Technical Report No. 152

EVALUATION OF BIOLOGICAL N_2 FIXATION

IN A GRASSLAND ECOSYSTEM

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ABSTRACT

EVALUATION OF BIOLOGICAL N_2 FIXATION

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A series of investigations were conducted to determine the significance of biological nitrogen fixation on the Pawnee Site, the intensive study area of the U. S. International Biological Program Grassland Biome. Additional studies were carried out to investigate the rates of N_2 fixation by leguminous plants as a function of plant age. The acetylene reduction assay, an indirect method of measuring N_2 fixation, was used in all studies. All calculations of N_2 fixation rates were based on a conversion factor of 1 mole of N_2 fixed/3 moles of ethylene produced. The acetylene reduction assay proved to be a sensitive method for measuring low rates of N_2 fixation.

Under field capacity moisture levels without an added energy source, asymbiotic N_2 fixation rates were < 50 $\mu g/m^2/day$, the lower limit of detection in the systems studied. With the addition of readily assimilable carbon, 0.1 to 0.2 mg N_2 was fixed/m^2/day. Under saturated moisture conditions without additional energy supplied, N_2 fixation rates were under 1 $mg/m^2/day$. With the addition of sucrose to saturated soil cores, N_2 fixation rates were approximately 0.1 to 0.2 $g/m^2/day$.

With the addition of sucrose, asymbiotic N_2 fixation rates were greatest (0.25 g/m^2/day) in saturated soils under a helium atmosphere; about equal (0.12 g/m^2/day) with soils at field capacity under a helium atmosphere and with saturated soils under an 80:20 helium: oxygen atmosphere; and lowest (0.15 mg/m^2/day) in soils at field capacity under an 80:20 helium:oxygen atmosphere. Readily assimilable sucrose added to saturated grassland soils at levels of 14-56 g C/m^2 resulted in the fixation of 9-10 mg N_2/g C.

Nitrogen fixation was severely restricted at temperatures of 16°C and lower. Because of the moisture and temperature conditions found at the Pawnee Site, N_2 fixation by heterotrophic organisms is probably of limited significance.

Studies showed that symbiotic N_2 fixation by leguminous plants could represent an important source of nitrogen on grassland ecosystems where legumes compose a significant fraction of the vegetation. Oxytropis sericea demonstrated an average N_2 fixation rate of 0.61 mg/plant/day. However, low legume plant densities on the Pawnee Site suggest that this source of nitrogen is not likely to be significant in this ecosystem.

In single plant comparisons of N_2 fixation rates, soybeans, sweetclover, sainfoin and alfalfa demonstrated maximum N_2 fixation (C_2H_2-C_2H_4 assay) rates of 4.7, 3.3, 3.0 and 2.6 mg/plant/day

respectively. Differences in the N_2 fixation rates between light and

dark incubations were significant for soybeans only.

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December, 1971

ACKNOWLEDGMENTS

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Chapter I

INTRODUCTION

Nitrogen is a key nutrient in a grassland ecosystem. Nitrogen

availability usually limits the productivity of grasslands whenever

sufficient moisture is present for rapid plant growth. In order to

predict the response of an ecosystem to changes in environmental

conditions or to manipulations imposed by man, an understanding

of the dynamics of the nitrogen cycle is essential.

A series of investigations were conducted to determine the

significance of biological nitrogen fixation on the Pawnee Site, the

intensive study area of the U.S. International Biological Program

Grassland Biome. Additional studies were carried out on cultivated

and forage legumes.

At the outset of these investigations, the biological fixation of

elemental nitrogen was suspected to be a major source of nitrogen

in the grassland ecosystem. Little information was available con-

cerning the probability of biologically significant amounts of nitrogen

fixation, either symbiotically or non-symbiotically, in a grassland

environment.

Since fixed nitrogen inputs were thought to be quite small, a

sensitive, rapid and economical method of measuring small amounts

of fixed nitrogen was required. The acetylene reduction assay met

these requirements and was used throughout the study.

Chapter II

LITERATURE REVIEW

The earth is surrounded by an envelope of air consisting of 79% molecular nitrogen. Although inert as far as plants, animals and most microorganisms are concerned, molecular nitrogen is converted to a combined form by a few specific microorganisms in symbiotic association with a higher plant or nonsymbiotically by free-living bacteria and blue-green algae. This conversion of gaseous nitrogen into a combined form readily assimilable by most plants and animals is the process of nitrogen fixation.

Sir Humphrey Davy (1821) in the 1821 edition of his textbook Elements of Agricultural Chemistry, was one of the first to suggest that plants may fix nitrogen when he wrote: "When glutenous and albuminous substances exist in plants, the azote they contain may be suspected to be derived from the atmosphere..." The German workers, Hellriegal and Wilfarth were the first, in 1886-1888 to report that nodule bearing legumes assimilated elemental nitrogen (Stewart, 1966 and Wilson, 1940). Shortly thereafter, in 1893, Winogradsky demonstrated nitrogen fixation by Clostridium pasteurianum, a free living bacterium (Delwiche and Wijler, 1956). Near the turn of the century, Beijerinek discovered the aerobic

nitrogen fixing bacteria Azotobacter, which was soon found to exceed

the clostridia in nitrogen-fixing efficiency (Jensen, 1965). Soon after

the discovery of Azotobacter, an intensive search for other nitrogen-

fixing organisms was begun. This search proved rather unfruitful

until the introduction of the N^{15} tracer techniques in the early 1940's

(Burris and Miller, 1941). Total nitrogen (Kjeldahl nitrogen) was the

principal method available to estimate nitrogen fixation rates prior to

the introduction of labeled nitrogen. Total nitrogen, as measured by

the Kjeldahl method, requires extensive replication and statistical

analysis (Burris and Wilson, 1957) because the amount of nitrogen

fixed is usually small compared to the total nitrogen present.

Depending upon the level of enrichment required for detection

and the isotope abundance level or concentration of N^{15} in the starting

material, the N^{15} technique can be anywhere from 40 to 1000 times as

sensitive as the Kjeldahl technique (Burris and Wilson, 1957, Hardy

et al., 1968, Steyn and Delwich, 1970 and Wilson, 1969).

Labeled nitrogen has found wide application in laboratory

studies to characterize properties of the nitrogenase system (Bergersen,

1965b and Koch, Evans and Russell, 1967a) and to elucidate the bio-

chemical reactions involved in the nitrogen fixation process

(Bergersen, 1965a).

The enzyme system responsible for the reduction of atmospheric

nitrogen to ammonia is called nitrogenase. Preparations of nitrogenase

from free living bacteria (Carnahan et al., 1960) and from root nodules (Bergersen and Turner, 1967 and Koch, Evans and Russell, 1967a) have been fractionated to give two components, both of which are essential for nitrogen fixation (Klucas et al., 1968). These components are proteins which have been named molybdoferrodoxin and azoferredoxin (Postgate, 1970 and Silver, 1967), but are more commonly referred to as the Mo-Fe protein fraction and the Fe protein fraction or simply as fraction 1 and fraction 2.

Preparations of nitrogenase, irrespective of origin reduce nitrogen to ammonia (Postgate, 1970). Such preparations also reduce other triply bonded substrates, such as acetylene (Dilworth, 1966 and Schollhorn and Burris, 1966 and 1967), cyanides, azides (Hardy and Knight, 1967), and nitrous oxide. Hardy and Knight (1967) and Postgate (1970) list the principal substrates that are reduced and their products in Table 1.

Table 1. Principal substrates and reduced products of nitrogenase.

$$N_2 \longrightarrow 2\ NH_3$$

$$C_2H_2 \longrightarrow C_2H_4$$

$$HCN \longrightarrow CH_4 + NH_3\ (+ \text{some } CH_3NH_2)$$

$$N_2O \longrightarrow N_2 + NH_3$$

$$H^+ \longrightarrow H_2$$
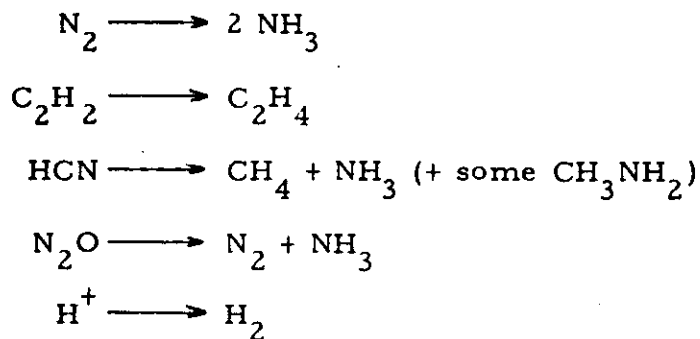
In enzyme preparations the evolution of hydrogen accompanies
all other substrate reductions (Postgate, 1969 and 1970). Hoch, Little
and Burris (1957) and Hoch, Schneider and Burris (1960) observed
hydrogen evolution from whole excised soybean root nodules. Wilson,
Umbreit and Lee (1938) and Wilson (1940) demonstrated that molecular
hydrogen acts as a specific, competitive inhibitor of nitrogen fixation
by nodulated red clover plants, and Wyss and Wilson (1941) found
molecular hydrogen to inhibit nitrogen fixation by Azotobacter in a
similar manner. Hardy and Burns (1968) and Hardy and Knight (1968)
have proposed a scheme (figure 1) for nitrogenase and its reactions
based on an electron activation, 2 site hypothesis, by which nitrogen
fixation and hydrogen evolution can take place concurrently. Burris
(1966 and 1969) and Bergersen (1969) propose a somewhat different
scheme for nitrogen fixation, but present information that supports
the scheme of Hardy and co-workers.

In the electron activation, 2 site hypothesis, electrons from a
suitable donor (Bergersen, 1970, Hardy and Burns, 1968 and Hardy
and Knight, 1968) react with an oxidized group of nitrogenase (assumed
to be a metal protein complex) represented by X_{ox} in figure 1, and
utilize ATP to produce ADP, inorganic phosphorus, and a reduced
group with increased reduction potential, represented by X_{red}. The
electron activating site operates at the same rate, independent of the
presence or absence of substrates and appears to be the limiting factor
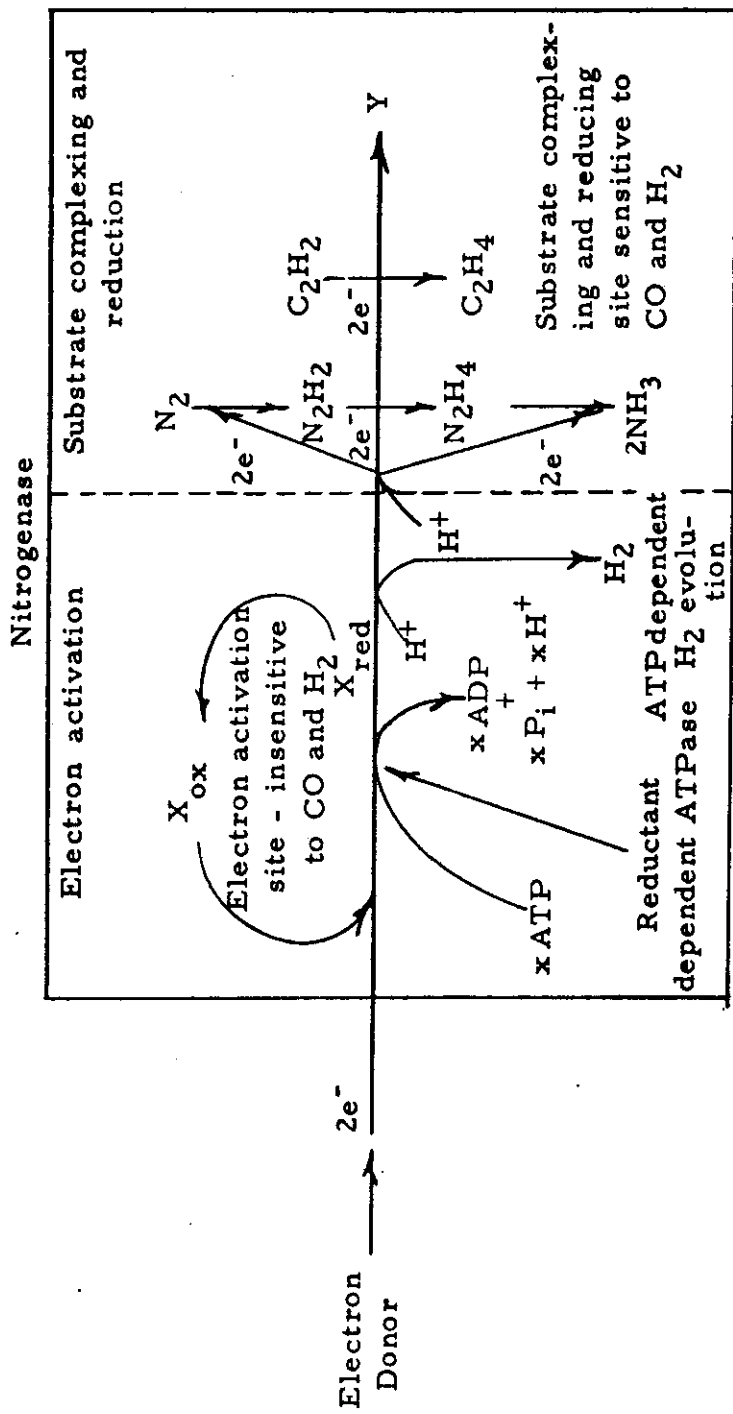
Fig. 1. Scheme for nitrogenase and its reactions based on the electron activation, two site hypothesis. (Adapted from Hardy and Burns, 1968 and Hardy and Knight, 1968).

in N_2 fixation (Hardy and Burns, 1968 and Hardy and Knight, 1968).

A second site, the substrate complexing and reducing site, represented by Y in figure 1, is distinguished from the electron activating site, X, on the basis of selective CO and H_2 sensitivity. When a substrate is present, X_{red} (which may be a protein metal hydride) donates some of its electrons for the reduction of the substrate. Electrons used for the reduction of substrates are, in general, equivalent to the decrease in electrons evolved as H_2. However, even at maximal rates of reduction of N_2 or other substrates, H_2 is still evolved. The variety of substrates reduced by nitrogenase and the variety of products produced, indicate the versatility of site Y with respect to both binding and reduction.

Ammonia is considered to be the key intermediate or the first stable product of the nitrogen fixation process (Bergersen, 1965, Wilson and Burris, 1953, and Zelitch et al., 1951). No stable or transient intermediate has been detected as yet. Alexander (1961) defines the key intermediate as the end-product of inorganic conversion and the point at which nitrogen is assimilated into organic molecules. Glutamic acid is formed as the primary organic compound by reductive condensation with α-keto-glutaric acid (see below). Other amino acids then arise by transamination and proteins by condensation of amino acids (Jensen, 1965).

$$\begin{array}{ccc}
\text{COOH} & & \text{COOH} \\
| & & | \\
\text{CO} \quad + \text{NH}_3 \xrightarrow[\substack{\text{glutamic acid} \\ \text{dehydrogenase}}]{(2\text{H})} & \text{H}_2\text{NCH} & + \text{H}_2\text{O} \\
| & & | \\
\text{CH}_2 & & \text{CH}_2 \\
| & & | \\
\text{CH}_2\text{COOH} & & \text{CH}_2\text{COOH} \\
\text{a- ketoglutaric} & & \text{glutamic} \\
\text{acid} & & \text{acid}
\end{array}$$
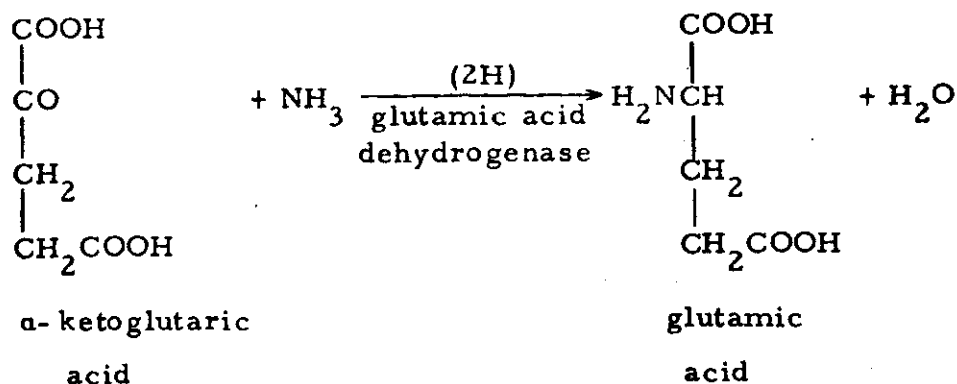
In the 30 years following Hellriegal and Wilfarth's linking of nodules on legumes to the fixation of atmospheric nitrogen, investigators sought to develop methods for the most efficient use of legumes. Some fundamental research was done on physiology of the bacteria, but few attempts were made to arrive at an understanding of the basic nature of the biological nitrogen fixation process (Wilson, 1940). In 1928, Drewes became the first investigator to provide concrete evidence that blue-green algae fix atmospheric nitrogen (Stewart, 1966).

From the early 1940's until 1966, the verification of biological nitrogen fixation by free living bacteria, blue-green algae, and symbiotic nitrogen fixing plants was made principally by the use of the N^{15} tracer technique. In 1966 it was found that nitrogenase preparations from Clostridium pasteurianum not only catalyzed the reduction of elemental nitrogen, but also the reduction of acetylene (Dilworth, 1966 and Schollhorn and Burris, 1966 and 1967). Dilworth found acetylene to be reduced to ethylene in a reaction analogous to the reduction of nitrogen. He also noted that the ethylene, once formed

is not further reduced and that it does not interfere with further reduction of acetylene. Schollhorn and Burris (1967) established the competitive nature of the inhibition of nitrogen fixation by acetylene. Carnahan and co-workers (1960) had described methods for extracting and activating the nitrogenase from Clostridium pasteurianum some six years earlier. Hardy and Knight (1967) suggested the utilization of the reduction of acetylene to ethylene and detection of ethylene by hydrogen flame ionization following gas chromotography, as a means of measuring nitrogenase activity. Stewart, Fitzgerald, and Burris (1967) tested the feasibility of the acetylene reduction technique, as it has become known, to provide an index of nitrogen fixation in the field. They found that the reduction of acetylene to ethylene could be employed as an index of nitrogen fixation in situ, in aquatic environments, in soils, and by nodulated plants. Hardy et al. (1968) carried out a comprehensive study of the relationship of acetylene reduction to nitrogen fixation under both laboratory and field conditions. Silver and Mague (1970) employed the acetylene reduction assay to assess the nitrogen fixing activity of excised nodules from Myrica cerifera, a symbiotic nitrogen-fixing, non-legume, while Koch and Evans (1966) used it on excised soybean root nodules. Howard et al. (1970) utilized the acetylene reduction technique to measure biological nitrogen fixation by blue-green algae in Lake Erie and by bacteria in sediment collected from the bottom of Lake Erie.

The reduction of N_2 to $2NH_3$ involves the transfer of six electrons, whereas the reduction of acetylene to ethylene requires the transfer of two electrons (Hardy and Knight. 1967, Schollhorn and Burris, 1967, Stewart, Fitzgerald, and Burris, 1967 and Steyn and Delwiche, 1970). On this basis, it might be anticipated that 3 moles of ethylene would be formed for each mole of N_2 fixed (Stewart et al., 1967). Values close to this theoretical value have been obtained by several investigators (Hardy et al., 1968, Klucas et al., 1968 and Schollhorn and Burris, 1967) using cell-free extracts of nitrogen-fixing organisms, but there is little information from intact living systems or complex soil systems (Bergersen, 1970 and Steyn and Delwiche, 1970). Bergersen (1970) calculated a ratio of ethylene produced to nitrogen-fixed ($C_2H_4:N_2$) of 3:1 for growing cultures of Klebsiella aerogenes under anaerobic incubation conditions. However, he noted that the ratio of ethylene produced to nitrogen-fixed for detached soybean root nodules ranged from 5.4 to 8.4 with a mean ratio of 6.6. Steyn and Delwiche (1970) report that in the majority of soil systems investigated the ratio of acetylene reduced to nitrogen fixed ranged from 3.0 to 4.5. The ratios of those soils outside of this range were all larger than 4.5.

Because the acetylene reduction technique is an indirect measure of N_2 fixation and because few correlation studies between acetylene reduction and N_2^{15} fixation by intact living systems and complex soil systems have been made, investigators are cautioned against using the acetylene reduction assay as an absolute quantitative measure of

N_2-fixation (Stewart et al., 1967 and Steyn and Delwiche, 1970).

Present information would indicate that the 3:1 ethylene to N_2-fixed

ratio would tend to overestimate biological nitrogen fixation rather

than underestimate it. Even if the acetylene reduction assay is not

entirely quantitative for all systems, it does provide a rapid, inex-

pensive and very sensitive means of making comprehensive studies

of organisms and conditions responsible for biological N_2-fixation.

The legumes, which are dicotyledonous plants of the family

Leguminosae, have, because of their importance to soil fertility,

received more attention to date than any other nitrogen-fixing group.

Nutman (1965) and Alexander (1961) provide extensive descriptions of

the nodulation process and of the bacteroids from the root nodules.

The bacterial genus Rhizobium, which is responsible for nodule

initiation and development is a common soil organism (Stewart, 1966

and 1967). Alexander (1961) notes that legumes grown on agricultural

soils have been reported to fix anywhere from 64 to 332 kg N/ha/year.

Sims et al. (1971) above ground biomass data shows that legumes

account for less than 3% in all cases and usually less than 1% of the

standing crop on the Pawnee Site, a shortgrass prairie and the intensive

study site of the International Biological Program grassland biome.

Clark and Paul (1970) suggest that legumes, although of minor im-

portance as structural components in grassland ecosystems, may

account for substantial accretions of nitrogen over a number of years.

Karraker et al (1950) found that although legumes fixed less total nitrogen per unit area when grown with grass than when grown alone, the amount fixed per unit of legume herbage was much larger. Whitehead (1970) in a review of the literature on symbiotic nitrogen fixation by grass-legume mixtures, concludes that the quantities of nitrogen fixed vary enormously, and that the potential fixation for any one site is largely governed by temperature and rainfall.

The ability of nodulated plants to fix nitrogen is not limited to the Leguminosae. Stewart (1967) noted that some non-leguminous angiosperms contribute as much nitrogen to the soils in which they occur as leguminous plants do. Bond (1967) notes that over 300 species in 13 genera might qualify as non-leguminous N_2-fixing plants. Not all of these species are known to be nodulated at the present time. The plants which have been examined are all woody perennials which bear root nodules inhabited, not by a Rhizobium, but by actinomycetes or streptomyces (Bond, 1967, Stewart, 1966, Webster et al., 1967, Wollum, Youngberg, and Gilmour, 1966 and Wollum and Youngberg, 1964). Allen and Allen (1965), Becking (1970), Bond (1967), Silver (1969) and Stewart (1966) have summarized the information available on many of non-legumes and list their geographical distribution and ecological habitats. In 1968 Farnsworth and Hammond (1968) reported nodulation by the sagebrush, Artemisia ludoviciana, from which they isolated an unidentified bacterial endophyte. They also reported

nodulation by prickly pear cactus of the _Opuntia_ genus. These findings

add two genera to the list compiled by Bond (1967) and could represent

an important source of nitrogen on grassland ecosystems. However,

neither species or genus have been found to fix nitrogen at this time.

Becking (1970), Bond (1967), Stewart (1967) and Wollum and Youngberg

(1964) note that the ecological importance of non-leguminous nodulating

plants lies in their pioneer properties and ability to build up soil nitro-

gen in areas low in combined nitrogen, so as to favor growth of other

associated or following plant species.

Until about 1950, _Azotobacter_ and _Clostridium_ were regarded as

the only two genera of nitrogen-fixing bacteria and because of their low

numbers in agricultural soils, were considered unimportant (Rice,

Paul, and Wetter, 1967 and Stewart, 1967). Non-symbiotic nitrogen

fixation requires the presence of readily available sources of energy

and is encouraged by low oxygen tensions (Jensen, 1965, Steyn and

Delwiche, 1970 and Stewart, 1966 and 1969). Energy material, mostly

in the form of grass roots and litter is abundant in grasslands, however,

aerobic conditions are also most likely to occur in soils of grassland

ecosystems (Porter, 1969). Jensen (1965) reported a nitrogen fixation

efficiency of 12-20 mg N fixed per gram of sugar consumed by

Azotobacter and 2-3 mg N fixed per gram of sugar consumed by

Clostridium. Rice and co-workers (1967) reported 42-52 kg N fixed/ha

on soils ammended with 1% or less ground wheat straw under field

capacity moisture levels, while 13-150 kg of N was fixed/ha on water-
logged soils. When soils were amended with 5-20% straw and incu-
bated under waterlogged conditions, rates of nitrogen fixation of 500-
1000 kg/ha were observed. These investigators attributed the high
fixation rates to a combination of aerobic conditions required to
break down the plant residues and anaerobic sites necessary for
significant fixation rates. Clostridium bacteria were identified as
being responsible for the high fixation rates. Delwiche and Wijler
(1956) found 386 kg N fixed asymbiotically/ha 15 cm, when soils were
amended with 7-8% sucrose and incubated under aerobic conditions.
They were unable to measure significant nitrogen fixation on soils
amended with straw. Using the N^{15} enrichment technique, Delwiche
and Wijler (1956) reported less than 0.17 kg N fixed/ha 15 cm on
photosynthesizing soil-lawn discs incubated for 31 days. However,
when the soil-lawn discs were inverted, 4.5 to 13.4 kg of nitrogen was
fixed/ha 15 cm. Knowles (1965) confirmed the results of Delwiche
and Wijler on a Quebec soil, when he reported that under aerobic
incubation conditions, significant nitrogen fixation was not detected
except where a carbohydrate source was added. Under aerobic con-
ditions with the addition of 1% glucose, Knowles calculated an annual
nitrogen fixation rate of 35 kg/ha, while under anaerobic conditions,
with and without the glucose ammendment, he reported 73 and 32 kg
N/ha respectively. Recently Steyn and Delwiche (1970) obtained rates

of 2 kg N fixed/ha/year on a California soil under native vegetation.
Using the N^{15} enrichment technique, they found annual non-symbiotic
N fixation rates of 4.8 kg N/ha on another California soil under irri-
gated perennial lawn grass. Steyn and Delwiche (1970) concluded that
soil moisture and soluble energy sources were the limiting factors
effecting asymbiotic nitrogen fixation. It would appear that the above
observations would support the conclusion that significant non-symbiotic
nitrogen fixation would be associated with periods or regions of
anaerobic conditions or localized soluble energy sources.

In contrast to the free-living nitrogen-fixing bacteria, the blue-
green algae or blue-green lichen crusts do not require organic mate-
rials as an energy source, but derive their energy through photo-
synthesis. Stewart (1966 and 1970) has summarized the numerous
species of blue-green algae which fix nitrogen in pure culture and
the contributions that these organisms might make in various environ-
ments. Porter and Grable (1969) found that algae contributed about
4 kg N/ha per 10 days in a wet mountain meadow where conditions
were ideal for algal growth. Mayland, McIntosh and Fuller (1966)
found semiarid desert algal crust organisms to fix nitrogen at a rate
of 0.18 kg N/ha of crust surface/day when continuously wet. Since
the algal crusts do not occupy the entire surface area under native
desert conditions, N_2 fixation would be much less _in situ._ Mayland
et al. (1966) noted an enrichment or uptake of N^{15} by _Artemisia_ sp.

growing on algal crusts, which he attributed to nitrogen that had been fixed and excreted by the algal crust. Recently, MacGregor and Johnson (1971) have found that following a rainfall, algal crusts may contribute 3-4 g N/ha/hr to a desert grassland. Nitrogen-fixing blue-green algae frequently occur in moist, slightly alkaline habitats where there is a shortage of combined nitrogen (Stewart, 1970). They have been noted as early colonizers and apparently play an important role in ecological succession. Porter (1969) and Mayland et al. (1966) point out that blue-green algal associations depend upon substantial moisture and that the duration of the nitrogen fixation period will be limited in grassland or desert ecosystems.

Nitrogen is a key element in a grassland ecosystem. Nitrogen availability almost universally limits the productivity of grasslands whenever sufficient moisture is present (Reuss, 1971). Considerable nitrogen cycling information is available on highly managed grass-legume ecosystems (Whitehead, 1970), while very little information is available on native rangelands. Annual inputs represent only a very small fraction of the total fixed nitrogen in a native range eco-system. Native range legumes are usually not abundant on intensively grazed systems and little is known concerning the probability of biolog-ically significant amounts of non-symbiotic nitrogen fixation. Because rates of biological nitrogen fixation on a semiarid grassland ecosystem are likely to be quite low, a sensitive means of measuring low rates

of N_2 fixation is required. The acetylene reduction assay lends itself

very well to this condition.

Chapter III

METHODS AND MATERIALS

Studies were conducted to assess biological nitrogen fixation on disturbed and undisturbed soil from a shortgrass prairie and on intact leguminous plants growing in soil. The acetylene reduction assay was employed to measure nitrogen fixation rates by these soil and soil-plant systems. In this procedure formation of ethylene (C_2H_4) from acetylene (C_2H_2) is used as an index of nitrogen fixation since only nitrogenase preparations, N_2-fixing bacteria, blue-green algae and symbionts reduce acetylene to ethylene. The sensitivity of ethylene detection by flame ionization as noted by Hardy and Knight (1967) and Hardy and co-workers (1968) is the principal advantage of the acetylene reduction assay.

Equipment

Fixation Chambers

The acetylene reduction assay requires that a closed system be maintained. The majority of investigators using the acetylene reduction technique (Hardy et al., 1968, Koch et al., 1966 and 1967b, Stewart et al., 1967, and Campbell and Evans, 1969) have used small incubation vessels from 5 to 50 cm^3 in volume. In order to assess the nitrogen-fixing activity of intact growing plants or relatively large soil cores, a closed system with a much greater volume is required.

Plexiglas fixation chambers (incubation chambers) were constructed
in such a manner that soils and intact growing plants could be placed
within a closed system. Two chamber sizes were constructed, one
21 cm in height with an empty volume of 1250 cm^3 and one 41 cm in
height with an empty volume of 2380 cm^3. The larger chamber facili-
tated large actively growing plants, while the smaller chambers were
adequate for non-symbiotic studies. The chambers were constructed
(see figure 2) by fusing an 8.6 cm inside diameter plexiglas cylinder
with a wall thickness of 0.60 cm to a plexiglas base 0.90 cm thick. A
1.90 cm thick flange was fused to the top of the cylinder. The flange
contained a machined groove in which a rubber o-ring is seated. A
1.20 cm thick circular plexiglas end-plate was bolted to the flange with
six bolts having wing-nuts to facilitate removal of the top. A needle
valve, through which the chambers were evacuated and gases admitted,
was threaded into the end-plate. A rubber serum cap was fitted into
a hole in the end-plate. One-half cm^3 samples of the atmosphere in-
side the chamber were taken with a Plastipak 1 cm^3 tuberculin syringe
through the rubber serum cap.

Manifold System

A manifold system was constructed so that up to six fixation cham-
bers could be evacuated and the required gases admitted simultaneously
by connection to the needle value located on the top of the chamber. The
manifold was connected to a mechanical vacuum pump, mercury
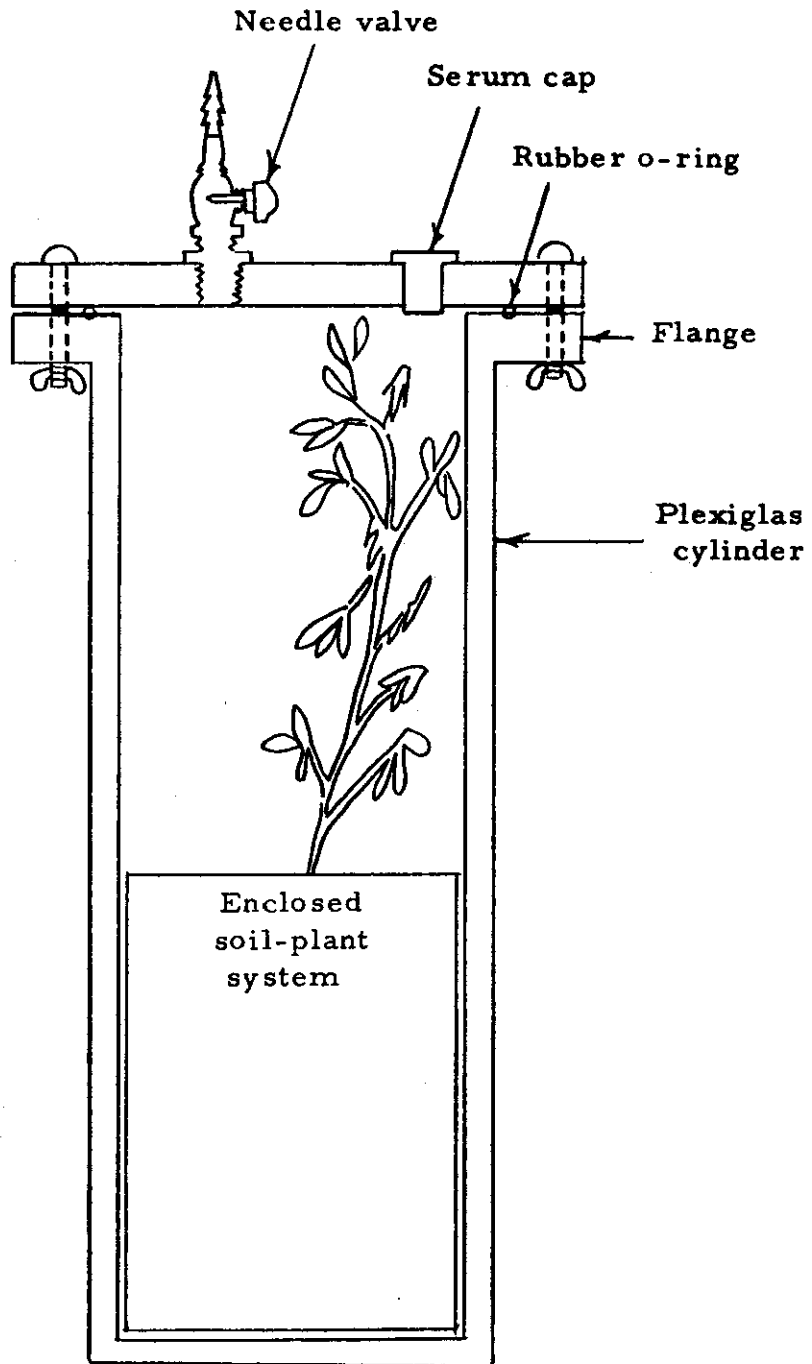
Fig. 2. Diagram of a gas-tight chamber for acetylene reduction assay of soil-plant systems.

manometer and to a source of helium, oxygen, and acetylene gas. The atmosphere in the fixation chambers was replaced with helium for anaerobic studies and with an 80:20 helium:oxygen mixture for aerobic studies. Acetylene was added to the fixation chambers until its partial pressure was equal to 5% of the total pressure. A positive pressure of 5 cm of Hg above the ambient atmospheric pressure level was maintained in the fixation chambers in order to minimize atmospheric contamination.

Successive evacuations were made on each series of chambers to remove N_2. Not all of the original N_2 could be removed by successive evacuations. However, based on partial pressures, the estimated Km (Michaelis-Menten constant) for acetylene is only about 5% that of N_2 (Hardy et al., 1968) i.e., the N_2-fixing enzyme complex has a higher affinity for acetylene than for N_2 at comparable partial pressures of the gases (Hardy et al., 1967 and Schollhorn and Burris, 1967). Thus slight air contamination is not a serious problem. Acetone was removed from the acetylene with a concentrated H_2SO_4 scrubber. Ethylene impurity in the acetylene was negligible in all systems except those with very low fixation rates. The gases required in the study were obtained commercially from the Matheson Company and from the Air Reduction Company.

Gas Chromatographs

Ethylene production was detected by gas chromatography (Hardy et al., 1968 and Koch et al., 1966) using an Aerograph

model 600-B gas chromatograph equipped with a hydrogen flame
ionization detector. A stainless steel column, 150 cm in length
and 0.32 cm in diameter, was packed with 80-100 mesh Porapak R.
Helium was used as the carrier gas and was passed through the
column at $40 cm^3$ per minute. The temperature of the column was
maintained between 50 and 70 degrees centigrade. Oxygen, nitrogen,
and carbon dioxide were measured using an Aerograph model A-90-P
gas chromatograph equipped with a thermal conductivity detector.
Stainless steel columns, 150 cm in length and 0.63 cm in diameter
were packed with 30-60 mesh Molecular Sieve 5A to separate oxygen
and nitrogen, while 100-200 mesh Porapak Q was used in separating
CO_2. Helium was used as the carrier gas and the column temperature
was maintained between 80-90 degrees centigrade. The detector output
was recorded on a Sargent model SR strip chart potentiometric
recorder.

Standardization and Calculations

A standard curve was constructed by plotting moles of ethylene/
sample as a function of peak height. The ethylene standard curves
prepared were linear with respect to detector response over an
ethylene concentration range of $5-300 \times 10^{-10}$ moles/sample. Quanti-
tative measurements of the ethylene concentrations in the fixation
chambers were obtained by multiplying the ethylene peak height by the

slope (moles ethylene/cm peak height) of the standard curve. The conversion of ethylene formed to nitrogen fixed was made on the basis of 1 mole of N_2 fixed/3 moles of ethylene produced, a theoretical value suggested by Hardy and co-workers (1968). All calculations were converted to nitrogen fixed/unit area (usually m^2 or ha) or to nitrogen fixed/plant. Rates of nitrogen fixation were determined by plotting nitrogen fixed or ethylene produced as a function of time and determining the slope of the linear portion of the curve after a short initial lag phase. Ethylene production by symbiotic systems was linear over the entire incubation period.

Soils

The principal soil used in these studies was an Ascalon sandy loam soil from the Pawnee Site, the intensive study site of the U.S. International Biological Program Grassland Biome located northeast of Nunn, Colorado. Soils from four comprehensive network sites were included in a related study. The comprehensive network sites were Ale (Washington), Cottonwood (South Dakota), Osage (Oklahoma), and Pantex (Texas). Soil texture and percent H_2O (by weight) at field capacity and at saturation are given for these soils in Table 2.

The classification, parent material and depth characteristics of soils from these sites are given by Reuss (1971).

Table 2. Soil texture and field capacity and saturation moisture percentages of soils from the Pawnee and comprehensive network sites.

Site	Soil Texture	% H_2O Field Capacity	% H_2O Saturation
Pawnee	sandy loam	19	30
Ale	silt loam	18	31
Cottonwood	sily clay loam	25	37
Osage	silty clay	39	55
Pantex	silty clay loam	27	50

Symbiotic Studies

Plant Species

Several plant species were grown in a growth chamber for periods of 10 weeks to 9 months. Rates of nitrogen fixation were established for the different plants at various stages of growth. The plant species studied included alfalfa (Medicago sativa L. var. Vernal), sweetclover (Melilotus officinales L. var. yellow blossom), soybeans (Glycine max Merr. var. Corsoy), sainfoin (Onobrychis viceaefolia Scop.) and Oxytropis sericea Nutt. The plants were germinated from seed and grown in 1 liter, cylindrical, paper cartons containing one kg of the Pawnee Site soil. No plant nutrients were added as fertilizer. Attempts to germinate Astragalus striatus were unsuccessful. All the plant species, except Oxytropis sericea, were inoculated with commercially available

Rhizobium inoculant. Alfalfa, sweetclover, and soybeans were maintained at approximately field capacity (19% H_2O by weight) throughout the experiment, by daily watering. Sainfoin and Oxytropis sericea, being more drought resistant, were maintained at slightly lower moisture levels.

The nitrogen fixing activity of selected symbiotic systems was observed in both the presence and absence of light. Maximum light intensity in the growth chamber provided up to 4000 foot candles of light. Light was excluded during the preparation and incubation of the dark runs by placing black polyethylene around and over the fixation chambers. Light and dark incubations were started midway through the 14-hour light period and midway through the 10-hour dark period of a 24 hour cycle, respectively.

Non-symbiotic Studies

Measurements of non-symbiotic nitrogen fixation on undisturbed soil cores were made on cores 8.1 cm in diameter. For studies of disturbed soil, weighed quantities of soil were placed in 1 liter cylindrical, paper cartons, 8.5 cm in diameter. Two moisture levels (field capacity and saturation) were imposed on the disturbed and undisturbed soil cores. Saturated moisture levels were determined by making a saturated paste and obtaining the moisture content gravimetrically. The quantity of water required to bring the overall soil

core to this moisture level was then added to the surface of the soil core. A soluble energy (carbon) source was applied to selected soils. This was accomplished by adding a quantity of a 2% (by weight) sucrose solution to the top of the soil core. The usual quantity of carbon added (as sucrose), was equivalent to 28 g/m^2. In the non-symbiotic systems, where respiration was likely to exceed photosynthesis, excess CO_2 was removed by placing a small vial of $2\underline{N}$ KOH in each fixation chamber.

Individual fixation chambers were connected to a source of O_2 through small specially constructed mercury check valves. As the O_2 in the fixation chamber was depleted by soil respiration, the CO_2 evolved was absorbed in the alkaline trap. This caused a slight pressure decrease which resulted in the automatic replacement of O_2 through the mercury check valve. This system worked well and generally maintained O_2 levels within $\pm$ 5% of ambient during runs that sometimes extended into days.

Both the disturbed and undisturbed soil cores and growing plants were placed in the fixation chambers and incubated with acetylene in combination with oxygen and helium. The length of time that the various systems were incubated varied from 4 hours for symbiotic systems to greater than 100 hours for some non-symbiotic systems. Ethylene production was measured at selected time intervals throughout the incubation periods.

The fixation chambers were placed in a Percival model MB-60 growth chamber during the incubation periods so that light and temperature could be maintained. A 14-hour light and 10-hour dark cycle was imposed on the non-symbiotic systems. Temperature was maintained at approximately $28^{\circ}C$ during the light period and $25^{\circ}C$ during the dark period. The growth chamber was operated at maximum light intensity during the light period.

At low levels of fixation, the acetylene reduction assay can easily detect rates of N_2 fixation as low as 1 g/ha/day (0.1 $mg/m^2/day$) (Reuss and Copley, 1969 and 1971 and MacGregor and Johnson, 1971). Duplicate samples often exhibit substantial variation and ranges of 30% of the mean between duplicates are not uncommon for the higher rates. When N_2 fixation rates are very low, ranges about the mean may be great, even though absolute variation is small.

Chapter IV

RESULTS AND DISCUSSION

Asymbiotic Studies

Pawnee Site

Moisture and energy source

Initial studies were conducted to measure the effect of moisture and energy levels on asymbiotic N_2 fixation. The soil cores were obtained from upland sites on an Ascalon sandy loam soil. The cover consisted of a mixture of buffalo-grass (Buchloe dactyloides Nutt.) and blue grama (Bouteloua gracilis Lag.). Duplicate soil-plant cores were incubated at room temperature (22-24°C) in fixation chambers containing an aerobic atmosphere. Treatments consisted of 2 levels of sucrose and 2 moisture levels. In systems without an added energy source, significant nitrogen fixation was not evident, the calculated rates being less than 50 µg N_2/m^2/day (0.5 g/ha/day) in soil cores at 5% and 18% (approximately field capacity) moisture levels. Because rates of N_2 fixation of this magnitude (<50 µg/m^2/day) are near the lower limits of detection by the acetylene reduction assay, the confidence associated with the precision of these measurements is low.

At field capacity moisture levels with 2 levels of sucrose-
carbon, the mean nitrogen fixation rates were 89 μg N_2/m^2/day with
the addition of 28 g C/m^2 and 547 μg N_2/m^2/day with the addition of
56 g C/m^2. The rates were determined by linear regression tech-
niques; the regression lines for the N_2 fixation rates at the 2 carbon
rates are shown in figure 3. The individual soil-plant cores were
quite variable. The highest single core N_2 fixation rate, achieved
in the presence of large amounts of readily assimilable carbon (56 g
C/m^2) represents the fixation of 880 μg N_2/m^2/day (0.88 kg N_2/ha/100
days). With the moisture and temperature conditions found at the
Pawnee Site, 100 days of maximal fixation per season is likely to
overestimate the amount of N_2 fixed.

Saturated sodded and bare soils

Figure 4 shows the quantity of N_2 fixed (presumably by free-
living bacteria) under saturated soil moisture conditions by duplicate
sodded and bare soil cores from the edges of a temporary water im-
poundment. Under these conditions, 250 μg N_2/m^2/day was fixed by
the bare soil cores while the average N_2 fixation in the sodded cores
was approximately 750 μg$/m^2$/day. The difference between the fixation
rates of the sodded cores and the bare soil cores can probably be
attributed to differences in available energy sources for heterotrophic
N_2-fixing bacteria. Root turnover and litter incorporation on sodded
areas would provide more carbohydrates (energy source) than would
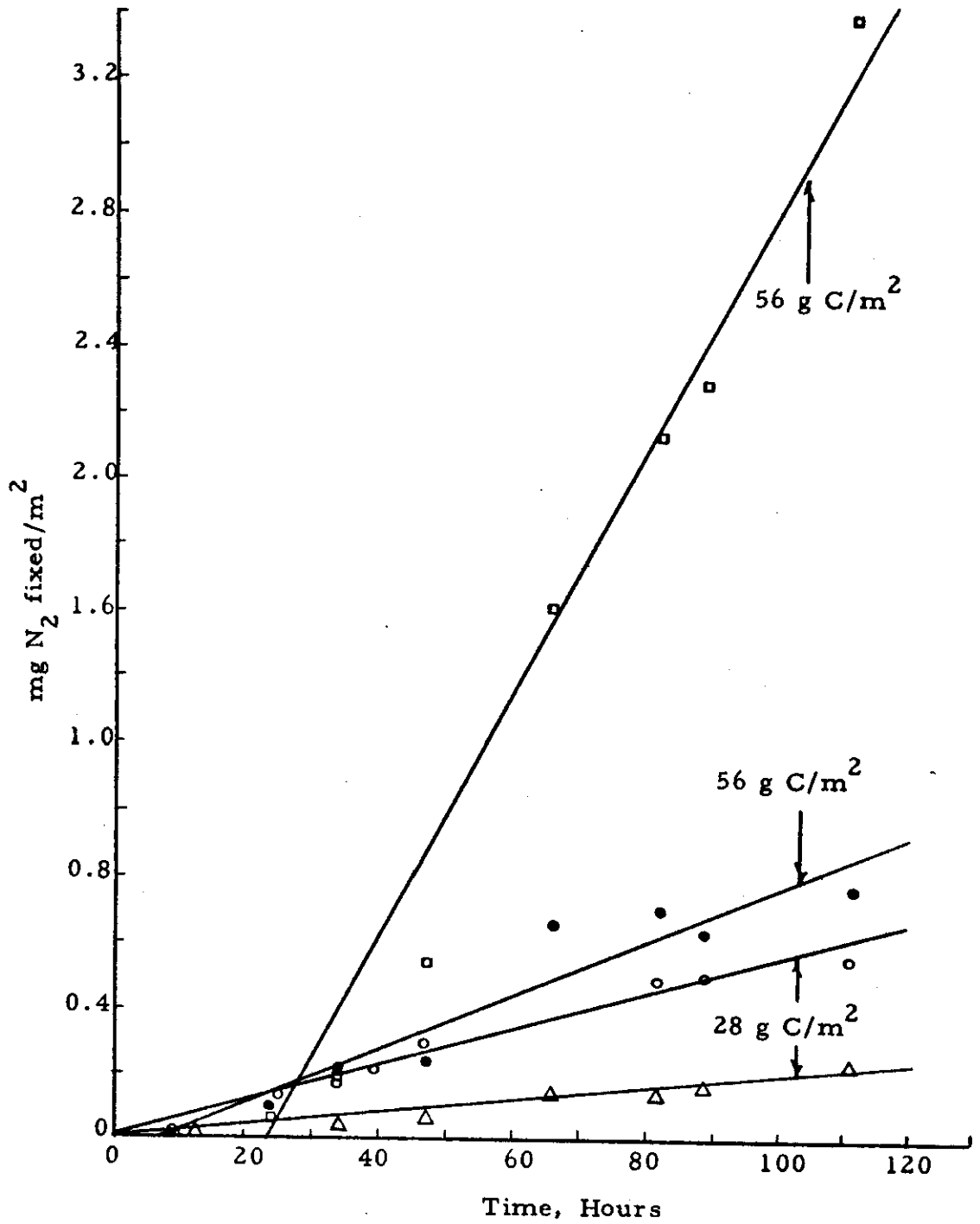
Fig. 3. Mean nitrogen fixation (C_2H_2-C_2H_4 assay) of duplicate soil cores under field capacity moisture conditions and with the addition of 28 and 56 g C/m^2 as sucrose.
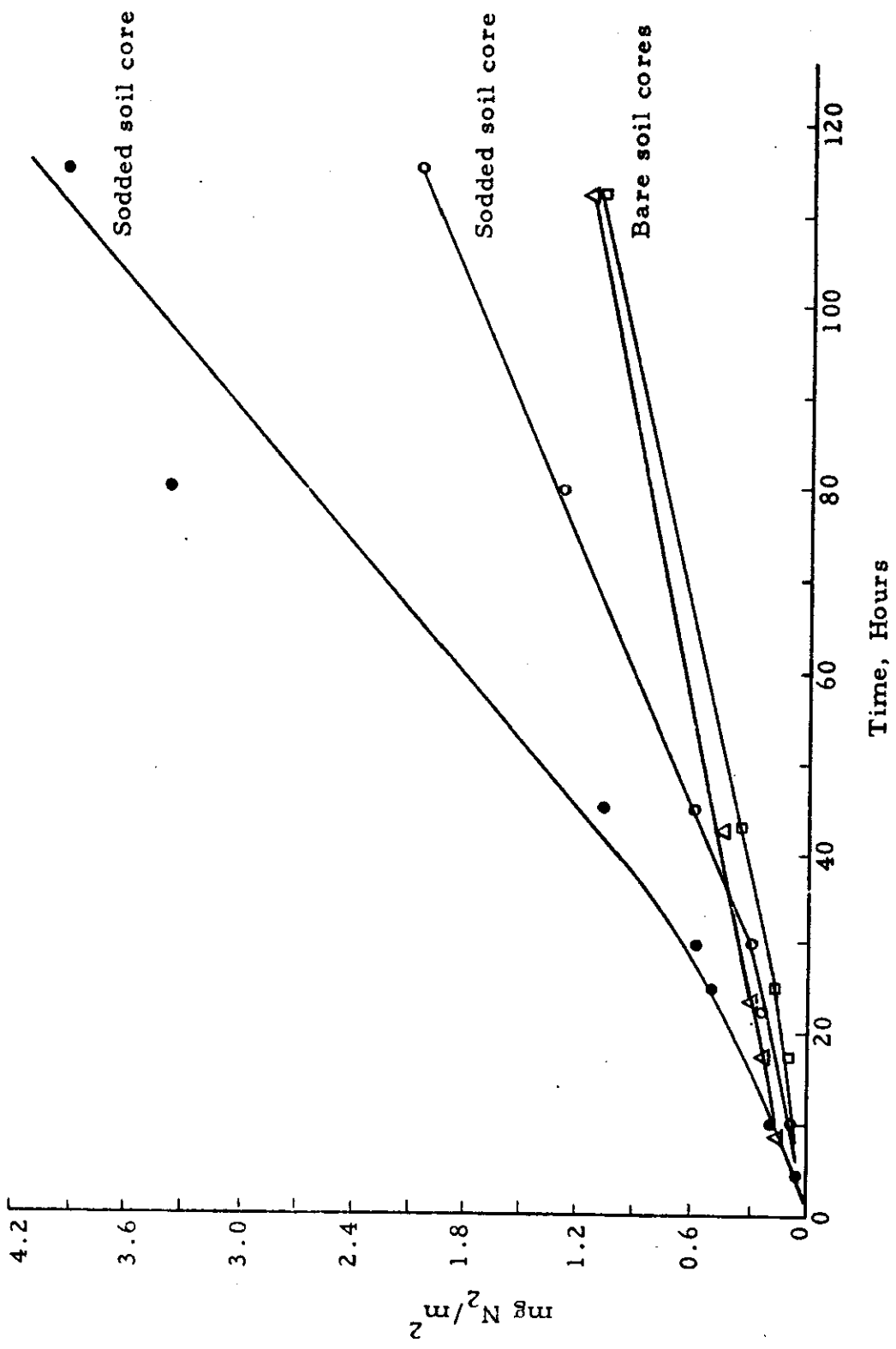
Fig. 4. Accumulated mg N_2 fixed (C_2H_2-C_2H_4 assay) /m^2 by saturated sodded and bare soil cores.

normally be available on bare soils. The variation in the replications

on the sodded cores is apparently due to variable microbial populations

or variable energy source rather than analytical error. Actually,

the disparity is quite small considering the low rate of fixation and

sensitivity of the measurement.

Saturated sodded and bare soils plus added energy source

Most free-living N_2-fixing microorganisms are heterotrophic

and inefficient users of carbohydrate, with only 1-20 mg of N_2 being

fixed per gram of carbohydrate utilized. Also, there is severe com-

petition for readily assimilable carbohydrate, not only by nitrogen-

fixing heterotrophs, but also by non-nitrogen-fixing organisms.

Readily assimilable carbohydrate is in short supply in most soils.

Several workers have shown that nitrogen fixation by free-living

bacteria increases markedly when carbohydrates such as sucrose are

added to soil (Delwiche and Wijler, 1956 and Knowles, 1965).

Studies were conducted to determine the effect of adding a

soluble energy source to saturated soil cores with and without a sod

cover on N_2 fixation. A 2%-sucrose solution was added to the surface

of soil cores in an amount which represented 28 g/m^2. Figure 5 shows

the results of adding this soluble energy source to saturated cores to

meet the metabolic requirements of the microbial population. The

nitrogen-fixing organisms responded to the addition of a soluble

energy source after an approximately 18-hour lag period. The slopes
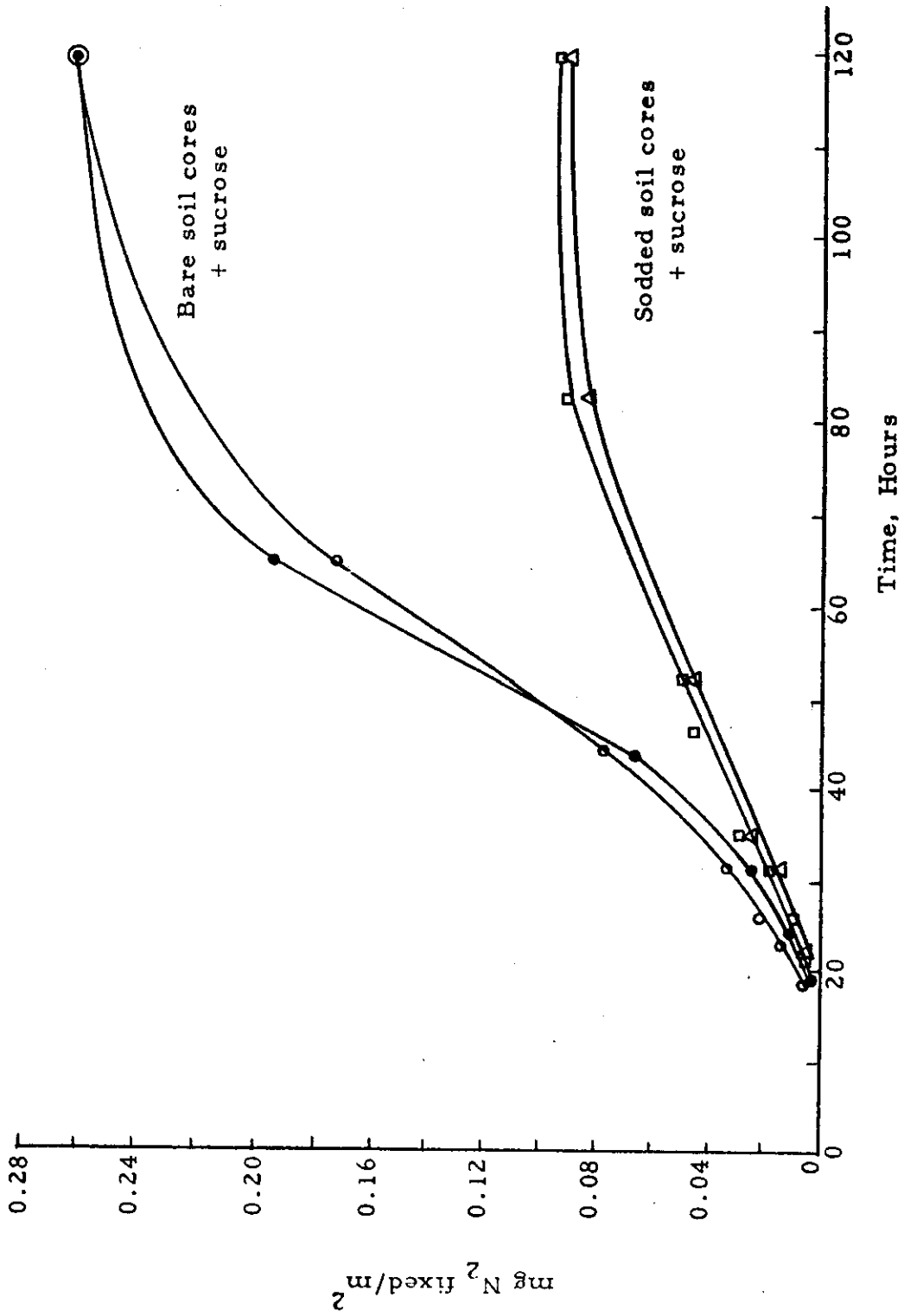
Fig. 5. Accumulated kg N_2 fixed (C_2H_2-C_2H_4 assay)/ha by saturated sodded and bare soil cores with the addition of 28 g sucrose-C/ha.

of the linear portion of these curves indicate a N_2 fixation rate of 0.11 g/m^2/day (1.1 kg/ha/day) for bare soil cores and 0.03 g/m^2/day (0.3 kg/ha/day) for sodded soil cores. Apparently, there was a greater competition among the soil microbial population for the added energy source in the cores with a sod cover. This resulted in a higher fixation rate by the bare soil cores and a better efficiency of carbohydrate utilization. The N_2-fixing efficiency, or the mg of N_2 fixed/g carbon added, was 9.3 for the bare soils and 3.3 for the sodded cores. These values are consistent with published values for nitrogen-fixing efficiencies of free-living N_2-fixing microorganisms (Jensen, 1965 and Stewart, 1967). Thus, if the lack of a suitable energy containing substrate is a limiting factor for nitrogen fixation, the addition of the equivalent of 28 g C/m^2 resulted in the fixation of approximately 100 mg N_2/m^2 (1 kg N_2/ha) for sodded cores and about 250-300 mg N_2/m^2 (2.5 to 3 kg N_2/ha) on bare soils.

Rate of energy source - Upland vs. Bottomland

Table 3 summarizes the results of an experiment conducted to evaluate the effect on N_2 fixation of adding sucrose to saturated upland and bottomland cores from the Pawnee Site. Both upland and bottomland sites consisted of mixtures of buffalo-grass and blue grama on Ascalon soil series. Figures 6 and 7 show the plots of the actual data for 3 levels of sucrose on bottomland cores and figure 8 shows the plots for 2 levels of sucrose on upland cores. On the bottomland

soil cores, the N_2 fixation rates and the total quantities of nitrogen fixed by duplicate cores were very similar and are shown by a single line. The duplicate cores were somewhat different on the upland cores, so both are plotted. Actually, the N_2 fixation rates of the duplicates are very similar on the upland cores, but the two cores exhibited slightly different lag phase durations and lower total nitrogen fixation. There seems to be a rather general pattern that longer lag phases are associated with lower total nitrogen fixation. Since the competition for readily assimilable sources of energy is quite keen between nitrogen-fixing heterotrophs and non-nitrogen-fixing organisms, a longer lag phase allows more of the added carbohydrate to be used by the non-nitrogen-fixing organisms.

Table 3. Nitrogen fixation (C_2H_2-C_2H_4 assay) on saturated upland and bottomland cores at different rates of added sucrose.

Source	Carbon Added	Maximum Fixation Rate	N_2 Fixed per g Carbon
	g/m^2	$g/m^2/day$	mg
Upland	14	No data	-
	28	.20	9
	56	.24	No data
Bottomland	14	.06	9
	28	.17	10
	56	.34	9
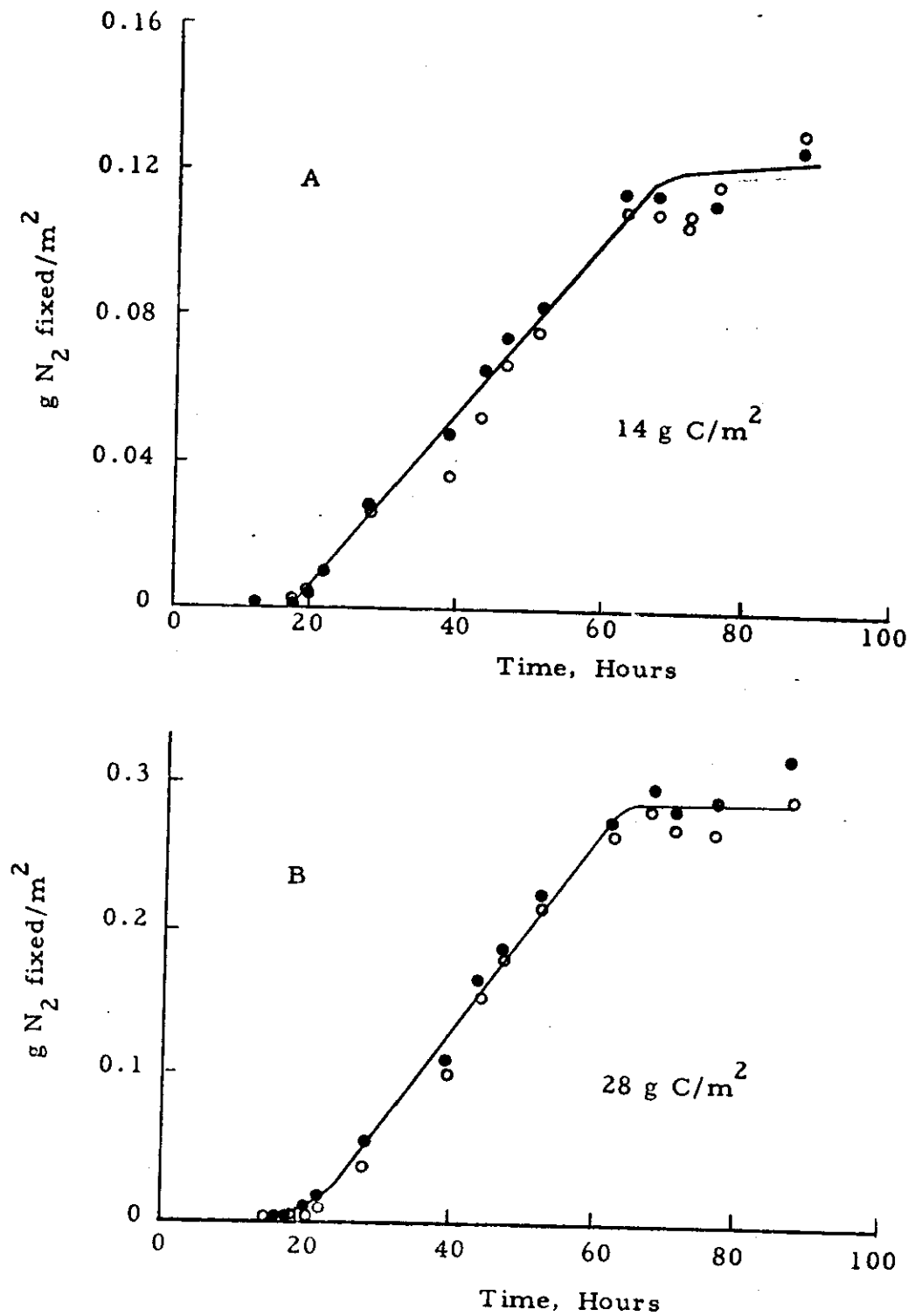
Fig. 6. Nitrogen fixation (C_2H_2-C_2H_4 assay) of duplicate cores of bottomland soils under saturated moisture conditions and with the addition of sucrose at 14 g C/m^2 (A) and 28 g C/m^2 (B).
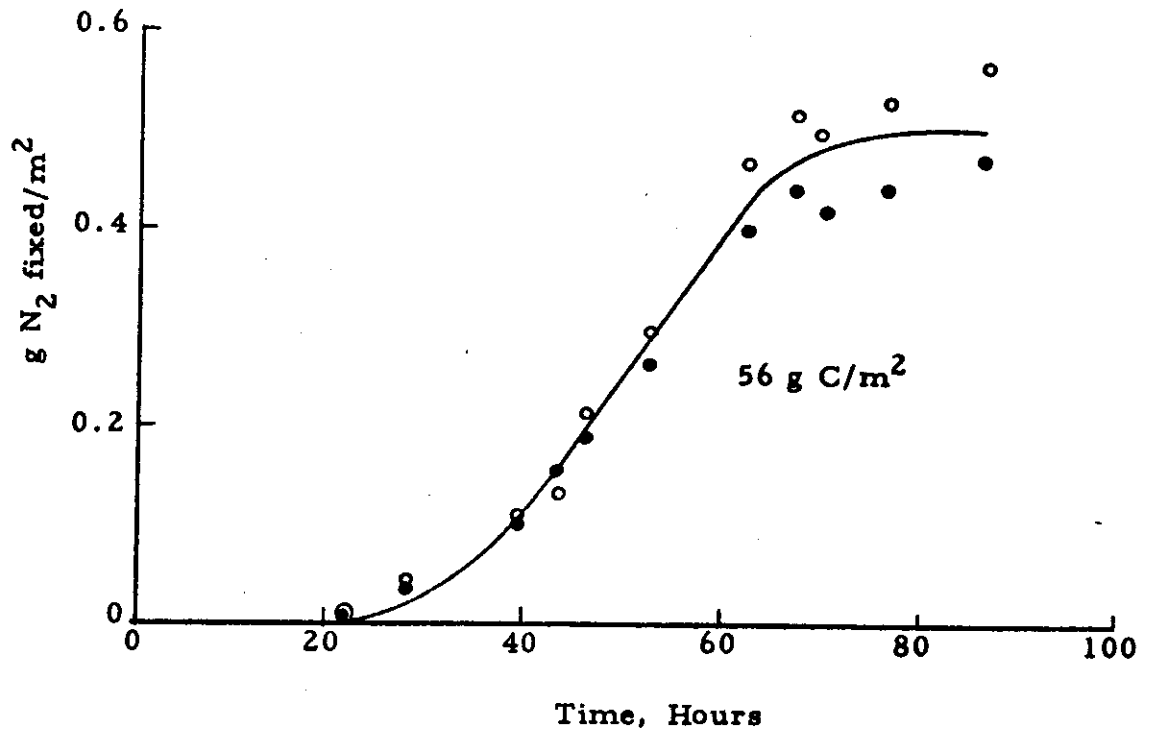
Fig. 7. Nitrogen fixation (C_2H_2-C_2H_4 assay) of duplicate cores of bottomland soil under saturated moisture conditions and with the addition of sucrose at the rate of 56 g C/m^2.
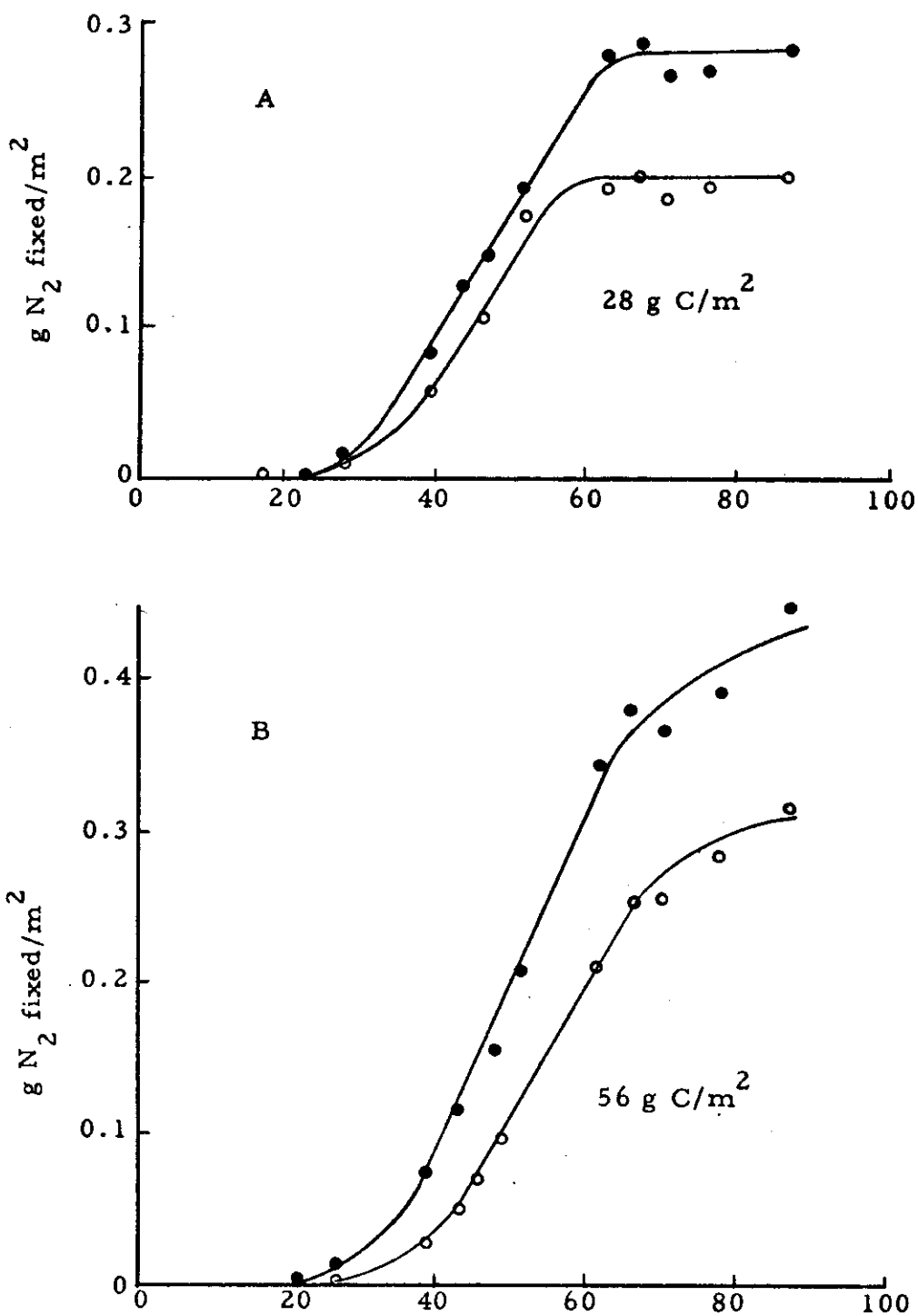
Fig. 8. Nitrogen fixation (C_2H_2-C_2H_4 assay) of duplicate cores of upland soil under saturated moisture conditions and with the addition of sucrose at 28 g C/m^2 (A) and 56 g C/m^2 (B).

Table 3 definitely indicates that on bottomland soils the higher fixation rates are associated with the higher levels of sucrose. Due to leaks in the fixation chambers, the data for the 14 g C/m^2 level on the upland soil cores is missing. Because of this, a distinct association between rates of N_2 fixation and levels of added sucrose cannot be detected for the upland cores. The maximum nitrogen fixation rates, obtained from the linear portion of the fixation curves, ranged from 0.06 $g/m^2/day$ to 0.34 $g/m^2/day$. In each case, the efficiency of carbon utilization in terms of N_2 fixed was 9-10 mg N_2 fixed/g C added. The efficiency value for the 56 g C to the upland soil is not given, because the amount of nitrogen fixed for this was still increasing at the time the incubation was terminated.

The atmosphere over these soil cores was maintained at approximately ambient O_2 levels, but the soils were kept under saturated moisture levels and relatively high levels of a readily assimilable carbon source were added. Previous experiments (experiment 2) have shown that under saturated moisture conditions without additional energy added, nitrogen fixation rates are generally under 1 $mg/m^2/day$. Also, under field capacity conditions and with the addition of a readily assimilable carbon source, rates of nitrogen fixation were generally less than 1 $mg/m^2/day$. Thus, high rates of nitrogen fixation can be achieved but apparently saturated moisture levels and a source of readily assimilable carbon are required.

The relatively low efficiency level of 10 mg N_2 fixed/g C added
is quite important. If 600 kg dry matter containing 50% C were decom-
posed under saturated conditions, we would only expect 3 kg N_2 to be
fixed. Actually, most decomposition probably takes place at moisture
levels at or below field capacity. The low rates of nitrogen fixed
when high levels of soluble carbon are added to soil cores at the field
capacity moisture level indicates that most decomposition is carried
out by non-nitrogen-fixing organisms.

Temperature effects

An experiment was conducted to study the effect of temperature
on nonsymbiotic nitrogen fixation. Soil-plant cores consisting of dup-
licate samples of 3 native plant species and duplicate bare soil cores,
two with disturbed Ascalon sandy loam soil and 4 from the edges of
temporary lake bottoms were examined for asymbiotic N_2 fixation.
Prior to incubation, all cores were saturated and all except one set
of lake bottom duplicates received sucrose in amounts equal to 32 g
C/m^2. A diurnal cycle of 12 hours of light and 12 hours of darkness
was established. Under maximum light intensity, the temperature in
the growth chamber reached $16^{o}C$, while during dark periods $0^{o}C$ was
maintained. The data from this experiment are summarized in Table 4.
Under this temperature regime, only one core attained a N_2-fixing
rate greater than 0.1 mg/m^2/day and the average was only 0.06
mg/m^2/day. Even though moisture and energy levels were adequate

Table 4. Nitrogen fixation rates (C_2H_2-C_2H_4 assay) in duplicate saturated soil cores held at $0^\circ C$ dark and $16^\circ C$ light for 138 hours followed by 120 hours at $27^\circ C$.

Description	$16^\circ C$ Light, $0^\circ C$ Dark 138 Hours			$27^\circ C$ 130 Hours		
	A	B	Mean	A	B	Mean
	$\mu g/m^2/day$			$mg/m^2/day$		
1. Disturbed upland soil, + sugar [1]/	60	10	40	20.4	10.5	15.4
2. Temporary lake bottom, no sugar	40	80	60	0.9	2.2	1.6
3. Temporary lake bottom, + sugar	180	90	140	10.2	6.4	8.4
4. Bachloe dactloides, + sugar	40	40	40	7.8	2.5	5.2
5. Agropyron cristatum, + sugar	40	10	30	3.4	1.3	2.4
6. Artemesia frigida, + sugar	50	50	50	5.7	2.6	4.2
Mean undisturbed uplands, with sugar			40			3.9

[1]/ Sugar treatment was the equivalent of 32 g C/m^2 applied as sucrose.

for optimum N_2 fixation, temperatures $16^\circ C$ and below severely restricted biological activity, so that for all practical purposes, nitrogen fixation was nonexistent.

After 138 hours the temperature was raised to 27°C. Measurable nitrogen fixation occurred, but at a much lower rate than would be expected for this temperature under saturated moisture conditions with the addition of a soluble energy source. There was considerable variation in duplicate cores under these conditions and consequently no conclusions can be made regarding the effects of different vegetative species on N_2 fixation. The six upland soil cores with vegetative cover averaged only 3.9 mg N_2 fixed/m^2/day as compared to 0.2 g N_2 fixed/m^2/day for upland soils (Table 3) on which no cold pretreatment was imposed. Thus, reduced temperatures not only stopped nitrogen fixation but reduced subsequent N_2 fixation rates after warming. The N_2 fixation rates of the soil cores obtained from the edges of temporary lakes averaged 1.6 mg/m^2/day without the addition of sugar; these N_2 fixation rates are higher than those of previous studies without added sugar (experiment 2). However, since temporary lakes do not constitute a significant fraction of the grassland area and since the rate is still quite low in absolute terms, this fixation rate probably does not have any significance in terms of nitrogen cycling in the overall grassland ecosystem.

Moisture - aeration experiment

The data in Table 5 summarize the results obtained from an experiment conducted to study the effect of moisture level and aeration status simultaneously. This experiment was designed to determine whether the low N_2 fixation rates in unsaturated soils noted in previous

Table 5. Mean N_2 fixation ($C_2H_2 - C_2H_4$ assay) rate as affected by
moisture status and atmospheric composition on disturbed
upland cores of Ascalon sandy loam soil (32 g sucrose-C/m^2
added).

Description	Atmosphere	Fixation Rate g N_2/m^2/day	Efficiency mg N_2/g C
Aerobic, saturated	80:20-He:0_2	0.12	10.1
Anaerobic, saturated	He	0.25	15.5
Aerobic, field capacity	80:20-He:0_2	0.00	-
Anaerobic, field capacity	He	0.12	5.2

experiments was due to anaerobic conditions or whether saturated

moisture conditions per se are necessary. The data are presented in

graphic form in figure 9 and are tabulated in Appendix Tables 1 and 2.

Average N_2 fixation rates of 0.15 mg/m^2/day were obtained at

field capacity moisture levels under a He:O_2 atmosphere. These rates

are similar to those obtained in experiment 1 under similar treatment

conditions. The highest N_2-fixing rate, 0.25 g/m^2/day, was attained

in saturated soils under a helium atmosphere. Nitrogen fixation rates

were approximately 0.12 g/m^2/day under both a helium atmosphere

at field capacity and under a He:0_2 atmosphere with saturated soil

moisture conditions, but in the saturated aerobic treatment more total

nitrogen was fixed than in the helium atmosphere at field capacity.
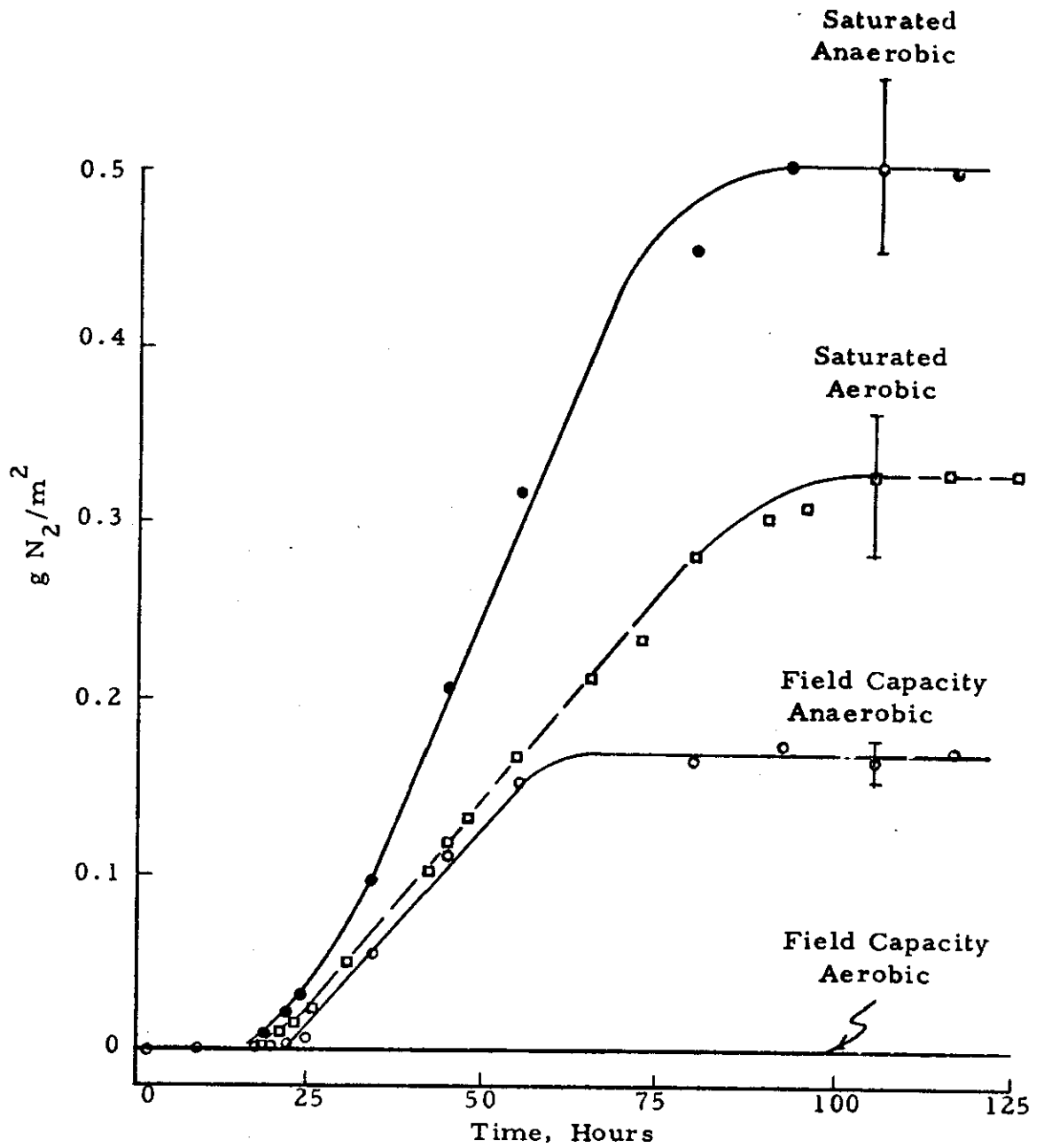
Fig. 9. Mean N_2 fixation (C_2H_2-C_2H_4 assay) as affected by moisture status and atmospheric composition on disturbed cores of Ascalon sandy loam soil amended with 32 g C/m^2 as sucrose. The vertical bars represent ± 1 S.E. of the mean.

Only in the soil in which oxygen was present was N_2 fixation very low.
This would indicate that the microorganisms responsible for nitrogen
fixation are largely anaerobic or require anaerobic conditions to fix
significant amounts of nitrogen. This conclusion is borne out in the
literature (Knowles, 1965, Rice et al., 1967 and Stewart, 1967).
The efficiency of the nitrogen-fixing organisms is greatest (15.5 mg
N_2 fixed/g C utilized) under saturated moisture conditions and with a
helium atmosphere. Their efficiency was lowest with field capacity
moisture conditions and under a helium atmosphere, with only 5.2 mg
N_2 fixed/g C utilized. Under saturated moisture conditions and with
an aerobic atmosphere an efficiency value of 10.1 mg N_2 fixed/g C
utilized was noted. This value agrees very well with previous experi-
ments under similar conditions.

Statistical analysis of such data is plagued with problems of
homogeneity of variances, but a reasonable evaluation of the variation
can be attained by considering the standard error of the mean at any
one point. The vertical bars on figure 9 at 105 hours representing
±1 standard error of the mean of the individual cores at that time
indicate that there is a high probability that the differences were due
to the treatments imposed.

Water extract energy source

Table 6 shows the rates of nitrogen fixation attained in disturbed
Ascalon sandy loam soil after the addition of a "standard" prairie hay

water extract. Clark (1970) indicates that coarse chopped samples of this "standard" prairie hay contained 10.5% soluble C. In this experiment, 100 g of finely ground prairie hay was shaken for 1 hour with 1 liter of cold water. The prairie hay water extract was applied to the soil surface at a level corresponding to the extract from 100 g dry plant material/m^2. Two moisture levels (field capacity and saturation) were included in the experiment. After an approximately 12-hr lag period, a fixation rate of 4.28 mg N_2 fixed/m^2/day (0.04 kg/ha/day) was attained in saturated soils with the prairie hay water extract. This rate was approximately 18 times greater than the 0.24 mg N_2 fixed/m^2/day rate attained under saturated moisture conditions without the prairie hay water extract. Apparently the N_2 fixing organisms were responding to a very small amount of readily assimilable C in the water extract. Using the cold-water soluble C percentage value given by Clark (1970) for coarse chopped prairie hay, an efficiency

Table 6. Asymbiotic nitrogen fixation (C_2H_2-C_2H_4 assay) rates on duplicate samples of Ascalon sandy loam soil following the addition of a prairie hay water extract.

Treatment	mg N_2 fixed/m^2/day		
	A	B	Mean
Saturation + water extract	3.62	4.94	4.28
Saturation	0.26	0.22	0.24
Field capacity + water extract	0.09	0.11	0.10

value of 0.35 mg N_2 fixed/g C utilized is obtained. This value is only about 1/30 of the efficiency value obtained with a soluble C source (Table 3). The addition of the water extract to soil at field capacity resulted in a fixation rate of 0.10 mg N_2 fixed/m^2/day. This rate is in agreement with rates obtained in previous experiments when a soluble energy source was added to soil at field capacity. Because of the lag period involved and the saturated moisture requirement, free-living N_2 fixers will probably not respond to carbon that might be flushed into the soil from plant residue during a rainstorm on the grassland.

Asymbiotic Studies

Comprehensive Network Sites

To complement the Pawnee Site investigations, asymbiotic nitrogen fixation studies were conducted on soils from four comprehensive network sites. The comprehensive network sites were Ale (Washington), Cottonwood (South Dakota), Osage (Oklahoma) and Pantex (Texas). The environmental conditions on these sites are quite different from those on the Pawnee Site, resulting in different types of grasslands. These types include Northwest bunchgrass (Ale), mixed prairie (Cottonwood), true prairie (Osage) and shortgrass prairie (Pantex). A more complete description of these soils is given in Table 2. Originally, it was hoped that undisturbed soil-plant cores with

maximum vegetative growth could be otained. However, due to difficulty in taking the cores in the field and in shipping them to the laboratory, the soil-plant cores were obtained in a somewhat disordered state.

Treatments on triplicate cores consisted of 2 moisture levels (field capacity and saturation) without an energy source and saturated cores with a soluble energy source (sucrose) added at the level of 28 g/m^2. All measurements were made on cores under an aerobic atmosphere.

Table 7 summarizes the magnitude of the N_2 fixation rates obtained from the comprehensive network site soil cores. Associated standard errors of the N_2 fixation rates are also given. The length of the lag period and the time course of the accumulation of fixed nitrogen for the 4 comprehensive network sites were similar to those demonstrated by soil cores from the Pawnee Site in earlier studies under similar treatment conditions. The Osage Site demonstrated the highest N_2 fixation rates under all treatment conditions. Under saturated conditions with the addition of 28 g/m^2 sucrose-C, N_2 fixation rates ranged from 221 $mg/m^2/day$ for Osage to 15 $mg/m^2/day$ for the Cottonwood Site. Under saturated moisture conditions without the addition of a soluble energy source, N_2 fixation rates ranged from 2.4 $mg/m^2/day$ for the Osage Site to approximately 0.4 $mg/m^2/day$ for both Ale and Pantex Sites. Under field capacity moisture levels

Table 7. Asymbiotic nitrogen fixation (C_2H_2-C_2H_4 assay) rates of comprehensive network soils under saturated moisture conditions, with and without the addition of sucrose and under field capacity moisture levels.

Site	Saturated + Energy		Saturated		Field Capacity	
	Rate	S. E.	Rate	S. E.	Rate	S. E.
	mg N_2 fixed/m^2/day					
Osage	220.7	14.1	2.42	1.35	0.99	0.23
Ale	93.9	29.3	0.35	0.04	0.11	0.08
Pantex	30.2	5.8	0.35	0.10	0.088	0.023
Cottonwood	14.6	5.8	0.48	0.20	0.085	0.011

without an energy source, N_2 fixation rates ranged from 1.0 mg/m^2/day for the Osage Site to approximately 0.1 mg/m^2/day for the remainder of the comprehensive network sites.

Comparison of the N_2 fixation rates of the comprehensive network sites with those of the Pawnee Site indicates that under saturated moisture conditions and with sucrose added, the N_2 fixation rates measured on the Pawnee Site are comparable to those of the Osage Site (Table 3). However, without a soluble energy source added to the soil cores, N_2 fixation rates of the Ale, Cottonwood, Pantex and Pawnee Sites are all very comparable. The N_2 fixation rates on the Osage Site under both saturated and field capacity moisture levels

without the addition of a soluble energy source were considerably greater than those observed on the Pawnee Site. The most probable explanation is that the soil of the Osage Site has a greater source of native carbon, a portion of which would be readily available to N_2-fixing organisms under ideal moisture and aeration conditions. Coupled with this, the Osage Site has a heavier textured soil (silty clay vs. sandy loam), than the Pawnee Site, which might result in localized anaerobic conditions in the Osage soil cores. However, if saturated or field capacity moisture conditions were to exist for a period of 100 days at the Osage Site, a total of 2.5 and 1.0 kg N_2 would be fixed/ha respectively.

Table 8 shows the N_2-fixing efficiency values (mg N_2 fixed/g C added) for the Pawnee Site and the 4 comprehensive network sites. The associated standard errors are also shown for the comprehensive network sites. Soil cores from the Osage Site gave a N_2-fixing efficiency value of 11.6 mg N_2 fixed/g C added, a value slightly higher than that found for soil from the Pawnee Site in previous studies. The Cottonwood Site demonstrated an extremely low efficiency value of 0.38 mg N_2 fixed/g of C added. This value is similar to that obtained for Pawnee Site soil with the addition of a prairie hay water extract. Since samples from all sites received the same level of soluble carbon in amounts which should overshadow any readily assimilable native carbon, the differences measured in efficiency of

Table 8. Efficiency of carbohydrate utilization in mg N_2 fixed/g C added as sucrose by comprehensive network site and intensive site soils.

Site	mg N_2 fixed/g carbon	
	Amount	Standard Error
Cottonwood	0.377	0.085
Pantex	2.14	0.17
Ale	4.28	1.21
Osage	11.64	0.61
Pawnee	9-10	

carbon utilization must be due to either differences in the heterotrophic N_2-fixing population or to competitive non-N_2-fixing organisms.

Early experiments demonstrated that ethylene was not absorbed on soil colloids. Recently, Abeles et al. (1971) reported that the soil acts as a sink for ethylene through microbiological degradation. These workers showed in a laboratory experiment that under aerobic conditions and following a lag period of 24 hours, ethylene disappeared from the air surrounding a soil at approximately 8 ppm/day. This concentration of ethylene is slightly below the detection limits of the equipment used in the present study. In incubation studies lasting 4-5 days the amount of ethylene degraded microbiologically (if indeed this is the case) probably could not be measured. Nevertheless, this

observation is of concern in long term asymbiotic N_2 fixation studies where N_2 fixation rates are below 50 $\mu g/m^2$/day and cannot be measured reliably. It should be noted that at N_2 fixation rates greater than 50 $\mu g/m^2$/day, the accumulation of ethylene is linear with time.

Certain plant tissues (particularly horticultural plants) produce extremely small amounts of ethylene. Ethylene acts as a regulator of fruit ripening and of vegetative and reproductive activities (Burg, 1962 and 1968). In early investigations on sodded soil-plant cores, the production of ethylene in the absence of acetylene could not be detected.

Symbiotic Studies

Preliminary legume studies

Single 30-day old potted plants of alfalfa (Medicago sativa L.) and sainfoin (Onobrychis viciaifolia Scop.) were placed in the fixation chambers for acetylene reduction assay. The N_2 fixed by duplicate alfalfa plants is portrayed graphically in figure 10 and is expressed as mg N_2 fixed/plant and as kg N_2 fixed/ha. These rates were calculated from single plants growing in a pot of soil with a surface area of 56.75 cm^2. Duplicate plants for each plant species gave almost identical rates, and excellent linear relationships were obtained in both cases. The N_2 fixation rates for both plant species are plotted on the same scale in figure 11. Single 30-day old alfalfa plants fixed N_2 at a rate of 1.09 kg/ha/day or 0.62 mg/plant/day, while single sainfoin
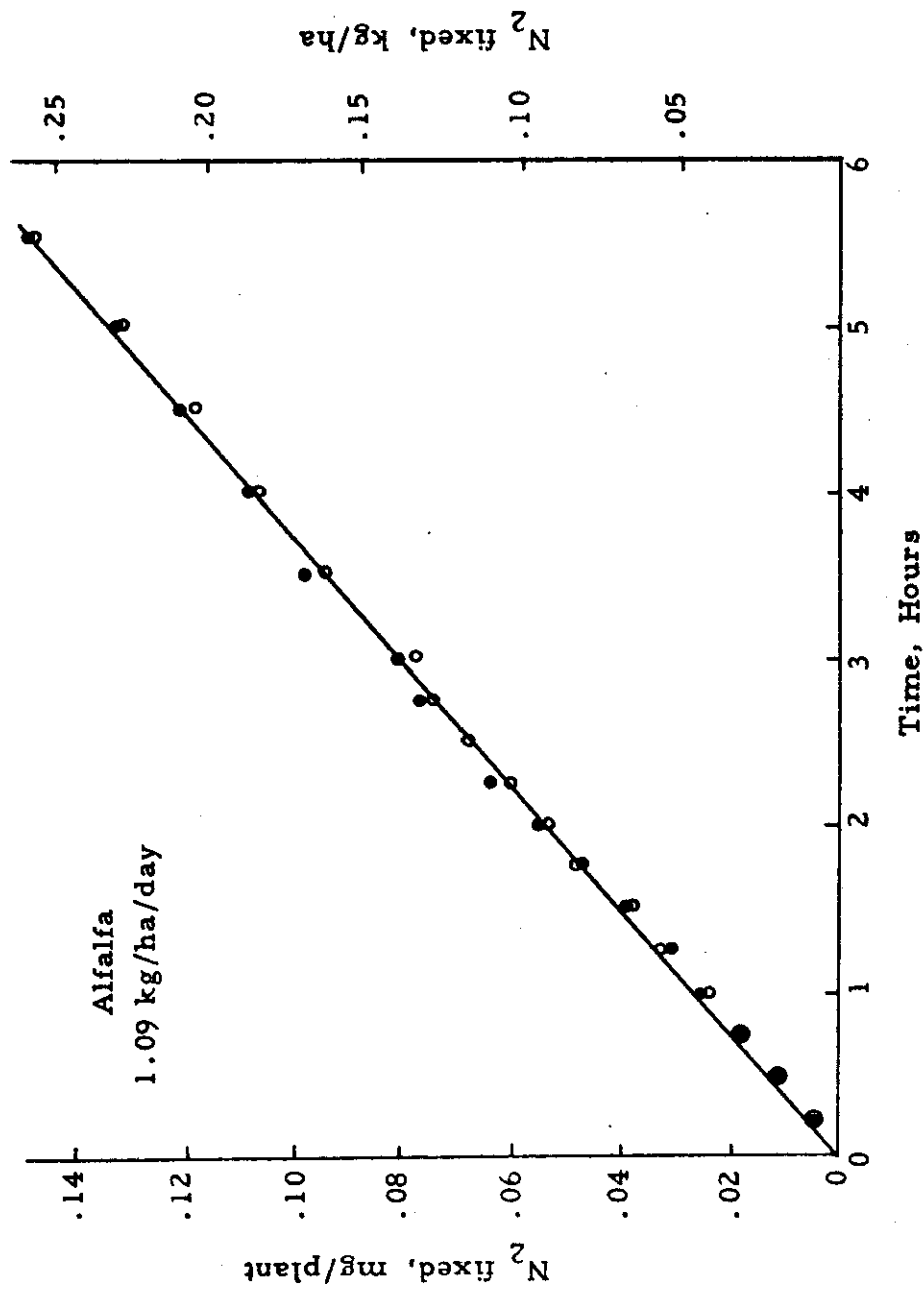
Fig. 10. Nitrogen fixation (C_2H_2-C_2H_4 assay) by duplicate 30 day old single potted potted plants of Medicago sativa L. (alfalfa).
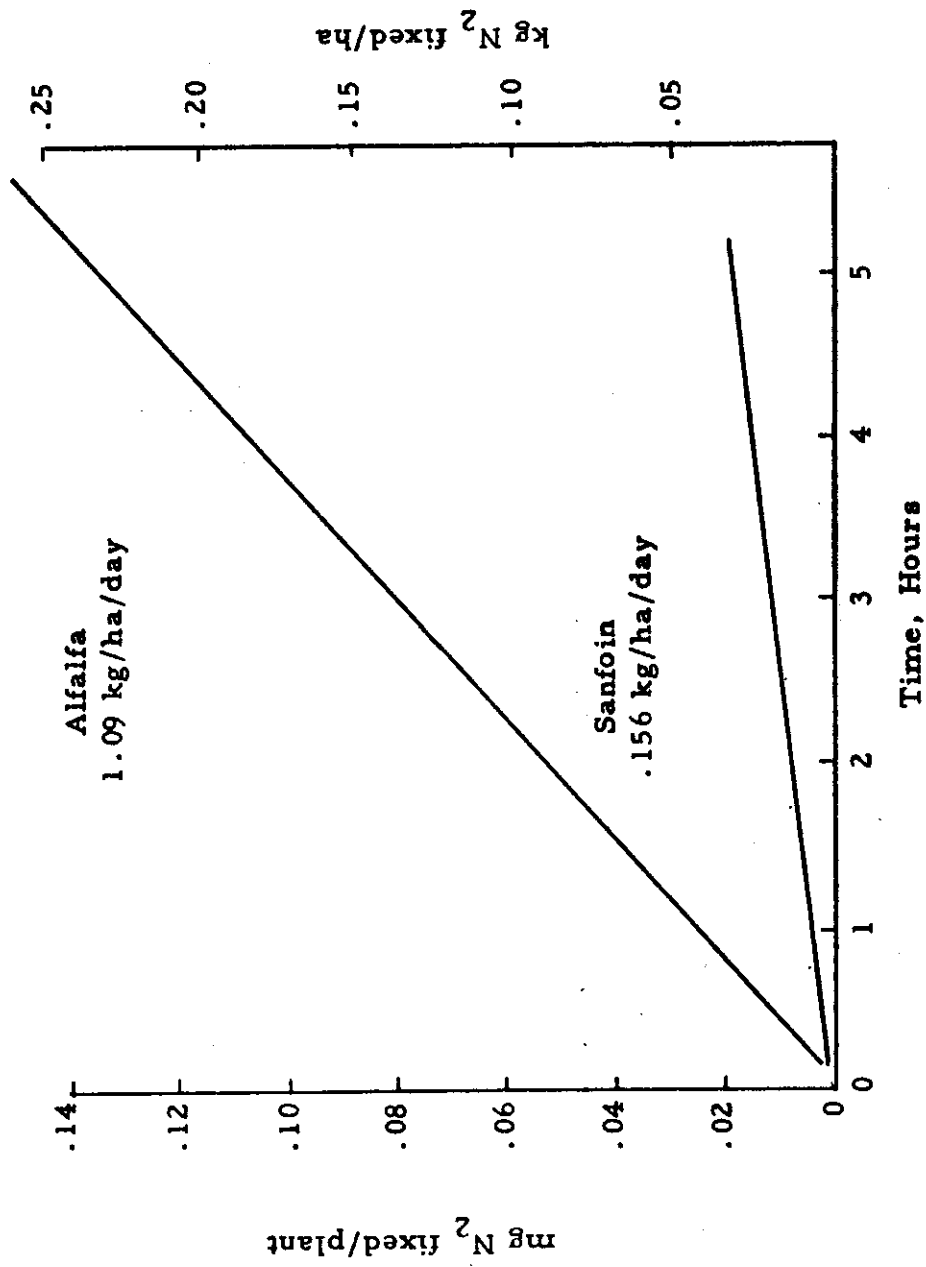
Fig. 11. Comparison of nitrogen fixation (C_2H_2-C_2H_4 assay) rates by single potted plants of Medicago sativa L. (alfalfa), and Onybrychis viciaefolia Scop. (sainfoin).

plants fixed N_2 at a rate of 0.156 kg/ha/day or 88.7 μg/plant/day.

Rates are given on a per plant basis because extrapolating the pot

area to a hectare gives a plant population of 1.76 x 10^6 plants/ha.

Previous attempts to measure nitrogen fixation rates on legumes

removed from the field were unsuccessful (Reuss and Copley, 1969).

Because of the excellent duplication on individual plants in this pre-

liminary study, a more comprehensive study involving more plant

species was designed to measure nitrogen fixation rates as the plant

matured.

Comprehensive legume study

Alfalfa, soybeans, sweetclover, sainfoin and Oxytropis sericea

were assayed for N_2-fixing activity at selected intervals. Nitrogen

fixation was measured during both a light and a dark incubation period

during 6 weeks of the overall 14-week experiment.

Duplicate plants of each species were examined for N_2 fixation

at each incubation period. Some typical plots of the accumulation of

ethylene or nitrogen fixed by sweetclover are shown in figure 12 and

13. The accumulation of ethylene and N_2 fixed/plant (based on a 3:1

conversion of ethylene produced to N_2 fixed) are plotted. The accumu-

lation of ethylene was linear over a 4-hr incubation period. Each

regression line represents one plant and has been fitted by least

squares. R^2 (coefficient of determination) values ranged from 0.97

to 0.99+ for individual plants. The duplication between individual
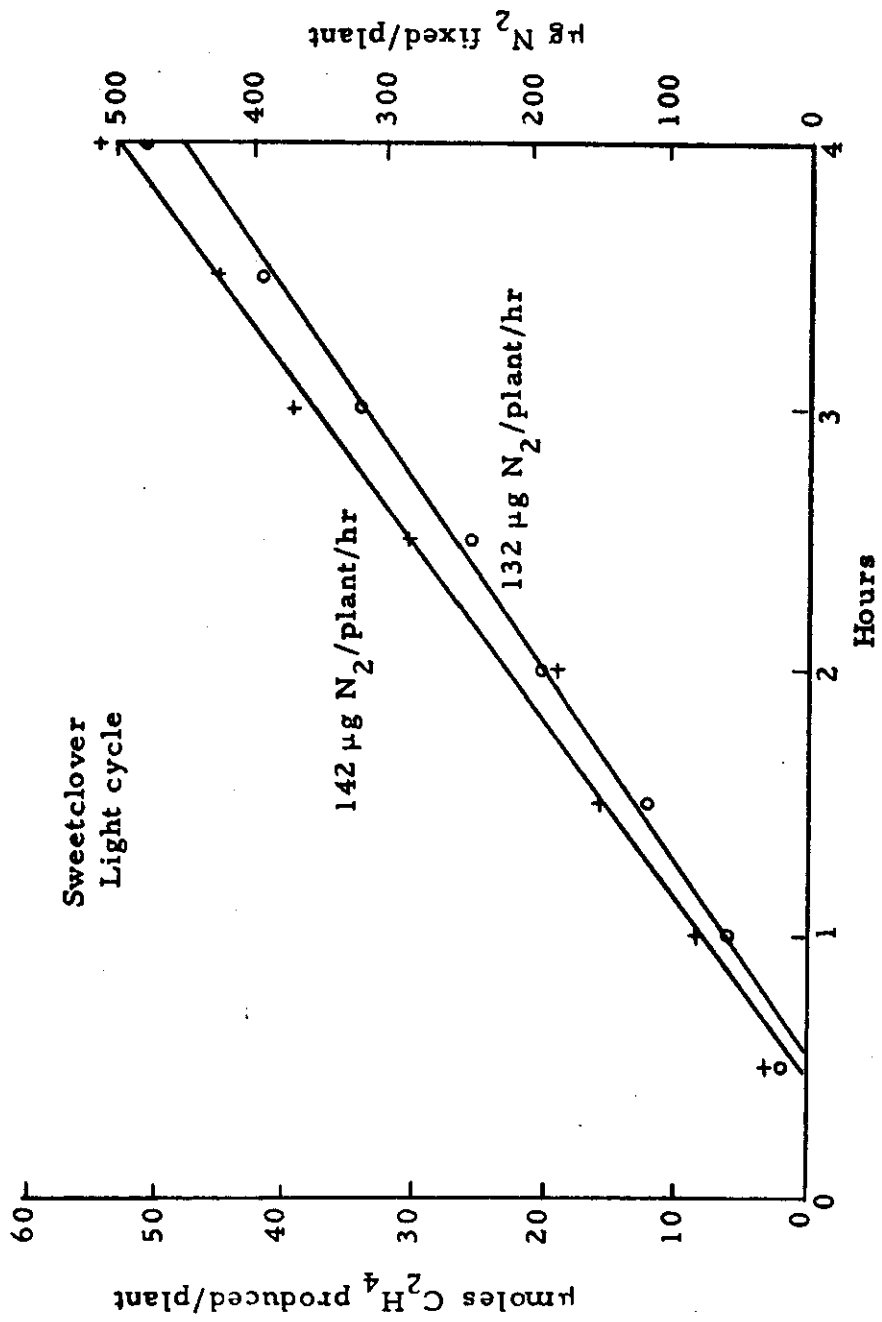
Fig. 12. Nitrogen fixation (C_2H_2-C_2H_4 assay) by duplicate single potted plants of *Melilotus officinales* L. (sweetclover) during a 4-hour light incubation.
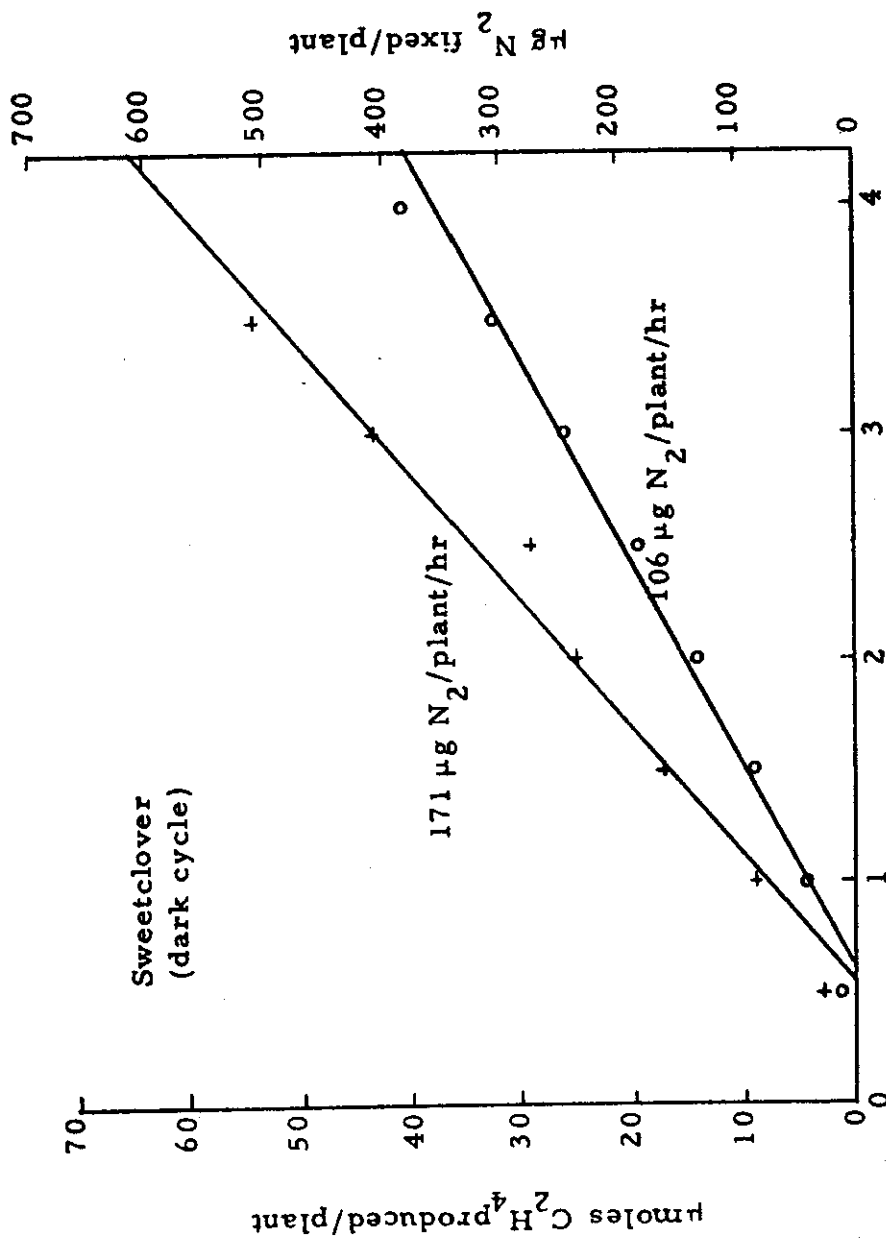
Fig. 13. Nitrogen fixation (C_2H_2-C_2H_4 assay) by duplicate single potted plants of _Melilotus officinales_ L. (sweetclover) during a 4-hour dark incubation.

plants was not nearly as good as that observed in preliminary studies for 30 day old alfalfa and sainfoin. A variation in nitrogen fixing rates is not surprising, but nevertheless was disappointing following the excellent duplication between plants in the preliminary legume study. The greater variation between plants during the dark incubation as illustrated in figure 13 for sweetclover was not a characteristic of dark incubations. Ethylene production or N_2 fixation is calculated on a per plant basis as μmoles ethylene produced/plant/hr and as μg N_2 fixed/plant hr. Table 9 shows the mean N_2 fixation rates for duplicate soybean, alfalfa and sweetclover plants as influenced by the age of the plants and by light and dark incubation conditions.

Table 10 summarizes the maximum N_2 fixation rates with associated standard errors obtained for the different legumes. Soybeans gave the highest N_2 fixation rate under light conditions while sweetclover exhibited the highest N_2 fixation rate under dark conditions. No dark measurements were taken on sainfoin. Oxytropis sericea did not exhibit any N_2-fixing activity during the first 6 months of growth. Differences in the N_2 fixation rates between light and dark incubations were significant for soybeans only. Summation of the weekly averages of C_2H_4 produced by single plants of each species and assuming specific plant populations, allows the calculation of an amount of N_2 fixed/ha/season. Based on a theoretical conversion factor of 1 mole of N_2 fixed/3 moles of C_2H_4 formed, alfalfa fixed

Table 9. Average µg N_2 fixed (C_2H_2-C_2H_4 assay)/plant/hr by duplicate <u>Glycine</u> <u>max</u> (soybeans), <u>Medicago</u> <u>sativa</u> (alfalfa) and <u>Melilotus</u> <u>officinales</u> (sweetclover) plants as influenced by plant age and light and dark incubation conditions.

Plant Age	Soybeans		Alfalfa		Sweetclover	
	Light	Dark	Light	Dark	Light	Dark
Weeks			µg N_2 fixed/plant/hr			
3	1.2	1.1				
4	13.8	29.2	0.9			
5	86.7	104.2	17.1	25.5	1.8	0.4
6	196.8	112.0	25.0	50.5	26.8	39.2
7	112.6	119.5	76.9	62.9	40.4	79.4
8	95.3	114.1	42.8	65.0	74.4	55.0
9	45.7		41.2	91.6	35.4	92.5
10	24.1		110.0	89.3	137.1	138.4
11	10.6		93.7		124.1	
12			88.7		104.2	
13			79.6			

60 kg N_2/ha/ 9 week season (5-14 weeks after germination) assuming 600,000 plants/ha; sweetclover fixed 33 kg N_2/ha/7 week season (6-13 weeks after germination) assuming 330,000 plants/ha; and soybeans fixed 36 kg N_2/ha/8 week season (4-12 weeks after germination) assuming 370,000 plants/ha. Weekly measurements of N_2 fixation

Table 10. Maximum symbiotic N_2 fixation (C_2H_2-C_2H_4 assay) rates for single potted legume plants of varying ages.

Species	Plant Age	Cycle	Rate	Standard Error
	Weeks		μg/plant/hr	
Soybeans	6	Light	196.8	7.7
	7	Dark	119.5	7.4
Alfalfa	10	Light	110.0	4.9
	9	Dark	91.6	11.5
Sweetclover	10	Light	137.1	5.2
	10	Dark	138.4	16.5
Sainfoin	14	Light	126.4	6.6

by individual alfalfa, sweetclover and soybean plants can be found in the appendix, Tables 3, 4, and 5. Rates of nitrogen fixation by sainfoin and Oxytropis sericea were not measured at regular intervals.

Figures 14 and 15 show the relationship of the plant age or stages of growth and the rates of nitrogen fixation for alfalfa and soybeans. Alfalfa being a perennial plant, maintains a high N_2 fixation for a longer period of time than do soybeans which complete their life cycle in one season. In general, alfalfa and sweetclover give parabolic type curves when N_2 fixation rates are plotted against plant age. Considerable variation in N_2 fixation rates between individual plants was observed with both of these plant species, especially with the sweetclover. The N_2 fixation rates of soybeans, when plotted
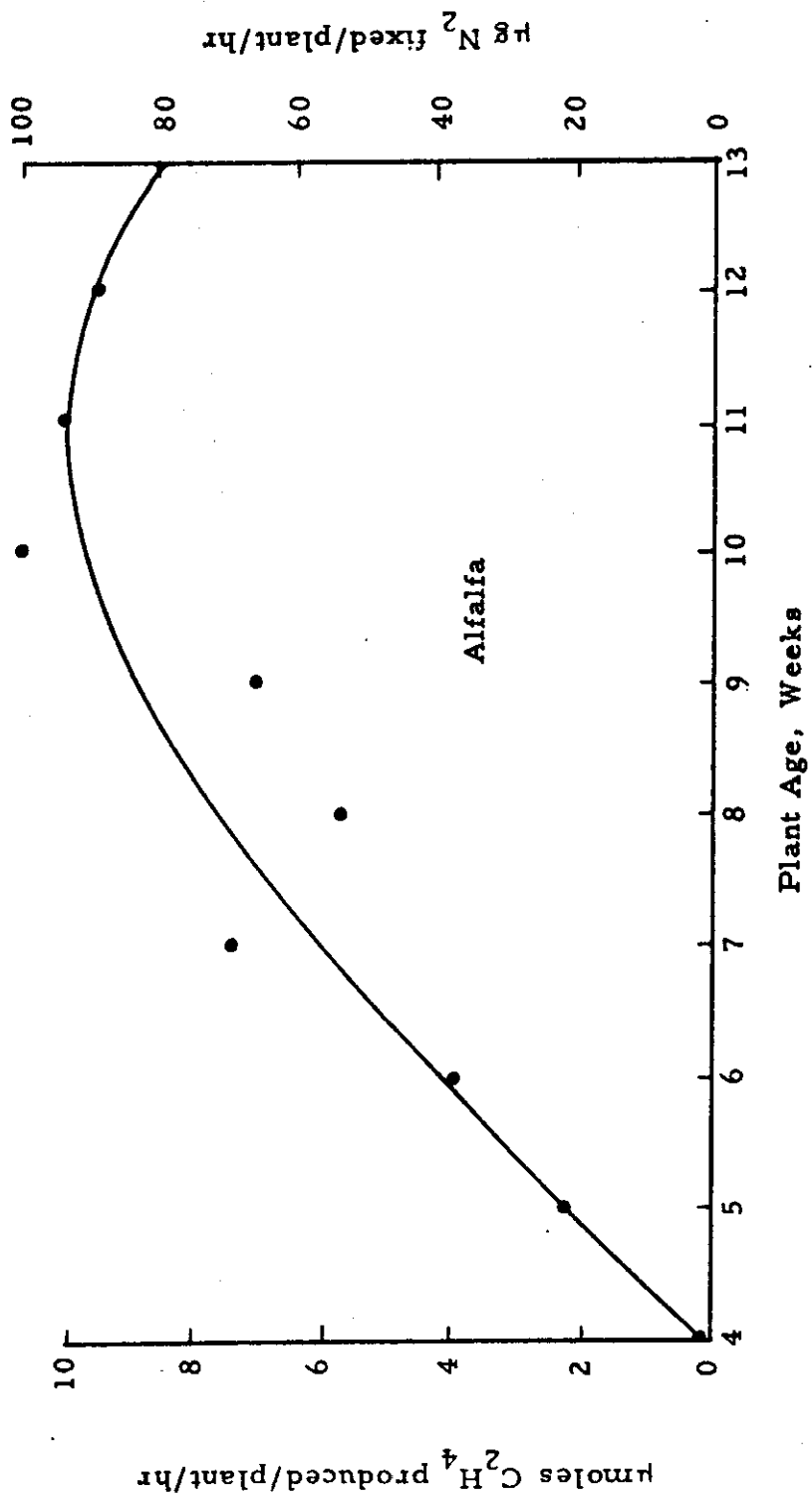
Fig. 14. Relationship of nitrogen fixation (C_2H_2-C_2H_4 assay) rate and plant age by _Medicago sativa_ L. (alfalfa) plants under both light and dark conditions.
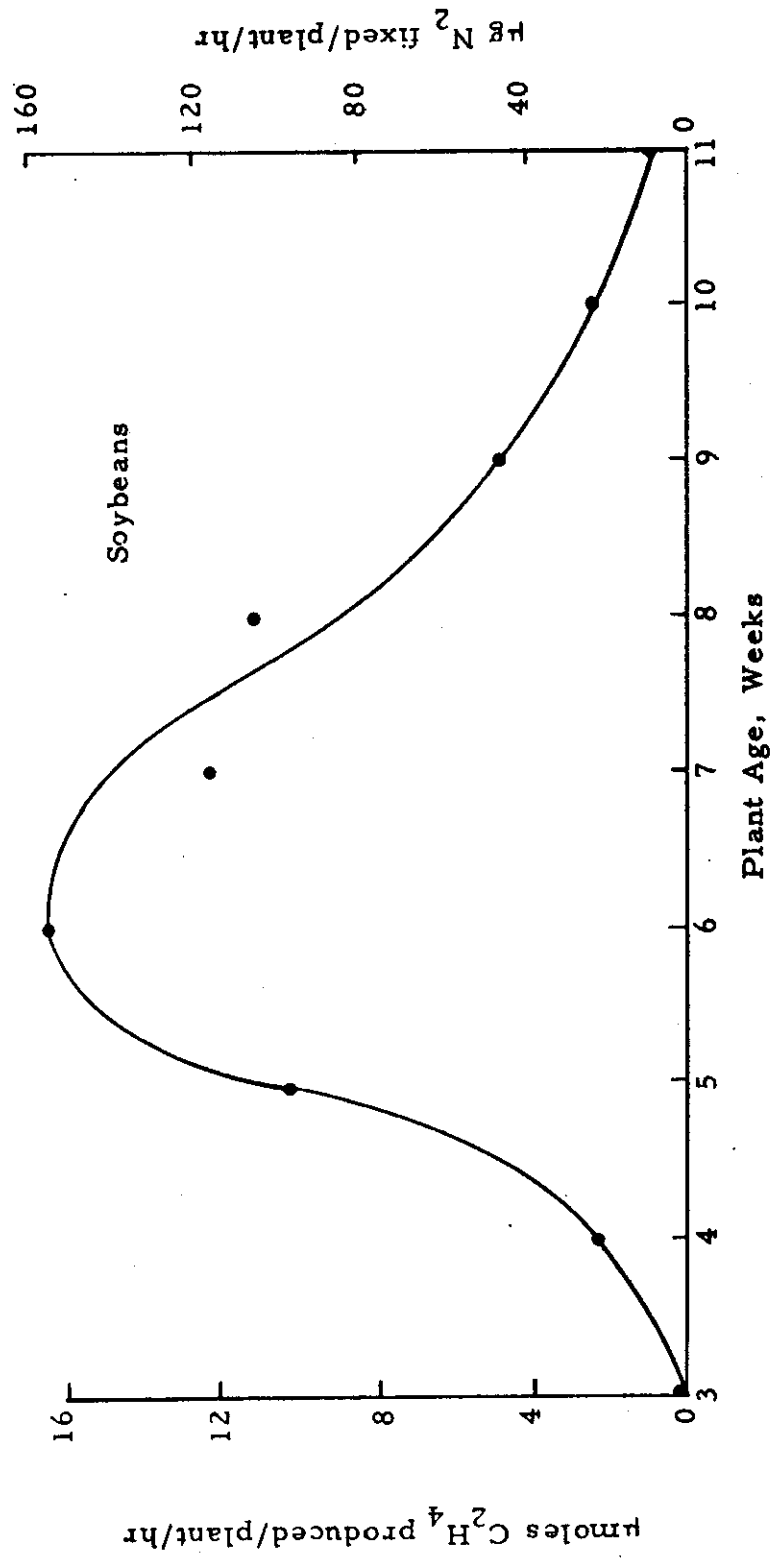
Fig. 15. Relationship of nitrogen fixation (C_2H_2-C_2H_4 assay) rate and plant age by *Glycine max* Merr. (soybean) plants under both light and dark conditions.

against plant age gives a bell-shaped curve. The plot of the relationship of nitrogen fixed by soybeans as a function of time or stage of development shows that N_2-fixing activity was initiated 3 weeks after germination, or about the time the soybean plants begin to flower. The plants began to set on seed pods between the 4th and 5th weeks, during which time the rate of N_2 fixation increased rapidly. A maximum N_2 fixation rate was reached 6 weeks after germination. The N_2-fixing activity decreased at a slightly slower rate than it increased. Plant senescence, as noted by the yellowing of leaves was initiated at the 8th week and the soybean root nodules had begun to decompose at the 10th and 11th weeks.

Nodules were collected from selected soybean plants as soon as collection became feasible, and continued until the experiment was terminated. The fresh weight of nodules collected/plant with time is tabulated in Appendix Table 6. The nodule fresh weight was slightly greater during the two weeks following the measurement of the maximum N_2 fixation rate. This would indicate that a larger fraction of the nodules were active at the earlier date.

Figure 16 shows the nitrogen fixation rates (in $\mu g/g$ fresh nodules/hr) plotted against the age of the soybean plants. The maximum N_2 fixation rate still occurs at 6 weeks post emergence, but the rate decreases faster initially, then trails off more slowly than when the N_2 fixation rates were expressed on a per plant basis.
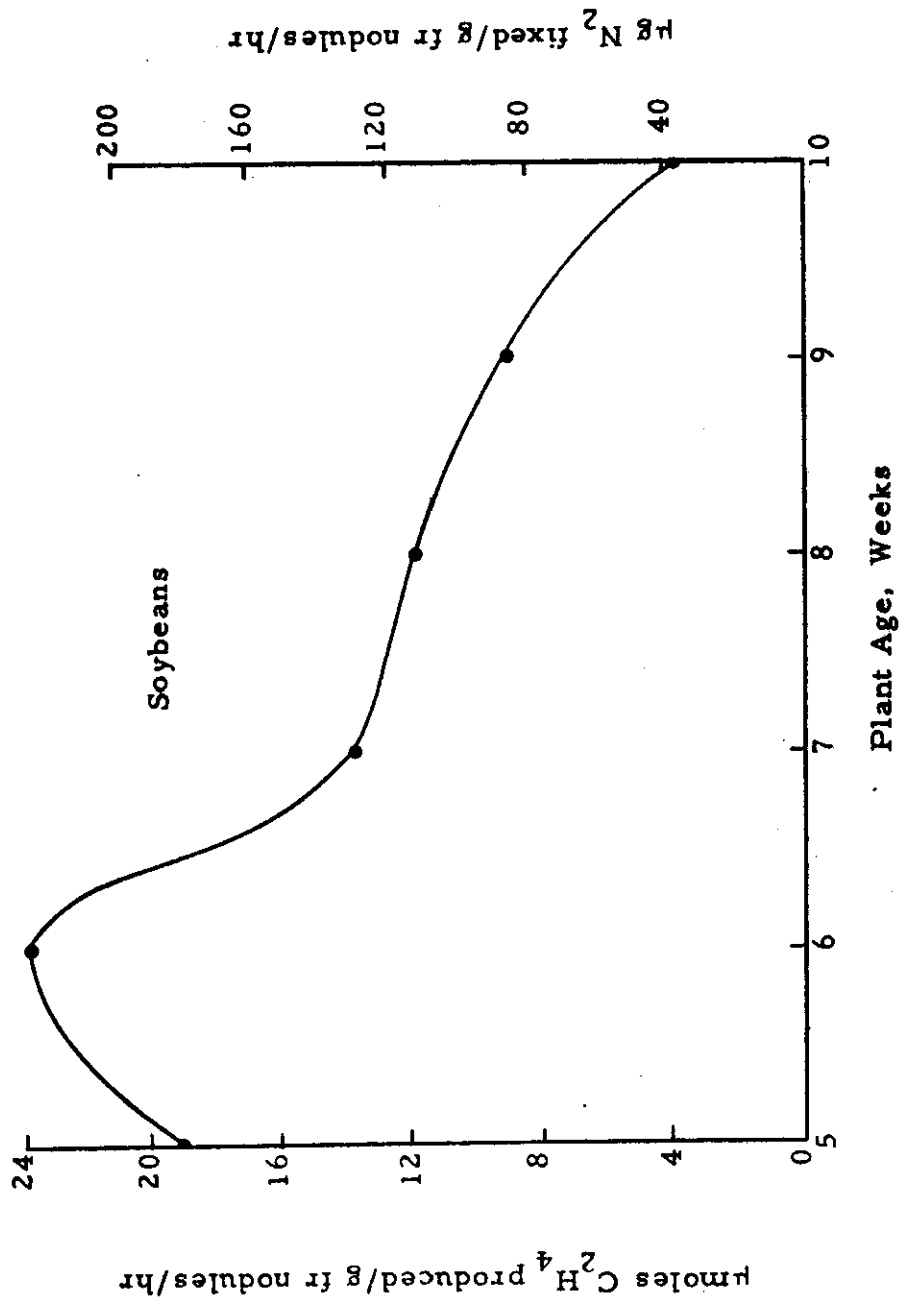
Fig. 16. Relationship of nitrogen fixation (C_2H_2-C_2H_4 assay) rate (expressed on a fresh nodule weight basis) and age of <u>Glycine max</u> Merr. (soybean) plants.

Appendix Table 6 lists the ethylene production and N_2 fixation rates (expressed on a fresh nodule weight basis), for individual soybean plants under both light and dark incubation conditions. Nodules were collected from soybean plants incubated under both light and dark conditions for a 3 week period. An analysis of variance indicates that the age of the plant, the light-dark conditions and the interaction (weeks x light-dark) term are highly significant. This data can be found in Appendix Table 7. Figure 17 shows a plot of the nitrogen fixation rates (expressed on a fresh nodule weight basis) for both light and dark conditions. Most of the significance revealed in the analysis of variance comes about as a result of the large differences in the light and dark nitrogen fixation rates at the 6th week, the date of the maximum N_2 fixation by the soybean plants. A probable explanation of this is that not enough photosynthates were available to maintain the higher N_2 fixation rate during the dark cycle.

Range legume, 9 months

Nine months after germination in the growth chamber, N_2-fixing activity was measured in the Oxytropis sericea plant species. Six plants demonstrated an average nitrogen fixation rate of 0.61 mg N_2 fixed/plant/day. Individual N_2 fixation rates are given in Table 11. Using this average fixation rate, 16 plants/m^2 would be necessary to obtain a N_2 fixation rate of 1 g/m^2/100 days. Sims et al. (1971), above-ground biomass data shows only 1 to 3 g of leguminous plants/m^2.
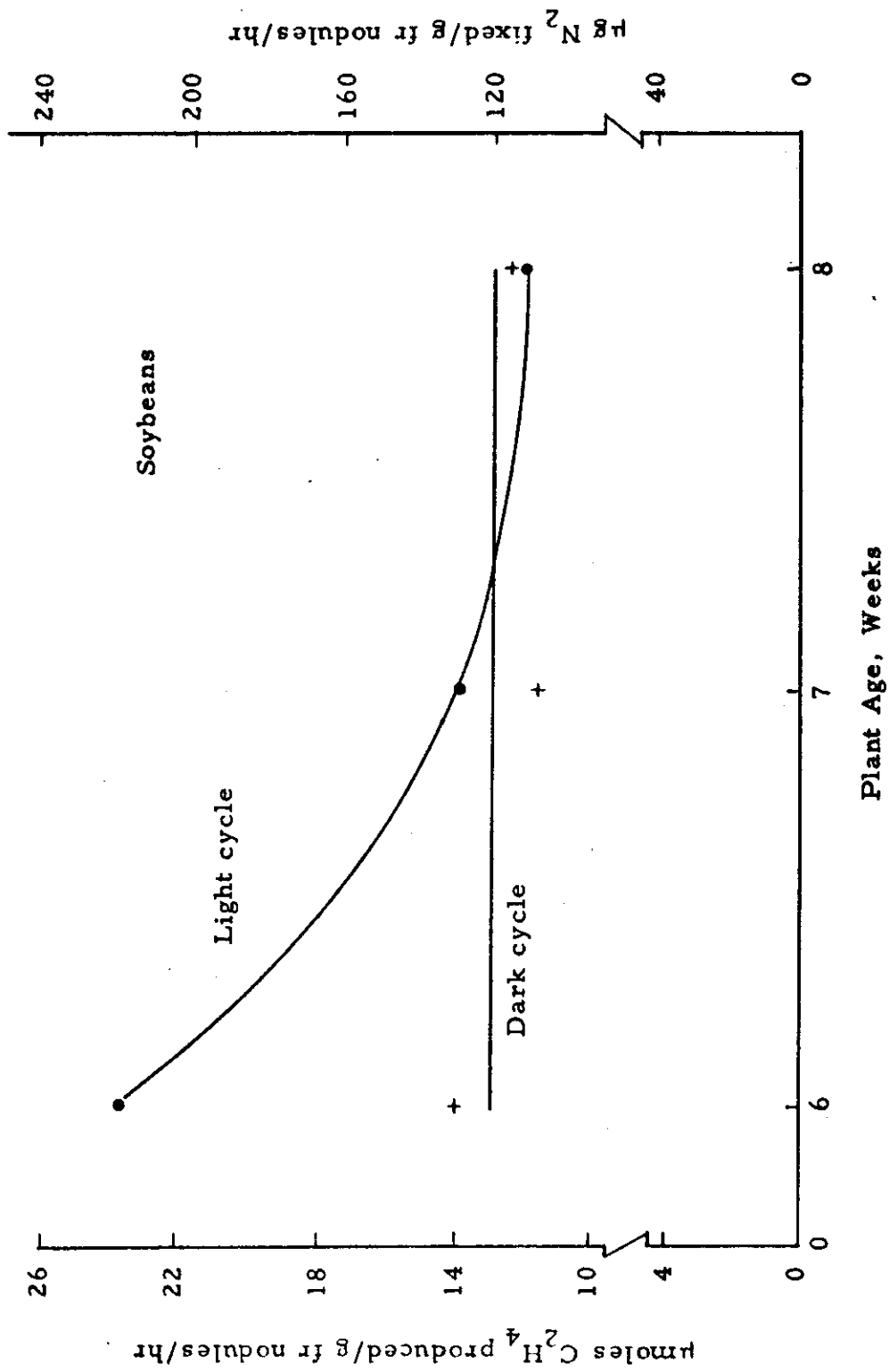
Fig. 17. Comparison of nitrogen fixation (C_2H_2-C_2H_4 assay) rate (expressed on a fresh nodule weight basis) during light and dark incubations by Glycine max Merr. (soybean) plants.

Table 11. Symbiotic nitrogen fixation (C_2H_2-C_2H_4 assay) rates of 9 month old <u>Oxytropis sericea</u> Nutt. plants.

mg N_2 fixed/plant/day	
1.01	
1.19	
	Mean 0.61
0.27	
0.48	
	S.E. 0.17
0.58	
0.15	

These <u>Oxytropis sericea</u> plants averaged approximately 5 g dry material/plant. Sixteen plants of this size per m^2 would account for 65-100% of the measured above-ground biomass on the Pawnee Site.

Conclusions regarding the Significance of Biological N_2 Fixation at the Pawnee Site

Investigations of biological N_2 fixation on the Pawnee Site would indicate that asymbiotic N_2 fixation is very low (<0.1 mg/m^2/day) under field capacity moisture levels, and would account for a maximum of 0.1 g/m^2/100 days under saturated moisture conditions. Saturated moisture conditions would not exist for extended periods, if at all on the Pawnee Site. Experiments involving the addition of

several levels of a soluble carbon source, show that 9-10 mg N_2 are fixed/g carbon added. Thus, even if large quantities of a soluble carbon source are present, only small amounts of nitrogen will be fixed. Grassland soils usually contain large quantities of carbonaceous material, however, only small amounts of carbon are readily assimilable at any one time.

The above-ground plant biomass data on the standing crop on the Pawnee Site would indicate a legume plant density considerably lower than one plant/m^2 (Sims et al., 1971). Single plants of sweetclover, a common legume on abandoned crop land in the Pawnee Site area and sainfoin, an introduced dryland legume are capable of fixing approximately 3 mg N_2/day. However, to be consistent with above ground biomass measurements, plant densities would have to be on the order of 1 plant/10 m^2. These legumes, would then, at the very best, fix only 0.25 kg N_2/ha/100 days. Because of low legume plant densities and temperature and moisture limitations at the Pawnee Site, it would appear that forage legumes or range legumes do not account for the fixation of significant quantities of nitrogen.

Investigations at the Pawnee Site would suggest a maximum total biological fixation of up to 0.1 g N_2/m^2/year. At the outset of these investigations it was suspected that biological fixation of elemental nitrogen would be the major source of nitrogen in the grassland ecosystem. However, Reuss (1971) indicates an input of 0.4 g N/m^2/year in precipitation at the Pawnee Site. He also noted a total annual flow

of 3.5 g N/m^2/year from the plant roots to the plant tops. The two most likely points of nitrogen loss in the grassland ecosystem are volitalization of N from animal wastes and losses during litter decomposition. If losses from these two processes are less than 0.5 g N/m^2/year (14% of the annual flow of N to the plant tops) the grassland ecosystem will be self-sustaining with regard to nitrogen.

Chapter V

SUMMARY

Studies were conducted on soils and soil-plant systems to assess
the importance of biological nitrogen fixation in grassland ecosystems.
Additional studies were carried out to investigate the rates of nitrogen
fixation by leguminous plants as a function of plant age. The acetylene
reduction assay, an indirect method of measuring N_2 fixation, was
used in all studies. This technique appears to be an effective index
for estimating low rates of N_2 fixation, for evaluating the effects of
environmental factors on N_2 fixation and for making comparisons
among different N_2-fixing symbionts.

Studies indicated that anaerobic conditions and a high level of
readily assimilable carbon were necessary for significant amounts
of nitrogen to be fixed by heterotrophic organisms. Saturated mois-
ture levels can satisfy the anaerobic requirement, however, this
condition is unrealistic in an ecosystem where moisture is limiting
and where infiltration of water is not restricted. Potential energy
material, mostly in the form of grass roots and litter is abundant
in grassland ecosystems. However, readily assimilable energy
sources are in short supply in most soils. Soluble energy sources
added to grassland soils as sucrose, at levels from 14-56 g C/m^2,

resulted in the fixation of 9-10 mg N_2/g C under saturated moisture conditions. Biological N_2 fixation by free-living heterotrophs is of limited significance in the grassland ecosystem.

Above-ground biomass studies show that leguminous plants usually account for less than 1% of the standing crop on the Pawnee Site (Sims et al., 1971). This percentage is equal to 1-2 g plant material/m^2. Single range legumes weighing approximately 5 g each demonstrated an average N_2 fixation rate of 0.61 mg/plant/day. One plant/m^2 would suggest a N_2 fixation rate of 0.61 mg/m^2/day, which could be significant if the duration of favorable environmental conditions was long enough. However, this plant density is not consistent with the above-ground biomass measurements of Sims et al. (1971). In addition to limiting the rate and duration of asymbiotic N_2 fixation on the Pawnee Site, temperature and moisture conditions would also restrict the duration of the N_2-fixing activity of leguminous plants. This coupled with low plant density, suggests that symbiotic nitrogen fixation is of limited significance in the grassland ecosystem.

In single plant comparisons of N_2 fixation rates, soybeans, sweet-clover, sainfoin and alfalfa demonstrated maximum N_2 fixation (C_2H_2-C_2H_4 assay) rates of 4.7, 3.3, 3.0 and 2.6 mg/plant/day respectively. Differences in the N_2 fixation rates between light and dark incubations were significant for soybeans only, and this was principally the result of large differences at one date.

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APPENDICES

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Appendix Table 1. Nitrogen fixed (C_2H_2-C_2H_4 assay) in triplicate disturbed cores of Ascalon sandy loam soil under a 80:20 He:O_2 atmosphere at saturation and field capacity moisture levels with 326 kg C/ha as sucrose added to all treatments. Values are g N_2 fixed/ha.

Time (Hours)	Field Capacity				Saturated			
	A	B	C	Mean	A	B	C	Mean
2.0	1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.6
18.5	2.6	2.6	2.2	2.5	46	64	61	57
19.5					74	102	88	88
21.0			2.2		107	144	117	123
23.0	3.2	3.2	3.2	3.2	166	223	173	187
26.0			3.2		254	343	253	283
31.0	3.9	3.9	4.2	4.0	471	621	450	514
42.0	3.9	3.9	5.1	4.3	1085	1228	880	1064
45.0					1291	1382	994	1222
48.0	4.5	4.8	5.8	5.0	1451	1525	1085	1354
55.0	4.5	4.8	6.4	5.2	1896	1902	1325	1708
66.0	4.5	4.8	7.1	5.5	2485	2433	1582	2167
72.0	4.8	4.8	7.4	5.7	2707	2559	1748	2338
79.0	5.1	5.1	8.7	6.3	3336	3084	2039	2820
90.0	6.4	6.1	9.0	7.2	3633	3370	2233	3079
95.0	6.4	6.1	9.0	7.2	3667	3450	2330	3149
104.0	6.4	6.1	9.3	7.3	3838	3519	2490	3282
115.0	6.7	6.4	9.3	7.5	3838	3553	2559	3312
125.0	7.1	6.4	10.0	7.8	3930	3667	2650	3315
162.0					3838	3587	2445	3290

Appendix Table 2. Nitrogen fixed (C_2H_2-C_2H_4 assay) in triplicate and duplicate cores of Ascalon sandy loam soil under a helium atmosphere at saturation and field capacity moisture levels with 326 kg C/ha as sucrose added to all treatments. Values are g N_2 fixed/ha.

Time (Hours)	Field Capacity				Saturated		
	A	B	C	Mean	A	B	Mean
2.0	1.6	1.6	1.9	1.7	2.1	2.1	2.1
9.0	2.2	2.2	2.2	2.2	2.8	3.2	3.0
19.5	4.8	5.5	3.2	4.5	108	82	95
22.5	30	46	13	30	222	185	204
24.5	69	108	49	75	353	276	314
34.0	489	678	549	572	1099	860	980
45.0	1001	1263	1099	1121	2335	1823	2079
55.0	1417	1705	1428	1517	3554	2814	3184
79.0	1674	1900	1479	1684	5012	4169	4590
92.0	1771	2003	1510	1761	5582	4557	5070
105.0	1685	1890	1459	1678	5514	4523	5018
116.0	1736	1952	1500	1729	5501	4580	5040

Appendix Table 3. Individual and mean data of μmoles C_2H_4 produced/plant/hr and μg N_2 fixed/plant/hr by <u>Medicago sativa</u> L. (alfalfa) as influenced by incubation conditions and plant age.

Plant age, Weeks	Incubation Condition	Ethylene produced μmoles/plant/hr		Nitrogen fixed μg/plant/hr	
		Individual	Mean	Individual	Mean
4	Light	0.11 0.09	0.10	0.99 0.81	0.90
5	Light	1.40 2.27	1.84	13.06 21.22	17.14
5	Dark	0.56 4.90	2.73	5.25 45.76	25.51
6	Light	4.43 0.92	2.68	41.40 8.57	24.99
6	Dark	4.65 6.17	5.41	43.48 57.66	50.57
7	Light	8.61 7.86	8.23	80.41 73.39	76.90
7	Dark	6.67 6.80	6.74	62.32 63.55	62.94
8	Light	3.99 5.18	4.58	37.24 48.35	42.80
8	Dark	4.60 9.31	6.96	42.98 87.01	65.00
9	Light	5.18 3.64	4.41	48.40 33.99	41.20
9	Dark	12.06 7.54	9.80	112.66 70.43	91.55

Appendix Table 3 (Continued).

Plant age, Weeks	Incubation Condition	Ethylene produced μmoles/plant/hr		Nitrogen fixed μg/plant/hr	
		Individual	Mean	Individual	Mean
10	Light	12.56 10.99	11.77	117.36 102.63	110.00
10	Dark	10.74 8.38	9.56	100.38 78.30	89.34
11	Light	11.95 8.12	10.03	111.64 75.82	93.73
12	Light	9.31 9.68	9.50	86.97 90.46	88.72
13	Light	6.33 10.70	8.52	59.17 99.97	79.57

Appendix Table 4. Individual and mean data of μmole C_2H_4 produced/ plant/hr and μg N_2 fixed/plant/hr by <u>Melilotus officinales</u> L. (sweetclover) as influenced by incubation conditions and plant age.

Plant age, Weeks	Incubation Condition	Ethylene produced μmoles/plant/hr		Nitrogen fixed μg/plant/hr	
		Individual	Mean	Individual	Mean
5	Light	0.38 0.02	0.20	3.50 0.17	1.84
5	Dark	0.02 0.07	0.05	0.21 0.64	0.43
6	Light	3.75 1.99	2.87	35.01 18.57	26.79
6	Dark	4.80 3.60	4.20	44.81 33.60	39.21
7	Light	6.69 1.95	4.32	62.53 18.24	40.39
7	Dark	3.74 13.25	8.50	34.90 123.83	79.37
8	Light	4.40 11.53	7.97	41.11 107.76	74.44
8	Dark	4.88 6.89	5.88	45.56 64.39	54.98
9	Light	4.01 3.57	3.79	37.45 33.34	35.40
9	Dark	12.58 7.22	9.90	117.55 67.45	92.50

Appendix Table 4 (Continued).

Plant age, Weeks	Incubation Condition	Ethylene produced μmoles/plant/hr		Nitrogen fixed μg/plant/hr	
		Individual	Mean	Individual	Mean
10	Light	14.10 15.24	14.67	131.75 142.39	137.07
10	Dark	11.36 18.26	14.81	106.10 170.64	138.37
11	Light	17.57 8.99	13.28	164.16 84.01	124.09
12	Light	7.81 14.49	11.15	79.96 135.34	104.15

Appendix Table 5. Individual and mean data of μmoles C_2H_4 produced/ plant/hr and μg N_2 fixed/plant/hr by <u>Glycine max</u> Merr. (soybeans) as influenced by incubation conditions and plant age.

Plant age, Weeks	Incubation Condition	Ethylene produced μmules/plant/hr		Nitrogen fixed μg/plant/hr	
		Individual	Mean	Individual	Mean
3	Light	0.18 0.08	0.13	1.71 0.76	1.24
3	Dark	0.01 0.22	0.12	0.09 2.10	1.10
4	Light	1.69 1.27	1.48	15.81 11.87	13.84
4	Dark	5.35 0.89	3.12	50.01 8.28	29.15
5	Light	9.79 8.75	9.28	91.49 81.83	86.66
5	Dark	11.28 11.03	11.15	105.39 103.01	104.20
6	Light	22.62 19.50	21.06	211.36 182.18	196.77
6	Dark	10.61 13.37	11.99	99.15 124.87	112.01
7	Light	9.92 14.19	12.06	92.69 132.58	112.64
7	Dark	11.42 14.17	12.79	106.69 132.34	119.52
8	Light	9.97 10.43	10.20	93.14 97.47	95.31
8	Dark	10.84 13.58	12.21	101.30 126.85	114.08

Appendix Table 5 (Continued)

Plant age, Weeks	Incubation Condition	Ethylene produced µmoles/plant/hr		Nitrogen fixed µg/plant/hr	
		Individual	Mean	Individual	Mean
9	Light	5.22 4.57	4.89	48.73 42.66	45.69
10	Light	0.90 4.27	2.58	8.42 39.86	24.14
11	Light	0.63 1.64	1.14	5.89 15.33	10.61

Appendix Table 6. Mg fresh nodules collected and individual and mean data of μmoles C_2H_4 produced/g fresh nodules/hr and μg N_2 fixed/g fresh nodules/hr by <u>Glycine max</u> Merr. (soybeans) as influenced by incubation conditions and plant age.

Plant age, Weeks	Incubation Condition	Mg fresh Nodules	Ethylene produced μmoles/g fresh nodules/hr Individual	Mean	Nitrogen fixed μg/g fresh nodules/hr Individual	Mean
4	Dark	445	12.03	10.89	112.38	101.69
		91	9.74		90.99	
5	Light	531	18.44	18.91	172.30	176.67
		452	19.38		181.04	
6	Light	946	23.91	23.82	223.42	222.53
		822	23.72		221.63	
6	Dark	887	11.96	13.87	111.78	129.61
		847	15.78		147.43	
7	Light	792	12.53	13.82	117.03	129.04
		940	15.10		141.04	
7	Dark	1083	10.54	11.43	98.51	106.80
		1150	12.32		115.08	
8	Light	727	13.71	11.94	128.12	111.52
		1027	10.16		94.91	
8	Dark	951	11.40	12.30	106.52	114.90
		1029	13.19		123.28	
9	Light	452	11.54	9.02	107.81	84.29
		702	6.50		60.77	
10	Light	590	1.53	3.86	14.27	36.06
		689	6.19		57.85	
11	Light	99	6.36	6.43	59.49	60.04
		253	6.49		60.59	

Appendix Table 7. The mean N_2 fixation (C_2H_2-C_2H_4 assay) rates expressed on a per g fresh nodule weight basis for Glycine max Merr. (soybeans) and analysis of variance.

	N_2 Fixation			
Weeks	6	7	8	Mean
	μg N_2/g fresh nodules/hr			
Light	23.8	13.8	11.9	15.2
Dark	13.9	11.4	12.3	12.5
Mean	18.8	12.6	12.1	13.9

Weeks	$P \leq$	.010
Light-dark	$P \leq$	.005
Weeks x Light-dark	$P \leq$	.025