckweed has been reported to range from 3 to 4% by a number of investigators. With the 3% N content, 2.0 g (dry weight) of duckweed would require 0.06 g of N m^{-2} day^{-1}. Taking an AR value of 7.6 μmol of C_2H_4 g (dry weight)$^{-1}$ day^{-1} from the present study and assuming the theoretical ratio of 3 mol of acetylene reduced per mol of dinitrogen fixed, approximately 71 μg of N was fixed per g of duckweed. Applying this to a standing biomass of 178 g (dry weight) m^{-2} gives 12.5 mg of N m^{-2} day^{-1}, or roughly 15 to 20% of the N requirement for a biomass production of 2 g (dry weight) m^{-2} day^{-1}. Clearly, there is room for variation in these figures, although the biomass value reported here agrees well with values reported by Rejmankova (14, 15). Nonetheless, they serve to estimate the potential contribution of associative nitrogen fixation to the nitrogen economy of duckweed mats.

In summary, these investigations have demonstrated that duckweed mats supported a mixed microbial population which was capable of nitrogen fixation. This population contained diazotrophic cyanobacteria and heterotrophic bacteria and fixed N_2 under light and dark conditions. N_2 fixation appeared to be enhanced when dense mats of the macrophytes were formed. At least a part of the nitrogen requirement (15 to 20%) for growth of these plants can be provided through these associations, which are of the nature of phylloplane and rhizoplane colonization (21).

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