New Phytol. (1982) **92**, 235–241

$^{15}N_2$ FIXATION AND MOLYBDENUM ENHANCEMENT OF ACETYLENE REDUCTION BY *LOBARIA* SPP.

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(*Accepted 31 May 1982*)

SUMMARY

The lichens *Lobaria pulmonaria* and *Lobaria oregana* were assayed for $^{15}N_2$ assimilation in order to confirm previous rates of nitrogenase activity measured by acetylene reduction. In addition, the effect of molybdenum on acetylene reduction activity was studied in both species. The molar conversion ratio (acetylene reduction rate divided by $^{15}N_2$ fixation rate) was 4·27 for *Lobaria pulmonaria* and 4·78 for *Lobaria oregana*. The rate of $^{15}N_2$ uptake decreased over time in both species. Addition of 1 p.p.m. molybdenum enhanced acetylene reduction by 180% in *Lobaria pulmonaria* and by 50% in *Lobaria oregana*. Optimum enhancement in *Lobaria oregana* was obtained with 1 p.p.m. molybdenum, while 10 p.p.m. may be inhibitory.

INTRODUCTION

Lobaria oregana (Tuck.) Müll. Arg. and *L. pulmonaria* (L.) Hoffm. are important members of the epiphytic community in old-growth Douglas fir trees [*Pseudotsuga menziesii* (Mirb.) Franco] in the Pacific Northwest, accounting for 35 to 60% of epiphyte biomass or about 5% of the foliar biomass, and 56% of total epiphyte nitrogen content (Pike *et al.*, 1972; Pike, Rydell and Denison, 1977). *L. pulmonaria* is also present on Willamette Valley white oak (*Quercus garryana* Dougl. *ex* Hook.), although in much smaller amounts (Pike, 1971). Both lichens contain a green alga as the primary phycobiont and *Nostoc* as the cephalodiate cyanophycobiont. Denison (1979), using the acetylene reduction technique, has estimated that *L. oregana* fixes 3·5 kg N ha^{-1} $year^{-1}$. In order to confirm this estimate of the contribution by these lichens to the nitrogen content of forest ecosystems, it is necessary to measure actual $^{15}N_2$ fixation rates in *Lobaria* to compare with known acetylene reduction rates (Hardy, Burns and Holsten, 1973). $^{15}N_2$ incorporation rates are presented here, as well as C_2H_2 reduction/$^{15}H_2$ fixation molar conversion ratios.

In addition to its importance as a micronutrient for growth in plants, molybdenum has long been known to have a role in nitrogen fixation. Bortels (1930) first reported that molybdenum is necessary for normal growth of *Azotobacter chroococcum* grown in nitrogen-free medium. Later molybdenum was shown to increase total

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Kjeldahl nitrogen content of *Anabaena* and *Nostoc* in pure culture in the absence of combined nitrogen, and in *Anabaena azollae* in symbiotic association with *Azolla carolinina* (Bortels, 1940). Dry wt and total nitrogen content of cultures of *Anabaena cylindrica* supplied with N_2 or NO_3^- increased in response to addition of molybdenum (Wolfe, 1954). A molybdenum requirement for optimal growth and total nitrogen content also has been reported for *Tolpothrix tenuis*, a nitrogen-fixing blue-green alga found in rice paddy soils, grown on N_2 or NO_3^- (Okuda, Yamaguchi and Nioh, 1962). These data suggest that molybdenum is necessary for nitrogen fixation and nitrate utilization in cyanobacteria. It is now known that molybdenum is a constituent of the Fe–Mo protein component of nitrogenase (N_2ase) and of nitrate reductase, and therefore this requirement is to be expected. Consequently, we have also investigated the effect of molybdenum on N_2ase activity in *Lobaria*.

MATERIALS AND METHODS

Acetylene reduction assays

Lobaria pulmonaria, located on *Quercus garryana* at Soap Creek, Corvallis, Oregon, was misted with creek water *in situ* each hour for 9 h. Samples were then collected and assayed within 4 h. *L. oregana* was collected from *Pseudotsuga menziesii* in the H. J. Andrews Experimental Forest, Cascade Range. Samples were taken to the laboratory, misted with rain water, and stored moist at 14 °C under fluorescent light (1000 lx) for 9 h before being assayed. Lichen discs (10 mm) were cut from outer thallus lobes, then random samples of 12 discs were placed into 13 ml vacutainer tubes. One millilitre of acetylene was added to each tube and the material was incubated for varying lengths of time in the light (3000 lx from cool white fluorescent) at 20 °C. Ethylene production was assayed using a Hewlett–Packard gas chromatograph with ethylene measured quantitatively using acetylene as the internal standard. The method was checked carefully using authentic ethylene and absolute calibration techniques. Corrections were made for temperature and pressure at the time of assay. Wet and dry wts of the discs were measured after acetylene incubation was terminated. All data presented are for lichens hydrated to an excess of 1·5 g H_2O g^{-1} thallus dry wt, which Denison (1979) has shown to be necessary for maximum N_2ase activity in *L. oregana*.

$^{15}N_2$ *fixation assays*

Nitrogen gas containing 95 atom per cent ^{15}N was purchased from Prochem Co. and mixed with oxygen to give an 80:20 mixture. Lichen material was prepared as above, tubes were evacuated for 30 s, then the $^{15}N_2/O_2$ mixture was added by syringe to atmospheric pressure. Tests indicated that a 30 s evacuation had no significant effect on acetylene reduction. Tubes were incubated as described above. Samples (0·2 ml) of the gas mixture in each tube were taken and assayed for $^{15}N_2$ and O_2 content using an MS10 mass spectrometer.

On completion of $^{15}N_2$ assay, acetylene was added to each tube to give a final concentration of 7 % and acetylene reduction was measured for 1 h. This amount of acetylene was shown to completely saturate lichen N_2ase. After determining lichen disc wet and dry wts, the material was digested in sulphuric acid (Kjeldahl method) using a copper and selenium catalyst. The digest was distilled, assayed for ammonia by titration and dried. The resulting ammonia was analyzed by mass spectrometry for isotope ratio by Dr B. B. McInteer, Los Alamos National Laboratory, New Mexico.

Molybdenum effects

Lichen thalli were sprayed
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Molybdenum effects

Lichen thalli were sprayed with molybdenum (normally 1 p.p.m.) in the form of aqueous sodium molybdate or with complete nutrient solution containing 0·2 p.p.m. molybdenum and all other essential elements but lacking nitrogen (designated minus N). Thalli were sprayed until the surface was saturated, then they were incubated for 24 to 48 h in the laboratory under fluorescent light (1000 lx) at 14 °C. Control material was similarly treated with distilled water. Samples were assayed for acetylene reduction using thallus discs as described above.

Results

$^{15}N_2$ assimilation

The results of $^{15}N_2$ uptake (Table 1) reveal that in *L. pulmonaria* a high rate of nitrogen fixation was maintained over the 3 h incubation period with a slight reduction in activity over time. In *L. oregana*, however, there is a substantial drop in activity in samples incubated for over 1·5 h. Acetylene reduction assays conducted for 30 to 45 min on the same lichen discs used for $^{15}N_2$ uptake, measured immediately following $^{15}N_2$ incubation (Table 1), suggest that some of the difference in $^{15}N_2$ fixation rates is due to variation in activity among groups of lichen discs. For each pair of tubes incubated for a given time during $^{15}N_2$ treatment, the relationship between $^{15}N_2$ fixation and acetylene reduction is similar. However, it is clear from the four acetylene reduction values for each species that $^{15}N_2$ fixation rates are inhibited at 3 h. The molar ratio for each sample, calculated by dividing μmoles of acetylene reduced by μmoles of N_2 fixed, confirms $^{15}N_2$ fixation inhibition over time because the ratios for both species incubated for 3 h are higher. The average values for molar ratios for 1·5 h incubation time are 4·27 for *L. pulmonaria* and 4·78 for *L. oregana*.

Effect of molybdenum on nitrogenase

L. pulmonaria was treated with 1 p.p.m. molybdenum solution, complete nutrient solution (minus N) or distilled water. The results in Table 2 show that molybdenum increased N_2ase activity by 180 %. It is also evident that the effect is specific for molybdenum in that the complete nutrient solution produced no stimulation apart from that attributable to molybdenum.

A similar experiment conducted on *L. oregana* treated with 1 p.p.m. molybdenum solution or distilled water and assayed for N_2ase activity after 1 and 2 h is reported in Table 3. Activities of *L. oregana* are generally lower than for *L. pulmonaria* and the molybdenum enhancement, although significant, was 50 % in this case.

The effect of molybdenum on *L. oregana* is quantitative as shown in Table 4 with a saturation at 1 p.p.m. and a possible inhibitory effect at 10 p.p.m.

Discussion

There are several reports of N_2ase activity measured by acetylene reduction for *Lobaria* species. Hitch and Millbank (1975) report N_2ase activity measured in the laboratory for *L. scrobiculata*, a non-cephalodiate species, collected in Scotland, at 290 nmol C_2H_4 g^{-1} h^{-1} at 25 °C. *L. pulmonaria* collected in North Carolina produced 963 nmol C_2H_4 g^{-1} h^{-1} at 20 °C in the laboratory (Kelly and Becker, 1975) and 12330 nmol C_2H_4 g^{-1} h^{-1} at 16 to 18 °C in the field (Becker, 1980).

Table 1. *$^{15}N_2$ uptake and acetylene reduction rates by* Lobaria *spp. Samples were incubated at 20 °C, in 80% N_2, 20% O_2 (86 atom % excess $^{15}N_2$) followed by 7% C_2H_2 in air*

Species	Dry wt of 12 discs (mg)	$^{15}N_2$ incubation time (h)	Atom % excess ^{15}N*	μg ^{15}N excess†	N_2 fixation (μg N g^{-1} h^{-1})	Acetylene incubation time (h)	Acetylene reduction (μg C_2H_4 g^{-1} h^{-1})	R‡
Lobaria pulmonaria	108	1·5	0·035	1·01	6·34	0·77	26·8	4·23
	142	1·5	0·049	1·74	8·36	0·78	36·0	4·31
	143	3·2	0·087	3·02	6·68	0·50	37·1	5·56
	141	3·2	0·062	2·14	4·79	0·53	28·6	5·96
L. oregana	109	1·5	0·008	0·216	1·35	0·72	6·68	4·95
	114	1·5	0·014	0·358	2·13	0·73	9·80	4·60
	121	3·8	0·017	0·459	0·99	0·67	7·54	7·62
	99	3·8	0·014	0·310	0·82	0·70	6·05	7·38

* Mean of two separate analyses on 12 half discs in each case.
† Corrected for initial enrichment of $^{15}N_2$ in incubating gas.
‡ Molar ratio of acetylene reduction and N_2 fixation rates.

Table 2. *Effect of molyb incubated for 1·5 h at 20*

Distilled w	
1·0 p.p.m.	
Complete n	

* Containing 0·2 p.p.m. molyb

Table 3. *Effect of molybd incubated at 20 °C in*

Minus molybdenum	
Plus molybdenum	

Table 4. *Effect of varying co* oregana *incubated at 20 °C*

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0	
0·1	
1·0	
10·0	

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Table 2. *Effect of molybdenum on nitrogenase activity of* Lobaria pulmonaria *incubated for 1·5 h at 20 °C in 7% acetylene. Means and standard errors* (n = *3*)

	N_2ase activity (nmol. C_2H_4 g^{-1} h^{-1})
Distilled water	460 ± 58
1·0 p.p. m. molybdenum solution	1282 ± 175
Complete nutrient solution*	1251 ± 132

* Containing 0·2 p.p.m. molybdenum and all other essential elements, but lacking combined nitrogen.

Table 3. *Effect of molybdenum (1 p.p.m.) on N_2ase activity of* Lobaria oregana *incubated at 20 °C in 7% acetylene. Means and standard errors* (n = *6*)

	Incubation time (h)	N_2ase activity (nmol C_2H_4 g^{-1} h^{-1})	Hydration (g H_2O g^{-1} dry wt)
Minus molybdenum	1	276 ± 69	2·90
	2	281 ± 74	
Plus molybdenum	1	416 ± 55	2·40
	2	417 ± 57	

Table 4. *Effect of varying concentrations of molybdenum on N_2ase activity of* Lobaria oregana *incubated at 20 °C in 7% acetylene. Means and standard errors* (n = *6*)

Molybdenum concentration (p.p.m.)	N_2ase activity (nmol C_2H_4 g^{-1} h^{-1})	
	1·3 h	2·8 h
0	280 ± 51	260 ± 44
0·1	330 ± 36	314 ± 34
1·0	392 ± 75	373 ± 72
10·0	316 ± 33	299 ± 32

These latter rates are much higher than the mean rates (*L. pulmonaria* 278 and *L. oregana* 350 nmol C_2H_4 g^{-1} h^{-1}) reported by Denison (1979) for a large number of samples collected in the Cascades and measured in the field over a temperature range of 0 to 15 °C (September 1976 to May 1978). In the present study, laboratory acetylene reduction rates at 20 °C were 460 and 279 nmol C_2H_4 g^{-1} h^{-1} for *L. pulmonaria* and *L. oregana*, respectively. The wide range in specific activity recorded for *Lobaria* spp. both within this study (cf. Tables 1 and 2) and between the above, reflects the range of conditions and preincubation times that have been used and little weight should be placed on the differences.

Stewart, Fitzgerald and Burris (1968) measured a conversion factor for acetylene reduced versus $^{15}N_2$ fixed by N_2ase for *Nostoc muscorum* of 3·6. The molar ratios presented in this report for *Nostoc* in symbiosis with *L. pulmonaria* (4·27) and *L. oregana* (4·78) are higher than Stewart's value and the theoretical value of three. A significant effect recorded here is that the nitrogen fixation rate drops dramatically during the incubation while acetylene reduction rate remains constant. The reasons for this are obscure but are unlikely to be due to incubation conditions

* Mean of two separate analyses on 12 half discs in each case.
† Corrected for initial enrichment of $^{15}N_2$ in incubating gas.
‡ Molar ratio of acetylene reduction and N_2 fixation rates.

as identical conditions were set up. It is possible that in very short assays a value closer to three may be achieved but further work needs to be done in estimating the real relationships in lichens.

Both *Lobaria* species pretreated with 1 p.p.m. molybdenum show a marked enhancement of N_2ase activity. This concentration may be optimal for *L. oregana* with a possible inhibitory effect at 10 p.p.m. While studying molybdenum enhancement of growth and nitrogen content in *Anabaena*, Wolfe (1954) tested molybdenum concentrations up to 10 p.p.m. and, although her various experiments are not directly comparable, 0·2 p.p.m. molybdenum produced slightly better growth than higher molybdenum concentrations. Allen and Arnon (1955) confirmed that molybdenum (0·01 p.p.m.) is necessary for growth of *Anabaena*, but they did not investigate other molybdenum concentrations. Okuda *et al.* (1962) tested molybdenum (10^{-4} to 10^{-1} p.p.m.) effects on *Tolypothrix tenuis* and reported maximum growth and nitrogen content in cultures grown on nitrogen with 0·01 p.p.m. molybdenum. The molybdenum requirement of cephalodiate blue-green algae in lichens may be higher than that of algal cultures because the lichen thallus may impede diffusion of the micronutrient to the algal cells. In addition, the green algal symbiont and, possibly, the mycobiont may require molybdenum for nitrate reductase. Little is known about the ability of lichenized fungi to utilize nitrate, but Ahmadjian (1964) has reported one lichen fungus (isolated from *Acarospora fuscata*) which showed maximum growth in cultures containing KNO_3 as the nitrogen source.

Preliminary analysis of incident rainwater and throughfall samples collected at the site where *L. oregana* was collected show no detectable molybdenum in incident water and 0·01 p.p.m. molybdenum in canopy throughfall. It is not known from which canopy component(s) molybdenum may be leaching, but it appears that molybdenum levels in water reaching thalli, even in the lower canopy, may be limiting to nitrogen fixation by *Lobaria* in Douglas fir canopies.

ACKNOWLEDGEMENTS

We gratefully acknowledge support from the following sources: National Science Foundation, U.S.A. for grants to Dr George C. Carroll for support of J. L. H., and to W. C. D.; University of Waikato for sabbatical leave support for W. B. S.; Orgeon State University School of Forestry for laboratory facilities for W. B. S. We sincerely thank Tom Verhoeven for technical assistance.

REFERENCES

AHMADJIAN, V. (1964). Further studies on lichenized fungi. *Bryologist*, **67**, 87–98.

ALLEN, M. B. & ARNON, D. I. (1955). Studies on nitrogen-fixing blue-green algae. I. Growth and nitrogen fixation by *Anabaena cylindrica* Lemm. *Plant Physiology*, **30**, 366–372.

BECKER, V. E. (1980). Nitrogen fixing lichens in forests of the southern Appalachian mountains in North Carolina. *Bryologist*, **83**, 29–39.

BORTELS, H. (1930). Molybdän als Katalysator bei der biologischen Stickstoff-bindung. *Arkiv für Mikrobiologie*, **1**, 333–342.

BORTELS, H. (1940). Über die Bedeutung des Molybdäns für stickstoffbindende Nostocaceen. *Arkiv für Mikorbiologie*, **11**, 155–186.

DENISON, W. C. (1979). *Lobaria oregana*, a nitrogen-fixing lichen in old-growth Douglas fir forests. In: *Symbiotic Nitrogen Fixation in the Management of Temperate Forests* (Ed. by J. C. Gordon, C. T. Wheeler & D. A. Perry), pp. 266–275. Oregon State University School of Forestry, Corvallis, Oregon.

HARDY, R. W. F., BURNS, R. C. & HOLSTEN, R. D. (1973). Applications of the acetylene-ethylene assay for measurement of nitrogen fixation. *Soil Biology and Biochemistry*, **5**, 47–81.

HITCH, C. J. B. & MILLBANK, J. W
heterocyst frequency in lichen
KELLY, B. B. & BECKER, V. E. (19
Lobaria pulmonaria, Sticta weig
OKUDA, A., YAMAGUCHI, M. & NIC
of molybdenum on the growth
Nutrition, **8**, 35–39.
PIKE, L. H. (1971). *The role of epip*
Ph.D. thesis, University of Or
PIKE, L. H., RYDELL, R. A. & DEN
biomass, surface area, and the
PIKE, L. H., TRACY, D. M., SHER
nitrogen of epiphytes from old
by J. F. Franklin, L. J. Demst
Experiment Station, Portland,
STEWART, W. D. P., FITZGERALD,
blue-green algae. *Archiv für M*
WOLFE, M. (1954). The effect of m
study of the molybdenum requ
Annals of Botany, **18**, 299–308

HITCH, C. J. B. & MILLBANK, J. W. (1975). Nitrogen metabolism in lichens. VII. Nitroenase activity and heterocyst frequency in lichens with blue-green phycobionts. *New Phytologist*, **75**, 239–244.

KELLY, B. B. & BECKER, V. E. (1975). Effects of light intensity and temperature on nitrogen fixation by *Lobaria pulmonaria*, *Sticta weigelii*, *Leptogium cyanescens* and *Collema subfurvum*. *Bryologist*, **78**, 350–355.

OKUDA, A., YAMAGUCHI, M. & NIOH, I. (1962). Nitrogen-fixing microorganisms in paddy soils. X. Effect of molybdenum on the growth and the nitrogen assimilation of *Tolypothrix tenuis*. *Soil Science and Plant Nutrition*, **8**, 35–39.

PIKE, L. H. (1971). *The role of epiphytic lichens and mosses in production and nutrient cycling of an oak forest*. Ph.D. thesis, University of Oregon, Eugene, Oregon.

PIKE, L. H., RYDELL, R. A. & DENISON, W. C. (1977). A 400-year-old Douglas fir tree and its epiphytes: biomass, surface area, and their distributions. *Canadian Journal of Forest Research*, **7**, 680–699.

PIKE, L. H., TRACY, D. M., SHERWOOD, M. A. & NIELSEN, D. (1972). Estimates of biomass and fixed nitrogen of epiphytes from old-growth Douglas fir. In: *Research on Coniferous Forest Ecoystems*. (Ed. by J. F. Franklin, L. J. Demster & R. H. Waring), pp. 177–187. Pacific Northwest Forest and Range Experiment Station, Portland, Oregon.

STEWART, W. D. P., FITZGERALD, G. P. & BURRIS, R. H. (1968). Acetylene reduction by nitrogen-fixing blue-green algae. *Archiv für Mikrobiologie*, **62**, 336–348.

WOLFE, M. (1954). The effect of molybdenum upon the nitrogen metabolism of *Anabaena cylindrica*. I. A study of the molybdenum requirement for nitrogen fixation and for nitrate and ammonia assimilation. *Annals of Botany*, **18**, 299–308.