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NITROGEN FIXATION IN GRASSLAND AND ASSOCIATED CULTIVATED ECOSYSTEMS

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SUMMARY

Nitrogen fixation in the natural, Agropyron–Koeleria grassland ecosystem was studied using the C_2H_2–C_2H_4 and N^{15} assays. Small soil samples and also undisturbed soil cores were used for analyses. Both techniques indicated that grassland and associated cultivated soils had low fixation rates (0.6–1.8 kg/ha per 28 days in the laboratory and, 1 kg/ha per season under actual field conditions). Algal colonies (*Nostoc* spp.) on the soil surface were active fixers when the surface of the grassland was moist. However, their small biomass limits the extent of fixation in most areas.

In native grassland, 16 legumes bore nodules. The three most common species *Vicia americana, Thermopsis rhombifolia* and *Oxytropis sericea,* all of which had active nodules, contributed 10 per cent of the total nitrogenase activity.

The non-legumes *Elaeagnus commutata* and *Shepherdia argentea* were profusely nodulated with active nodules, but were confined to specific habitats. No nodules were found on *Artemisia* or *Opuntia* spp.

The major, heterotrophic, asymbiotic bacteria in the soil were clostridia. These utilize substrates produced by aerobic cellulose and hemicellulose degrading organisms to fix N in anaerobic microsites. The C_2H_2:N_2 reduction ratio was 3 to 1 in large, aerobic core samples, but was greater under waterlogged conditions where high fixation rates occurred.

INTRODUCTION

A thorough understanding of the factors affecting nitrogen accumulation and distribution is important in interpreting the fertilizer requirements of crops and the cycling of nitrogen in nature. Interest centers on the accretion of nitrogen per unit area per unit time, and on possible means of increasing nitrogen accretion. In many soils of the semi-arid regions, small grain pro-

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duction under cultivation usually prevails and few legumes are utilized. In associated grassland soils, where some legumes are present, the relative significance of both symbiotic and asymbiotic nitrogen fixing processes must be fully assessed. This situation dictates the need for *in situ* field measurement of nitrogen fixation, identification of the organisms responsible, and determination of the parameters which regulate the nitrogen-fixing activity of these organisms.

Removal of N is small in native grasslands, yet they respond strongly to addition of fertilizer-N. This suggests that nitrogen availability is one of the major factors affecting productivity of these sites even though there usually is a fairly large amount of organic-N in the system.

In native pastures legumes usually have a sparse distribution and lack vigour. Nodulated non-legumes are present in specific habitats [7 13], although their N fixing capacity has not been confirmed. Recent reports of root nodules on the semi-arid genera Opuntia (prickly pear cactus) and Artemisia (pasture sage) [3] may be relevant as both plants are common in native grasslands of the Great Plains of North America.

Algal crust organisms have been implicated in N fixation in many environments including semi-arid rangeland [5 8 12]. Heterotrophic N-fixing bacteria are ubiquitous in soil, and where the annual increments of N are small, they may assume a relative importance. Given adequate energy-rich material and anaerobic or partially anaerobic conditions, prairie soils have been shown to fix substantial quantities of N [9]. This paper describes a study of the nitrogen fixing potential of the natural Agropyron–Koeleria grassland ecosystem and a wheat summerfallow system. Fixation by heterotrophic, free-living micro-organisms, nodulated non-legumes and legumes, and blue-green algae were investigated to assess their relative contribution. Fixation was measured by increase in total N by Kjeldahl techniques, by the N^{15} isotopic method, and by the C_2H_2–C_2H_4 assay.

MATERIALS AND METHODS

Soil

The majority of samples were obtained from the Matador Field Station of the International Biological Programme. The site situated in Southern Saskatchewan, Canada has clay soils (Sceptre association). The soil has a pH of 7.5 (water saturated paste), the organic carbon is 2 per cent and total nitrogen 0.22 per cent.

Kjeldahl techniques

N fixation was determined in 0.6 g samples of soils amended to contain 0, 20, 50 and 80 per cent straw under waterlogged conditions. The samples, contained in 20 cc bottles, were incubated at 28°C. Analysis was by standard Kjeldahl digestion, followed by distillation of the ammonia into 2 per cent boric acid and electrometric titration [2].

N^{15} techniques

Small soil samples (3.0 g) amended with 3 per cent straw, were incubated at field moisture content for 28 days in an atmosphere containing N_2^{15} (20.32 per cent abundance). Total N was determined by the Kjeldahl method and N^{15} was determined on a MAT GD–150 mass spectrometer.

Undisturbed cores, 15 cm diameter by 60 cm height, were obtained from native grassland and cultivated sites. The cultivated soils were amended with 448 g/m^2 straw and all received sufficient moisture to bring the upper 15 cm to field capacity. The cylinders were sealed by a screw-on base, and by perspex caps with an O-ring seal. The samples were exposed to an atmosphere containing 20 per cent O_2, 10 per cent N_2 (3.29 atom % excess N^{15}) and 70 per cent He, and incubated for 28 days with the O_2 level being maintained between 15–21 per cent, and the temperature 19–28°C with a 16 hour day length. After incubation, plant materials and the soil were sampled, dried and analysed for total N and N^{15}.

Acetylene reduction assay

The technique was similar to that of Hardy *et al.*[4] and Stewart *et al.*[14]. Test materials were exposed to an atmosphere containing 20 per cent O_2, 70 per cent He, and 10 per cent C_2H_2. The container used varied with the sample size. Gas samples were taken by a gas-tight syringe through a rubber serum cap for analysis of C_2H_4, using a Varian Aerograph gas chromatograph. This technique was used with small soil samples, with excised nodules from legumes and non-legumes, with soil cores, and with samples of blue-green algal colonies. The nodule samples were exposed for short periods of less than one hour. Soil samples were exposed for 24 to 48 hours. To define the moisture status under which they fix N, algae were wet to saturation and exposed to C_2H_2 daily for one to four hours as they dried from saturation

to air-dry. The sample size was 0.20 g air-dry (0.176 g oven dry, 50 per cent ash) and the atmosphere contained 5 per cent CO_2.

Nitrogenase activity under field conditions was determined during 1970 by taking 6 cm by 12.5 cm samples with a hydraulic coring unit. The cores were exposed to a C_2H_2–O_2–Ar mixture in micro-canopies made from glass jars. Each jar was sealed with a rubber ring and brass base fitted with a serum cap. This was placed in the soil with the glass surface acting as the top of the canopy. Partial shading was used to maintain the inside temperature close to that of the surrounding soil. The amount of nitrogenase activity in the dark was determined by painting the exposed area of some of the jars with aluminum paint. The rate of C_2H_4 production was used to calculate nitrogenase activity using a theoretical ratio of 3 to 1, between C_2H_4 production and nitrogenase activity.

RESULTS

Asymbiotic fixation

Amendment with high levels of crop residues and waterlogged conditions under a normal atmosphere stimulated active fixation in a range of soils (Table 1). Most rapid fixation occurred with 50 per cent straw (data not shown) which showed a 10 per cent increase in total N over 28 days incubation under waterlogged conditions. Incubation of soils with 3 per cent straw but at moisture levels equivalent to field capacity resulted in lower amounts of nitrogen fixation than those reported by Rice *et al.*[9]. However, the previously reported results were obtained under conditions where partial anaerobiosis could have occurred.

TABLE 1

Nitrogen fixation in disturbed soil samples amended with straw and incubated 28 days

Soil type	Texture	N content before incubation mg N/g	N fixed with 3% straw field cap μg/g*	N fixed with 20% straw waterlogged μg/g**
Chestnut	Clay	2.45	0.01	68
	Loam	2.26	0.11	420
	Sandy Loam	2.14	0	280
Chernozem	Loam	4.01	0.03	180
	Sandy Loam	3.36	0	30
Luvisolic	Loam	2.21	0.06	0

* Detn with N^{15}.

** Detn by Kjeldahl.

TABLE 2

Acetylene reduction by small soil samples of Sceptre clay

Soil	C_2H_2 reduction n moles/core/h*	Nitrogenase activity g/ha per 28 days
Virgin (field moisture)	0	0
(wet)	0.48	0.10
Cultivated (field moisture)	0	0
(wet)	0.15	0.03

* core 2 cm × 15 cm.

There were large differences in the amount of nitrogen fixation in the different soils, with the medium textured soils generally showing the highest fixation rates.

A large number of soil samples obtained during 1968 and 1969, with a 2-cm corer, and exposed to C_2H_2 did not show significant nitrogenase activity when incubated at field moisture levels. Wetting the soil to field capacity, however, often resulted in activity levels that were detectable but very low (Table 2).

The use of soil cores large enough to take a representative, relatively undisturbed sample has been found very useful in microbiological analysis of soils [1]. During the 1970 growing period, 6 cm diameter, 12.5 cm deep soil cores were obtained at different intervals, and exposed to C_2H_2 under field conditions. The average monthly acetylene reduction under field conditions are shown in Table 3. The values for May were obtained in the latter part of the month

TABLE 3

Average nitrogenase activity, under field condition, of soil plus vegetation cores from Sceptre clay (expressed as g N-fixation potential per hectare per hour)

Sampling month 1970	Virgin		Cultivated (light)
	Exposed to Light	Dark	
May	0.29	nd	nd
June	0.20	0.12	nd
July	0.38	0.16	0.002
Aug.	0.05	0	0.01
Sept.	0.02	0	0

nd = not determined

and are probably high. The data for individual cores showed a great deal of variability which does not show in the tables since the averages were determined from 15 to 25 separate cores. Where results for individual cores were high, examination usually showed the presence of nodulated legume roots or algal crusts. These however were infrequent. Summation of the data indicates a total fixation of 0.71 kg per ha for the virgin soil during the growing season.

Incubation in the dark gave lower results. It had been hoped that this would indicate heterotrophic fixation, however, preliminary work with algal crusts indicate that they can reduce acetylene for some time after being placed in the dark. Adsorption of ethylene does occur to a small extent, however, no corrections for adsorption were made for these data. Results from irrigation experiments indicated a 10- to 20-fold rise in nitrogenase activity 40 hours after irrigation. The level slowly dropped to normal during the next 7 to 10 days.

The results of two experiments, using 15-cm diameter soil cores of both cultivated and virgin Sceptre clay containing growing plants but incubated in the growth chamber, corroborated the field data (Table 4). In cultivated soils with no history of fertilizer addition, fixation was detected to a depth of 7.5 cm with a total of 0.06 g N per m^2 in 28 days. Fixation in the virgin soil was greater but was restricted to the surface 2.5 cm. The fixation in the surface of the

TABLE 4

Nitrogen fixation in undisturbed cores of Sceptre clay at field capacity (using N^{15} techniques), in the growth chamber

Soil	Depth (cm)	Final H_2O (%)	Total N (%)	Final mineral N		kg/ha in 28 days	
				NH_4^+-N µg/g	NO_3^--N µg/g	N fixed	Total
Cultivated	0–2.5	38.8	0.291	13.7	8.0	0.15	
	2.5–7.5	38.0	0.278	15.3	11.0	0.44	0.59
	7.5–15	35.3	0.255	7.8	5.8	0.00	
Virgin	'crust'	n.d.	0.732	n.d.	n.d.	0.48	
	0–2.5	45.1	0.364	13.5	20.5	1.04	
	2.5–7.5	38.0	0.253	6.2	9.1	0.00	1.52
	7.5–15	36.3	0.176	4.4	4.5	0.00	

n.d. Not determined.

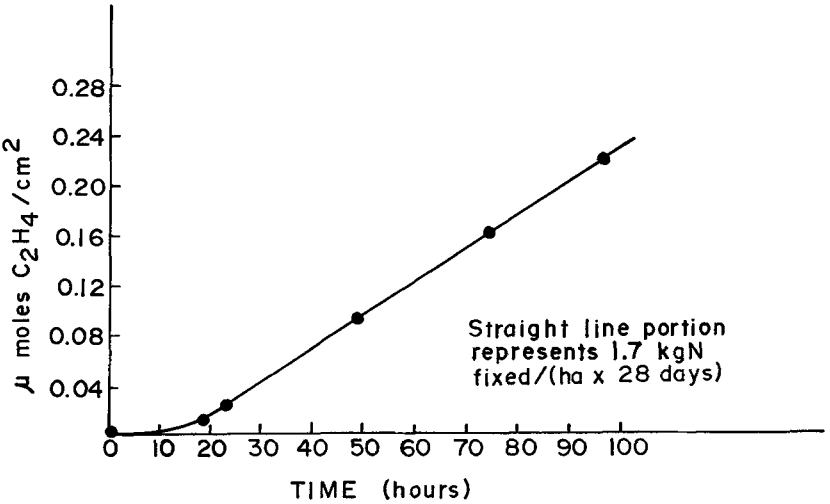

Fig. 1. C_2H_2 to C_2H_4 reduction by a Sceptre soil core (15 cm × 60 cm) as a function of time.

virgin could indicate the use of litter by the N-fixing organisms. More likely, it is attributable to blue-green algae such as Nostoc which are distributed on the surface, as well as being present as a visible crust. Cores of the virgin soil were also assayed with the C_2H_2 method. The cumulative C_2H_2 to C_2H_4 reduction is shown in Figure 1. The straight line portion of this curve represents 1.7 kg N fixed/ha per 28 days. The theoretical conversion factor of 3 moles C_2H_2 reduced for each mole of N_2 fixed was used in calculating this value.

The effect of the plant cover on acetylene reduction was determined in 10 cm × 30 cm deep cores. Four vegetation types were studied in the growth chamber-grass mixture, *Vicia americana* (American vetch), *Opuntia polyacantha* (prickly pear cactus), and *Artemisia cana* (pasture sage). *Vicia americana* showed the highest rate of acetylene reduction (Table 5). However, all samples produced ethylene, with *Artemisia cana* and the grass mixture producing more than the cactus. *Vicia* and *Astragalus* occurring in equal proportion, accounted for the majority of legumes in the grazed prairie with a total occurrence of 2.3 plants per m^2. In the ungrazed prairie, *Vicia* predominated with a total legume occurrence of 1.4 plants per m^2. Calculation of the fixation potential

TABLE 5

Nitrogenase activity of undisturbed (15 cm diam. × 30 cm depth) soil cores of different natural vegetation on Sceptre clay

Species	Area occupied %	C_2H_4 production, μM/g soil Time of incubation (hrs)		Nitrogen fixation	
		24	48	μg N/15 cm core per 28 days	gN/ha per 28 days
Opuntia polyacantha	negligible	204	276	15	—
Artemisia cana	6	558	861	46	22
Grass mixture	90	505	688	37	261
Vicia americana and *Astragalus spp.*	2	1444	3115	160	35.3
Others Composite	2	—	—	32*	5.0

* Average of non leguminous area.

by a composite of plants based on their density on the site indicates a nitrogenase activity equivalent to 0.34 kg N per ha during 28 days activity equal to that occurring during the analyses period in the growth chamber.

The Nostoc crust represents an efficient system on a weight basis, *i.e.* it had a 3 per cent increase in nitrogen during the 28-day incubation period. Analysis of the crust from the virgin core in the N^{15} experiment indicated a fixation of 8.6 μg N per g of oven dry material per day, which agrees with the 7.5 μg N per g per day which was calculated from the results reported by Mayland *et al.*[6] for similar materials. Samples uncontaminated with soil and incubated at high water content, were capable of fixing 30 μg N per g of dry material per hour.

When conditions were dry, the crusts dry out and become nactive, but within minutes of being rewetted they become green and active. The factor limiting N fixation would be the duration of active fixation. This is determined by the time the colonies remain moist after rainfall or dew. Nitrogenase activity was high at the saturation moisture level (580% H_2O, Table 6), activity peaked at 450 per cent H_2O and declined during the drying period until the crust was apparently dry (16%). The colonies dried initially on the outer surface, they thus continued to be active even though superfically dry at 45–70 per cent H_2O. The biomass of Nostoc crust on the overall project site has been estimated at

TABLE 6

Acetylene reduction by *Nostoc spp.* crusts at varying moisture contents. C_2H_4 production ($\mu M/g$)*

Time (h)	580	525	465	410	340	260	200	140	70	45	16
		Moisture content (%)									
1	0.24	2.17	2.74	2.43	0.86	1.38	1.58	1.04	0.56	0.00	0.00
2	1.00	n.d.	6.63	6.28	2.41	3.11	3.29	2.16	0.98	0.03	0.00
3	2.62	7.47	9.75	9.15	3.96	4.80	4.87	3.57	1.66	0.13	0.00
4	5.00	11.26	12.91	13.15	5.55	7.52	6.79	4.66	2.35	0.26	0.00

* Conversion factor for μg N/g is $\times 9.3$.

0.23 g per m^2. This means that a maximum of 5.2 mg N per m^2 could be fixed during 28 days. Nostoc occurs in heavier concentrations on specific areas of the site such as eroded slopes. On these areas it attains a concentration of 4 g per m^2 indicating a much higher fixation potential at these sites.

Symbiotic nitrogen fixation

A total of 16 legume species found on the Matador site were examined and found to bear nodules although in some instances the nodules were sparse. These species were: *Astragalus missouriensis, A. dacyglottis, A. pectinatus, A. tenellus, A. bisulcatus, A. gilviflorus, A. striatus, Hedysarum cinerescens, Psoralea argentea, Ps. esculenta, Glycorrhiza lepedota, Petalostemon* sp., *Oxytropis sericea, Vicia americana, Thermopsis rhombifolia* and *Medicago sativa.* All nodules tested with the C_2H_2 assay gave positive results (Table 7). However only three of the species are common on the experimental area, the remainder are confined to coulees and hillsides.

Two species of nitrogen fixing, nodulated non-legumes occurred in the area, *Elaeagnus commutata* (wolf-willow) and *Shepherdia argentea* (buffalo berry). Nitrogenase activity by the profuse nodules was confirmed by acetylene assay. The nodules were obtained from relatively dry soil in the summer; addition of H_2O greatly enhanced activity (Table 7).

Following reports of nodulation in cactus and *Artemisia* spp. roots [3] other non-legumes were investigated in the field. The following species were studied but no nodules were identified. *Opuntia polyacantha, O. fragilis, Mamillaria vivipara, Artemisia*

TABLE 7

Acetylene reduction by excised nodules of legumes and non-legumes

Species	C_2H_4 production μM/g o.d. nodule per h
Thermopsis rhombifolia	31,000
Psoralea argentea	2,400
Astragalus dacyglottis	260
Oxytropis sericea	5,600
Vicia americana	5,160
Elaeagnus commutata (field moisture content)	130
Elaeagnus commutata (moist)	2,850
Shepherdia argentea (field moisture content)	150
Shepherdia argentea (moist)	11,760

cana, Artemisia frigida. Small protuberances on the roots of the three cactii species could not be positively identified as nodules. Acetylene assays confirmed these observations in that no nitrogenase activity occurred, except in the case of soil-root samples. No other species in the area were found to have root nodules.

Organisms and biological processes involved in asymbiotic nitrogen fixation

The addition of a substrate such as straw and incubation under waterlogged conditions in an atmosphere containing 20 per cent O_2 resulted in high fixation rates, *i.e.* up to 800 kg/ha (Table 1). This indicated the possibility of significant fixation in microsites or at specific times in the field, and made it possible to study the factors affecting nitrogen fixation in model systems. The C_2H_2 assay demonstrated two maxima of nitrogenase activity in waterlogged soil straw and sand-clay-straw mixtures which were incubated for 28 days [10].

The nitrogen fixation in the model systems was attributed to a degradation of the straw by hemicellulases and cellulases produced by the aerobic, heterotrophic microflora. The intermediates produced were then utilized by the clostridia under anaerobic conditions for nitrogen fixation.

During incubation, clostridia numbers increased from 10^3 to 10^7 per g in a soil-straw model system [11]. Of 35 clostridia isolates, 31 were found to reduce C_2H_2. Azotobactor were not present in the systems, however, a large number of other organisms were

capable of growing on mineral agar to which no nitrogen had been added (NFA plate count). These included large spore-forming rods, non-spore forming rods, pseudomonus-like rods, cocci and some actinomycetes. None of these organisms were capable of reducing C_2H_2 [11].

Rice and Paul [10] found that in this system the (C_2H_2 reduction): (N_2 fixation) ratio did not approximate the theoretical ratio of 3 to 1, unless the system was shaken anaerobically in the presence of N_2 after aerobic preincubation. Calculations showed that the N_2 in solution could become limiting under the conditions of incubation, whereas C_2H_2, which is more soluble in water, was not.

DISCUSSION

Virgin grassland systems may be considered as systems in equilibrium, where the nitrogen-loss mechanisms are of minor importance and nitrogen input requirements are low. This was found to be the case in this study where nitrogen fixation was found to approximate 1 kg per ha per annum. The cultivated soils also have been found to fix low quantities of nitrogen asymbiotically. Under the present extensive method of cultivation, nitrogen input requirements are still low. This must be attributed to the fact that reserves of nitrogen built up during the virgin condition have been adequate to supply the crops for 40 to 70 years after initial cultivation.

The algal genus, Nostoc, was shown to be an active fixer of N and to be widespread over the virgin site. The factors limiting its contribution of combined N are its relatively small biomass over the majority of the site, and the limited time during which moisture conditions are suitable.

The usual major contributers of N fixation, the legumes, could be of importance in the virgin grassland. Three species of legume, *Vicia americana*, *Thermopsis rhombifolia*, and *Oxytropis sericea*, are common on the experimental area although their abundance seldom exceeds one plant per m^2. Nodulated non-legumes occur commonly in coulees and alongside watercourses adjacent to the experimental site. As they are extensively nodulated, and as the nodules are active fixers, these species must be important contributers of N in the localized areas.

Organisms associated with the cacti are not considered to fix appreciable N. Acetylene assays of soil cores containing artemisia reduced slightly more acetylene than cores containing grass mixtures. However, in N^{15} studies, artemisia plants from cores that had been incubated with N_2^{15} showed no enrichment with N^{15} indicating that fixation, if any, by organisms associated with Artemisia was very low. In general, the report of nodulation on Opuntia and Artemisia species [3] is not confirmed.

The other input of nitrogen into this system is the amount coming in via rainfall. Analysis for total N, ammonia N, and nitrate N were conducted according to Bremner [2]. Expressing the data for total N on a kg/ha basis indicated that slightly more N would be brought down by precipitation during the year than was attributed to N-fixation.

The asymbiotic organisms responsible for fixation were shown to be blue-green algae and members of the clostridia. The clostridia have a very high potential for fixation under specific conditions. This involves the availability of an energy supply and waterlogged conditions. In a system such as this, aerobic organisms partially degrade the cellulose and hemicellulose of the residue. The resulting intermediates provide the energy for fixation by the anaerobic clostridia. Nitrogen fixation under these conditions would have to occur in microsites under specific conditions such as immediately after a rain or irrigation when partial anaerobiosis exists.

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