Plant Physiol. (1974) 53, 820–824

The *Azolla, Anabaena Azollae* Relationship

II. LOCALIZATION OF NITROGENASE ACTIVITY AS ASSAYED BY ACETYLENE REDUCTION[1]

Received for publication November 8, 1973 and in revised form January 16, 1974

GERALD A. PETERS AND BERGER C. MAYNE
C. F. Kettering Research Laboratory, Yellow Springs, Ohio 45387

ABSTRACT

Anaerobic (microaerophilic) acetylene reduction by *Azolla caroliniana* Willd. was dependent on light and saturated at approximately 450 foot candles. Maximum rates of acetylene reduction were 60 nmoles/mg chlorophyll·minute. However, rates of 25 to 30 nmoles/mg chlorophyll·minute were more common.

The growth of *Azolla* for 35 days with nitrate or urea as a nitrogen source decreased the rate of acetylene reduction approximately 30% compared to controls grown on nitrogen. Prolonged growth on nitrate or urea (6–7 months) resulted in a 90% decrease in the rate of acetylene reduction.

The inhibition of acetylene reduction by 3(3,4-dichlorophenol)1,1-dimethylurea (12 μM) was not pronounced until the *Azolla* became depleted of the reserves formed during photosynthesis. The interval required for this depletion was dependent upon pretreatment and varied from 2 to more than 12 hours. Oxygen evolution was inhibited 75% in 10 minutes by the same concentration of 3(3,4-dichlorophenol)1,1-dimethylurea.

The addition of oxygen, 20% volume per volume, resulted in a 30 to 40% decrease in the rate of acetylene reduction and the onset of 3(3,4-dichlorophenol)1,1-dimethylurea inhibition was more rapid then under microaerophilic conditions. The aerobic dark reduction of acetylene was from 10 to 30% of the rate of aerobic reduction in the light.

Acetylene reduction activity was absent in fronds freed of the symbiotic algae and present in isolated *Anabaena azollae*. This study shows that the alga is the agent of acetylene reduction and suggests that there is considerable transport of metabolites between the fern and the blue-green alga.

There is an abundance of indirect evidence, spanning at least 6 decades, that the algal-containing *Azolla* is capable of assimilating atmospheric N_2 (1, 13, 18, 19). This evidence was obtained by experiments in which the fern was grown in N_2-free solution and subsequent N_2 increases determined by Kjeldahl analysis. More recently, Johnson *et al.* (13) reported that cobalt, which is an essential element for other N_2-fixing organisms, is required for the symbiotic growth of *Azolla* in the absence of a combined N_2 source. In a comprehensive review of the biology and agronomic significance of *Azolla*, which emphasizes the literature in relation to N_2 fixation and the practical significance of *Azolla*, Moore (18) reported evidence of N_2 fixation by nonaxenic *Azolla* using ^{15}N.

There are unconfirmed reports of independent growth (18, 26) and N_2 fixation (26) by the isolated microsymbiont *Anabaena azollae*. Others state that the microsymbiont cannot be grown apart from the host (1, 15, 19, 21). To the best of our knowledge the isolated alga does not currently exist in any culture collection and, other than the unconfirmed report by Venkataraman (26), no evidence has been presented that the isolated alga fixes N_2. Therefore, while it is generally concluded, and reasonably so, that the blue-green alga is the organism responsible for fixation, as stated by Moore (18), there is no conclusive evidence for this.

The few studies which have been conducted suggest that the *Azolla-Anabaena azollae* symbiosis is capable of fixing N_2 in the field to the same order of magnitude as a leguminous crop (18). In many areas, including the United States, *Azolla* is a water weed and is considered a nuisance (5). On the other hand, in Asia and other areas, it is used as a fodder and as a green manure (18).

This manuscript reports results of initial studies on acetylene reduction by the symbiotic association. It provides evidence that the symbiotic alga is the agent of acetylene reduction and if acetylene reduction is a valid assay of the ability to fix N_2, the site of N_2 fixation.

MATERIALS AND METHODS

Azolla plants, nutrient solutions, growth conditions, isolation of the symbiotic algae, and Chl determinations were as described in the preceding paper (20).

Chemicals. DCMU was obtained as Diuron from the E. I. duPont de Nemours Company. Polyvinylpyrrolidone (PVP-40) was obtained from the Sigma Chemical Company. All other chemicals were reagent grade. The gas mixtures were obtained from Matheson Gas Products.

Acetylene Reduction Assays. Three to five fronds were incubated under either 1% CO_2, 10% acetylene in argon (microaerophilic conditions) (24) or with oxygen added to 21% (v/v) (aerobic conditions) in calibrated 25-ml Erlenmeyer flasks fitted with serum caps and containing 5 ml of the desired nutrient solution. The flasks were evacuated and flushed prior to filling to a slightly positive pressure. The flasks were illuminated from the bottom with a bank of General Electric white fluorescent lights. Screens were used to lower the intensity for the light intensity curve. The intensity for all other assays was maintained at approximately 750 ft-c. Incubations were carried out at 23 C.

The gas phase was analyzed for ethylene by gas chromatography using a Hewlett-Packard (Model 700) dual flame gas chromatograph with a hydrogen flame ionization detector and a 3.2 mm, 2-m long column containing 80 to 100 mesh Pora-

[1] Contribution No. 510 from the Charles F. Kettering Research Laboratory.

pak N. The oven temperature was 60 C and helium was the carrier gas. The columns were calibrated with "Mini-Cal" calibration standards obtained from Applied Science Laboratories, Inc. Duplicate or triplicate samples were assayed from each flask at each time interval and two to six flasks were employed for each specific treatment.

For time course studies, the gas samples removed for analysis at each interval were replaced with an equivalent volume of the appropriate nutrient solution and the serum caps were lightly coated with rubber cement. In the inhibitor studies the solution added back contained the same final concentration as that present in the flask. Solvent controls were used where necessary.

RESULTS

Light Intensity Curve and General Comments. The dependence of microaerophilic (24) N_2 fixation (acetylene reduction) on light intensity by the *Azolla-Anabaena azollae* symbiotic association is shown in Figure 1. Saturation was attained at 450 ft-c. The rates obtained in the light are linear over a 24-hr period (Fig. 2) and, as shown in Table I, dark fixation under anaerobic conditions is negligible. The values of ethylene production presented in Figure 1 and in all subsequent figures and tables are expressed on the basis of *total Chl, i.e.*, fern plus blue-green alga, unless stated otherwise. This results in lower rates than would be obtained if presented only on the basis of Chl from the blue-green alga. The number of algal cells and their Chl content per leaf cavity, and therefore per frond is variable. This variability is in part responsible for the variations in the rates obtained from duplicate flasks under identical conditions. A 2- to 3-fold difference is often obtained in the rates for different experiments. Rates as high as 60 nmoles/mg Chl·min have been obtained, but the usual rate was from 25 to 30 nmoles/mg Chl·min.

The fronds contained 0.5 to 1 mg total Chl/g fresh weight with roots removed, regardless of the combined N_2 content of the growth medium. These data are not in agreement with Johnson *et al.* (13). They found a decrease in the Chl content of *Azolla* grown on medium with nitrate compared to those grown on N_2.

Effect of Combined Nitrogen Sources on Nitrogen Fixation. Fronds containing the blue-green symbiont were grown on several different combined N_2 sources and were assayed for acetylene reduction. An inhibition of nitrogenase activity was expected, since Stewart *et al.* (23) had shown that free-living blue-green algal cells grown on nitrate lost their capacity to reduce acetylene within 1 week. This was presumably due to an inhibition of nitrogenase synthesis by nitrate and a dilution of the preformed nitrogenase as growth continued. Bone (2), however, has presented data indicating that in *Anabaena flos-aquae* urea did not repress the synthesis of nitrogenase, but did effectively inactivate it. He found a rapid loss of activity, since the rate of inactivation was greater than the rate of synthesis.

Table I shows that nitrate and urea decreased but did not suppress the capacity for acetylene reduction in *Azolla*. The fronds were grown on media containing combined N_2 as nitrate, urea or ammonium for a minimum of 35 days and transferred to new media for the assays. Fronds grown on urea or nitrate reduced acetylene at a rate 70% as great as those grown on N_2. Under the experimental conditions employed, only ammonium chloride was clearly inhibitory to acetylene reduction and it did not support growth of the fronds as well as the other nitrogen sources used. These results raised the question as to whether the fronds were capable of utilizing combined N_2. Therefore, a series of experiments was performed in which N_2 was excluded. Flasks containing equivalent numbers of fronds and the appropriate media were purged and continuously bubbled with a mixture of 1% CO_2, 20% oxygen in argon. Within 1 week, the fronds on N_2-free media were dying and after 15 days they were dead. At this time, the fronds growing on the ammonium medium were also beginning to show signs of necrosis. No attempt was made to de-

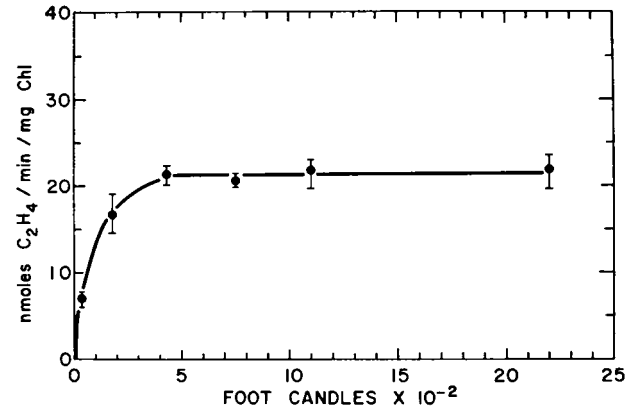

FIG. 1. Nitrogenase activity (acetylene reduction) as a function of light intensity in the symbiotic association under microaerophilic conditions. Plants were incubated at 23 C for approximately 12 hr at the light intensities shown.

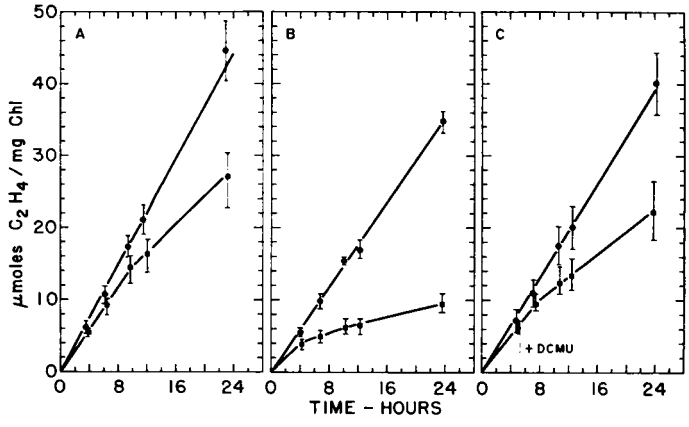

FIG. 2. Effect of DCMU and light or dark pretreatment on acetylene reduction by the symbiotic association under microaerophilic conditions. A: Fronds maintained under light (750 ft-c), aerobic conditions 12 hr prior to incubation under microaerophilic conditions. ●: control; ■: DCMU-treated; 12 μM added at time zero. B: Fronds maintained in darkness under aerobic conditions for 12 hr prior to incubation, otherwise as for A. C: Same as B except fronds left in light 5 hr before adding DCMU.

Table I. *Effect of Combined Nitrogen Sources on Acetylene Reduction*

Nitrogen Source	Rate of C_2H_4 Produced	SD	No.
	nmoles/mg total Chl·min		
N_2	27.1	5.3	7
Nitrate	18.5	3.8	6
Urea	18.4	2.4	7
Ammonium	0.3	0.2	10
N_2-dark control	0.09	0.08	4

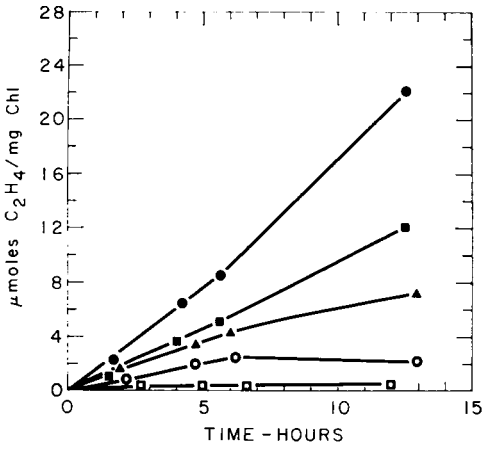

FIG. 3. Acetylene reduction by *Azolla* fronds in the light under aerobic (■) and microaerophilic (●) conditions and the effect of DCMU (12 μM) on acetylene reduction under aerobic conditions in the light (▲), all after a 12-hr preincubation in air and light. Aerobic dark acetylene reduction after a 12-hr preincubation in air and light (○) and after 12 hr in air and dark (□).

termine if ammonia would serve as a N_2 source under other culture conditions. Acetylene reduction by fronds from the nitrate and urea flasks was approximately the same as found in Table I, 18 and 25 nmoles/mg Chl·min, respectively.

The doubling time of the nitrate- and urea-grown fronds under these conditions was about 7 days, essentially the same as that under air. The fronds on the nitrate and urea media had the same rate of acetylene reduction per mg Chl after a 4-fold increase in biomass, indicating that nitrogenase was being synthesized in the presence of urea or nitrate. A possible explanation for the difference between *Azolla* and free living algae is that the algal cells in the leaf cavity are not in direct contact with the medium, and therefore are presumably exposed only to metabolized forms of urea or nitrate taken up by the frond.

After prolonged growth on urea or nitrate, *i.e.*, 6 to 7 months, much lower rates of acetylene reduction were found (3–5 nmoles/mg Chl·min). Whether this is the result of an inhibition of the nitrogenase by combined N_2, inactivation, or simply a decrease in the number of algal cells per frond has not yet been determined.

DCMU Sensitivity of Nitrogenase Activity: Dependence Upon Previous Light or Dark Treatment. Cox and Fay (6) and Bothe and Loos (3) reported that in short term studies on *Anabaena cylindrica* DCMU inhibited CO_2 fixation but had little or no effect on acetylene reduction. In longer term experiments (25) or after a pretreatment in the dark (16) a distinct inhibition of acetylene reduction was observed. These results suggested that photosynthesis supplies a pool of reductant which can be used to reduce N_2. Photosystem I may function by mediating a flow of electrons from carbon compounds to nitrogenase or may on the other hand only provide ATP via cyclic photophosphorylation. In either case, acetylene reduction in the presence of DCMU should proceed only until the reserve of organic substrates is no longer available.

The effect of 12 μM DCMU on acetylene reduction by the *Azolla-Anabaena azollae* symbiosis under microaerophilic conditions (24) is shown in Figure 2, A, B, and C. In Figure 2A, the fronds were in continuous light for 12 hr prior to the addition of DCMU. The rate of acetylene reduction was linear for 13 hr and nitrogenase activity was still present at 24 hr. In Figure 2B, the fronds were maintained in complete darkness for 12 hr prior to the addition of DCMU. In this case, acetylene reduction declined markedly after 4 hr but proceeded at a greatly diminished rate during the next 20 hr. In Figure 2C, the fronds were again kept in darkness for 12 hr. Acetylene reduction in the light was followed for 5 hr prior to adding DCMU. The rate remained constant for approximately 3 hr after adding DCMU and then decreased to a new linear rate which was slightly less than the terminal rate of the DCMU-treated fronds in Figure 2A. Controls, run under identical conditions, show that the rates of acetylene reduction were linear over the entire time course.

These results are in general agreement with those reported by Lex and Stewart (16) for the free living blue-green alga *Anabaena cylindrica*. Their results indicated that under certain conditions a pool of carbon compounds supplied reductant for nitrogenase activity. When the pool was high, acetylene reduction proceeded in the presence of DCMU and the elimination of PS II had little or no effect. When the pool was low, acetylene reduction was inhibited by DCMU. These pools were apparently metabolized in the dark. The products furnished by photosynthesis were carbon skeletons which provided a source of reductant and/or ATP for nitrogenase activity (16). In the symbiotic association, however, we are dealing with two photosynthetic organisms. In view of the long time intervals (Fig. 2A), before there was any indication of an inhibition of nitrogenase activity by DCMU, it seemed likely that there was cross-feeding of carbon reserves from the fern to the alga. Therefore, we attempted to supply exogenous substrates to the fronds after inhibition of acetylene reduction by DCMU was evident. Neither 1% (w/v) sucrose, glucose, fructose nor 0.1% (w/v) glycerol had any effect on restoring acetylene reduction. These carbon sources have been shown to support heterotrophic or photoheterotrophic growth of some blue-green algae (10–12, 14, 17) and sucrose supports growth of some fern gametophytes (8). It is possible that there was little or no uptake of the exogenous compounds by the fronds under the experimental conditions employed.

In order to inhibit electron transport in the blue-green alga, DCMU must be transported via the roots or penetrate through the non-chlorophyllous ventral lobe and the walls of the cavity. Slow absorption may in part explain why there is a lag of approximately 3 hr (Fig. 2B) before there is a discernible inhibition. In preliminary experiments on the symbiotic association, oxygen evolution was 75% inhibited within 10 min by a comparable DCMU concentration. CO_2-fixation, which is saturated at 750 ft-c, showed a 70% inhibition by DCMU after 15 min.

The Effect of Oxygen on Acetylene Reduction. The addition of oxygen to 20% v/v, (aerobic conditions) and incubation in the light resulted in rates, over a 12-hr period, which were generally 30 to 40% less than those obtained under microaerophilic conditions in the light (Fig. 3). In aerobic cultures incubated in the light with 12 μM DCMU acetylene reduction was inhibited more rapidly than under anaerobic conditions. Under aerobic conditions in the dark, fronds which were in continuous light for 12 hr had rates of acetylene reduction from 10 to 30% (3–8 nmoles C_2H_4/mg Chl·min) of the aerobic-light rate and decreased after 6 hr. Fronds which were maintained in darkness for 12 hr prior to the beginning of the aerobic-dark incubation period showed that the rates of acetylene reduction became non-linear within 2 hr. These results suggest that oxygen-dependent acetylene reduction in the dark is also dependent upon endogenous carbon reserves. Dark acetylene reduction under anaerobic conditions is negligible.

Absence of Acetylene Reduction in Fronds Free of the Algae. *Azolla* fronds freed of the symbiotic algae as described

previously (20) were assayed for acetylene reduction. Both aerobic and microaerophilic conditions were employed. No indication of nitrogenase activity was detected in these fronds.

Acetylene Reduction by the Isolated Alga. The symbiotic blue-green algae isolated by fractionation and gradients (20) gave some acetylene reduction, while no acetylene reduction was found in the chloroplast fraction. The rates were very low and became non-linear within 2 hr. There were a variety of factors which might have contributed to this low activity, including osmotic effects, cold inactivation during the centrifugation, and shearing during the initial preparation which results in shortened filaments. There is some evidence that shearing decreases rates of acetylene reduction (9).

Another procedure for isolating the symbiotic blue-green, which eliminates these problems, was developed. This is the roller method (20), which involved neither cold nor osmotic effects. With care it was possible to obtain long filaments of the alga which were virtually free of fern chloroplasts by the same criterion employed for determining the purity of the gradient fractions. The alga was isolated in a N_2-free medium employed for the growth of many free living blue-green algae, BG11 (22), containing 1% (w/v) PVP-40. The inclusion of the latter increased the activity. The alga was placed in calibrated flasks containing N_2-free BG11 media. Under aerobic conditions, the rates of acetylene reduction were less than those obtained under microaerophilic conditions.

A rate of 45 nmoles C_2H_4 produced/mg algal Chl·min under microaerophilic conditions is the best we have obtained. This is only 20% of the rate we expected on the basis of our estimate of the rate of fixation by the alga in the frond. This estimate is based on our determination (20) that the Chl *a* of the algae comprises 7.5 to 15% of the total Chl in the symbiotic association. Therefore, rates of acetylene reduction obtained with intact association would be 6 to 13 times greater if presented on the algal Chl only. The inclusion of 1% fructose or 1% glucose in the incubation medium was inhibitory under aerobic conditions, as was fructose under microaerophilic conditions. Glucose under microaerophilic conditions had no effect as compared to the control.

DISCUSSION

The results of this investigation provide evidence that the symbiotic alga is the site of acetylene reduction (nitrogenase activity). This was shown using both the symbiotic association and the isolated algae. The absence of acetylene reduction was directly correlated with the absence of the symbiotic algae.

The relationship between the alga and fern has been referred to as either a space parasitism or a symbiotic association (15, 18). These studies have provided evidence that the fern at least, and probably the alga as well, benefit from the association. It is, therefore, a true symbiosis in the refined sense. The fern, in the absence of the alga, does not reduce acetylene and cannot grow on a N_2-free medium. Therefore, in the combined state, the growth of the fronds on N_2-free medium appears to be dependent upon N_2 fixed by the alga.

Inhibitors such as DCMU must pass through the fern before contacting the algae. This could account for a part of the long delay before any effect of DCMU on acetylene reduction is observed. However, initial experiments have shown that DCMU inhibited oxygen evolution by the frond within 10 min and CO_2 fixation after 15 min. DCMU inhibited acetylene reduction in cells which were maintained for 12 hr in darkness within 4 hr *versus* 10 to 12 hr before any effect was seen on fronds which were kept in continuous light. Thus DCMU reaches the algal cells in the leaf cavity within 4 hr. The fact that the fronds kept in light were inhibited only slightly after 12 hr, and continued to reduce acetylene for 24 hr at a relatively constant rate would indicate a large endogenous supply of carbon compounds capable of providing reductant. In agreement with the results found in free living blue-green algae, *Anabaena cylindrica* (16), our results indicate a reserve of endogenous reductant. Our studies suggest an even larger reserve than would be expected to occur in the small quantity of symbiotic alga contained in the fronds. As suggested indirectly by Moore (18), it is probable that there is a cross-feeding of carbon skeletons from the fern to the blue-green alga. This, along with dark, aerobic, acetylene reduction, implies that within the cavity the alga is capable of either heterotrophic or photoheterotrophic growth. It should be noted that this is not improbable, since Khoja and Whitton (14) reported that at least nine genera are capable of dark heterotrophic growth, six of which grow in the dark in the absence of combined N_2. The endophytic blue-green alga, *Nostoc sp.*, isolated from the coralloid roots of a cycad, was shown to be capable of dark heterotrophic growth (4, 10), and more recently to grow photoheterotrophically utilizing a wider variety of exogenous carbon sources (11). The unicellular blue-green, *Agmenellum quadruplication*, which is capable of rapid photoheterotrophic growth at light intensities which do not support autotrophic growth, does not grow heterotrophically in the dark (12). Therefore, we are planning additional studies, utilizing $^{15}N_2$-, $^{14}CO_2$-, and ^{14}C-labeled compounds, to show the extent of cross-feeding.

Obligate photoheterotrophy would help to explain why the alga has not been maintained in independent culture with conventional methods. Although our fronds are free of epiphytic contaminants and are unialgal with respect to the symbiont, neither the symbiont-containing nor symbiont-free fronds are axenic. We have isolated the symbiont from the cavity by a variety of sterile techniques, including micromanipulation, and attempted to culture them on numerous media under a very large number of conditions. In each case, when an exogenous carbon source was supplied, the cultures were eventually overrun by bacterial contamination. The contamination was more prevalent under aerobic conditions with a combined N_2 source than under anaerobic or aerobic conditions in N_2-free medium. In the latter cases, bacterial growth was linked to the amount of inoculum. Microscopy revealed that the bacteria initially aggregated around the blue-green algal cells. Acetylene reduction assays were conducted on subcultures of the bacterial contaminants after transfer to medium free of combined N_2. There was no indication of nitrogenase activity. Individual leaflets of the symbiont-free fronds squashed in medium with a carbon source also result in bacterial growth.

The absence of acetylene reduction by the bacterial contaminants is important with respect to the aerobic dark fixation observed in the fronds. These results indicate that it is the algal cells and not contaminating bacteria which are responsible. Acetylene reduction in the dark is in some way linked to aerobic metabolism of the alga and is dependent upon the presence of oxygen and carbon reserves. This oxygen dependence has been shown for dark fixation in free living blue-green algae (7, 16) and, as noted previously, dark heterotrophic growth in the absence of combined N_2 has been shown in several genera of blue-greens.

In conclusion, these studies provide the initial characterization of the N_2-fixing symbiotic association utilizing modern assay methods. Although no direct determination of N_2-fixation was made and the algal fraction was not free of bacterial contaminants, the results of these studies strongly indicate that

Anabaena azollae is the N_2-fixing agent in the association and that there is metabolite interaction between the two photosynthetic organisms. Additional studies on physiological aspects of the symbiotic association are in progress.

Acknowledgments—The authors wish to acknowledge the excellent over-all technical assistance of Mrs. Joan Schnieders and Mr. Robert Hart.

LITERATURE CITED

1. Bortels, H. 1940. Über die Bedeutung des Molybdäns für die stickstoffbindende Nostocaceen. Arch. Mikrobiol. 11: 155-186.
2. Bone, D. H. 1972. The influence of canavanine, oxygen, and urea on the steady-state levels of nitrogenase in *Anabaena flos-aquae*. Arch. Mikrobiol. 86: 13-24.
3. Bothe, H. and E. Loos. 1972. Effect of far red light and inhibitors on nitrogen fixation and photosynthesis in the blue-green alga *Anabaena cylindrica*. Arch. Mikrobiol. 86: 241-254.
4. Bowyer, J. W. and V. B. D. Skerman. 1968. Production of axenic cultures of soil-borne and endophytic blue-green algae. J. Gen. Mikrobiol. 54: 299-306.
5. Cohn, J. and R. N. Renlund. 1953. Notes on *Azolla caroliniana*. Amer. Fern. J. 43: 7-11.
6. Cox, R. M. and P. Fay. 1969. Special aspects of nitrogen fixation by blue-green algae. Proc. Roy. Soc. London Ser. B 172: 357-366.
7. Donze, M., J. Haveman, and P. Schiereck. 1972. Absence of photosystem 2 in heterocysts of the blue-green alga *Anabaena*. Biochim. Biophys. Acta 256: 157-161.
8. Edwards, M. E. and J. H. Miller. 1972. Growth regulation by ethylene in fern gametophytes. II. Inhibition of cell division. Amer. J. Bot. 59: 450-457.
9. Fay, P. and S. A. Kulasooriya. 1972. Tetrazolium reduction and nitrogenase activity in heterocystous blue-green algae. Arch. Mikrobiol. 87: 341-352.
10. Hoare, D. S., L. O. Ingram, E. L. Thurston, and R. Walkup. 1971. Dark heterotrophic growth of an endophytic blue-green alga. Arch. Mikrobiol. 78: 310-321.
11. Ingram, L. O., J. A. Calder, C. van Baalen, F. E. Plucker, and P. L. Parker. 1973. Role of the reduced exogenous organic compounds in the physiology of the blue-green bacteria (Algae): photoheterotrophic growth of a "heterotrophic" blue-green bacterium. J. Bacteriol. 114: 695-700.
12. Ingram, L. O., C. van Baalen, and J. A. Calder. 1973. Role of the reduced exogenous organic compounds in the physiology of the blue-green bacteria (Algae): photoheterotrophic growth of an "Autotrophic" blue-green bacterium. J. Bacteriol. 114: 701-705.
13. Johnson, G. V., P. A. Mayeux, and H. J. Evans. 1966. A cobalt requirement for symbiotic growth of *Azolla filiculoides* in the absence of combined nitrogen. Plant Physiol. 41: 852-855.
14. Khoja, T. and B. A. Whitton. 1971. Heterotrophic growth of blue-green algae. Arch. Mikrobiol. 79: 280-282.
15. Lang, N. J. 1965. Electron microscopic study of heterocyst development in *Anabaena azollae* Strasburger. J. Phycol. 1: 127-134.
16. Lex, M. and W. D. P. Stewart. 1973. Algal nitrogenase, reductant pools and photosystem 1 activity. Biochim. Biophys. Acta 292: 436-443.
17. Miller, J. S. and M. M. Allen. 1972. Carbon utilization patterns in the heterotrophic blue-green alga, *Chlorogloea fritschii*. Arch. Mikrobiol. 86: 1-12.
18. Moore, A. W. 1969. *Azolla*: Biology and agronomic significance. Bot. Rev. 35: 17-34.
19. Oes, A. 1913. Über die Assimilation des freien Stickstoffs durch *Azolla*. Zeitschr. Bot. 5: 145-163.
20. Peters, G. A. and B. C. Mayne. 1974. The *Azolla, Anabaena Azolla* Relationship. I. Characterization of the Association. Plant Physiol. 53: 813-819.
21. Shields, L. M. and L. W. Durrell. 1964. Algae in relation to soil fertility. Bot. Rev. 30: 92-128.
22. Stanier, R., R. Kinisawa, M. Mandel, and G. Cohen-Bazire. 1971. Purification and properties of unicellular blue-green algae (order Chroococcales). Bacteriol. Rev. 35: 171-205.
23. Stewart, W. D. P., G. P. Fitzgerald, and R. H. Burris. 1968. Acetylene reduction by nitrogen fixing blue-green algae. Arch. Mikrobiol. 62: 336-348.
24. Stewart, W. D. P. and M. Lex. 1970. Nitrogenase activity in the blue-green alga *Plectonema boryanum* strain 594. Arch. Mikrobiol. 73: 250-260.
25. Stewart, W. D. P. and H. W. Pearson. 1970. Effects of aerobic and anaerobic condition on growth and metabolism of blue-green algae. Proc. Roy. Soc. Lond. B. 175: 293-311.
26. Venkatarman, G. S. 1962. Studies on nitrogen fixation by blue-green algae. III. Nitrogen fixation by *Anabaena azollae*. Indian J. Agr. Sci. 32: 22-24.