Technical Report No. 253

PHYLLOSPHERE NITROGEN FIXATION IN VEGETATION OF THE NORTHERN ROCKY MOUNTAINS: A PRELIMINARY STUDY

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U.S. International Biological Program

April 1974

TABLE OF CONTENTS

ABSTRACT

Preliminary studies of phyllosphere nitrogen fixation in dryland plants were made because this source of fixed nitrogen appears important in more moist habitats. After exposure to an atmosphere enriched with 1.7% ^{15}N for 48 hours at 18°C and 100% humidity leaves of *Triticum aestivum* (wheat), *Bromus inermis* (smooth brome grass), and *Poa pratensis* (Kentucky blue grass) were enriched by 0.06% to 0.11%. Under these conditions microbes associated with the leaves of these plants apparently fixed nitrogen at rates of 0.010 to 0.025 mg/g of leaf/hour. If we assume that such conditions prevail in vegetation during 400 hours of the year, and that there are 100 g of leaf material/m^2 we would expect phyllosphere nitrogen fixation of 10 kg/ha/year. (These assumptions are subject to confirmation of the nitrogen fixing potential through use of the acetylene method.) Lower fixation rates observed in range and forest plants suggest that man has selected for this trait of nitrogen fixing through his choosing and breeding crop plants that have this propensity.

INTRODUCTION

Despite denitrification, leaching, and harvest, soil nitrogen may increase by 50/kg/ha/year under native vegetation, tree crops, or herbaceous crops of the temperate zone (Stevenson 1959). Edmisten (1970) reports gains of 161 kg/ha/year in a tropical rain forest. In most instances the amounts of nitrogen added in rainfall, by nonsymbiotic microorganisms, and by legumes (which are sparse or absent) are insufficient to account for such gains (Stevenson 1959; Copley and Reuss 1972).

Work reported here suggests that significant amounts of such nitrogen fixation may occur on the leaves of dryland grasses. Phyllosphere nitrogen fixation has already been reported in several studies made in more mesic habitats. The tropical and subtropical families Rubiaceae, Myrisinaceae, and Dioscoreaceae have leaf nodules containing bacteria capable of fixing nitrogen in pure culture. (Silver 1971; Bond 1967). Blue-green algae in the leaves of some cycads, ferns, liverworts, and lichens fix nitrogen (Silver 1971). Less exact associations of nitrogen-fixing microbes and higher plants at the leaf surface (phyllosphere) have been reported for tropical plants (Ruinen 1961, 1965, 1970; Edmisten 1970), subtropical plants (Vasantharajan and Bhat 1968), and temperate mesophytes (Lipman and Taylor 1922; Ruben, Hassid, and Kamen 1940; Stevenson 1959; Ruinen 1965; Jones 1970).

Significant nitrogen fixation is also associated with the roots of nonleguminous plants (Silver 1971). Besides legumes, several angiosperm genera including *Alnus, Arctostaphylos, Ceanothus, Cercocarpus, Dryas, Eleagnus, Myrica, Purshia,* and *Sheperdia* have nodulated roots which are presumed to fix nitrogen (Silver 1971; Bond 1967). Nitrogen fixation

may also occur in some higher plant-microbe associations of the root nodules of Cycads, Podocarps, and Araucarias (Silver 1971; Allen and Allen 1965) and the rhizosphere. Except at the soil surface where light may serve as an energy source for blue-green algae (Alexander and Schell 1973), nonrhizosphere binitrogen fixation is limited by lack of energy.

We set about to learn whether there are associations of nitrogen fixing microorganisms on the surfaces of predominant plant species of the mountain grassland ecosystem. These results are reported herein.

METHODS

Samples of widespread Montana plants were exposed to atmospheres enriched with ^{15}N, were digested, and had their ^{15}N enrichments determined.

Plant shoots were collected from fields and forests within 30 km of Bozeman, Montana and transported to our laboratory with their bases in water. Collections for experiments 1, 2, 3, and 4 were made in May 1971, May 1973, July 1973, and August 1973, respectively. The plants studied included (i) the grasses, *Triticum aestivum* L., *Hordeum vulgare* L., *Poa pratensis* L., *Bromus inermis* Leyss., *Festuca idahoensis* Elmer, *Agropyron spicatum* (Pursh) Scribn. & Smith, and *Stipa viridula* Trin.; (ii) the dicot, *Salix* spp.; and (iii) the conifers, *Pinus contorta* Dougl., *Pseudotsuga menziesii* (Mirb.) Franco, and *Abies lasiocarpa* (Hook.) Nutt.

Cut plants were transferred within 3 hours to chambers containing ^{15}N gas and held there for 48 hours. Conditions during exposure were (Appendix I): 18° to 26°C, high humidity, 12 to 24 hours of light per day, oxygen available (19%+), and ^{15}N-available (1.7% enrichment). ^{15}N, 95% atom excess, was supplied by Bio-Rad Laboratories, Richmond, California.

After exposure, the plants of experiments 3 and 4 were dried, weighed, triturated, and digested in hot H_2SO_4. Nitrogen contents were determined by the Kjeldahl method (Taras et al. 1971) and samples were acidified with H_2SO_4, dried, and stored in small vials until their ^{15}N contents were determined. Sample nitrogen was then converted to nitrogen gas by addition of sodium hypobromide solution and determined on a Pieka MS-IO mass spectrometer (3 to 10 replications). The percentage of ^{15}N was calculated by the equation:

$$(\text{mass } 29/\text{mass } 28/[2 + (\text{mass } 29/\text{mass } 28)] \times 100.$$

Experiments 1 and 2 were treated similarly except that dry weight was not determined.

^{15}N enrichments were calculated by subtracting the 0.38% ^{15}N measured in standard ammonium sulfate from the percentage of ^{15}N found in the plant material studied. ^{15}N and total nitrogen fixation rates were calculated as explained in Table 1.

One might guess that the nitrogen-fixing organisms were loosely bound on the leaf surface. To test this assumption, ^{15}N contents of plants washed with detergent (Triton X-100) before exposure and unwashed plants were compared. ^{15}N contents of other plants washed after exposure and the ^{15}N content of the wash water were determined as a second test of the same hypothesis. Aside from these washings the plants were treated as described above.

RESULTS AND DISCUSSION

Live leaves of three cultivated grasses, *T. aestivum*, *B. inermis*, and *P. pratensis* harvested in August were enriched with ^{15}N after exposure to the ^{15}N enriched atmospheres (Table 1). Leaves of other plants

Table 1. Nitrogen enrichment of plant material exposed for 48 hours to atmospheres enriched by 1.7% ^{15}N in the light, at 18-24°C, and at 100% humidity.

Plant*	Season and† experiment	^{15}N‡ enrichment (%)	$\frac{mg\ N}{g\ leaf}$ **	$\frac{\mu g\ ^{15}N}{g\ leaf \times hr}$ ††	$\frac{\mu g\ N}{g\ leaf \times hr}$ ‡‡
Agropyron spicatum	spring-2	0.06	8.1	0.10	5.9
	summer-3	0.00	8.1	0.00	0.0
Bromus inermis	summer-3	0.09	9.0	0.17	10.0
Festuca idahoensis	spring-1	0.07	12.0	0.18	0.2
	summer-3	0.00	12.0	0.00	0.0
Poa pratensis	summer-3	0.07	11.9	0.18	10.6
Triticum aestivum	summer-4	0.11	18.1	0.43	25.4

*Plant species showing no enrichment were: Experiment 2 *Stipa viridula* (live and dead); Experiment 3 *Festuca idahoensis* and *Agropyron spicatum*; Experiment 4 *Hordeum vulgare*, *Salix* sp., *Pinus contorta*, *Abies lasiocarpa*, *Pseudotsuga menziesii* (leaves and rotting sapwood): in every chamber where fixation occurred on one plant species, it failed to occur on others.

†Experiment 2 may be less reliable than experiments 1, 3, and 4 since digestions and Kjeldahl nitrogen determinations were made by graduate students in a biochemistry course.

‡Compared to standard ammonium sulfate $(NH_4)_2SO_4$ read as 0.41% nitrogen-15 in experiments 1 and 2 and as 0.38% in experiments 3 and 4.

**% N = (mg N/g) × 0.1

††(enrichment % × µg N/g = µg ^{15}N fixed in 48 hours)/48 hours.

‡‡Micrograms ^{15}N fixed per hour × total atmospheric N/^{15}N enrichment. 95% of the atmospheric binitrogen in experiment 1 was ^{15}N while 1.7% of it was in experiments 2, 3, and 4.

exposed to the same gas, in the same chamber, and at the same time were not so enriched so contamination of the gas by other nitrogen forms does not explain the enrichment. *H. vulgare* was not so enriched. Nitrogen fixation by *Triticum*, *Hordeum*, *Dactylis glomerata* L., and *Tripsacum laxum* Nash has been reported from field studies (reviewed by Stevenson 1959), solution culture studies (Lipman and Taylor 1922; Stevenson 1959), ^{13}N studies (Ruben, Hassid, and Kamen 1940), and observation of nitrogen-fixing organisms on their leaves (Ruinen 1970). The fixation was associated with the leaves of *Tripsicum* (Ruinen 1961), the roots of *Dactylis* (Stevenson 1959), and with no specific plant parts in *Triticum* and *Hordeum*.

Nitrogen-fixing organisms were less numerous or less active on the leaves of native grasses (Table 1); perhaps plant breeders have selected for nitrogen-fixing ability as they selected for yield. Small enrichments were observed in *F. idahoensis* and *A. spicatum* leaves collected in May, but no fixation occurred on plants collected in August. Such seasonality might be expected if nitrogen is fixed by microorganisms located on the leaf surface since the frequency and length of moist conditions (and therefore population sizes) are lower in the summer than in the spring. No fixation was associated with *S. viridula* leaves collected in the spring. Last year's *Stipa* straw was not enriched after exposure to ^{15}N gas; one unreplicated measurement of *A. spicatum* suggests that nitrogen-fixers are found on its old flowering culms (Table 2).

Leaves of one dicot (*Salix* sp.) and three conifers (*P. menzeisii*, *P. contorta*, and *A. lasiocarpa*) were not enriched with ^{15}N after

Table 2. Effects of washing on the mean ^{15}N enrichments (%) of plants exposed to atmosphere enriched with ^{15}N gas.*,†

Plant	Unwashed	Washed before exposure	Washed after exposure	Wash water after exposure
Agropyron spicatum	0.05	0.16	0.04	0.09
Agropyron spicatum (dead)	0.19	0.02	0.00	--
Festuca idahoensis	0.06	0.00	--	--
Stipa viridula	0.00	0.00	0.02	0.01
Stipa viridula (dead)	0.00	--	--	--

*Plants were collected in May 1973 and left untreated or washed. Some plants were washed before exposure and some after exposure. The washing process consisted of a brief soak in solution, a light rub, and a rinse. Wash water from plants washed after exposure was also analyzed for nitrogen enrichment.

†Digestions and Kjeldahl nitrogen determinations were performed by graduate students in a plant biochemistry course.

exposure to ^{15}N gas (Table 1). The lack of fixation by these plants may be due to the dryness of the sites and the season in which they were collected. Nitrogen-fixing organisms have been associated with leaves of a variety of tropical to moist temperate dicots including species of *Morus, Prunus, Phaseolus,* and perhaps *Epilobium* (Ruinen 1961, 1965; Stevenson 1959; Vasantharajan and Bhat 1968). In temperate conifers nitrogen fixation has been associated with leaves of *P. menziesii* (Jones 1970) and roots of *Pinus radiata* (Stevenson 1959).

Nitrogen fixation by leaves or roots of higher plants is likely due to prokaryotes on or in them. This conclusion may be drawn from attempts to demonstrate nitrogen fixation by higher plants grown under sterile conditions (compare Burris 1941 with Ruben, Hassid, and Kamen 1940) or from surveys of their nitrogen-fixing microbial associates (Ruinen 1961, 1965, 1970; Vasantharajan and Bhat 1968; Silver 1971). Nitrogen-fixing bacterial associates include *Azotobacter, Beijerinckia, Aerobacter, Pseudomonas, Sprillum, Klebsiella, Bacterium, Mycobacterium,* and *Chromobacterium* species (Ruinen 1961, 1965, 1970; Vasantharajan and Bhat 1968; Silver 1971). Nitrogen-fixing blue-green algal associates include *Nostoc, Calothrix,* and *Anabena* (Silver 1971).

Organisms of the leaf surface are probably dependent on dew or rain for growth (Ruinen 1961) while those organisms inside the leaf have longer periods of activity. Certainly the nodule-forming bacteria of the Rubiaceae, Myrisinaceae, and Dioscoreaceae are internal; Jones' (1970) inability to scrub nitrogen-fixers from leaves of *Pseudotsuga* suggest that some of them may be internal. The lower rates of fixation associated with washed than with unwashed leaves of *F. idahoensis*

(Table 2) suggest that nitrogen-fixers may be loosely attached to its surface. If *A. spicatum* does indeed fix more ^{15}N gas when its leaves are washed in detergent before exposure (Table 2) this might be due to better exposure of the internal organisms to the gas by removal of parts of the cuticle. The heavy enrichment of wash water relative to plant material when plants are washed after exposure to ^{15}N suggests that some, but not all, phyllosphere organisms are loosely bound at the leaf surface. This observation is consistant with the hypothesis, presented above, that phyllosphere nitrogen-fixing populations are smaller or less active in August than in May because they are exposed to more severe drought in the summer.

The possible agricultural and ecological significance of nonleguminous nitrogen fixation is suggested by observations of increases in soil nitrogen at rates of 30 to 50 kg/ha/year and even 160 kg/ha/year (Stevenson 1959; Edmisten 1970). One may speculate on the significance of phyllosphere nitrogen fixation as a component in these processes: (i) at 18°C and approximately 100% humidity we observed nitrogen fixation at rates of 10-25 µg N/g dry leaf/hour (Table 1), (ii) most grasslands have over 100 g leaf material/m^2 (total biomass = 200 to 5000 g/m^2, Whittaker 1970) and might therefore fix 2.5 mg N/m^2/hour, and (iii) if such fixation occurs during 400 hours in a year, it would provide 10.0 kg of N fixed/ha/year. In the Gallatin Valley (Belgrade Airport) temperatures exceed 40°C and humidities exceed 80% during approximately 600 hours (Appendix II); these measurements were made at approximately 6.4 m and are surely a minimal estimate of high humidity periods in the vegetative layer. If moist periods were longer or if organisms are sheltered inside the leaf

or in leaf sheaths (Ruinen 1970), fixation periods might be considerably longer. (iv) Nitrogen fixation rates of this magnitude may be compared with that associated with lightning (0.5 to 4 kg/ha/year: Stevenson 1959; Reuss 1971), with legumes on dry steppes (0.3 kg/ha/year Copley and Reuss 1972), or with asymbiotic microbes (considerably less than 1 to 2 kg/ha/year: Copley and Reuss 1972; Paul, Myers, and Rice 1971).

Experiments in progress are designed to determine: (i) whether wheat plants grown under sterile conditions will fix nitrogen and (ii) how fixation rates are effected by varying the temperature. We hope to confirm and extend our results by using the acetylene method in the summer of 1974.

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APPENDIX I.

APPROXIMATE ENVIRONMENTAL CONDITIONS DURING EXPOSURE OF PLANTS TO ^{15}N ENRICHED ATMOSPHERE

Experiment	1	2	3	4
Temperature (°C)	22	26	18	18
Light* hrs/day	12	24	16	16
Water†	None	SB	SB	SB
Humidity (%)	100	100	100	100
^{15}N, % of atm	1.95	1.95	1.95	1.95
^{14}N, % of atm	Trace	78.1	78.1	78.1
CO_2, % of atm	Trace	1.7	1.7	1.7
O_2, % of atm	98	19.3	19.3	19.3

*Lights were normal lab florescent lights, a bank of florescent lights, and both florescent and incandescent lights in experiments 1, 2, 3, and 4.

†SB = stem base in water, In experiments 3 and 4 the water was covered with a thin layer of mineral oil.

APPENDIX II.

HOURS WITH HUMID CONDITIONS AND THEIR TEMPERATURES AT BELGRADE, MONTANA.*

Table 1. Humidity = 100% at 6.4 m.

Temperature (°C)	Month												
	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Tot
-30 to -20	--	--	--	--	--	--	--	--	--	--	--	--	--
-20 to -10	2	--	--	--	--	--	--	--	--	--	1	4	7
-10 to 0	--	--	--	--	--	--	--	--	--	2	0	2	4
0 to 5	6	--	3	--	11	--	--	--	--	1	3	--	24
5 to 10	--	--	--	--	4	6	--	--	6	--	--	--	16
10 to 15	--	--	--	--	3	24	--	3	1	--	--	--	31
15 to 20	--	--	--	--	--	--	--	--	--	--	--	--	--

Table 2. Humidity exceeds 80% at 6.4 m.

Temperature (°C)	Month												
	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Tota
-30 to -20	7	--	5	--	--	--	--	--	--	--	---	32	44
-20 to -10	86	66	32	37	--	--	--	--	--	17	62	202	602
-10 to 0	75	132	79	59	--	--	--	--	30	85	174	123	657
0 to 5	34	39	53	125	109	36	10	--	88	148	56	11	709
5 to 10	4	--	--	44	142	150	19	2	68	26	--	--	455
10 to 15	--	--	--	--	18	64	19	21	28	--	--	--	150
15 to 20	--	--	--	--	--	--	2	25	--	--	--	--	27

*Humidities and temperatures were recorded at hourly intervals at 6.4 m. The figures presented are a direct tabulation of those data: if the humidity was 100% on the hour, it was assumed to be for the whole hour. The length of humid periods may have been overestimated by this method. The frequency and length of humid periods was likely higher in the vegetation than at 6.4 m.