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USE OF AN INTACT SOIL CORE - ACETYLENE REDUCTION ASSAY
TO ESTIMATE IN SITU NITROGEN FIXATION RATES

by

CHRISTI ANN NORDSTROM

A thesis submitted in partial fulfillment
of the requirements for the degree
Master of Science
Major in Microbiology
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1986

USE OF AN INTACT CORE - ACETYLENE REDUCTION ASSAY
TO ESTIMATE IN SITU NITROGEN FIXATION RATES

This thesis is approved as a creditable and independent investigation by a candidate for the degree, Master of Science, and is acceptable for meeting the thesis requirements for this degree. Acceptance of this thesis does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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TABLE OF CONTENTS

	Page
ACKNOWLEDGEMENTS	i
TABLE OF CONTENTS	ii
LIST OF FIGURES	iii
LIST OF TABLES	v
LIST OF COMMONLY USED ABBREVIATIONS	vi
I. INTRODUCTION	1
II. LITERATURE REVIEW	3
III. PURPOSE	20
IV. DEVELOPMENT OF AN INTACT SOIL CORE ASSAY FOR MEASUREMENT OF ACETYLENE REDUCTION ...	22
A. INTRODUCTION	22
B. MATERIALS AND METHODS	26
C. RESULTS AND DISCUSSION	33
D. CONCLUSIONS	53
V. FIELD SURVEY	54
A. INTRODUCTION	54
B. MATERIALS AND METHODS	55
C. RESULTS AND DISCUSSION	60
VI. CONCLUSIONS	80
VII. LITERATURE CITED	82

LIST OF FIGURES

	Page
FIGURE 1. Effect of incubation atmosphere on ethylene production rates	34
FIGURE 2. Effect of varying concentrations on ethylene production rates	36
FIGURE 3. Effect of incubation time on ethylene production rates	37
FIGURE 4. Effect of soil moisture on ethylene production rates	39
FIGURE 5. Effect of carbohydrate amendment on ethylene production rates	41
FIGURE 6. Effect of incubation temperature on ethylene production rates	43
FIGURE 7. Effect of incubation temperature on ethylene production rates	44
FIGURE 8. Effect of autoclaving on ethylene production	46
FIGURE 9. Effect of freezing on ethylene production rates	48
FIGURE 10. Effect of freezing on ethylene production rates	50
FIGURE 11. Effect of preincubation for 15 hours on ethylene production rates	52
FIGURE 12. Diagram illustrating the locations of the field sites used in the ethylene production study	56
FIGURE 13. Rates of ethylene production at three locations in a corn field	61
FIGURE 14. Rates of ethylene production at three locations in an alfalfa field	62
FIGURE 15. Rates of ethylene production at three locations in a barley field	63

FIGURE 16.	Rates of ethylene production and soil moisture for a native grassland site ...	65
FIGURE 17.	Rates of ethylene production and soil moisture for a native grassland site ...	66
FIGURE 18.	Rates of ethylene production and soil moisture for a corn field	67
FIGURE 19.	Rates of ethylene production and soil moisture for a corn field	68
FIGURE 20.	Rates of ethylene production and soil moisture for a corn field	69
FIGURE 21.	Rates of ethylene production and soil moisture for a corn field	70
FIGURE 22.	Rates of ethylene production and soil moisture for a rye field	71
FIGURE 23.	Rates of ethylene production and soil moisture for a barley field	72
FIGURE 24.	Rates of ethylene production and soil moisture for a wheat field	73
FIGURE 25.	Rates of ethylene production and soil moisture for an alfalfa field	74

LIST OF TABLES

	Page
TABLE 1. Calculations to determined ethylene production rates	29
TABLE 2. Comparison of two types of syringes for measurement of ethylene	32
TABLE 3. Statistical analysis using linear regression with ethylene production and soil moisture	75
TABLE 4. Statistical analysis using linear regression with ethylene production and sampling time	76
TABLE 5. Statistical analysis using linear regression with ethylene production and cropping type	77
TABLE 6. Statistical analysis using linear regression with ethylene production and cultivation management	79

COMMONLY USED ABBREVIATIONS

C_2H_2	acetylene
C_2H_4	ethylene
N_2	nitrogen
H_2O	water
ha	hectare (2.47 acre)
ml	milliliter
AR	argon
m	meter
wt	weight
r	radius
h	height
pi	value equal to 3.14
ppm	parts per million
atm	atmosphere
GC	gas chromatograph

I INTRODUCTION

Availability of nitrogen to the plant is a paramount concern in agricultural production today. Crop yields are most often dependent on the level of available nitrogen than any other element. Nitrogen can be supplied to the soil environment by either fertilizers or by the natural processes of decomposition of nitrogen containing compounds and biological fixation of nitrogen.

The most abundant source of nitrogen on the earth is the atmosphere. Although the atmosphere is approximately 79% nitrogen, this form is generally unavailable for plant use (11). Atmospheric nitrogen contains two nitrogen atoms held together by a triple bond which must be reduced before the resulting NH_3 can be assimilated by the plant. The conversion of atmospheric N to a usable form - ammonium via ammonia by microbial populations is termed nitrogen fixation. This process is not only an energy requiring reaction but occurs under a limited set of environmental conditions provided by only a few biological systems. Lightning and combustion reduce small quantities of atmospheric nitrogen but the majority of nitrogen transferred on a global scale from the atmosphere to the soil is by microbial organisms.

Biological nitrogen fixation can be performed by either symbiotic, associative or free-living microorganisms.

Plant roots produce nodules which are the site of fixation when infected by these symbiotic fixers. Associative fixers do not invade the plant root but reside in the root zone in close contact with the plant. Free-living fixers are not directly associated with any plant but are distributed throughout the soil profile. However the greatest numbers are found in the rhizosphere or the zone of soil inhabited by plant roots.

Other microbes are responsible for further cycling of nitrogen. Ammonia produced from fixation is oxidized to nitrite then nitrate by a process termed nitrification. Nitrate can undergo reduction to nitrite and ammonia or reduction through denitrification, resulting in the release of some form of nitrogen gas - N_2O , NO_2 , NO and N_2 into the atmosphere.

Nitrogen fixation represents a key link in the cycling of nitrogen both on a global and on an individual plant scale. An understanding of nitrogen fixation and the factors that affect this process are essential to maintaining adequate plant production to feed an evergrowing world population.

II LITERATURE REVIEW

A. Developments in nitrogen fixation prior to 1960

The phenomenon of nitrogen fixation was first suggested by researchers in 1836 (42). Evidence supporting the hypothesis of nitrogen fixation developed slowly over the next 50 years. During 1886-1888, nodules were described as inhabited by a bacterium and assimilating elemental nitrogen. Nitrogen fixation was first demonstrated in a free-living bacterium, Clostridium pastorianum (now C. pasteurianum) in 1893. In 1928 blue-green algae, without the presence of bacteria, were shown to fix nitrogen (42).

The earliest method for detecting nitrogen fixation was vigorous plant growth in nitrogen free medium (43). This method was replaced with Kjeldahl digestion in 1883 which measured total nitrogen content of the plant. Kjeldahl analysis does not reliably detect small nitrogen increases in materials with an initially high nitrogen content (1, 8, 42). Burris and Miller applied ^{15}N techniques to study biological nitrogen fixation in 1941. In this method the heavy isotope replaces a portion of the ^{14}N molecule in a test atmosphere. As the N_2 gas is reduced changes in production of the products formed are quantified using a mass spectrophotometer. Close agreement between calculated and observed values indicated there is no selection of one isotope form of N_2 over another (7). The

use of ^{15}N introduced a sensitive and reliable assay for nitrogen fixation, especially for experiments on media or materials high in nitrogen (1, 7, 8, 28, 42). Using ^{15}N and Kjeldahl analysis, addition of straw was observed to increase fixation rates in water logged soils, but only when oxygen was present (33).

B. Developments in nitrogen fixation during the 1960's

Methods to obtain cell-free extracts of free-living nitrogen fixers were developed in 1960 (42). Cell-free extracts allowed direct study of the nitrogen fixing enzyme system. Difficulties were encountered in cell-free extract experiments because of the need for high sensitivity of detection over a short period of time. A new isotope technique, involving the much shorter lived isotope ^{13}N was developed in 1961 (42). Although ^{13}N is highly sensitive, its use was not widespread. Drawbacks included a 2 hour limit on experiments because of the short half-life of the ^{13}N , high background counts in controls unless thoroughly flushed with oxygen and the high cost of the necessary equipment (42). ^{15}N continued as the technique of choice, being applied to plant-soil systems in general and specifically to nonsymbiotic fixation in soil and plant-soil systems (28, 35). The observation that ammonia was the apparent product of fixation in cell-free extracts led to the development of another technique (14). Ammonia analysis

was less sensitive than isotopic methods but proved very important in the progress of nitrogen fixation research. Using cell-free extracts of Clostridium pasteurianum, Dilworth et al. determined the requirements of nitrogen fixation, ATP, Mg^{++} , H_2 and ferredoxin and discovered H_2 , CO and N_2O inhibited nitrogen fixation (14, 15).

A major turning point in nitrogen fixation research came in 1966 when acetylene was observed to inhibit nitrogen fixation in cell-free extracts (15). Mass spectrometry revealed acetylene was reduced to ethylene and not ethane by the cell-free extract-nitrogenase enzyme system. Using manometric techniques and mass spectrometry acetylene reduction was shown to have the same biochemical requirements as nitrogen reduction (fixation) (15, 22, 36). The ability of the nitrogen fixing enzyme to reduce other substrates led researchers to look for a new method to follow nitrogen fixation through the use of one of these alternate substrates (6). In 1967 Stewart et al. reported that in situ studies of nitrogen fixation using acetylene reduction had been conducted in three ecosystems: Lakes, soils and soils with symbiotic nitrogen fixing plants. The technique was described as simple, requiring minimal equipment, extremely sensitive, ideal for in situ studies, and a good replacement for the more expensive and less sensitive ^{15}N . The validity of the acetylene reduction assay was verified by comparisons with ^{15}N results (44).

In 1968 the methodology, characteristics, and application of acetylene reduction was presented as an assay for nitrogen fixation (21). The acetylene-ethylene assay was applied to bacterial cultures and cell-free extracts in the laboratory and with legumes and free-living bacteria in situ. The general method involved closed system incubation of the culture or sample in the presence of acetylene. The amount of acetylene added was determined by the nitrogenase saturation point of the particular system in question, in cells saturation is 0.025 to 0.1 atmosphere acetylene, in roots 0.025 to 0.2 atmosphere (21). After incubation, C_2H_2 and C_2H_4 were quantified in atmosphere samples using a gas chromatograph with a H_2 flame ionization detector.

The relationship between acetylene and nitrogen was studied extensively in Azotobacter preparations (21). These gasses produced identical responses including ATP requirement, linear time course, optimum pH, sigmoidal relationship between rate and enzyme concentration, inhibition of H_2 evolution, competitive inhibition by CO , insensitivity to NH_4^+ , similar activation energies, and requirement for both protein fractions of the enzyme. Acetylene reduction values also correlated well with the literature showing Kjeldahl determined fixation rates (21).

A technique was developed to study in situ activity of nodulated plant roots, plant root soil bores, soil bores and pond water using acetylene reduction (21). The

methodology was carefully designed to minimize sample alteration and maintain the temperature, moisture and porosity of the system from which the sample was taken. The acetylene-ethylene assay had numerous advantages over other methods measuring fixation. Acetylene reduction was determined to be 10^6 more sensitive than Kjeldahl analysis and 10^3 more sensitive than ^{15}N . Acetylene reduction was also rapid, simple and inexpensive to run. No mechanical or chemical manipulation of the sample was needed. The reduction of acetylene to ethylene paralleled the reduction of gaseous dinitrogen to ammonia and was universal with the ability to fix nitrogen. Kjeldahl digestion was described as time consuming and insensitive, ^{15}N as complex and expensive, ^{13}N as inappropriate for any longterm experimentation and very expensive. Acetylene reduction does have two disadvantages, its indirectness of measure and its explosive nature (21).

In his review of nitrogen fixation in 1969, Burris discussed the characteristics of the nitrogenase enzyme system (6). The system was found to contain a labile Fe protein fraction and a stable Fe-Mo protein fraction. Researchers determined that 4 ATP's were transferred in nitrogen fixation for each pair of electrons added, reflecting the high energy needed to break the triple bond (6).

Silver reviewed nitrogen fixation in nonleguminous

plants, applying the use of the acetylene reduction assay (39). In assaying nodules this technique was considered an ideal tool in assessing the nitrogen input by fixation because of special problems which existed. The acetylene reduction assay was readily adapted to a mobile field laboratory to keep exposure of the nodules to oxygen at a minimum and maintain the integrity of the nodules (39).

Other investigations continued to compare the acetylene reduction assay with other commonly used indicators of nitrogen fixing ability or effectiveness (38). In nodules, acetylene reduction rates correlated well with leghemoglobin content which also parallels nitrogen fixation rates determined by ^{15}N and the Kjeldahl procedure as shown by Schwinghamer, Evans and Dawson. Rates in mutant Rhizobium strains were compared all correlated with plant dry weight, and total nitrogen content. Visual classification of nodules and plant appearance also correlated with acetylene reduction rates. Acetylene was found valid as a measure of nitrogen fixation and became the method of choice for comparison of bacterial strains (38).

Validity of the acetylene reduction assay was further demonstrated by the agreement between results from positive and negative free-living microorganisms suspected of nitrogen fixation using both ^{15}N and acetylene assays (31, 41). The acetylene-ethylene technique allowed

researchers to determine a list of organisms capable of fixing nitrogen and to evaluate factors affecting nitrogen fixation such as energy sources, oxygen, combined nitrogen and concentrations of Fe, Mo and H ions. Since these free-living, heterotrophic bacteria require an available exogenous energy source, fixation rates were higher in soils containing plants which provided root excretions and plant residues. Oxygen levels vary with the oxygen requirements of the organism. Anaerobic and facultatively anaerobic organisms require strict anaerobic conditions. Aerobes require oxygen for growth but the amount present affects the rates of fixation. The concentration of ammonium and nitrate present usually inhibits fixation. Researchers concluded that in situ studies using the acetylene reduction assay enabled determination of the contribution of free living nitrogen fixers to soil fertility and to study seasonal variations in nitrogen fixing activity (31, 41).

Brezonik and Harper used the acetylene-ethylene assay to study the presence of nitrogenase in lakes having an extensive anoxic environment (4). Results indicated acetylene reduction did occur in the anoxic environment of these lakes and generally increased with water depth. Because no other enzyme but nitrogenase was known to reduce acetylene, the expense of ^{15}N and the accepted validity of the acetylene reduction assay as a measure of fixation, the researchers did not verify their results with ^{15}N (4).

Other investigators used the acetylene technique to determine the availability of phosphorus in lakes (43). Limiting phosphorus had been shown to limit algal blooms. Management to limit and control algal blooms would be simplified if phosphorus levels could be readily determined. Measurement of acetylene reduction rates when the system was phosphorus starved were compared to rates when phosphorus was added. Up to this time the method of acetylene reduction assay involved the evacuation of the gas phase surrounding the sample. These researchers claimed the presence of gaseous nitrogen did not effect acetylene reduction when sufficient acetylene was present (43).

An extensive study was conducted in California comparing ^{15}N rates with acetylene reduction rates in soils (46). Both methods were used for samples collected and assayed monthly for a year. A modified sampling technique was developed. Soil cores were taken with a cork borer resulting in a 1 cm diameter core 8 cm long. Cores were placed in 1.5 x 10 cm test tubes. A 1 cm paper disk was placed on the core to prevent contact between the soil and rubber serum stopper (46). Rates varied from 5 kg nitrogen per hectare per year in a favorable environment to 2 kg nitrogen per hectare per year in an arid environment.

A comparison was made between the amount of acetylene reduced and the amount of nitrogen fixed. The conversion factor ranged from 8:4 to 3:1. They proposed

that ethylene could be metabolized; adsorbed to the soil surfaces, and that the rates of acetylene reduction became nonlinear with time to explain the variation (46). Kavanagh and Postgate reported the possible absorbance and subsequent release of CH_4 , C_2H_2 , and C_2H_4 by rubber closures. They claimed the carryover of these hydrocarbons could lead to false positives and slightly higher rates. They recommended the use of 'suba seals' which showed the least absorption or disposal of rubber closures after use (24).

C. Developments in nitrogen fixation during the 1970's

During the 70's, research continued on factors affecting acetylene reduction rates. Soils were preincubated under aerobic, anaerobic or oxygen gradient atmospheres to determine the effect of various levels of oxygen. Following preincubation, acetylene was added to the soil assay chambers and samples were taken. Aerobic preincubation, with aerobic assay, and anaerobic preincubation with aerobic assay gave very little activity. Aerobic preincubation with aerobic assay resulted in a 24 hour lag phase whereas anaerobic preincubation with anaerobic assay gave a shorter lag phase and the highest overall values of acetylene reduction. In oxygen gradient studies, the highest acetylene reduction rates were produced under 0.18 atm O_2 ; the activity markedly declined at O_2 partial pressures of 0.2 atm. The lag phase in these

gradient studies was longer than 24 hours (6).

Another group studied the effects of long-term preincubation under acetylene (12). One treatment was incubated several hours under 10% C_2H_2 in air, another treatment was alternated every 30 min between 10% C_2H_2 in air and ambient air and a final treatment (control) was incubated without C_2H_2 in ambient air. These treatments were applied to two species of algae and two species of bacteria. Acetylene reduction activity was greatly enhanced by long-term incubation under acetylene. Discontinuous incubation under acetylene, alternating between acetylene containing and nonacetylene containing atmosphere, acetylene produced similar increases in activity. Long-term pretreatment with acetylene studied on one of the algae was found to depress N_2 incorporation and photosynthetic CO_2 fixation. This long-term preincubation was thought to cause nitrogen depletion and according to David and Fay (12) should be limited to a brief period, long enough to allow enzyme adaptation and saturation. The researchers concluded that long term incubation under acetylene might grossly overestimate the acetylene reduction-nitrogen fixation ratio.

A variety of factors affecting acetylene reduction were studied, including addition of carbohydrate, moisture levels and light (30, 31). Addition of glucose or other carbohydrate sources such as plant residue was found to

enhance acetylene reduction regardless of other treatments. One percent glucose gave higher ethylene production values than 2.5 percent glucose. Ethylene production was significantly increased in samples incubated in light versus dark. The higher moisture level produced the same effect over the lower moisture level (30). Water logged soils also showed enhanced acetylene reduction (31). Stress due to water excess on nitrogen-fixing root nodules was found to adversely affect acetylene reduction rates. Similar patterns were seen in N_2 incorporation on nodules using .5 N_2 . Nitrogenase activity ceased, respiratory rates became very low and structural changes were noted (40).

Experiments were conducted to study the effects of the gas phase on acetylene reduction. In root nodule assays, rates were not affected by the presence or absence of air (40). Studies on eutrophic Wisconsin lake samples determined that the presence of nitrogen in the lakes did not affect acetylene reduction when sufficient acetylene was present (45). This practice also simplified field work by eliminating the need for gassing equipment (45). Previously the ambient atmosphere, including N_2 was removed, not only because nitrogenase was known to be oxygen labile but because N_2 inhibited acetylene reduction (36). Aeration treatments were also applied to soil systems. The treatments were ambient air, argon and oxygen and anaerobic (argon). There was no effect or change in acetylene

reduction by altering the composition of the gas phase (30).

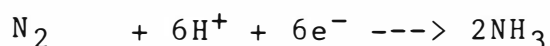
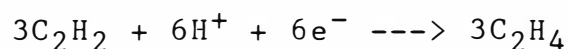
The aerobic-anaerobic interface was an important consideration in measuring nitrogen fixation, directly or indirectly. Materials such as straw, leaf litter and roots were broken down under aerobic conditions and anaerobic microsites provided proper conditions for anaerobic bacteria to fix nitrogen (51).

Applications of acetylene reduction included physiological studies of nitrogen fixing bacteria, investigations on blue-green algae and legume symbionts, studies of non-legume symbionts, refining the list of nitrogen fixing organisms and in situ measurements in many habitats (19). In grassland and associated cultivated ecosystems, acetylene reduction was used to determine the input of nitrogen by biological fixation. Medium textured soils generally yielded higher rates of ^{15}N fixed. Using ^{15}N , virgin soil showed higher rates up to 2.5 cm in depth than cultivated soils with no history of fertilization reduced nitrogen to 7.5 cm (31). The effect of core sizes were also studied by acetylene reduction. Individual, smaller cores gave variable results so larger cores, 6 cm diameter by 12.5 cm length, were suggested to provide more representative samples (31).

Acetylene reduction was used to show that bacteria in the root zone of rice were responsible for fixation not the root tissue as first believed (34,52). This assay was

also applied to algal nitrogen fixation. Algal fixers were found to contribute about two-thirds of the nitrogen fixed, indicating rhizosphere bacterial fixers played an important role (16,34). Maize and tropical grasses were also shown to have nitrogenase in their rhizosphere (16). Later, nitrogen fixation, as determined by acetylene reduction, was confirmed in the rhizosphere of two tropical grasses by ^{15}N incorporation (13).

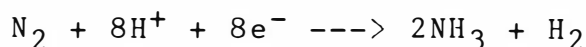
The conversion factor for acetylene reduced to nitrogen fixed became a major point of concern for the determination of actual nitrogen inputs by fixation. The chemical conversion factor appeared to be 3:1 (2), using the formulas



A wide range of acetylene reduction rates was calculated using ^{15}N determined nitrogen fixation rates. In soybean nodules the calculated ratios ranged from 2.71:1 to 4.2:1 and in soils from 3:1 to 25:1 (2). Fundamental differences exist between the reactions which can greatly affect the conversion factor: C_2H_2 is 65 times more soluble in H_2O than N_2 contributions are made by fixed nitrogen to protein synthesis but no contribution is made with acetylene reduction, they have differing abilities to enter the system, and nitrogenase is unstable (19, 23).

Hydrogen evolution became a suspected factor when it

was determined to be generally associated with nitrogen fixation in symbionts and blue-green algae. The assumption that the nitrogenase enzyme was responsible for producing H_2 and not the classical hydrogenase enzyme and the knowledge that acetylene inhibited H_2 evolution suggested electrons were diverted from N_2 to H^+ (3, 20, 23, 32, 37). With the N^2 part of the equation rewritten as



the conversion factor was raised to 4:1 (32). One group claimed 40% to 60% of the electrons are lost through H_2 evolution (37). If half the electrons were diverted from N_2 to H^+ , the ratio could go as high as 6:1. Some nonleguminous symbionts and a few leguminous symbionts gave ratios very close to 3:1, suggesting they have evolved some mechanism which minimizes electron diversion to H^+ and thus hydrogen production (17). It became apparent that the conversion factor was dependent upon the organism and the experimental conditions. Conversion factors needed to be calculated by comparison of acetylene reduction rates with ^{15}N rates for each system studied (32, 37).

D. Development in nitrogen fixation in the 1980's

Research in the 80's verified some earlier findings and contradicted others. When comparing time courses, a lag period was seen in soil cores. Slow diffusion of acetylene throughout the core seemed to be the cause. The diffusion

rate was affected by soil textures with and without plants. Minimum diffusion occurred between midnight to 6 am and maximum diffusion between noon to 6 pm (25, 34, 48, 49). Postgate described the 10 to 15 h lag phase as oxygen interference (34). Transferring soil from its natural environment increased the oxygen content, even if placed in an anaerobic environment. This excess oxygen interfered with and switched off the nitrogenase activity. As microorganisms of the soil system used up the oxygen, nitrogenase was slowly turned on (34). Preincubation of soil was not recommended to overcome the problem, whether due to diffusion or oxygen interference, because erroneous high estimates could result (49).

Additional applications were found for acetylene reduction. Nitrogen fixing bacteria were isolated from the rhizosphere of temperate cereals and forage grasses. One group of isolates was found to have a special requirement for vitamin B₁₂ (26). Acetylene reduction was used to estimate nitrogen fixation in association with the phyllosphere of plants. Plants included citrus and mulberry leaves, douglas fir, maize and Guatemala grasses, rice and cotton (29).

In contradiction to earlier studies, Lethbridge et al. (25) reported the acetylene reduction assay was invalid for low rates of nitrogen fixation, although $^{14}\text{C}_2\text{H}_2 \rightarrow ^{14}\text{C}_2\text{H}_4$ values were within experimental error. It was

possible for endogenous ethylene to accumulate, such an occurrence could overestimate activity and even indicate activity where none was present (25).

Another group reported that acetylene was decomposed in soil, serving as an energy source for some microorganisms (47). The addition of a carbon source slowed adaptation of the organisms for acetylene decomposition. Loss of acetylene in the system led to incomplete saturation of nitrogenase and erroneous results. To avoid problems, they suggested using fresh samples not previously exposed to acetylene (47).

Nitrogen fixation was estimated in alfalfa using three different methods to increase the reliability of the acetylene reduction determined value (27). The three methods were in situ acetylene reduction, ^{15}N isotope dilution with two reference crops and total nitrogen difference. Isotope dilution was also applied to soils containing soybean plants. The soil was labeled with $(^{15}\text{NH}_4)_2\text{SO}_4$ or ^{15}N labelled plant material (9). Although this technique offered a method to obtain estimates of fixation throughout the growing season, problems existed. The amount of nitrogen fixed was determined by taking the total N in the nitrogen fixing crop and subtracting (% fertilizer uptake by the fixing crop/100)(soil nitrogen pool with the nonfixing crop plus to the amount of fertilizer added) (50). Determination of the soil nitrogen pool was

the major problem encountered with the ^{15}N fertilizer dilution method because a non-fixing control crop with similar rooting patterns and similar nitrogen uptake was necessary for this calculation (9, 27, 50).

III PURPOSE OF THIS STUDY

Most of the methods used to measure nitrogen fixation have been either 1) laborious and expensive as is the case with ^{15}N or 2) not sensitive as with Kjeldahl digestion. The acetylene reduction assay, which is sensitive, inexpensive and simple, is based on the fact that acetylene is reduced to ethylene by the nitrogenase enzyme. Nitrogenase is the only enzyme known to reduce acetylene to ethylene. As ethylene appears to accumulate results can be expressed as either acetylene reduction or ethylene production.

When acetylene reduction has been measured in soils, to indicate the rate of nitrogen fixation, the assays generally included the use of disturbed or manipulated soil samples. Disturbed soil samples are those whose integrity has been changed by either mixing, amending with nutrient additives or changing the soil moisture status. Changes from the original state of the soil could markedly effect the rate of nitrogen fixation. The technique of acetylene reduction is not widely accepted for field in situ or undisturbed assays of soil. The acetylene reduction technique for measuring nitrogen fixation using undisturbed intact soil cores is evaluated in this study. Specific considerations include 1) environmental factors affecting the soil core assay for nitrogenase activity.

and 2) determination of relationships between vegetation and its impact on acetylene reduction (nitrogen fixation).

IV DEVELOPMENT OF AN INTACT SOIL CORE ASSAY FOR MEASUREMENT OF ACETYLENE REDUCTION

A. Introduction

Intact soil cores have been used in many aspects of soil sciences but very little application has been made in studying nitrogen fixation or acetylene reduction. Soil has often been removed from its natural habitat and mixed with substrates and water prior to analysis. These assays often represent a potential activity and not an actual activity determination. Interest in in situ assays to provide realistic determinations presents problems to the researcher. One is the need to keep the system as close to field conditions as possible.

Organisms and processes are dependent upon the presence or absence of oxygen. Nitrogen fixing organisms fall into three categories: 1) strict aerobes - those requiring oxygen to survive, 2) facultative anaerobes - those capable of growth in the presence or absence of oxygen and 3) strict anaerobes - those which survive only in the absence of oxygen. The enzyme responsible for nitrogen fixation (acetylene reduction) is oxygen labile, thus the process of acetylene reduction can take place only when the enzyme is protected from oxygen (20). Aerobic organisms have developed sophisticated mechanisms of protecting their

nitrogenase from the oxygen they require for survival. The importance of oxygen to acetylene reduction was investigated in a study comparing reaction rates under aerobic and anaerobic incubation.

The saturation point for the nitrogen fixing enzyme is 0.03 to 0.10 atmosphere acetylene (21). Researchers have reported using up to 0.50 atmosphere acetylene on cell free extracts without detectable inhibition of nitrogenase (21). The whole cell system may be adversely affected by use of excess acetylene. Because all enzyme reactions are controlled in some way by substrate concentrations, the concentration of acetylene added will greatly affect ethylene production rates and hence estimates of nitrogen fixation rates. Since measurement of ethylene production is the basic purpose of this research, it was very important to determine the concentration of acetylene to use in the intact core assay.

Another parameter which affects an enzyme reaction rate is the time of incubation or how long the enzyme is allowed to react with the substrates. Long incubation periods could result in an accumulation of end products which may inhibit the reaction. Inaccurate conclusions about the rate of ethylene production would then be evident. However the incubation period must be of sufficient length to allow for a detectable level of product to accumulate. Determination of the optimum incubation time for ethylene

production using the intact soil core assay was achieved.

In any biological assay moisture is a controlling factor in enzymatic reduction rates. Excess moisture could eliminate the oxygen from the microsites within the soil. Lack of sufficient moisture will inhibit cellular and enzymatic activities related to growth, maintaining only those necessary to sustain life. In any biological assay moisture is a controlling factor in enzymatic reduction reaction rates. Because nitrogen fixation is a high energy process and is intimately involved with growth, this activity may be influenced by available soil moisture.

Nitrogen fixation (ethylene production) is a heterotrophic process which requires a readily available carbohydrate source. Levels fluxuate greatly in soil due to plant litter inputs and decay, it is important to establish the impact of soil carbohydrate levels on ethylene production. Glucose is considered the "universal" carbohydrate of soils as most biological systems utilize glucose in preference to other carbohydrates.

Bacteria function over a wide range of temperature. As soil temperature varies with climate so does the soil microorganisms. Bacteria within each temperature growth range have their own temperature for optimal activity. At this temperature they grow fastest and their enzymatic activity is at its highest. Those living below 0°C to 20°C with a 15°C optimum are psychrophilic, from 15°C to 45°C are

mesophilic, and from 45°C to 50°C and above are thermophilic (5). The nitrogen fixing enzyme will be affected by the soil temperature.

Intact soil cores allow the researcher to maintain the integrity of the soil and soil structure with only minimum disruption due to a slight compaction. This technique also provides a means of taking a larger number of replications to improve statistical reliability of results. The use of intact soil cores is relatively new to acetylene reduction (nitrogen fixation) studies. Therefore an assay had to be developed for use in soil systems. A number of environmental factors, which vary greatly in soils, need to be evaluated for their effect on the measurement of acetylene reduction before the intact core technique can be applied to soil systems in general.

B. Materials and Methods

1. The assay chamber

Intact soil cores were removed using a stainless steel plunger-type soil corer. Soil cores (2.54 cm diameter) were taken at a depth of 5 cm and two cores were added to each 50 cc plastic disposable syringe barrel. A #6 rubber stopper was used to close the large opening. A rubber serum septa (7 x 13 mm) was placed on the needle attachment site to facilitate headspace sampling for gas chromatographic analysis and evacuation of the reaction vessel atmosphere.

2. Assay atmosphere

For some studies it was necessary to have an anaerobic or oxygen-free (inert) atmosphere. Evacuation and replacement of the atmosphere in the syringe was performed on a manifold apparatus connected to a pump and an Argon tank. Syringes were evacuated and filled with Argon five times, allowing the pressure to reach 0.90 atmosphere each time (the limitation of the equipment). Pressure was measured using a long, bent glass tube and a container of mercury connected to the manifold apparatus. After evacuation and replacement of the atmosphere with argon a quantity of acetylene was added through the septum stopper to give the desired final concentration. Acetylene was

added in a like manner to assay chambers with ambient atmosphere.

3. $C_2H_2-C_2H_4$ determinations using gas chromatography

Aliquots of the syringe-core headspace were injected into a Varian model 3700 Gas Chromatograph (GC), with a hydrogen flame ionization detector. A stainless steel column containing Porapak R was used for capture and was operated at $50^{\circ}C$. Nitrogen served as the carrier gas at a flow rate of 30 ml min^{-1} . The resulting acetylene and ethylene peaks were plotted on a Sargent-Welch model SRG recorder. A standard concentration curve was established using an equal dilution of ethylene and acetylene in air.

Soil cores, still in the syringe but with the stoppers removed, were weighed then dried in a Frigidaire Household Microwave oven using the #6 or bake setting (17). Syringes were turned every 3 to 4 min to avoid melting of the plastic. Syringe-soil cores were weighed after cooling, and samples were considered dry when a constant weight was obtained.

4. Calculation of acetylene reduction rates

From the results of varying concentrations of an ethylene standard injected into the GC, a linear regression comparing peak height and ethylene concentration was used to

develop the standard curve. The Y values represented peak height in cm and the X values were the concentration of ethylene injected. The sample peak heights were used in a linear regression equation to provide a calculated value for the concentration of ethylene in an injection.

Determination of the final rate of acetylene reduction (ethylene production) for a given core were obtained using the formulas in Table 1. The calculations took into account bulk density, porosity, and moisture of the soil to arrive at the final value. To determine wet weight and dry weight of the soil alone, an average weight of 12.71 grams was subtracted for the syringe weight. An average value of 2.65 g cm^{-1} particle density for eastern South Dakota soils was obtained from Gary Lemme, associate professor in Plant Science, SDSU.

Values are reported as micromoles of ethylene produced per hectare per day and could be interpreted as rates of nitrogen fixation. Until the proper conversion factor can be determined for these soils it would be inappropriate to convert from acetylene reduction values to nitrogen fixation rates.

Table 1. Calculations to determine micromoles of ethylene (C_2H_4) produced per hectare (ha) per day.

ppm	determination from a standard curve and linear regression
injection size	l/amount injected
ppm C_2H_4 /sample/day	ppm/ 10^6
mls C_2H_4/m^2 /day	mls air/syringe/ $2^a/(4.5*10^{-4})^b$
moles C_2H_4 /ha/day	mls C_2H_4/m^2 /day* $10^{4c}/24560^d$
micromoles C_2H_4 /ha/day	moles C_2H_4 /ha/day* 10^6
% moisture	((wet wt-dry wt)/dry wt)*100
bulk density	dry wt/volume of soil ^e
volume 1 soil core	$\pi*r^2*h$
% H_2O by volume	% moisture*bulk density
porosity	$100\% - (\text{bulk density}/2.65^f)*100$
% air filled volume	porosity-% H_2O by volume
air filled pores	volume 1 core*(%air filled volume*100)
mls air/syringe	$(70^g - \text{volume 1 core}) + \text{air filled pores}$

a converts to 1 core basis

b converts area of the top of 1 core into hectares

c meters squared per hectare

d milliliters ethylene per mole

e based on 2 cores

f average particle density in this region

g total volume in milliliters of the syringe

5. Soil sample collection

Soil cores were collected from either a corn field located at the Agronomy farm near campus or from an undisturbed native grassland site near Oakwood Lakes State Park. Soils collected from the Agronomy Farm were amended with 0.1% glucose to enhance activity by mixing the sample with an aqueous solution of glucose. Controls were prepared by mixing an equal volume of water with a duplicate soil sample. The soil was mixed in a clear plastic container from which soil cores were taken after the substrate was mixed with the appropriate volume of water. In this same manner soils were prepared with elevated moisture levels.

6. Determination of optimum column temperature

To determine the most appropriate column temperature for gas chromatographic separation of acetylene and ethylene peaks, an acetylene-ethylene standard was injected at different column temperatures ranging from 40 to 95°C. A column temperature of 50°C was chosen for all subsequent analyses as it was the temperature at which optimum resolution of acetylene and ethylene peaks were observed.

7. Use of syringes for injections

A 1 cc disposable tuberculin syringe was compared with a Hamilton gas tight syringe for reproducibility of gas sample volumes. Using the syringes, injection volumes of

0.25 ml from a standard ethylene concentration were analyzed (Table 2). As differences between the reproducibility of the two syringes appear insignificant the disposable syringes were therefore used throughout this study for the transfer of small gas volumes.

8. Statistical analysis

Selected data were analyzed using SYSTAT (SYSTAT, Inc., 603 Main St., Evanston, Il. 60202), a statistical program for the IBM personal computer, made available by W. Kennedy Gauger, Associate Professor in Microbiology, SDSU. Tests included linear regression, t-test, Duncan's multiple range, ANOVA and LOWESS, a robust nonlinear data smoothing procedure. Significance at the 0.05 level is indicated by * and at the 0.01 level or less by **.

Table 2. Comparison of two types of syringes for measurement of ethylene.

SYRINGE	PEAK HEIGHT	STANDARD DEVIATION	RANGE
Disposable Tuberculin	6.98	0.34	7.32 - 6.63
Hamilton Gas tight	6.93	0.40	7.33 - 6.54

C. Results and Discussion

1. Incubation atmosphere

Agricultural soil from the SDSU Agronomy farm was mixed and amended with 0.1% glucose. The soil was placed in syringes and the appropriate atmospheric manipulations performed. Samples incubated anaerobically were evacuated and filled with Argon five times. The aerobic (samples with oxygen present) were incubated in an ambient atmosphere. Five percent acetylene was added to each syringe. Determinations of the acetylene reduction rates were made after 20 hour incubation at 22°C. The data, presented in Fig. 1, indicate the highest rates of ethylene production occur in the aerobically incubated syringes. T-test analysis shows aerobically incubated samples produce significantly higher rates ($p < 0.001$) than anaerobically incubated samples. There appears to be a higher population of aerobic nitrogen fixers but there is a detectable population of anaerobic fixers as indicated by the activity measured under anaerobic incubation.

2. Concentration of incubation atmosphere

Three separate experiments were conducted where different concentrations of acetylene were added to their respective intact cores within the assay chambers. Soil for

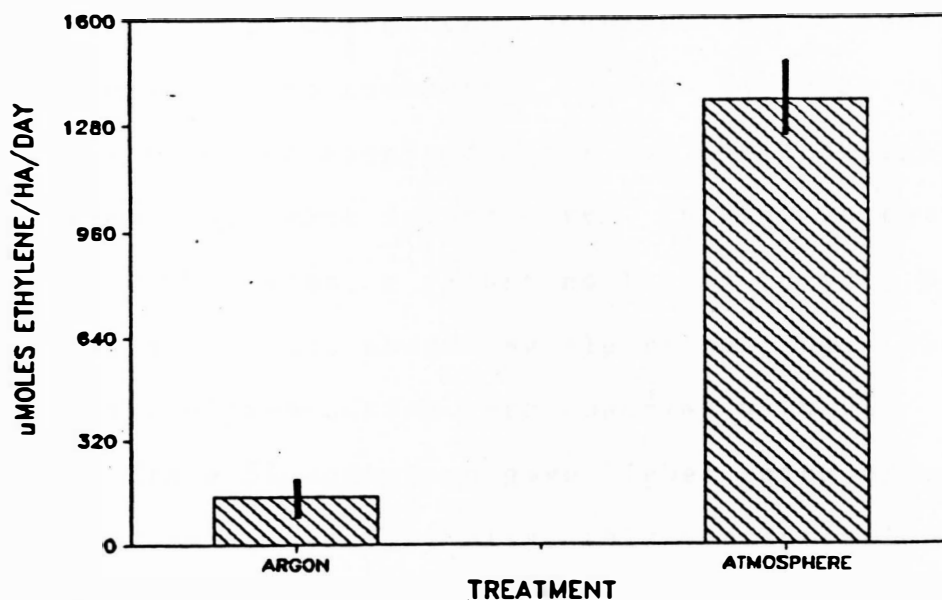


Figure 1. Effect of incubation atmosphere on ethylene production from soil collected from the SDSU Agronomy Farm. Soils were amended with 0.1% glucose and 5% acetylene (C_2H_4) was added to the resulting atmosphere. Values reported are means of five determinations and standard error is indicated by vertical lines. Results from a t-test analysis are $t = 8.794$ and p is less than 0.001**.

the three studies was collected from the Agronomy farm, mixed and amended with 0.1% glucose. Concentrations of acetylene, based on the total volume of the syringe, ranged from 0% to 20%. All cores were incubated for 24 hours at 22°C. The results are summarized in Fig. 2a, each value represents a mean and standard error for the ethylene production rate for each intact core. In Fig. 2b this data is analyzed with Lowess, a robust nonlinear locally weighted regression scatter plot smoothing algorithm (10). Highest rates of ethylene production were observed with 5% acetylene. Since 5% acetylene gave higher rates of ethylene production as shown statistically, this concentration of acetylene was used in subsequent assays, both laboratory and field studies.

3. Time of incubation

A series of soil cores were collected from a native grass site near Oakwood State Park and incubated under an ambient atmosphere to which 5% acetylene was added. Samples were incubated for 0 to 32 hours at 22°C. Ethylene production rates were determined for four syringes at each injection time of 0, 4, 8, 12, 16, 20, 24, 28, and 32 hours. The resultant time course is presented in Fig. 3. Linear regression indicates a positive correlation between time and activity. Analysis shows a significant difference of rate over time ($p < 0.001^{**}$).

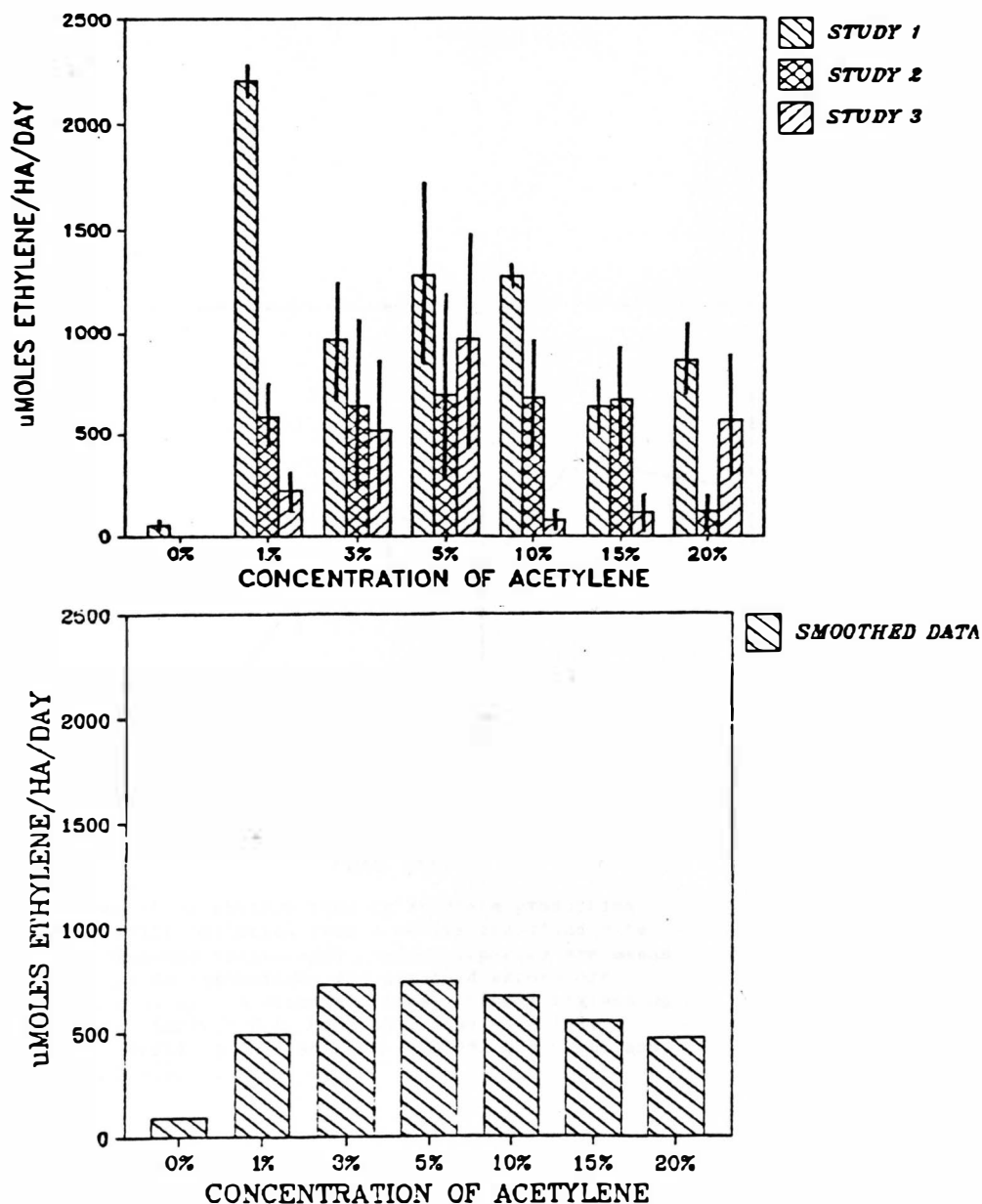


Figure 2. Effect of varying acetylene concentration (upper) on ethylene productions from soil collected from the SDSU Agronomy Farm. Soils were amended with 0.1% glucose. Values reported are means for the determinations for three separate experiments. Standard error is indicated by the vertical lines. The lower figure represents the same data after smoothing with LOWESS.

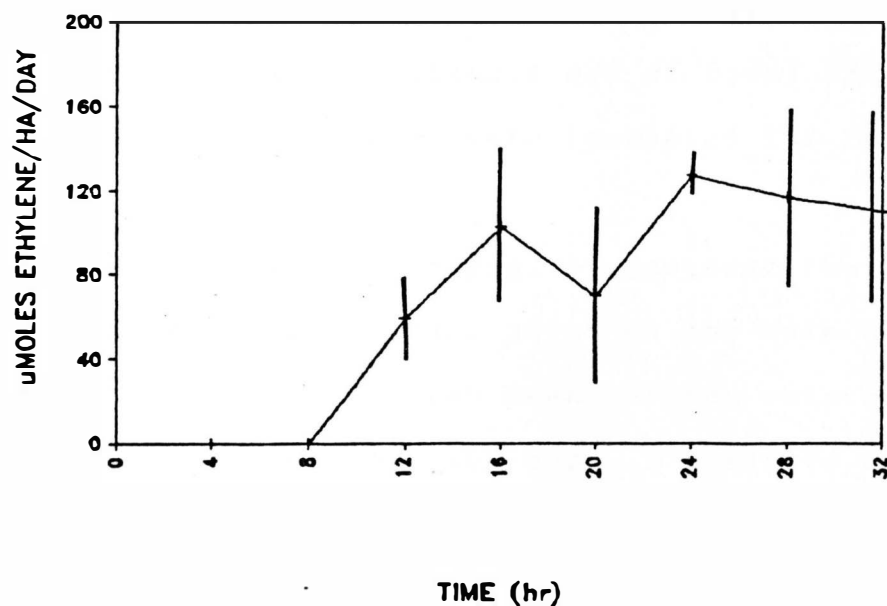


Figure 3. Effect of incubation time on ethylene production from soils collected from a native grassland site near Oakwood State Park. Values reported are means of four determinations and standard errors are indicated by the vertical lines. Linear regression results include $C_2H_4 = 17.5551 \text{ Time} - 22.792$, $F_2 = 30.511$, p is less than 0.001^{**} , $r = 0.688$ and $r^2 = 0.473$.

4. Moisture

Soil from the Agronomy farm was collected, mixed and amended with 0.1% glucose dissolved in different volumes of water: 40 ml, 34 ml, 25 ml, or 10 ml which resulted in 27.9%, 25.5%, 21.9%, and 18.7% moisture respectively. The soil moisture value of an unamended fifth set of eight cores (two cores per syringe gives a set of four) was 17.6%. The entire series of syringes were incubated for 21 hours at 22°C.

Data presented in Fig. 4 represent the results of soil moisture in the intact cores on the rate of ethylene production. The calculated mean percent moisture is indicated at the top of each bar. A positive correlation value ($r = 0.929$) was calculated, as soil moisture increased, ethylene production rates increased. Analysis using linear regression also indicates a significant difference in activity over milliliters water added. It would be expected that excess soil moisture, approaching saturation, would retard ethylene production because of the displacement of oxygen from the microsites in the soil. Thus excess soil moisture values were not compared in this study, rather the values tested were to represent a range of normally encountered soil moistures in this area.

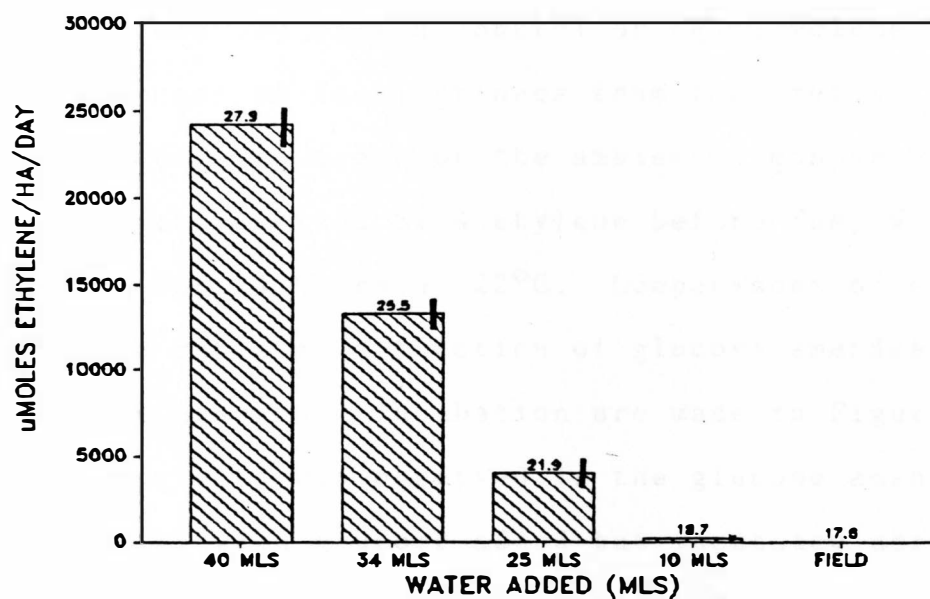


Figure 4. Effect of soil moisture on the rate of ethylene production from soils collected from the SDSU Agronomy Farm. Glucose (0.1%) was amended to each sample and values reported are a mean of four determinations. Standard errors are indicated by the vertical lines. Analysis with linear regression gave $C_2H_4 = -10136.907 \text{ mls} + 6156.705$, $F_2 = 113.418$, p is less than 0.001**, $r = 0.929$ and $r^2 = 0.863$.

5. Carbohydrate amendment

The effect of soil glucose levels on ethylene production rates was measured on a series of soil cores collected from the Agronomy farm. Half the soil was amended with 0.1% glucose dissolved in water while the other half of the soil received only a control or equal volume of water. The atmosphere of four syringes from each set were evacuated and replaced with Argon or the ambient atmosphere was left. All syringes received 5% acetylene before they were incubated for 24 hours at 22°C. Comparisons of ethylene production rates as a function of glucose amendments and aerobic or anaerobic incubation are made in Figure 5. Highest activity was observed in the glucose amended soils. Soil samples with glucose added but incubated aerobically had higher rates of ethylene production than did those incubated anaerobically. This substantiates the earlier observation on the effect of anaerobic incubations of soil cores for ethylene production determinations but more importantly indicates that ethylene production in soil may be limited by available carbohydrates. This may account for the extreme variability of ethylene production often encountered within a given site.

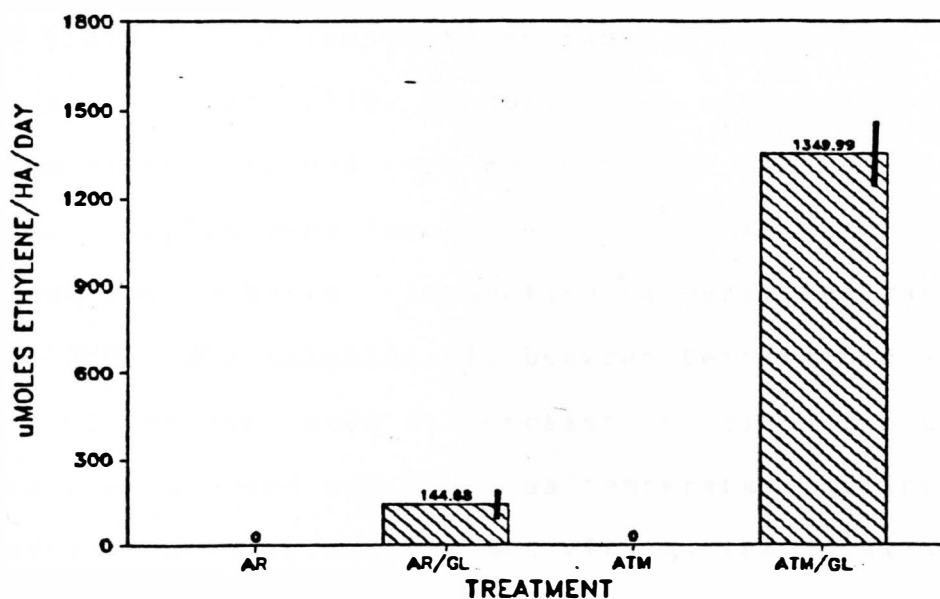


Figure 5. Effect of carbohydrate amendment (GL = glucose) and anaerobic incubation (AR = argon atmosphere, ATM = ambient atmosphere) on ethylene production from soil collected from the SDSU Agronomy Farm. Mean values from four determinations are listed at the top of each bar and standard errors are indicated by the vertical lines.

6. Temperature of incubation

The effect of incubation temperature on ethylene production rates was investigated on two different soil systems. In the first study (Fig. 6), soil samples were taken from the SDSU Agronomy farm and amended with 0.1% glucose. Samples were incubated with an ambient atmosphere and 5% acetylene at temperatures ranging from 0°C to 60°C. In the second study (Fig. 7) soil cores were collected from a native grass site and were not mixed or amended with glucose. Samples were incubated aerobically with 5% acetylene for 24 hours. Incubation temperatures ranged from 0°C to 55°C. The relationship between temperature and rates is typical, as indicated by correlation values for both studies ($r=0.395$ and $r=0.738$), as temperature of incubation increases so does activity. Activity varies significantly with time as shown by linear regression analysis. What is not typical of the response is the high activity observed at the elevated temperatures, those beyond the mesophilic range. This phenomenon is explored further in the following section.

7. Activity at high temperature

From the previous study, it was observed that soil ethylene production rates were highest when the incubation temperature exceeded 50°C. Because of the climate of South Dakota, naturally occurring soil temperatures of this level

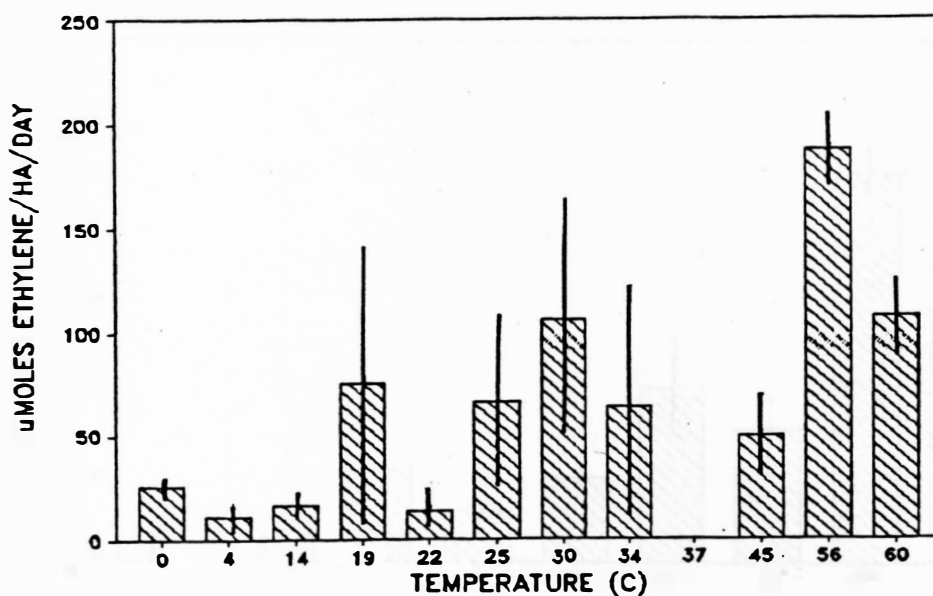


Figure 6. Effect of incubation temperature on ethylene production from soils collected from SDSU Agronomy Farm. Samples were amended with 0.1% glucose. Mean values for four determinations are presented and standard errors are indicated by the vertical lines. Analysis with linear regression gave $C_2H_4 = 8.867 \text{ Temp} + 2.293$, $F = 8.488$, $p = 0.006^{**}$, $r^2 = 0.395$ and $r^2 = 0.156$.

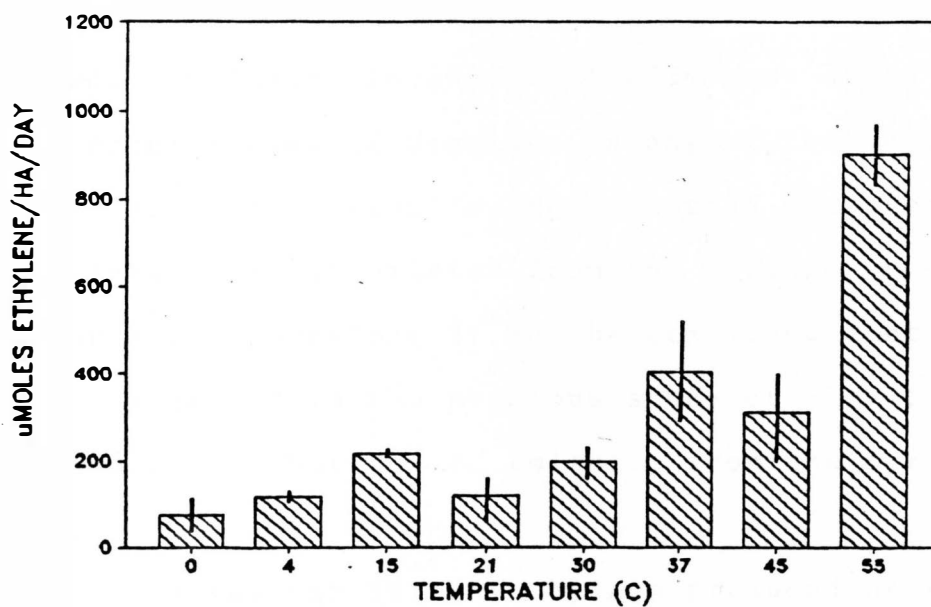


Figure 7. Effect of incubation temperature on ethylene production from soils collected from a native grassland site near Oakwood State Park. Mean values for four determinations are presented and standard errors are indicated by the vertical lines. Analysis with linear regression gave $C_2H_4 = 87.946 \text{ Temp} + -102.978$, $F = 35.848$, p is less than 0.001, $r = 0.738$ and $r^2 = 0.544$.

would be highly unusual and organisms capable of growth at these elevated temperatures may not be found in South Dakota soils.

To determine if ethylene is emitted from the syringe at high temperatures, sets of four syringes, each containing either 10% acetylene, 10% ethylene, or 10% of both, were prepared. Following incubation for 24 hour at 55°C, there was no accumulation of ethylene in any of the reaction atmospheres. These results indicate that measurable amounts of ethylene were not emitted from the syringe at elevated temperatures. Therefore it may be concluded that the ethylene measured in the previous study at elevated temperatures was not due to emission from the syringe itself.

To establish if the ethylene produced at elevated temperatures was a function of the soil, soil samples were collected from a native grassland site. One set of 10 cores (equivalent to 5 syringes) was autoclaved at 121°C for 1 hour in glass beakers then aseptically transferred to sterile syringes. The second set was collected directly into five sterile syringes. Both sets were incubated with 5% acetylene in ambient atmosphere at 55°C for 24 hours. Data in Fig. 8 indicate that high activity was observed in the nonautoclaved soil cores whereas minimal activity was observed in the autoclaved samples. This minimal activity indicates a lack of sterility reached by a 1 hour

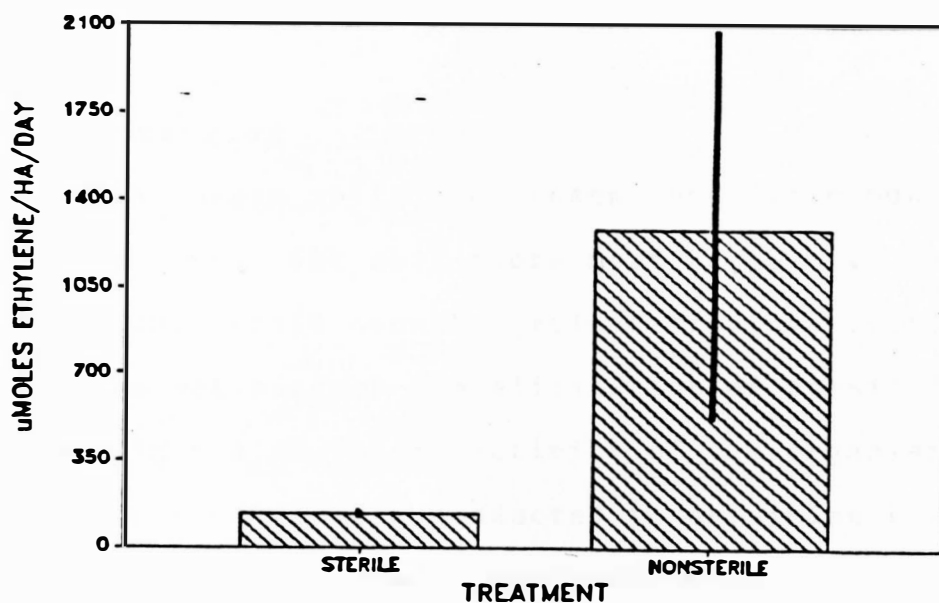


Figure 8. Effect of autoclaving (sterility) on ethylene production from soil collected from a native grassland site near Oakwood State Park. Means for five determinations are presented and standard errors are indicated by the vertical lines. Results from a t-test analysis are $t = 1.352$ and $p = 0.213$.

autoclaving process. Since the production of ethylene is depressed in the autoclaved soils it can be concluded that a biological component in the soil is responsible for ethylene production.

8. Freezing

South Dakota soils are frozen about five months out of any given year. The soil flora must be able to survive freezing or they would soon be extinct from the system. Survival does not suggest the elimination of possibilities for changes in the enzymatic activity of the organisms present. Two studies were conducted to determine how freezing of soil affects ethylene production rates. In the first study soil from the Agronomy farm was mixed and amended with 0.1% glucose. One set of 4 syringes was prepared with 5% acetylene in an ambient atmosphere then incubated for 20 hours at 22°C. The second set of syringes was frozen for 1 week then thawed and incubated in the same manner as the first set. The data are shown in Fig. 9. A t-test indicates there is no statistically significant difference between the treatments.

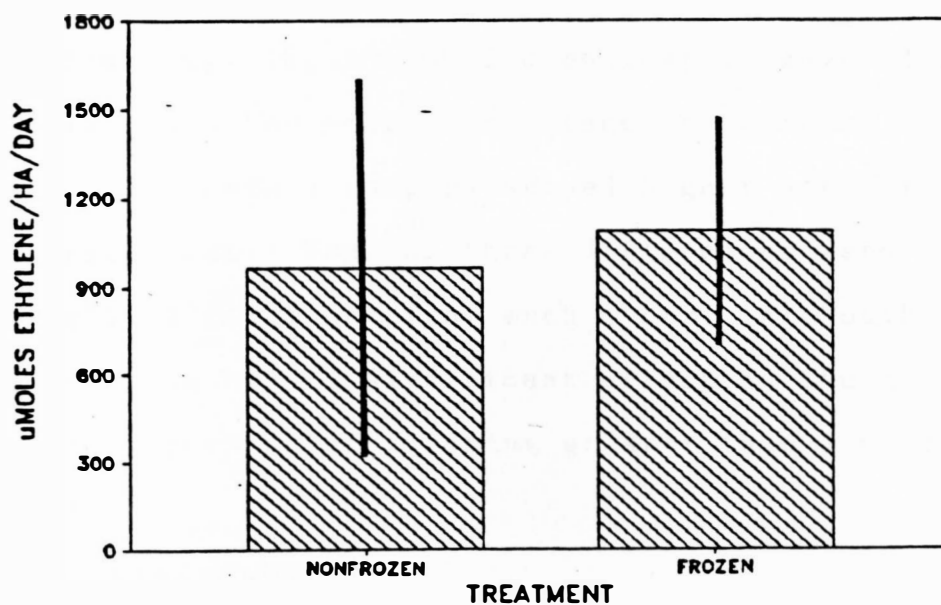


Figure 9. Effect of freezing on the ethylene production rates of soil collected from the SDSU Agronomy Farm. Values represent means for four determinations and standard errors are indicated by the vertical lines. Results from a t-test analysis are $t = 0.142$ and $p = 0.888$.

The second study involved a longer frozen period. Samples for this study were collected from two native grass sites (OW and BLg). Sixteen cores from each site were frozen 1 month, while another sixteen cores were incubated 24 hours at 22°C with ambient atm and 5% acetylene. The frozen set was thawed and then incubated in the above manner. From Fig. 10, the OW frozen samples gave slightly higher rates than the nonfrozen (standard) samples. In contrast, BLg standard samples showed higher activity than did the frozen set. None of these differences were statistically significant from each other. Although it appears freezing has no significant effect on the three soil types tested, periods of freezing greater than 1 month were not tested.

9. Preincubation

Some researchers have reported preincubation of their soils before addition of acetylene to determine rates of ethylene production (11, 49). The purpose of preincubation is to allow build up of a microbial population to obtain a higher, more easily detected rate. To determine the effect of preincubation using the intact core assay, forty soil cores collected from the Agronomy farm were amended with 0.1% glucose. Half of the samples were assayed by adding 5% acetylene under an ambient atmosphere for a 20 hour incubation at 22°C. The remainder of

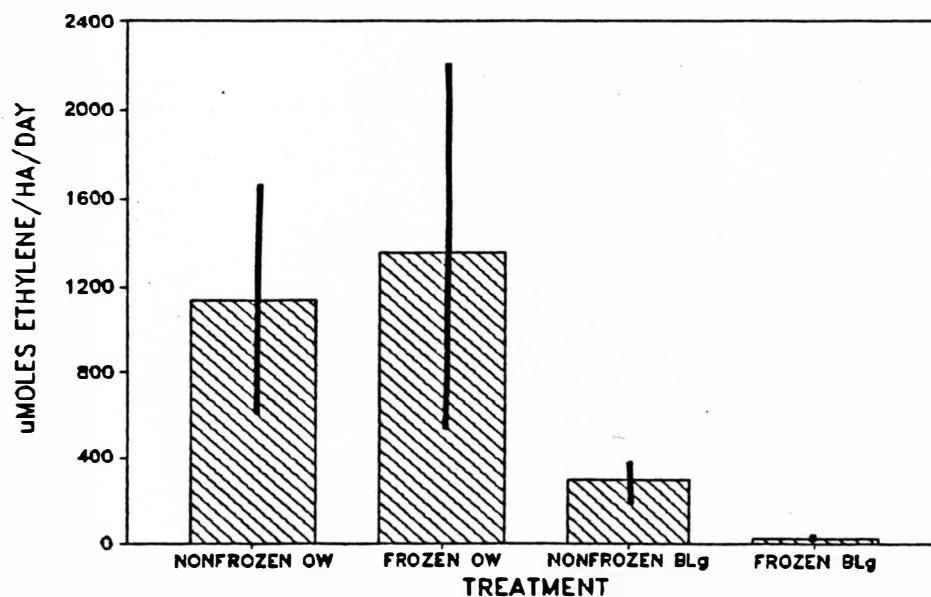


Figure 10. Effect of freezing on the rate of ethylene production from soils collected from two native grassland sites (OW and BLg) near the Oakwood Lakes State Park. Means of eight determinations are presented as well as standard errors as indicated by the vertical lines. ANOVA results were $F = 1.501$, $p = 0.236$ and there was no significant difference at either site.

the samples were "preincubated" 15 hours at 22°C with ambient atmosphere before the addition of 5% acetylene. Preincubation for 15 hours prior to analysis produced lower ethylene production rates as shown by t-test analysis ($p = 0.033$) and is not a viable alternative to the design for the intact core assay for ethylene production (Fig. 11).

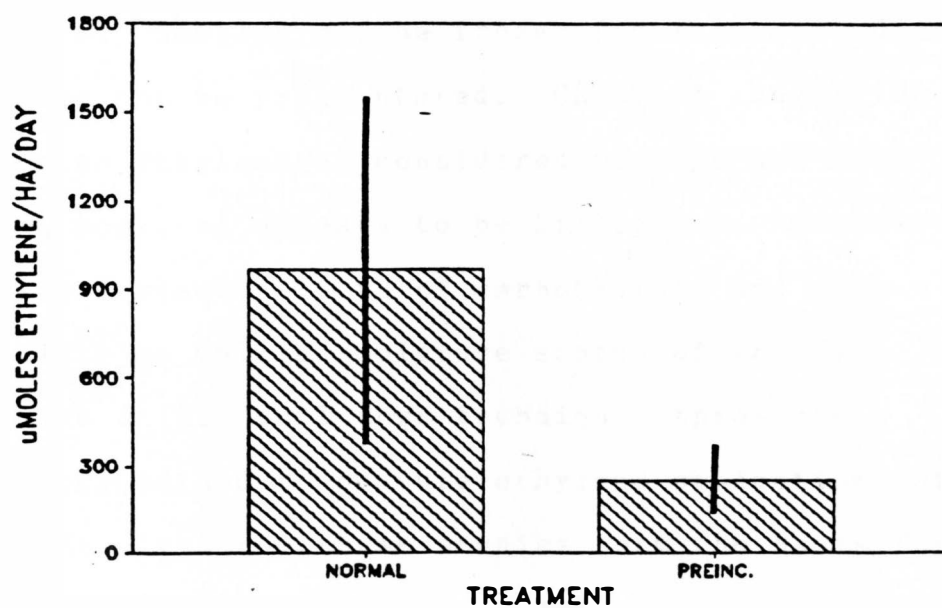


Figure 11. Effect of preincubation for 15 hours on the rate of ethylene production from glucose amended soils collected from the SDSU Agronomy Farm. Data represents means for five determinations and standard errors are indicated by the vertical lines. Results from a t-test analysis are $t = 2.231$ and $p = 0.033$.

D. Conclusions

When using the intact soil core technique for measuring ethylene production a concentration of 5% acetylene should be used and the length of incubation should be 24 hours. Samples may be frozen for future studies but they should not be preincubated. Chemical conversion of acetylene to ethylene is considered minimal and any reduction measured appears to be biological. Observed rates adequately reflect effects of carbohydrate and oxygen availability as well as moisture status of the soil.

The intact soil core technique represents a satisfactory method to measure ethylene production rates in soils. Results from these studies form the basis for establishing an intact soil core assay for ethylene production from field soils.

V FIELD SURVEY

A. Introduction

Research on nitrogen fixation using an acetylene reduction (ethylene production) assay has focused on microbial laboratory cultures and disturbed soil systems. Application of this assay to a field system has been limited, yet field determinations of microbial processes in their natural environment are important. Determination of field activity rates can lead to practical and efficient means of managing the soil nitrogen availability to the plant. The knowledge of biological nitrogen fixation incorporated with current farming practices will lower operating costs and environmental damages such as pollution while at the same time producing higher yields of foodstuffs.

Measurement of ethylene production using intact soil cores was applied to survey the nitrogen fixing activity in field systems in eastern South Dakota. The purpose of this field survey was to establish rate trends of ethylene production within a field and to indicate changes which occur due to a variety of factors; both natural and man-induced.

B. Materials and Methods

1. Study Sites

Eight locations in eastern South Dakota were selected for the field survey of ethylene production rates. A total of 10 sites at the 8 locations provided 4 vegetation types - native grasses, corn, small grain and alfalfa. With the exception of the native grass sites, each site and vegetation type represented typical agricultural management practices for the area. All soils were well drained silt loams that had received continuous management.

The 8 locations are designated with a code which corresponds to a point on Fig. 12. The 12 sampling sites are as follows:

- OW undisturbed native grass site located in
 Oakwood State Park
- BLg native grass pastureland in which grazing
 is not continuous
- BL(c) a corn field with samples taken on a
 hilltop
- MS a corn field which received 0 lbs N
 fertilizer and was chisel plowed. This site
 was the only non-private location as it was
 operated by SDSU research personnel.
- JW a sloping corn field that was planted late
 in the 1985 growing season

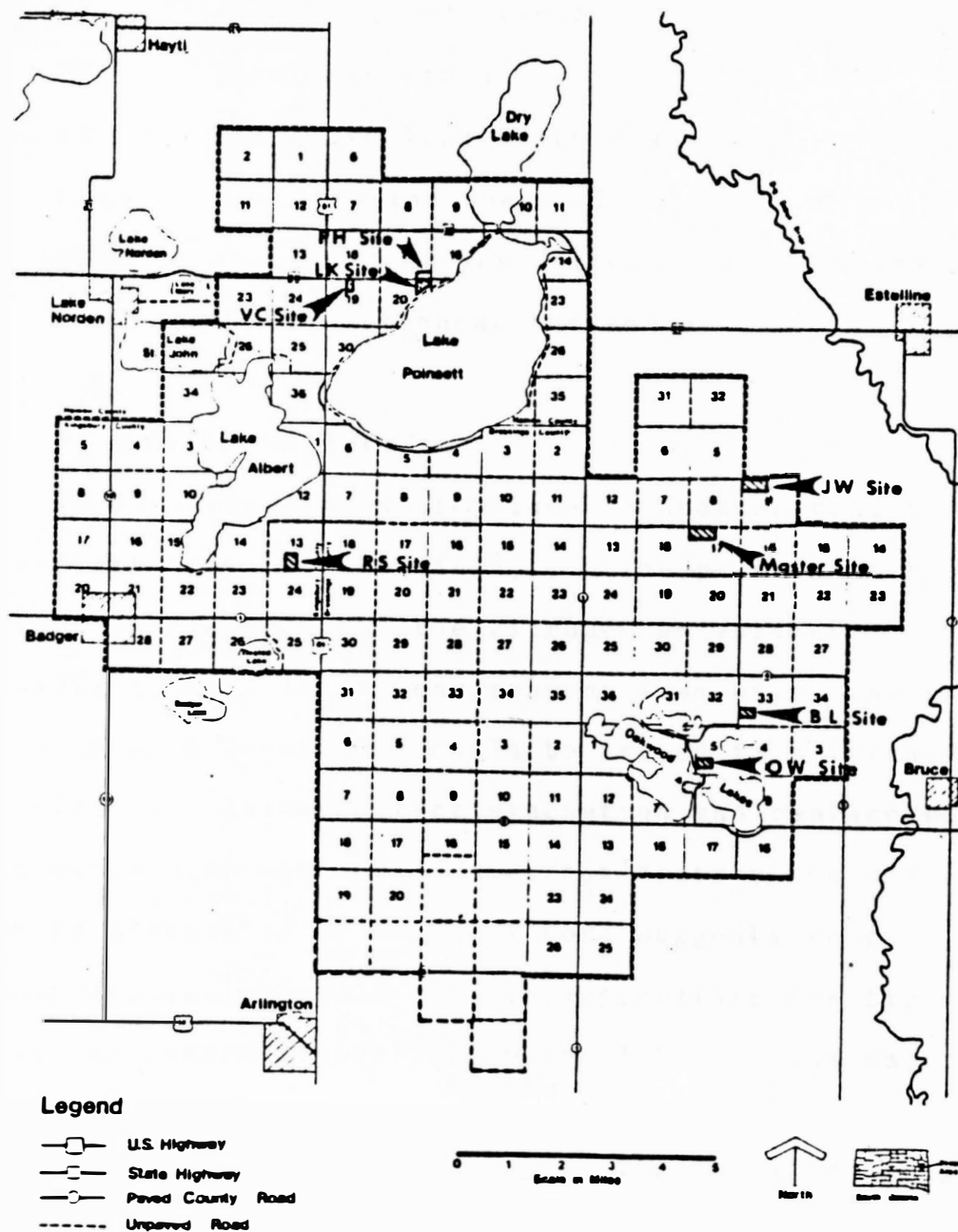


Figure 12. Diagram illustrating the locations of the field sites used in the ethylene production study.

RS	a rolling corn field
VC	level rye field
PH	gently rolling beardless barley
LK(w)	level spring wheat field
LKa	alfalfa that was cut and baled several times throughout the summer

2. Sample Collection

To determine the appropriate incubation conditions which reflect those in the field, consideration must be given to the organisms and the nitrogenase enzyme responsible as well as the environment from which the sample came. Anaerobic incubation could be argued to eliminate aerobic fixers. Assuming that evacuation and replacement of ambient atmosphere with Argon does not destroy the oxygen containing microsites of the soil core suggests that anaerobic incubation would be more appropriate for field studies. In contrast aerobic (ambient) incubation may produce artificially higher rates by eliminating the natural competition which exists between soil flora. At 5 cm depth (the depth of the soil cores) oxygen will not be in the abundance provided by the aerobic assay in the 70 ml syringes. Anaerobic incubation was chosen as the better estimate of the field conditions in the intact soil core system.

Soil samples were randomly taken at each site with a hand operated plunger type metal corer. Two of the resultant cores (each 2.5 cm in diameter by 5 cm in length) were placed in a 70 ml (total capacity) disposable syringe. The syringe, minus the plunger, was fitted at the tip with a 13mm serum stopper for addition and removal of gas samples. A rubber stopper (#6) was fitted into the open plunger end. For the row crop sites, one core was taken within a row and one was taken between rows. Samples were transported to the laboratory for immediate analysis.

3. Analysis

Headspace samples from the syringes were injected into a Varian model 3700 Gas Chromatograph equipped with a hydrogen flame ionization detector and a stainless steel column, packed with Porapak R and run at 50°C. The carrier gas of nitrogen was set at a flow rate of 30 ml min⁻¹, Ethylene and acetylene peaks were recorded on a Sargent Welch model SRG recorder. Equal dilutions of ethylene and acetylene in air served as the standard, this standard was used to establish a standard concentration curve.

Soil cores were dried in a Frigidaire Household microwave oven at the #6 or bake setting in the plastic syringes but with the stoppers removed. To avoid melting of the plastic, syringes were turned every 3 to 4 minutes. Dryness was defined when a constant weight was achieved when

the samples were weighed after cooling.

Calculations were performed as stated in Chapter IV section B and part 4. Linear regression was used to determine the standard curve equation and the parts per million of ethylene contained in the sample injection. Further calculations are listed in Table 1 of that section.

C. Results

1. Field sampling scheme

Within a single field or site there exists a great diversity in soil and soil microbial populations. The location and number of samples taken must be considered as a variable in any field assay of microbial activity. This study was designed to compare ethylene production rates across selected sites field. Sampling locations were determined by variations in field topography. Comparisons are made between sites within three fields in Figure 13, 14, and 15. Statistical analysis using Anova shows no significance between samplings within any of these fields, that is the location of the sample did not significantly affect ethylene production. Because of the variation in standard errors between sampling locations within a given field it would not be feasible to extrapolate rates to an entire field. Therefore comparisons, using the intact core technique, are limited to a given, finite location.

2. Field studies

The studies previously discussed have addressed how individual factors affect ethylene production rates while using the intact core system. The purpose of the study described in this section is to determine ethylene production rates within several field locations over a

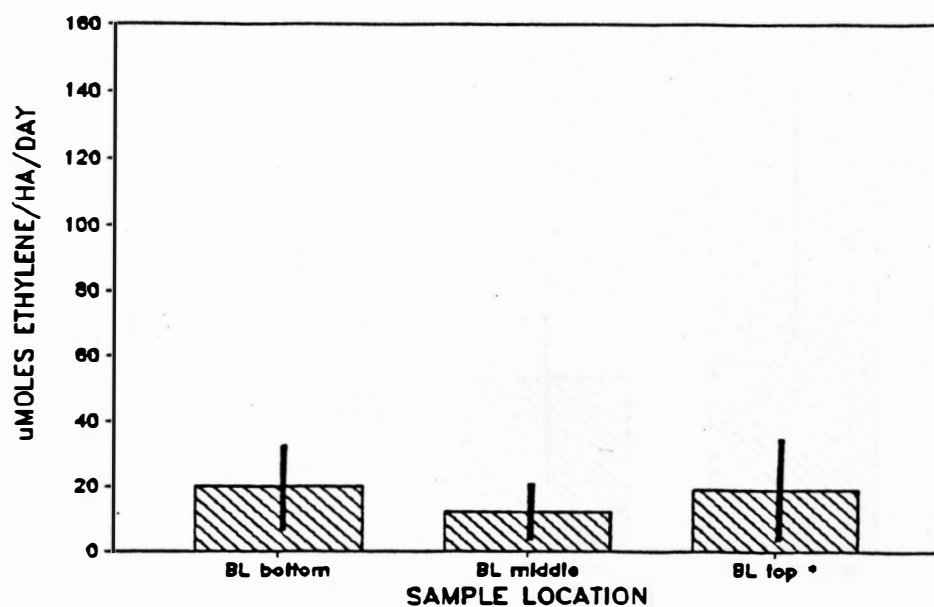


Figure 13. Rates of ethylene production at three loactions in a corn field in July. Values are means of four determinations an standard errors are indicated by the vertical lines. ANOVA results are $F = 0.125$, $p = 0.885$. There is no significant difference between locations.

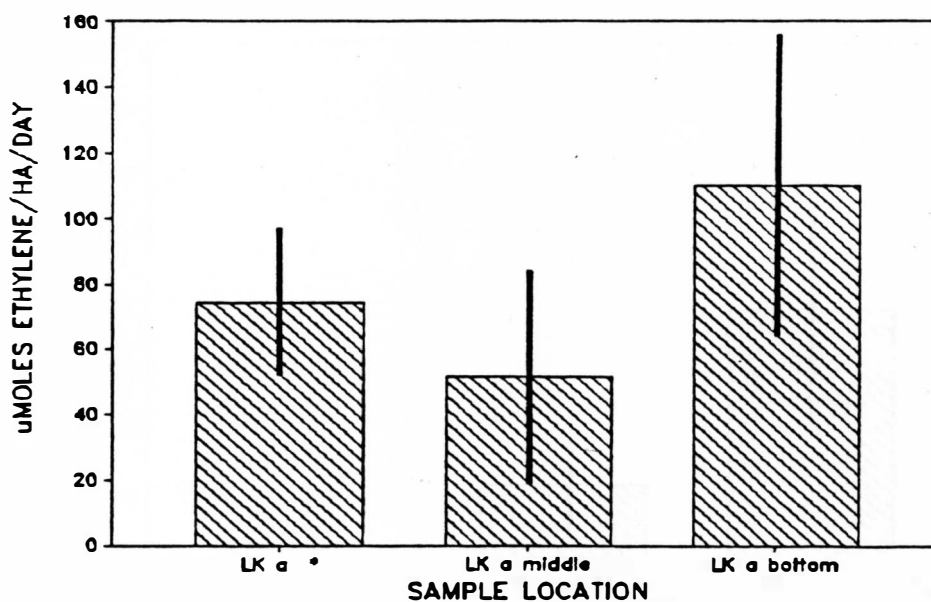


Figure 14. Rates of ethylene production at three locations in an alfalfa field in July. Values are means of four determinations and standard errors are indicated by the vertical lines. ANOVA results are $F = 0.717$, $p = 0.514$. There is no significant difference between locations.

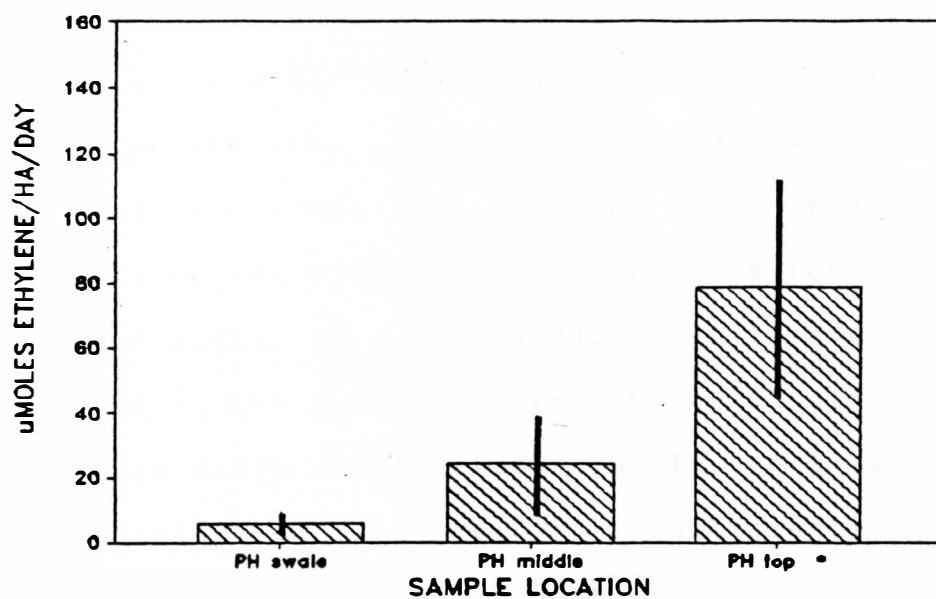


Figure 15. Rates of ethylene production at three locations in a barley field in July. Values are means of four determinations and standard errors are indicated by the vertical lines. ANOVA results are $F = 3.005$, $p = 0.100$. There is no significant difference between locations.

growing season. The great diversity of the soil system makes extrapolation to large units such as to a field scale inaccurate.

Cores were taken within a predesignated area at each of the ten sites over 13 dates. Results are illustrated in Figures 18 - 27. In each figure ethylene production rates are shown in the upper drawing and available soil moisture determined at the time of sampling in the lower one.

Statistical analyses included linear regression of ethylene production with moisture for all sites combined and for each individual site (Table 3). Significant variation was observed in the pooled set and on the PH site data. In the remaining sites no significant differences were noted between activity and moisture. The coefficients of determination indicate other factors beside moisture are affecting ethylene production rates. In Table 4 the activity for each site is compared to sampling date by linear regression. All sites except Lk a show a significant variation between activity and sampling time. Correlation is greater than 0.6 for these sites, indicating sampling time is an important factor in determination of ethylene production rates.

To better understand the data the sites were pooled according to crop type, that is grass, corn and small grain. The regression of sites within the cropping type are compared in Table 5. Corn and small grain

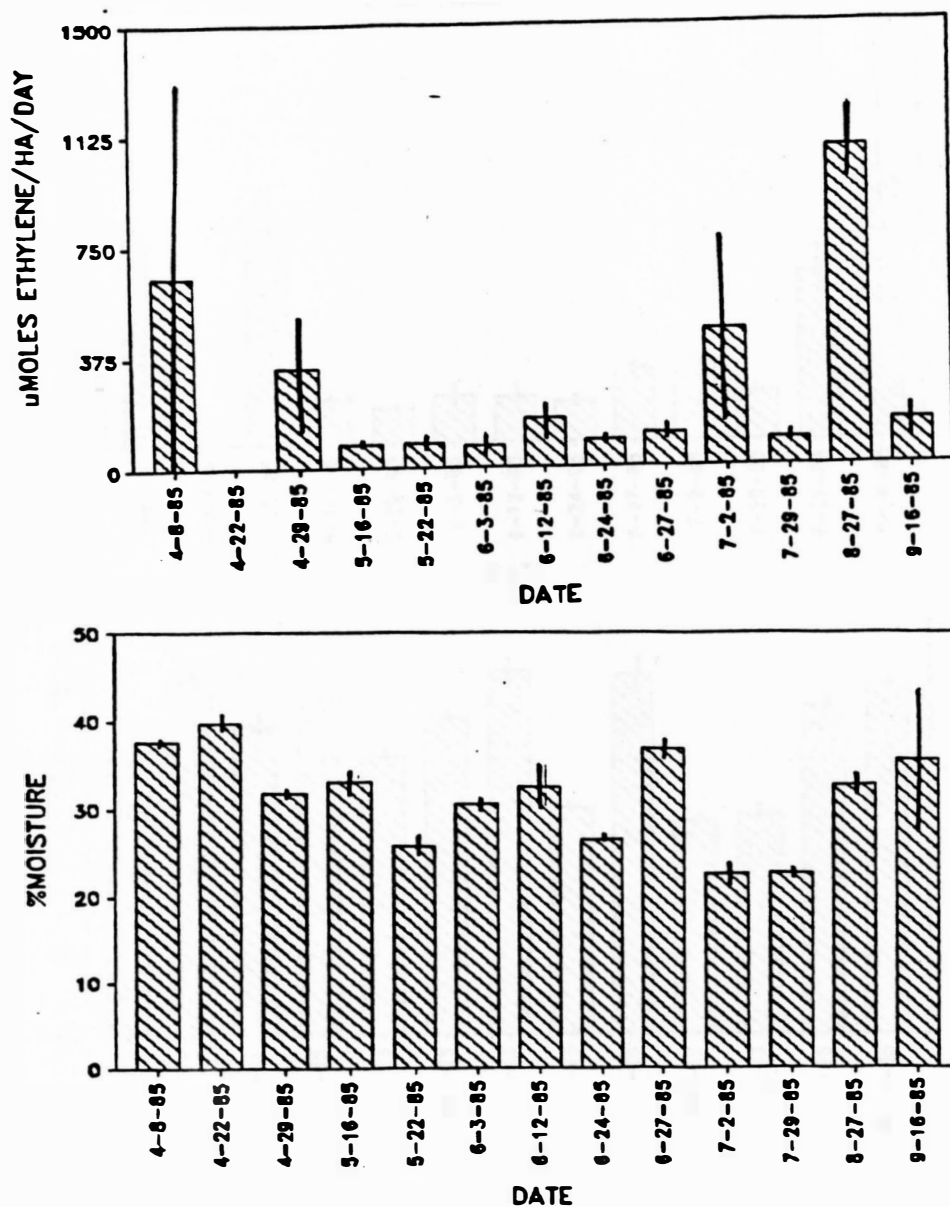


Figure 16. Rates of ethylene production (upper) and soil moisture (lower) for a native grassland site (OW) during the 1985 growing season. Values reported are means for four determinations and standard errors are indicated by the vertical lines.

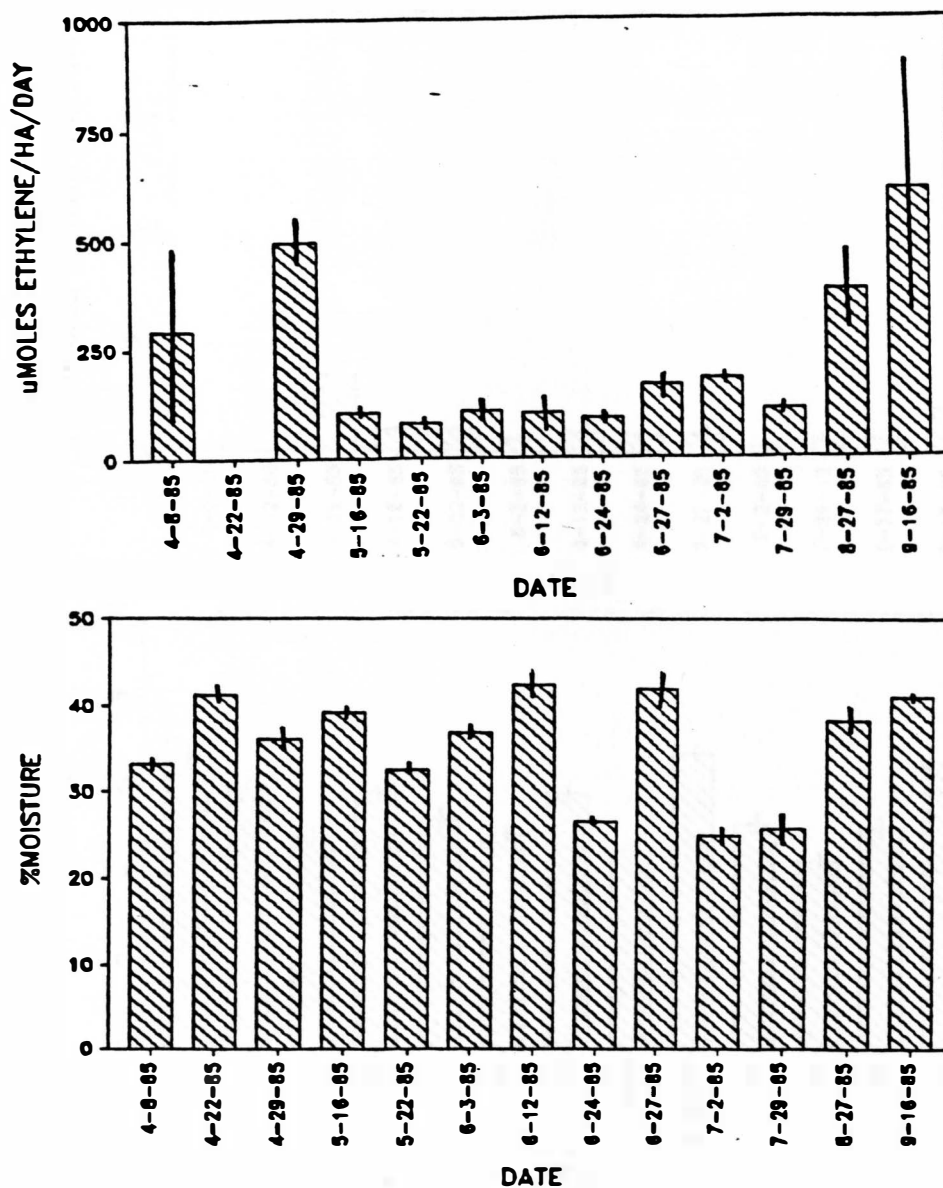


Figure 17. Rates of ethylene production (upper) and soil moisture (lower) for a native grassland site (BLg) during the 1985 growing season. Values reported are means for four determinations and standard errors are indicated by the vertical lines.

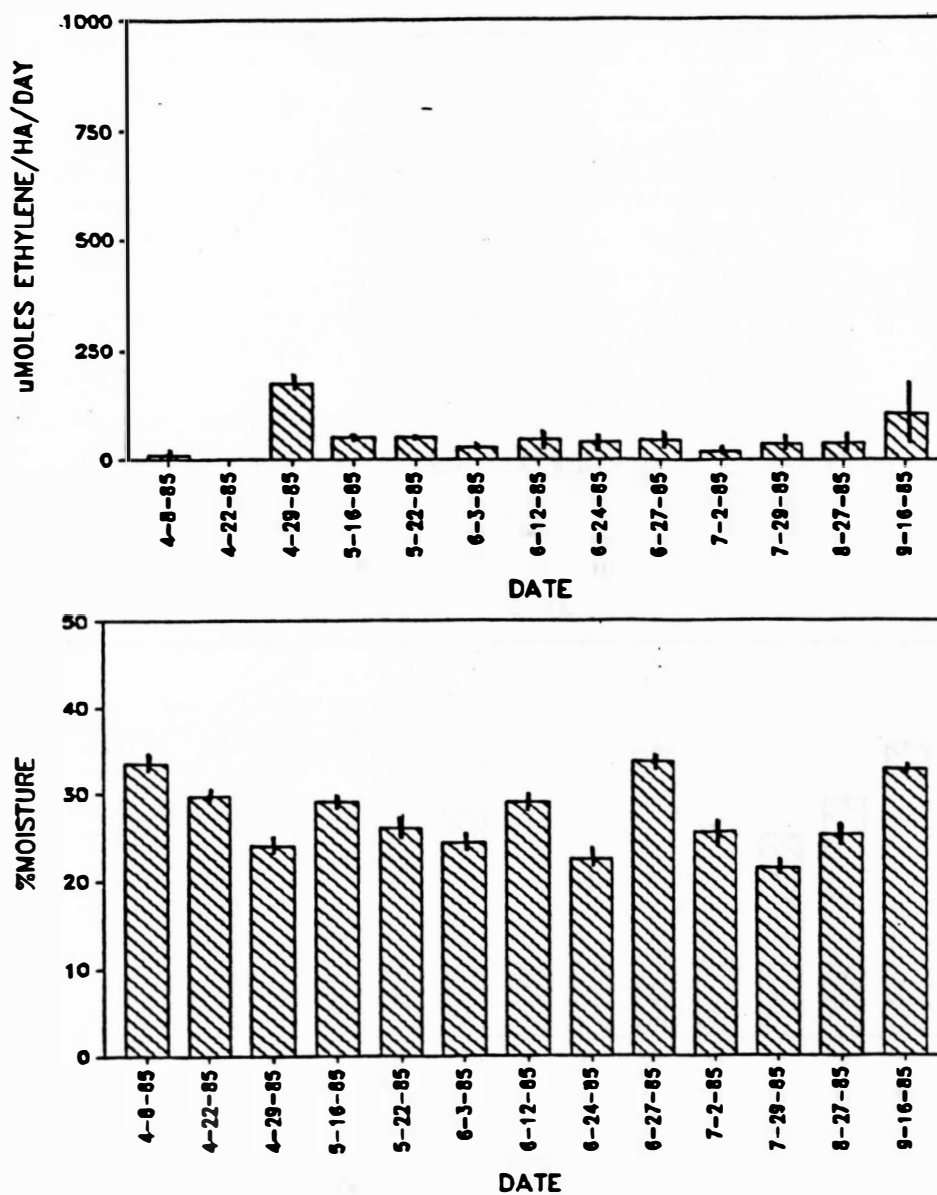


Figure 18. Rates of ethylene production (upper) and soil moisture (lower) for a corn field (BL) during the 1985 growing season. Values reported are means for four determinations and standard errors are indicated by the vertical lines.

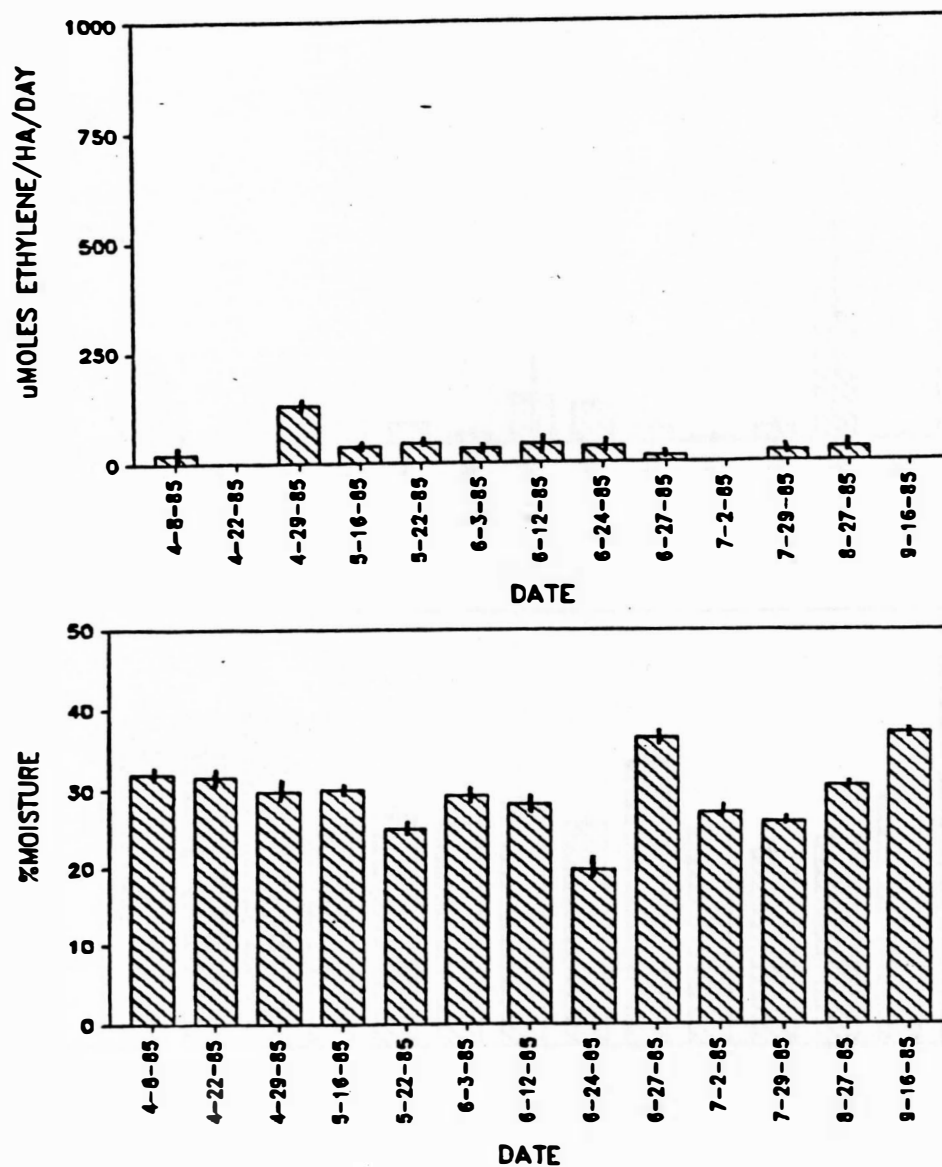


Figure 19. Rates of ethylene production (upper) and soil moisture (lower) for a corn field (MS) during the 1985 growing season. Values reported are means for four determinations and standard errors are indicated by the vertical lines.

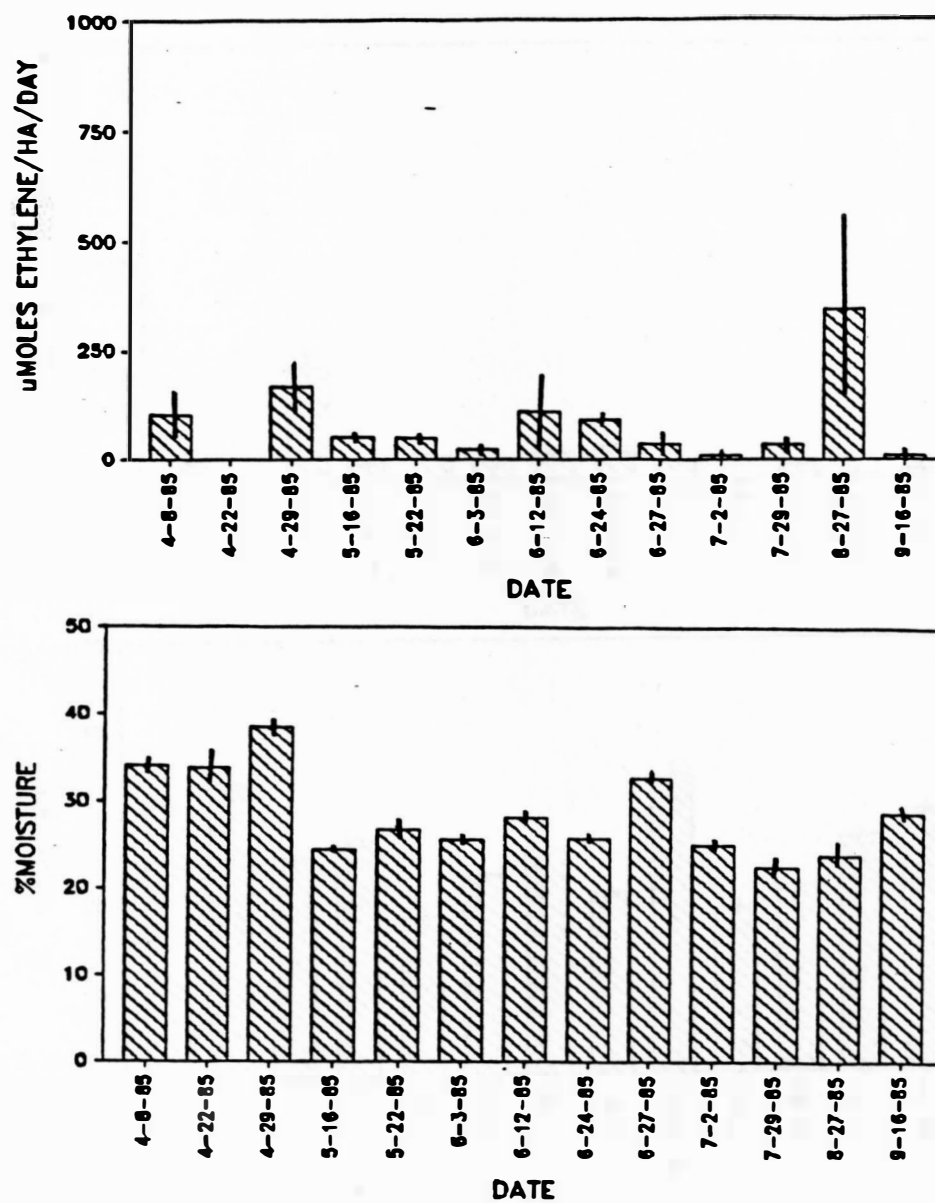


Figure 20. Rates of ethylene production (upper) and soil moisture (lower) for a corn field (JW) during the 1985 growing season. Values reported are means for four determinations and standard error is indicated by the vertical lines.

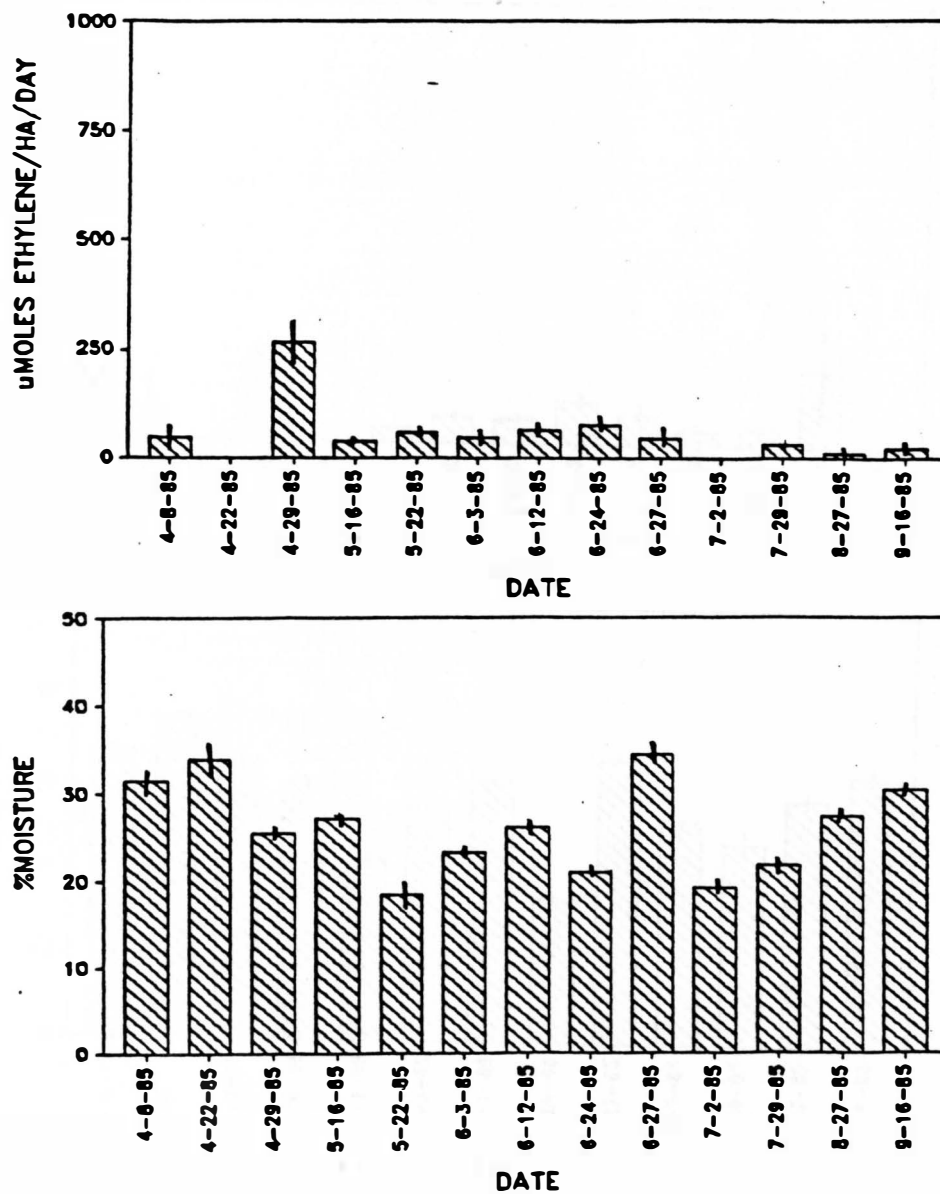


Figure 21. Rates of ethylene production (upper) and soil moisture (lower) for a corn field (RS) during the 1985 growing season. Values reported are means for four determinations and standard errors are indicated by the vertical lines.

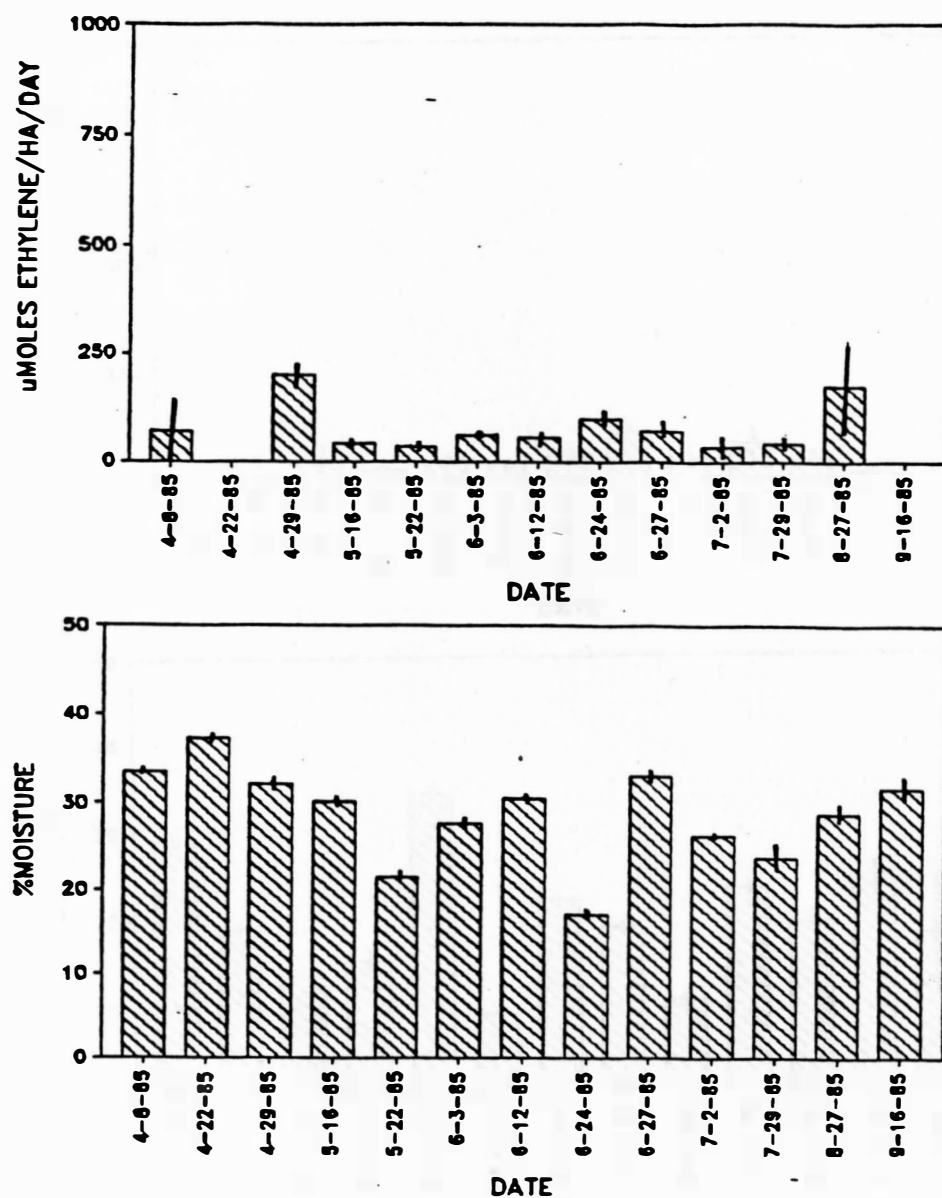


Figure 22. Rates of ethylene production (upper) and soil moisture (lower) for a rye field (VC) during the 1985 growing season. Values reported are means for four determinations and standard errors are indicated by the vertical lines.

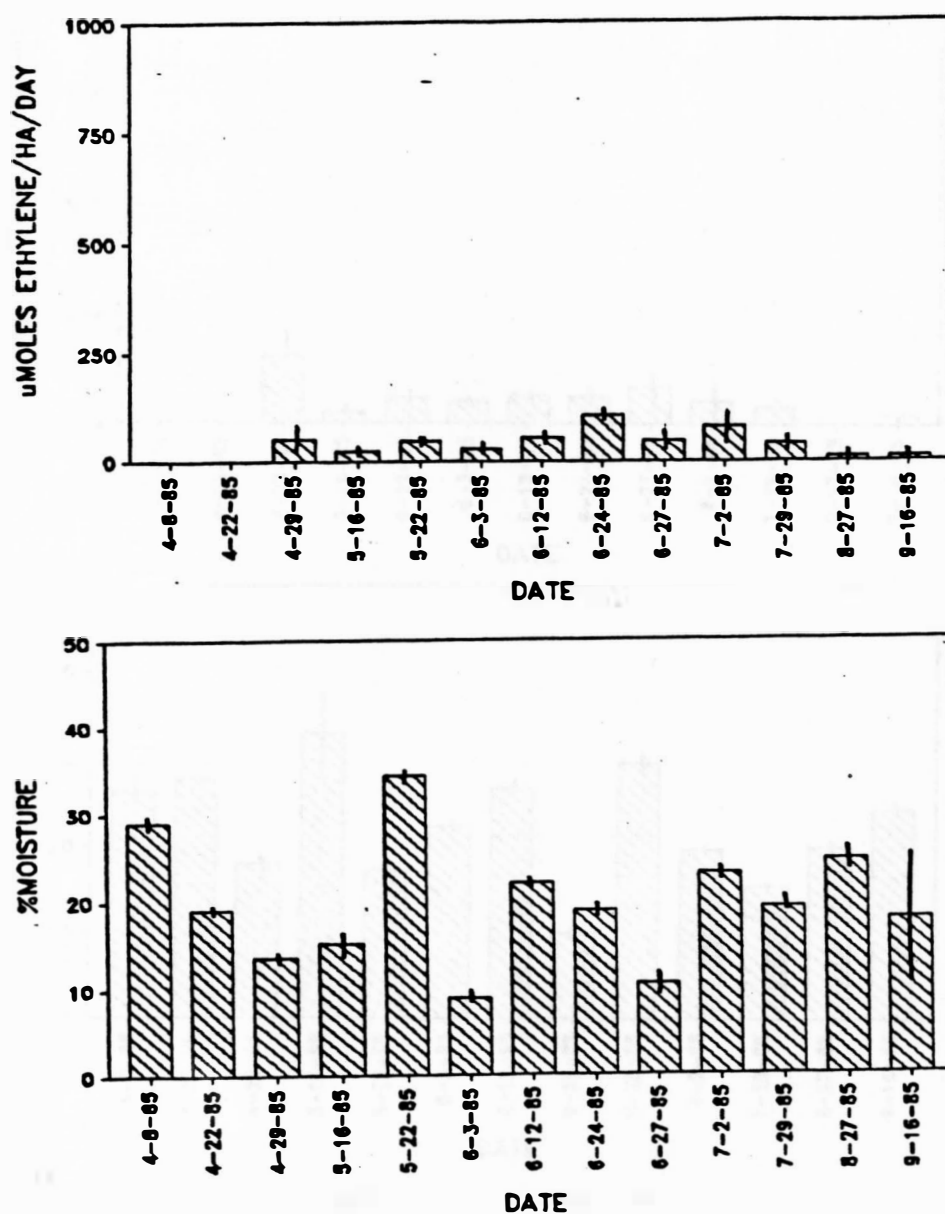


Figure 23. Rates of ethylene production (upper) and soil moisture (lower) for a beardless barley field (PH) during the 1985 growing season. Values reported are means for four determinations and standard errors are indicated by the vertical lines.

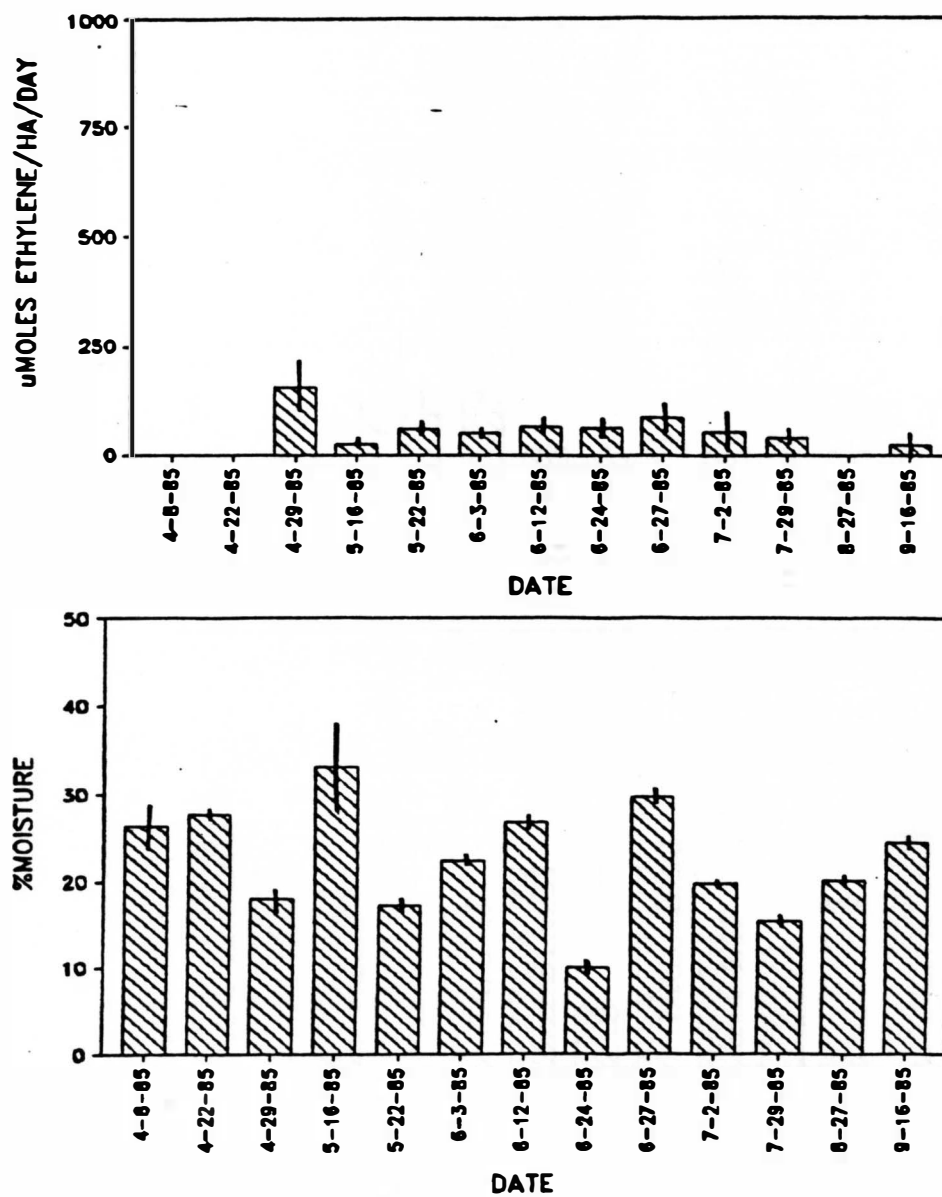


Figure 24. Rates of ethylene production (upper) and soil moisture (lower) for a spring wheat field (LK) during the 1985 growing season. Values reported are means for four determinations and standard errors are indicated by the vertical lines.

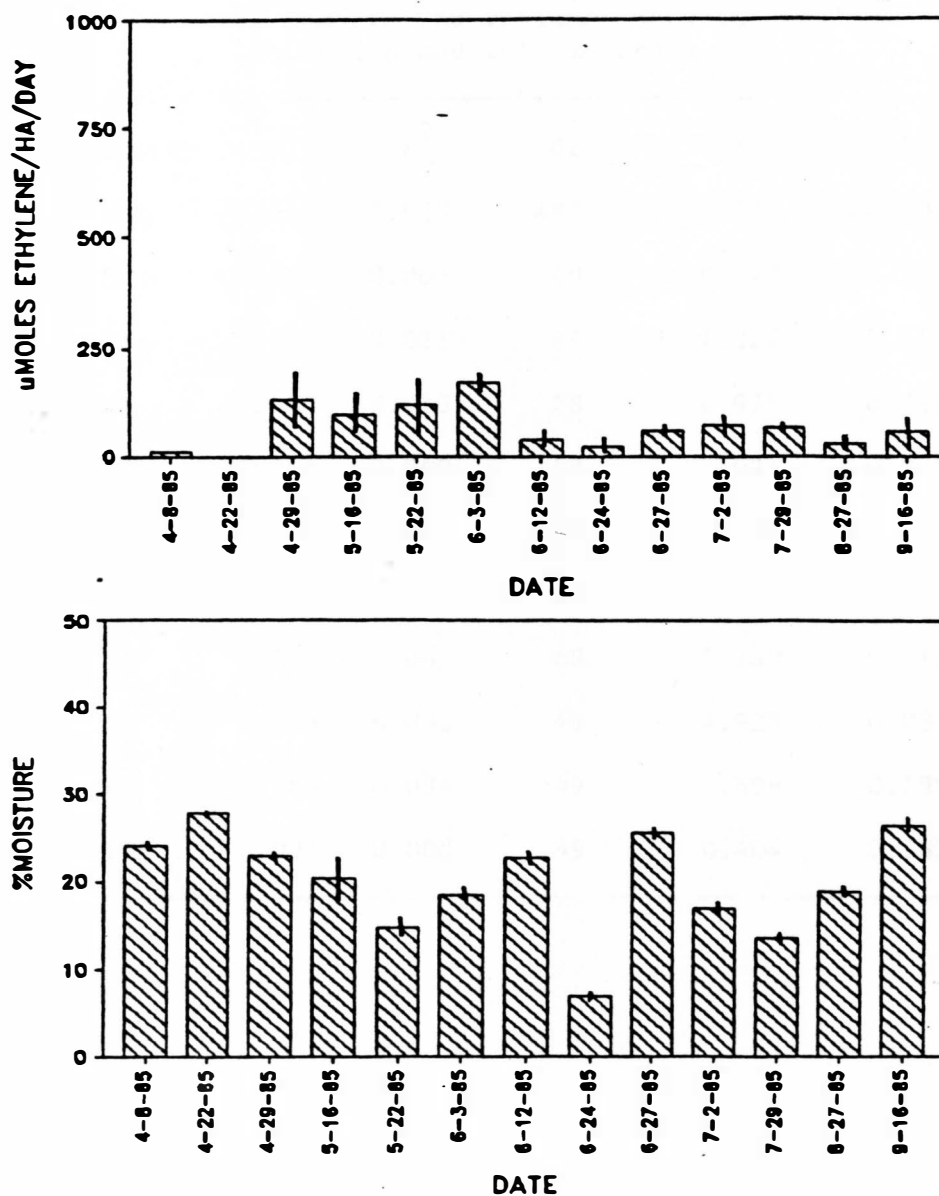


Figure 25. Rates of ethylene production (upper) and soil moisture (lower) for an alfalfa field (LRa) during the 1985 growing season. Values reported are means for four determinations and standard errors are indicated by the vertical lines.

Table 3. Statistical analysis using linear regression to compare the variance between ethylene production and soil moisture.

C ₂ H ₄ vs moisture	r	r ²	df	F	P
all	0.168	0.023	497	14.373	< 0.001**
OW	0.055	0.003	49	0.147	0.707
Blg	0.144	0.021	49	1.022	0.317
Bl	0.143	0.020	48	0.976	0.328
MS	0.246	0.060	48	3.021	0.089
JW	0.064	0.004	49	0.199	0.658
RS	0.061	0.004	49	0.178	0.675
VC	0.054	0.003	49	0.140	0.710
PH	0.305	0.093	49	4.923	0.031
LK	0.185	0.034	49	1.699	0.199
LKa	0.091	0.008	49	0.404	0.582

Table 4. Statistical analysis using linear regression to compare the variance between ethylene production and sampling time.

SITE	MEAN	r	r ²	df	F	P
OW	262.075	0.712	0.507	49	3.173	0.003**
B1g	212.087	0.740	0.548	49	3.741	<0.001**
B1	52.211	0.731	0.534	48	3.435	0.002**
MS	34.019	0.883	0.779	48	10.601	<0.001**
JW	80.769	0.630	0.397	49	2.032	0.049*
RS	58.066	0.900	0.810	49	13.132	<0.001**
VC	69.194	0.700	0.490	49	2.965	0.006**
PH	40.054	0.703	0.494	49	3.008	0.005**
LK	50.191	0.717	0.514	49	3.263	0.003**
LKa	70.760	0.484	0.234	49	0.942	0.518

Table 5. Statistical analysis using ANOVA to compare ethylene production rates between sites within a cropping type (corn, grass and small grain).

CROP	r	r^2	df	F			P		
				SITE	SAMPLING	S*S ^a	SITE	SAMPLING	S*S
CORN	0.719	0.518	197	3.537	6.447	1.893	0.16**	<0.001**	0.004**
GRASS	0.721	0.520	99	0.997	4.693	1.918	0.321	<0.001**	0.046*
GRAIN	0.718	0.515	149	4.180	5.142	2.005	0.18*	<0.001**	0.008**

a interaction of site and sampling

sites show significant variation between sites and samplings. Grass sites show significance only between samplings. Rates from one grass site is not significantly different from the rates on the other grass site.

As a final comparison sites were pooled to represent cultivated and noncultivated management. These results (from an Anova) are shown in Table 6. Noncultivated systems have statistically higher rates of ethylene production than do cultivated systems.

The interpretation of this data is limited because of the relatively small number of random samples used in the studies. For this reason interpretations in this study are based on trends between vegetation types and moisture values and not on the actual values for a particular field or site. Although values were low, trends were still evident.

Table 6. Statistical analysis using ANOVA to compare ethylene production rates between cultivated and noncultivated systems

Cultivated vs noncultivated	Cultivated	Sampling	C*S ^a
F	129.303	16.259	12.383
F	< 0.001 ^{**}	< 0.001 ^{**}	< 0.001 ^{**}

a interaction of cultivation and sampling

VI. CONCLUSION

Ethylene production is an easily applied method for estimating nitrogen fixation and is especially useful in the field. Other techniques are expensive and cumbersome to use but measuring ethylene production allows for the handling of much larger number of samples with relative ease.

Intact soil cores proved efficient in conducting a field survey of various locations and vegetation types. Rates of ethylene production were low but the sensitivity of the ethylene production assay lends this technique to field assays where ^{15}N techniques may not produce a measurable product. Results give a good indication of nitrogen fixation because nitrogenase is the only enzyme known to reduce acetylene.

Many factors affect acetylene reduction rates as demonstrated in the studies reported in this thesis. The intact soil core technique is a useful, meaningful method for measuring ethylene production in fields soils. However comparisons should be limited to specific site to site or time to time comparisons. In additional field studies, consideration must be given to vegetation type and environmental conditions: Moisture, temperature, and presence of energy substrates such as carbohydrates.

As resources become more limited, incorporation of biological processes in agriculture will become more and

more important. Enhancement of nitrogen fixation can be an advantage for the farmer and consumer alike. Knowledge of field determined estimates of nitrogen fixation can result in refined standards for farm management which might include quantity and quality of tillage, fertility, time of planting and irrigation application.

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