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The University of Oklahoma, Ph.D., 1975
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THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

ACETYLENE REDUCTION (NITROGEN FIXATION) AND OLD FIELD SUCCESSION IN CENTRAL OKLAHOMA

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

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BY

LAWRENCE A. KAPUSTKA

Norman, Oklahoma

1975

ACETYLENE REDUCTION (NITROGEN FIXATION) AND

OLD FIELD SUCCESSION IN CENTRAL OKLAHOMA

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ABSTRACT

The nitrogen fixing activities of two old field succession plots (P_1-pioneer weed stage, P_2-annual grass stage) and one prairie site (P_3) were estimated using the acetylene reduction assay.

Measurements were taken of fresh soil cores as well as soil cores amended with glucose.

Fresh soil samples generally had very low activity. The average pM $C_2H_4 \cdot g\ soil^{-1} \cdot hr^{-1}$ for each site was P_1, 23; P_2, 15; P_3, 83. The amended treatment had substantially greater activity with the average pM $C_2H_4 \cdot g\ soil^{-1} \cdot hr^{-1}$ being P_1, 2447; P_2, 2764; P_3, 7097.

Statistical comparisons were made between C_2H_2 reduction of fresh and amended soils, soil moisture, soil temperature, Azotobacter density, and blue green algal density. The bluegreen algal density was highly correlated ($r = 0.735$) with C_2H_2-reduction in P_1 nonamended soils but not with P_2 or P_3 soils. Nonamended activity was also weakly correlated with soil moisture in all successional stages.

N_2-fixing organisms isolated from the soils were Azotobacter chroococcum, Enterobacter aerogenes, Clostridium sp., Nostoc sp., and Anabaena sp. It was inferred that the anaerobic forms progressively increased in density through the successional stages. Selected phenolic acids, ferulic, sinapic, p-coumaric, vanillic, gallic, ellagic, and chlorogenic were found to be most inhibitory to E. aerogenes and least inhibitory to Clostridium sp.

The data support a prior hypothesis that relates the longevity of the P_2 seral stage to a biological suppression of the accumulation of soil nitrogen.

ACETYLENE REDUCTION (NITROGEN FIXATION) AND OLD FIELD SUCCESSION IN CENTRAL OKLAHOMA

CHAPTER I

INTRODUCTION

Nonsymbiotic biological nitrogen fixation in soils is limited to relatively few genera of four major systems, heterocystic bluegreen algae, aerobic bacteria, anaerobic bacteria, and fungi (Stewart, 1966). The rate of nitrogen fixation in soils is a function of the density of nitrogen fixing organisms and the moderating influences of soil moisture, soil temperature, soil chemistry, available energy, and biological interference (Jensen, 1965).

The revegetation of abandoned fields in central Oklahoma has four prominent successional stages: (i) a pioneer weed stage which lasts 2-3 yr; (ii) an annual grass stage lasting 9-13 yr; (iii) a perennial bunch grass stage which lasts 40 yr or more; and (iv) the climax prairie (Booth, 1941). Numerous investigations have been conducted by Rice and colleagues in an attempt to identify the factors responsible for this successional pattern (Rice, 1974).

Jenny (1941), Hobbs and Brown (1957), and Hass, Evans, and Miles (1957) described the rapid and substantial loss of nitrogen from soils that occurs during cultivation. An important feature of

secondary succession of abandoned fields is the replenishment of mineral nitrogen. Rice, Penfound, and Rohrbaugh (1960) reported that the order in which species of seed plants invade abandoned fields in central Oklahoma is the same as the order based on increasing requirements for nitrogen. This observation prompted a series of investigations that demonstrated the inhibition of nitrogen-fixing organisms by extracts of pioneer plants. Rice (1964, 1965b, 1965c, 1969) found that extracts of several low nitrogen-requiring pioneer species in revegetating old fields inhibit Azotobacter and Rhizobium. He found also that six plant species important in the first two stages of succession inhibit nodulation, hemoglobin formation, and nitrogen fixation in legumes (Rice, 1968, 1971). The inhibitory phenolic compounds p-hydroxybenzaldehyde, chlorogenic acid, isochlorogenic acid, β-resorcylic acid, p-coumaric acid, and ferulic acid were isolated and identified from seed plants of the first and second successional stages (Abdul-Wahab and Rice, 1967; Rice, 1965a, 1965c; Rice and Parenti, 1967; Wilson and Rice, 1968). Many phenolic compounds have been recovered with organic solvents from soils and litter at concentrations higher than required to inhibit plants or microorganisms (Blum and Rice, 1960; Rice and Pancholy, 1973; Turner and Rice, 1975). Commercial preparations of several of the compounds added to growth media at concentrations found in the soils have been shown to inhibit the growth of nitrogen-fixing bluegreen algae (Parks and Rice, 1969) and the infectivity of Rhizobium (Blum and Rice, 1969).

Several species of the pioneer weed stage are autotoxic and toxic to other species of that stage but do not adversely affect

Aristida oligantha Michx., the chief dominant of Stage 2 (Abdul-Wahab and Rice, 1967; Olmsted and Rice, 1970; Parenti and Rice, 1969; Wilson and Rice, 1968). This results in the rapid transition from Stage 1 to Stage 2. As previously mentioned, members of the first two stages are inhibitory to nitrogen fixers. This presumably helps maintain a low nitrogen condition which in part explains the protracted successional process of abandoned fields.

The study reported here was initiated to (1) measure the nitrogen fixing capacity of fresh and amended soils of the pioneer weed, annual grass, and prairie stages, and (2) assess the degree of inhibition of nitrogen fixation of soil isolates by suspected inhibitor compounds. This was accomplished by the acetylene reduction assay for nitrogen fixation (Hardy et al., 1968; Hardy et al., 1973) which provides an accurate and rapid estimation of the rate of nitrogen fixation in soils.

CHAPTER II

METHODS

Three sites near Norman, Oklahoma designated P_1 (pioneer weed), P_2 (annual grass), and P_3 (prairie) (Rice and Pancholy, 1972) were selected for this study. Vegetational composition of the plots is summarized in Table 1.

At least once each month beginning in April 1974, fifteen randomly selected soil cores 2.0 cm diam x 5.0 cm were collected from each study site. Within 1 hr after collection the samples were placed in uniform conditions at 22 $\pm$ 2 C. Nitrogen fixation was estimated by the acetylene reduction assay of Hardy et al. (1968) as modified by Brouzes and Knowles (1973) and Stutz and Bliss (1973). The incubation period was 6 hr after which the ethylene content was determined with a Becker 409 gas chromatograph equipped with a column 1/8 in. x 2 m containing Porapak N. The weight of the soil samples was recorded for subsequent gravimetric determination of the percentage of soil moisture. In order to assess the potential nitrogen fixation of the soils under anaerobic conditions, 10 ml of 2% glucose solution were added to each sample. The containers were flushed with N_2 gas and allowed to incubate for 36 hr at room temperature before determining acetylene reduction as previously described. Following the assay the

Table 1. Vegetational composition of the study sites. Data given only for those species having 3 % or more of the total vegetational composition.[1]

Species	P_1	P_2	P_3
Acacia angustissima	2.9		
Ambrosia psilostachya	5.1		
Andropogon gerardii			22.4
Aristida oligantha	4.6	63.3	
Coreopsis tinctoria	29.6	6.8	
Cyperus sp.	8.4		
Conyza canadensis	3.5		
Erigeron strigosus	3.2	7.9	
Haplopappus ciliatus	13.9	14.5	
Panicum virgatum			11.2
Plantago purshei	4.6		
Sabatia campestris	6.9		
Schizacharium scoparium			42.4
Sorghum halepense	8.0		
Specularia perfoliata	4.2		

[1]Vegetational sampling of P_1 was in June, 1974 using the point-frame technique (Whitman and Siggeirsson, 1954). Sampling of P_2 and P_3 was in June, 1971 by Rice and Pancholy (1972) using the same method. Identifications based on Waterfall (1966) with recent nomenclature of Corell and Johnston (1970).

soils were oven dried at 105 C for 48 hr and the percentage of soil moisture was calculated. Acetylene reduction was expressed as pM C_2H_4 · g $soil^{-1}$ · hr^{-1}.

The temperature of the soil at 5 cm depth was recorded for each sample date.

Each month the number of Azotobacter/g of soil in the top 5 cm was determined from dilution plate counts with a Burk's minimal salts medium (Wilson and Knight, 1952). Also, the number of bluegreen algae per gram of soil was estimated using a modified direct count technique of Sharabi and Pramer (1973). Five grams wet weight of soil from the 0-1 cm depth were placed in 45 ml water and mixed for 10 min with a magnetic stirrer. A 2-ml aliquot was added to 2 ml of 5% gelatin and 0.1 ml of the suspension was spread uniformly on each of 10 cover glasses (22 mm x 22 mm). Fifty fields (430x) on each slide were selected on a stratified random basis for counting. The number of bluegreen algae/g soil was computed from the mean count per field, the number of fields per area of the cover glass, the soil dilution, and the percent moisture of the soil sample.

Microorganisms capable of nitrogen fixation were isolated from the soils. Azotobacter chroococcum Beij. was isolated on Burk's medium and Enterobacter aerogenes Hormaeche and Edwards and a species of Clostridium were isolated on the Line and Loutit medium (1971) as modified by Koch and Oya (1974). The diagnostic tests of Buchanan and Gibbons (1974) were used to identify these bacteria (Table 2). Stock cultures of the isolates were maintained in 250 ml erlenmeyer flasks each containing 50 ml of the selected medium. Azotobacter cultures

Table 2. Summary of diagnostic tests used in the identification of soil isolates.[1]

Organism	Characteristics Plus Tests and Results[2]
Azotobacter chroococcum	Aerobe, C_2H_2 reduced, diplococcus with massive sheath, water-soluble pigments absent, insoluble pigments brown-dark brown, motile, peritrichous flagella, utilize starch and mannitol.
Enterobacter aerogenes	Facultative anaerobe, C_2H_2 reduced, rods <1-1 μ x 1-3 μ, occurring singly, encapsulated, lacks spores, motile with peritrichous flagella, gram negative. Positive: catalase, nitrate reduction, gelatin liquification [(+)], Simmons citrate, acetyl methyl carbinol, ornithine decarboxylase, lysine decarboxylase. Negative: urease, indole production, H_2S production, methyl red, arginine dihydrolase. Fermentation Acid and Gas: mannose, arabinose, glucose esculin (+), lactose (+), glycerol, raffinose, maltose, salicin, mannitol, sucrose Acid only: inositol

Table 2. Continued

Organism	Characteristics Plus Tests and Results[2]
Clostridium sp.	Obligate anaerobe, C_2H_2 reduced, rods <1 μ x 3-9 μ, occurring singly or in chains, the cells straight or curved, endospores terminal, motile with peritrichous flagella, gram positive, catalase negative. Positive: gelatin liquification (+), methyl red Negative: urease, indole production, H_2S production, acetyl methyl carbinol. Fermentation Acid and Gas: mannose, raffinose, maltose, mannitol (+), sorbitol (+), sucrose, glucose. Negative: esculin, arabinose, lactose, glycerol, inositol, salicin.

[1] Buchanan and Gibbons (1974)

[2] (+) = slow, [(+)] = very slow

were placed on a reciprocal shaker set at 120 rpm, whereas the anaerobes were maintained in stationary flasks. The Clostridium cultures were kept anaerobic by a 1-cm layer of liquid paraffin.

The organisms were tested for their ability to grow in the presence of suspected inhibitor compounds released during microbial decomposition of lignin or tannin (Sarkanen and Ludwig, 1971; Rice and Pancholy, 1972). The acids tested were ferulic, sinapic, vanillic, p-coumaric, gallic, ellagic. In addition chlorogenic acid, a secondary metabolite leached from several P_1 species, was used. The initial concentration tested was 10^{-4}M. Two milliliters of inoculum from the log phase of growth ($\sim 2 \times 10^6$ cells for A. chroococcum and $\sim 5 \times 10^6$ cells for E. aerogenes and Clostridium sp.) were introduced to 50 ml of the selected medium. Three to seven replicates were used for each test. Growth was measured as an increase in turbidity with a Spectronic 20 spectrophotometer. Comparisons between test treatments and controls were made from 96-hr optical density values for A. chroococcum and E. aerogenes whereas Clostridium sp. was measured after 7 days. The stationary growth phase was achieved after approximately 110 hr for A. chroococcum and E. aerogenes and approximately 10 days for Clostridium sp. In the cases where inhibition was detected, additional tests were performed at lower concentrations of the acid in order to determine the lowest level that resulted in a significant reduction of growth.

Aqueous soil extracts from the three plots were prepared by mixing soil in distilled water (1:3, w/v). After 1 hr the mixture was decanted and the liquid was centrifuged at 5000 g for 10 min. The remaining clay in the supernatant was removed with a Seitz filter. The

soil water extracts were analyzed for pH, organic carbon (Piper, 1942); Ca, Mg, and K (Perkin-Elmer, 1971); readily available phosphate (King, 1932); and osmotic pressure by use of a microvolt psychrometer (Wescor Inc., 1970). The extracts were used to prepare culture media to test for the possible relative effects of water soluble inhibitor compounds in the three soils. Three Azotobacter media were made by adding either 20 g sucrose/liter water extract, 20 g sucrose plus 1/2 the concentration of Burk's ingredients/liter extract or 20 g sucrose plus the full compliment of Burk's ingredients/liter extract. Media for the anaerobes were prepared in the same manner but with 10 g glucose/liter with Line and Loutit ingredients. The media were adjusted to pH 7 and steam sterilized for 1 hr. Incubation and measurement of growth were as previously described.

CHAPTER III

RESULTS

The fluctuation in acetylene reduction by the nonamended (NA) soils from one sample date to the next was striking (Fig. 1). In May, 1974 in all three study sites and again in Feb., 1975 in P_3 the C_2H_2-reduction activity reached extremely high values in comparison with the rest of the year. The duration of the peak periods of activity is difficult to ascertain. However, they do not last longer than two weeks as indicated by proximate sampling dates in this study. Based on the growth rates of microorganisms in culture, these accelerated rates probably exist for no more than one week. Nevertheless with activity at the elevated rates as in P_3 for May, 1974, as much nitrogen would be fixed in one week as in the remainder of the year at the typically low levels of activity.

The average yearly rate of C_2H_2-reduction in pM $C_2H_4 \cdot g\ soil^{-1} \cdot hr^{-1}$ in the top 5 cm for the three sites was P_1, 23; P_2, 15; P_3, 83. Analysis of variance indicates that each of these mean values is significantly different from the other two at the 0.01 level. The addition of nitrogen to the soil can be estimated from the theoretical relationship of 3M C_2H_2 reduced equals 1M N_2 fixed. The rates of addition to the top 5 cm for the three sites in kg $N \cdot ha^{-1} \cdot yr^{-1}$

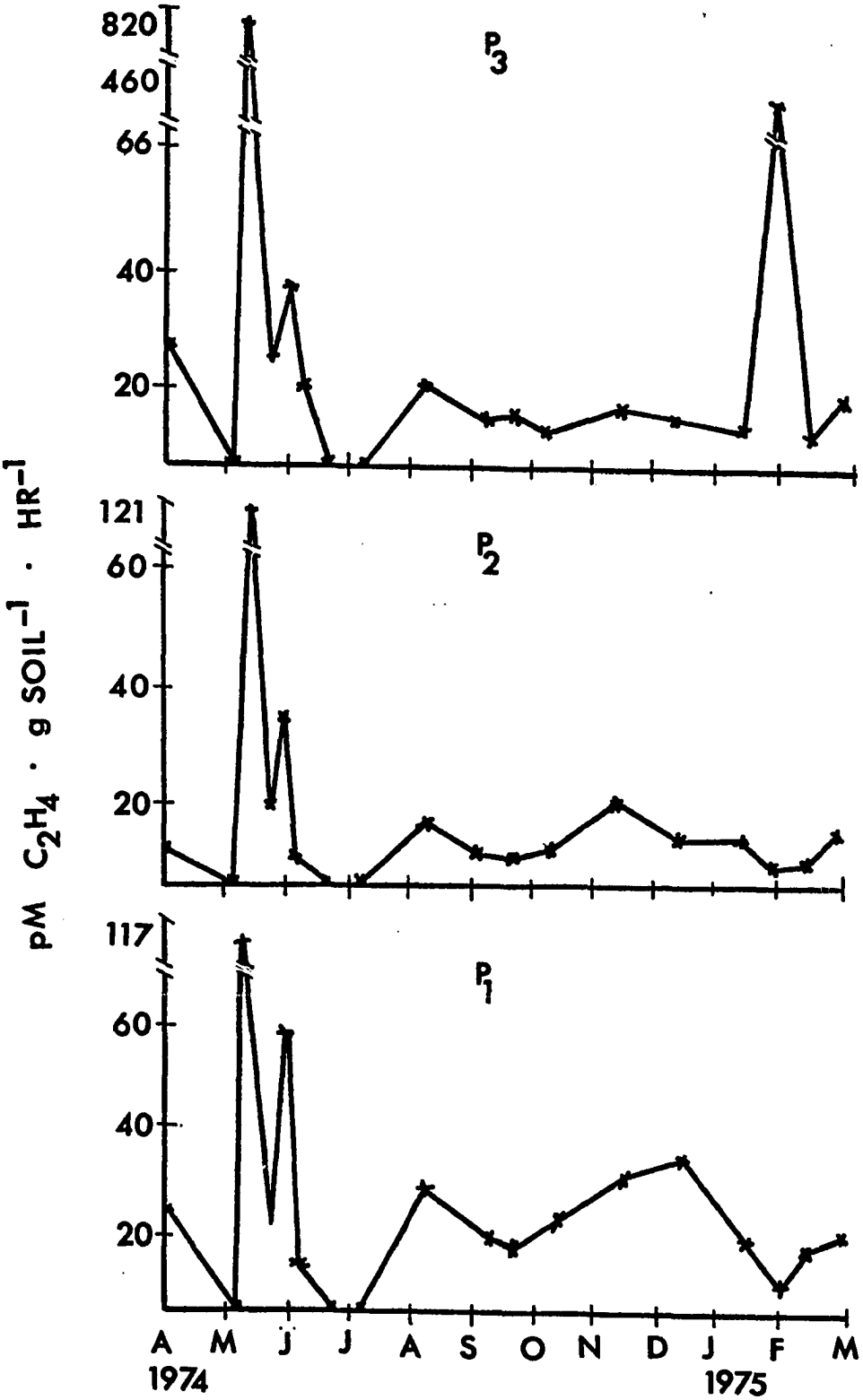

Fig. 1. C_2H_2 reduction of nonamended soils.

(adjusted for the bulk density of the soils) are P_1, 1.2; P_2, 0.7; P_3, 3.5.

The C_2H_2-reduction activity in the 2% glucose amended soils (2% G) (Fig. 2) was substantially greater than in the NA soils, and exhibited less fluctuation between sample dates. The average μM $C_2H_4 \cdot g\ soil^{-1} \cdot hr^{-1}$ for the three sites was P_1, 2447; P_2, 2764; P_3, 7097. Again, each mean was significantly different from the other two at the 0.01 level. The amount of stimulation of C_2H_2 reduction occurring as a result of amending the soils was different for the three sites. P_3 soils were stimulated the least and P_2 was enhanced the most. The Wilcoxson signed rank test indicated the differences in stimulation were significant at the 0.01 level or better.

The number of bluegreen algae $\cdot$ g $soil^{-1}$ (Fig. 3, Table 3) was highest in P_1 and can be attributed to the greater availability of light at the soil surface than in either P_2 or P_3 sites. The total number of cells is in close agreement with those found in Virginia soils (Bailey and Stauffer, 1975) determined by a MPN technique. In their study approximately 50% of the population was Nostoc. The density of individual taxa was not determined in this study. However, in the identification of the heterocystic bluegreen algae in the soils, Nostoc was the most frequently encountered genus with Anabaena quite common.

The density of Azotobacter (Fig. 4, Table 3) increased progressively through the successional stages.

The mean and range of the percentage of soil moisture for the three sites were P_1, 17.2 (1.0-28.9); P_2, 19.3 (1.9-28.0);

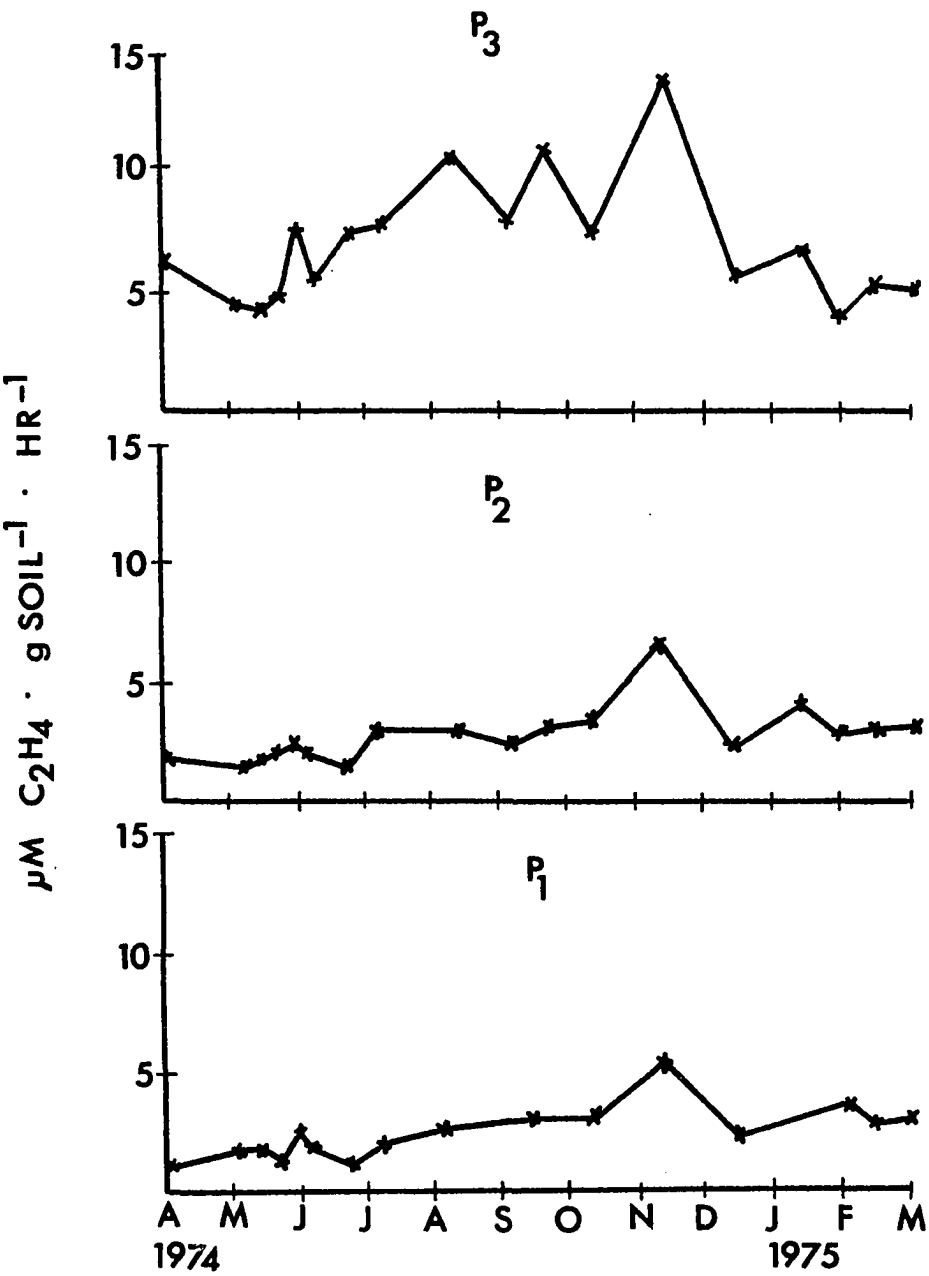

Fig. 2. C_2H_2 reduction of 2% glucose amended soils

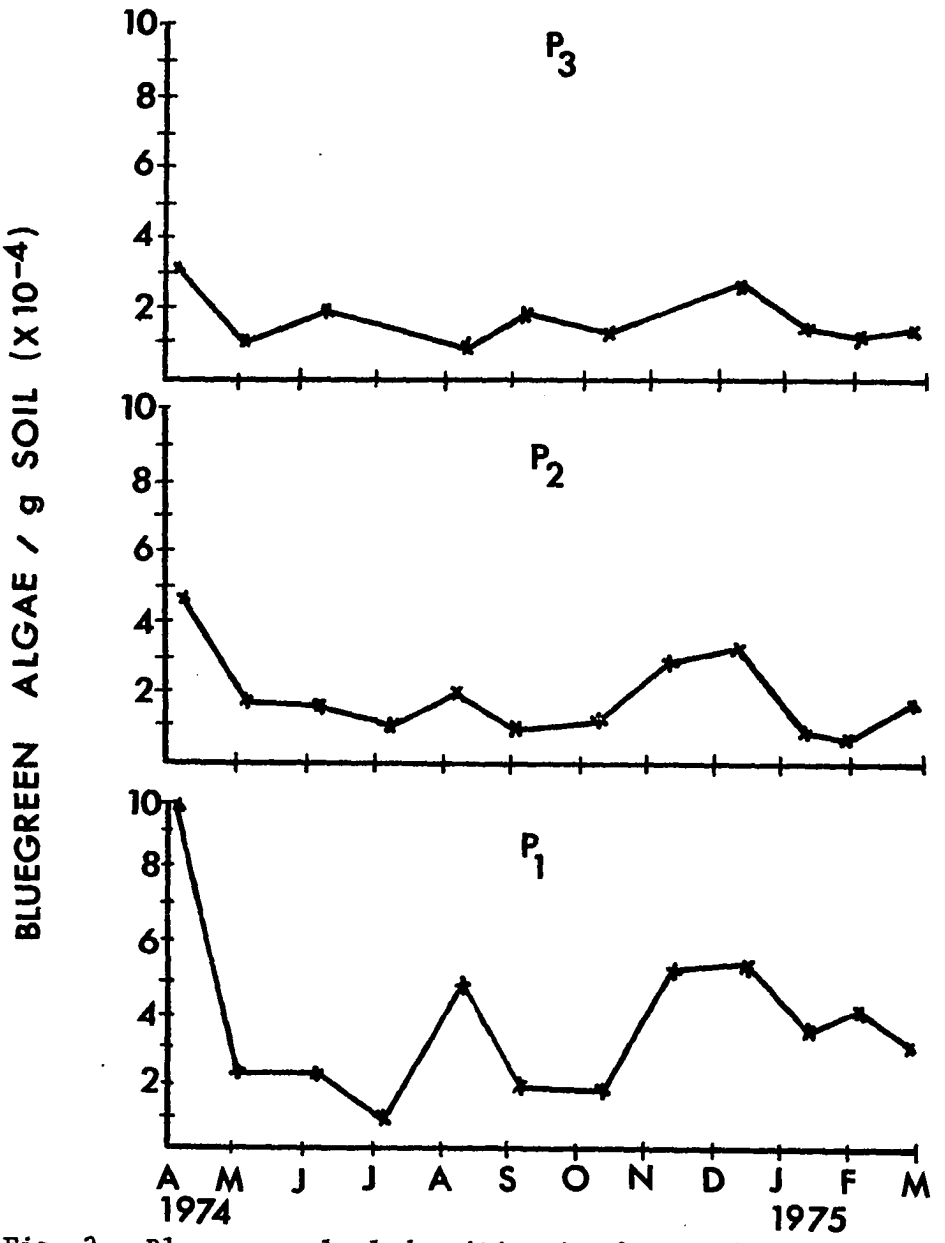

Fig. 3. Bluegreen algal densities in the top 1 cm of soil at the three sites.

Table 3. Mean values of *Azotobacter* and bluegreen algal densities and acetylene reduction rates for the three sites.

Factor	Seral Stage		
	P_1	P_2	P_3
Azotobacter · g soil^{-1}	24500[b]	28000[c]	38750
Bluegreen algae · g soil^{-1}	37000[ab]	19500	18500
pM C_2H_4 · g soil^{-1} · hr^{-1}			
non-amended	23[ab]	15[c]	83
2% glucose	2447[bd]	2764[c]	7097
2% G/NA	106.4[ab]	184.3[c]	85.5

a. Difference between P_1 and P_2 significant at 0.01 level or better.

b. Difference between P_1 and P_3 significant at 0.01 level or better.

c. Difference between P_2 and P_3 significant at 0.01 level or better.

d. Difference between P_1 and P_2 significant at 0.05 level or better.

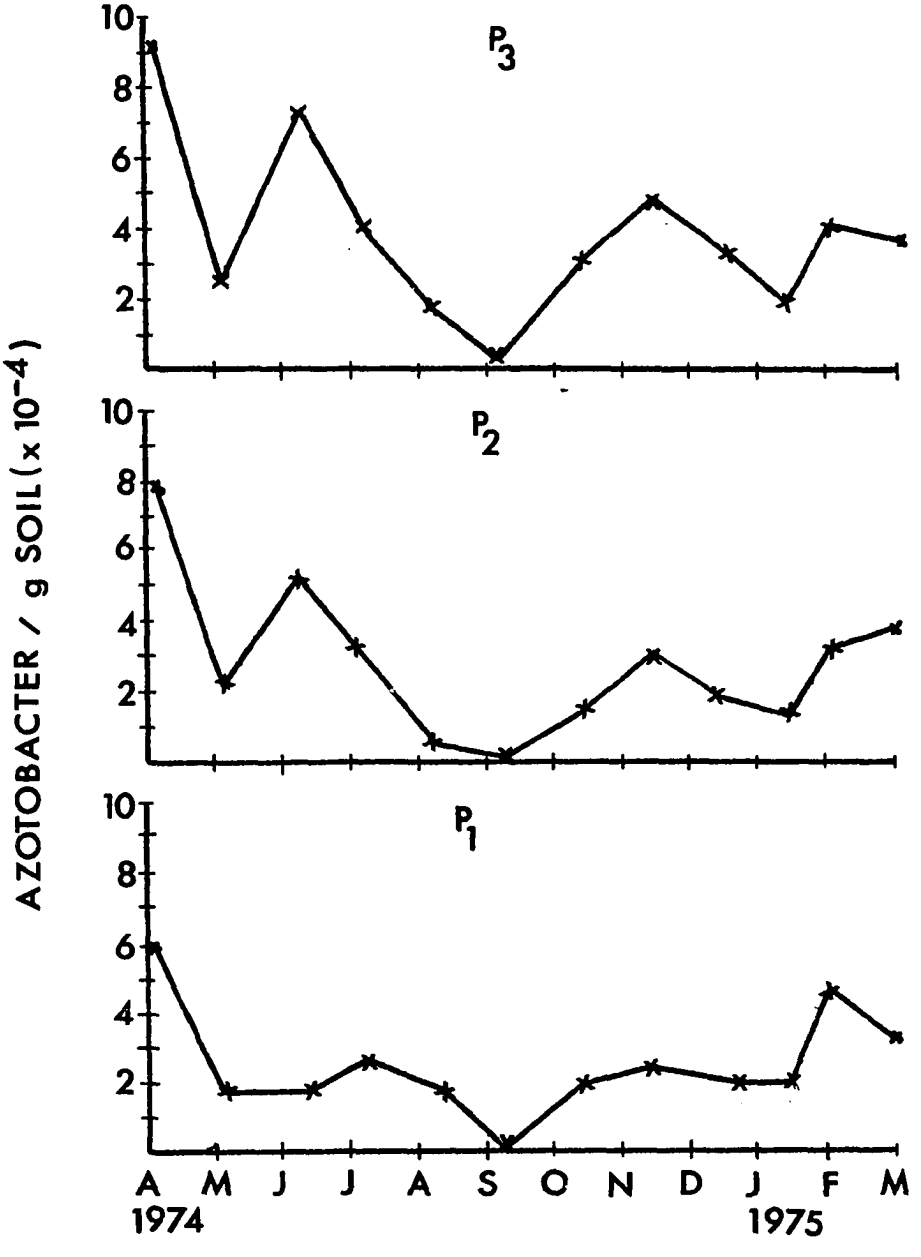

Fig. 4. Azotobacter densities in the top 5 cm of soil at the three sites.

P_3, 18.4 (3.6-26.8). The means and ranges of soil temperature were P_1, 19.0 (0-33); P_2, 16.1 (1-33); P_3, 15.9 (2-35).

First and second degree polynomial regressions between soil temperature, soil moisture, density of Azotobacter, density of bluegreen algae, C_2H_2-reduction NA, and C_2H_2-reduction 2% G were calculated for each of the three study sites (Table 4). Azotobacter density was not correlated with C_2H_2 reduction in any of the three sites. Bluegreen algal density was correlated with C_2H_2-reduction in NA soil in the P_1 by a second degree regression. The linear regression did not provide a significant relationship, but this was due to a single data point (April, 1974). When this point is left out of the calculation the linear relationship becomes significant ($r = 0.735$) and this is not improved by the second degree regression. The April, 1974 bluegreen algal density was abnormally high in comparison with the remainder of the year whereas the C_2H_2-reduction activity was typical. The high density may have been a result of sampling error. Another plausable explanation is that the high algal density may have been due to an unusually high level of nonheterocystic bluegreen algae. It is also interesting to note that the bluegreen algal density in P_1 was highly correlated with soil moisture and soil temperature with a second degree regression whereas the density of bluegreen algae in P_2 and P_3 and Azotobacter density in all three sites was not correlated with either soil moisture or temperature.

C_2H_2 reduction in NA soil and soil temperature were not correlated in any of the sites whereas soil moisture was correlated with C_2H_2 reduction in all three sites. The only other significant

Table 4. Regression statistics of C_2H_2 reduction and selected factors for the three sites.

X	Y	First Degree r	First Degree Equation	Second Degree r	Second Degree Equation
P1					
[1]Soil Moist.	NA	0.344	Y = 1.155 + 2.515 X	0.362	Y = -5.437 + 0.044 X - 0.023 X^2
[2]Bluegreen Algae	NA	0.735	Y = -1.230 + 0.001 X	N.I.	
[1]Soil Moist.	2% G	0.350	Y = 1482.24 + 56.084 X	0.357	Y = 1720.761 + 1.244 X + 1.833 X^2
[3]Soil Temp.	2% G	-0.663	Y = 3632.102 + 66.049 X	0.730	Y = 3124.081 + 45.202 X - 3.404 X^2
P2					
[1]Soil Moist.	NA	0.284	Y = -3.011 + 0.953 X	N.I.	
[1]Soil Moist.	2% G	0.287	Y = 1798.939 + 49.969 X	N.I.	
[3]Soil Temp.	2% G	N.S.		0.558	Y = 3498.973 - 9.480 X - 1.462 X^2
[1]NA	2% G	N.S.		0.234	Y = 2259.269 + 71.967 X - 0.628 X^2
P3					
[1]Soil Moist.	NA	0.190	Y = -66.348 + 7.750 X	N.I.	
[1]NA	2% G	-0.214	Y = 7490.933 - 4.77[illegible] X	0.238	Y = 7616.223 - 12.375 X + 0.010 X^2

1 200 d.f.

2 11 d.f.

3 17 d.f.

N.S. = not significant

N.I. = correlation not improved over the first degree

relationships were C_2H_2 reduction in 2% G soil and soil temperature in the P_1 and P_2 sites. Both were described best with a second degree regression.

The effect of suspected inhibitor compounds was quite variable for the three species tested. In all cases, except ferulic acid on A. chroococcum, inhibition was essentially complete at a concentration of 10^{-4} M (Table 5). Of the three isolates tested, Clostridium sp. was inhibited the least while E. aerogenes was inhibited the most. The variance in the growth of Clostridium sp. generally was quite large and may account for difficulties in statistically detecting inhibition. Therefore it is possible that other culture techniques that reduce the variance would show inhibition at lower concentrations. However, clostridia are notoriously ubiquitous in soil habitats apparently independent of soil properties (Jensen, 1965) and would presumably be immune to these inhibitors.

The growth of soil isolates in media prepared from soil water extracts was different for the three sites. In the case of A. chroococcum the water extracts showed increased growth over the normal minimal salts Burk's medium (Fig. 5). Growth increased with the addition of minerals with maximum growth of those media tested attained with the addition of 1/2 the concentration of Burk's ingredients. These data suggest that all three soils have growth stimulators for Azotobacter as well as a deficiency of readily available minerals. E. aerogenes achieved less growth in media with soil extracts than in controls of Line and Loutit media made with distilled water (Fig. 6). Maximum growth in soil extracts was obtained however with the addition

Table 5. The lowest molar concentrations of suspected inhibitors that significantly reduced the growth of soil isolates.

Inhibitor	Test Organism		
	A. chroococcum	E. aerogenes	Clostridium sp.
ferulic acid	$> 10^{-4}$	10^{-6}	10^{-4}
sinapic acid	5×10^{-5}	10^{-6}	10^{-5}
p-coumaric acid	5×10^{-5}	10^{-6}	10^{-5}
vanillic acid	10^{-6}	10^{-6}	10^{-4}
chlorogenic acid	10^{-6}	10^{-6}	10^{-4}
gallic acid	10^{-6}	10^{-6}	10^{-5}
ellagic acid	10^{-6}	10^{-5}	10^{-4}

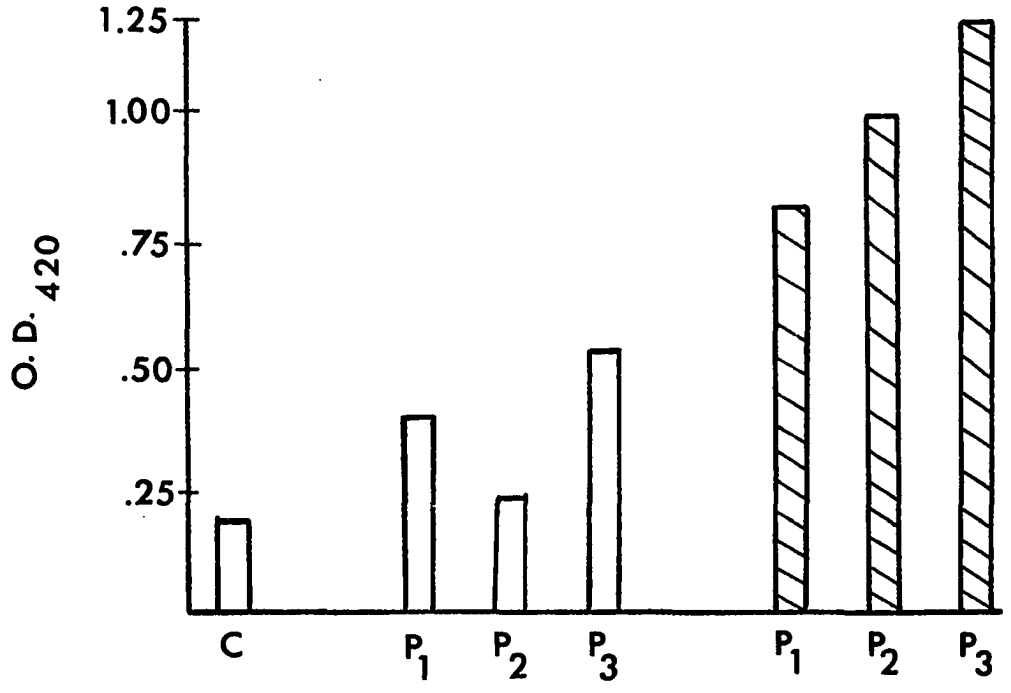

Fig. 5. Growth of A. chroococcum on soil water extracts. Open bars - C, Burk's minimal salts medium with distilled water; P_1, P_2, P_3, respective extracts + 20 g sucrose/l; Crosshatch bars - P_1, P_2, P_3, respective extracts + 20 g sucrose/1 + 50% Burk's ingredients.

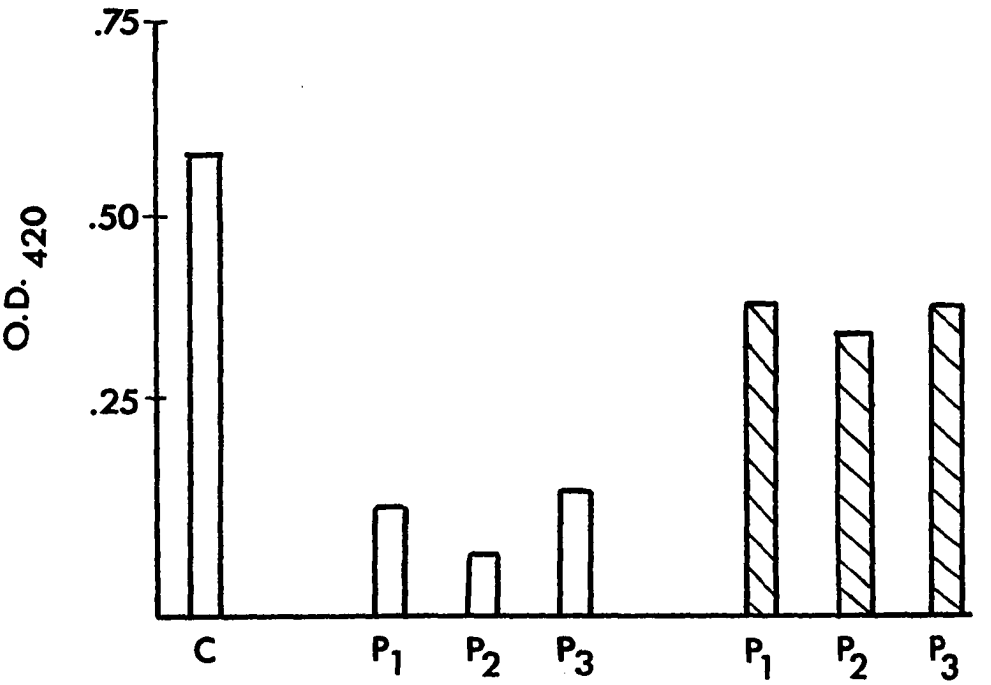

Fig. 6. Growth of E. aerogenes on soil water extracts. Open bars - C, Line and Loutit medium with distilled water; P_1, P_2, P_3, respective extracts + 10 g glucose/l; Closed bars - P_1, P_2, P_3, respective extracts + 100% Line and Loutit ingredients.

of the full complement of Line and Loutit ingredients. This again indicates a deficiency of minerals required for optimal growth, but it also points to the presence of inhibitors of E. aerogenes. A partial chemical analysis of the water extracts indicated that P_2 is slightly lower in readily available Ca and K than either P_1 or P_3 and slightly lower in readily available phosphate than P_3 (Table 6). On the other hand readily available Mg was highest in P_2. The inhibitory activity of the soil extracts was not due to the osmotic pressure because all had relatively low pressures and P_2 had the lowest of all.

The effect of soil extracts on Clostridium sp. was not examined because of the high variance in growth previously mentioned.

Table 6. Partial chemical analysis of the soil water extracts from the three sites.

Test	Seral Stage		
	P_1	P_2	P_3
pH	6.8	6.4	6.7
Organic C (%)	0.004	0.007	0.017
Ca (ppm)	2.6	0.9	12.2
Mg (ppm)	6.4	12.1	9.1
K (ppm)	0.6	0.3	2.7
$PO_4^{\equiv}$ (ppm)	16	21	24
Osmotic Pressure (atm)	-0.35	-0.30	-0.95

CHAPTER IV

DISCUSSION

In the past five years there have been several studies on the rates of C_2H_2 reduction of various soils. In situ measurements of nonamended soils are typically low, especially for cultivated fields. The rates of addition of nitrogen to the soil in this study are in general agreement with other reports for nonsymbiotic fixation in grasslands. Paul et al. (1971) reported 1 kg N · ha^{-1} · yr^{-1} and Vlassek et al. (1973) calculated 2 kg N · ha^{-1} · yr^{-1} in the top 12.5 cm for Canadian grasslands with slightly less activity in cultivated soils. Steyn and Delwiche (1970) obtained rates of 2-5 kg N · ha^{-1} · yr^{-1} in the top 35 cm in California soils. Balandreau and Dommergues (1973) quoted rates of 1-3 kg N · ha^{-1} · yr^{-1} for temperate grasslands.

Bailey and Stauffer (1975) stressed the importance of bluegreen algae in cultivated soils. The strong correlation between bluegreen algal density and C_2H_2 reduction in P_1 nonamended soil suggests a similar importance in the pioneer weed stage in Oklahoma. Later stages have lower bluegreen algal densities and show no correlation with C_2H_2-reduction activity.

Azotobacter densities were not correlated with C_2H_2 reduction activity in any of the sites which supports the general contention

of Jensen (1965) and others that this bacterium is relatively unimportant in the addition of nitrogen to soils.

Of the other possible genera of nitrogen fixers, only Enterobacter and Clostridium were isolated in this study. No attempt was made to determine the densities of these anaerobes. Both genera have been considered to be the principle nonsymbiotic nitrogen fixers found in soils. Koch and Oya (1974) identified E. aerogenes, E. cloacae and Klebsiella pneumonia as major constituents in Hawaiian grassland soils. Rice et al. (1967) and Mayfield and Aldworth (1974) concluded that clostridia are the chief nonsymbiotic nitrogen fixers in several Canadian soils.

Chang and Knowles (1965), Rice et al. (1967), Brouzes et al. (1969), and O'Toole and Knowles (1973) noted marked increases in nitrogen fixation following the addition of carbohydrates to soils. Anaerobic forms appear to be responsible for a substantial portion of this fixation. The nature of clostridial endospores insures the survival of a great percentage of the population during periods of adverse soil conditions. During incubation of the amended soils, metabolic activity is restored to the cells. Consequently there is less seasonal fluctuation in C_2H_2 reduction of amended soils. The differences in C_2H_2-reduction activity that exist between sites in this study suggest an increase in the density of clostridia and possibly Enterobacter in the later stages of succession.

Mayfield and Aldworth (1974) found that microsites exist in small soil aggregates that are conducive to the metabolism and growth of aerobes on the particle surface and to anaerobes within the matrix

of the particle. They concluded that in most nitrogenase assays where O_2 is not purged, both the aerobes and anaerobes are actively fixing nitrogen. Yet a comparison of the nonamended and 2% glucose amended assays in this study indicate no correlation in P_1, a second degree negative correlation in P_2, and first and second degree negative correlations in P_3. Apparently the higher periods of activity are a result of aerobic forms or bluegreen algae or both contributing a larger part of the fixation. Since the nonamended activity was not correlated with density of the bluegreen algae or Azotobacter in P_2 or P_3 the rise in activity must be a result of greater efficiency of the organisms without an accompanying increase in population density.

A common characteristic of all heterotrophic nitrogen fixers is that they are most efficient in metabolizing carbohydrate materials (Jensen, 1965). As succession proceeds organic carbon accumulates in the soil. Pancholy and Rice (1973) reported 0.561, 0.589, and 0.885% organic carbon for P_1, P_2, and P_3 respectively. They suggested on the basis of levels of activity of soil enzymes that the chemical composition of the organic materials is quite different between sites. Since then Rice and Mallik (personal communication) have demonstrated a sharp decrease in the carbohydrate/lignin ratio of underground plant material as succession proceeds toward the climax vegetation. They have also found a marked increase in activity of invertase, amylase, and cellulase when underground plant material from the pioneer weed stage (P_1) is added to climax soil which is usually low in activity of these enzymes. Yet the total magnitude of nitrogen fixation is greatest in the climax prairie, even though the heterotrophic nitrogen fixers would be most

efficient in the early stages of succession with the greater availability of simple carbohydrate metabolites.

There are several possible reasons for the low levels of nitrogen fixation in cultivated and recently abandoned fields. These habitats are characterized by larger fluctuations in environmental conditions compared with later seral stages which would influence the activities of microorganisms. The presence of inhibitor compounds in the soils released from plants through leaching and decomposition may account for the reduced rates of nitrogen fixation observed in the P_1 and P_2 sites. The inhibition of growth by pure chemicals in culture media occurs at concentrations equal to or more dilute than those recovered from soils. Wang et al. (1971) reported 0.36-1.67 μmoles p-hydroxybenzoic acid/100 g soil, 0.34-2.13 μmoles p-coumaric acid/100 g soil, 0.17-0.43 μmoles vanillic acid/100 g soil, and 0.11-0.24 μmoles ferulic acid/100 g soil. Synergistic effects of phenolic compounds may cause them to be even more inhibitory. The information from present soil water extract studies suggests the influence of inhibitor compounds on E. aerogenes. This may be considered even more significant since many compounds are inhibitory while attached to colloidal micelles apart from the soil solution. For instance, gallotannic acid, when added to soil at 30 ppm resulted in decreased nodulation of beans, but the compound could not be recovered from the soils until 400 ppm were added (Blum and Rice, 1969).

The net accumulation of soil nitrogen is dependent on the balance between competing processes of the nitrogen cycle. Nitrification can lead to the loss of soil nitrogen because of the ease with

which nitrate is leached from soils. Rice and Pancholy (1972, 1973, and 1974) demonstrated higher levels of nitrifiers in P_1 and P_2 than in P_3. The combined effect of high rates of nitrification and low rates of nitrogen fixation serve to keep soil nitrogen at low concentrations.

It was hypothesized earlier that the maintenance of the low nitrogen condition had a major influence in prolonging the longevity of the annual grass seral stage. Evidence for this was based on the nitrogen content of the soils, the nitrogen requirements of the climax species, and the production of large quantities of compounds by the pioneer weeds and Aristida oligantha inhibitory to Azotobacter, Rhizobium and bluegreen algae. In this study A. chroococcum, E. aerogenes and a species of Clostridium were added to the list of nitrogen fixing species inhibited by these phenolic compounds. Furthermore, the rates of acetylene reduction (and therefore nitrogen fixation) for the three sites is consistent with the proposed effect of these inhibitors. Therefore, these findings further support the hypothesis explaining the slow rate of old field succession in central Oklahoma.

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