THE EFFECT OF NUTRIENTS, PHENOPHASE, AND

TEMPERATURE ON THE NITROGEN-FIXING

ACTIVITY OF SELECTED LEGUMES

By

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ACKNOWLEDGMENTS

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CHAPTER I

INTRODUCTION

It has been known for many years that a major portion
of nitrogen which is consumed by plants is made available to
them through biological nitrogen fixation (Postgate, 1974a).
Much of this fixation is accomplished by leguminous plants
in association with certain bacteria.

With the advent of commercial fertilizers, nitrogen in
a form available to plants is now usually applied directly
to the soil. As a result, studies in biological nitrogen
fixation have been somewhat neglected, especially in the
prairie legumes (Nutman, 1971).

Since many types of nitrogenous commercial fertilizers
require manufactured energy in their production, the
increased use of legumes as the nitrogen source could result
in considerable energy conservation.

The extensive use of nitrogenous fertilizers during the
past few years has resulted in much run-off and erosion of
nutrients from the soil. These nutrients eventually enter
streams and lakes resulting in eutrophication, and often in
dissolved oxygen deficiency. Such oxygen deficiency there-
fore adversely affects growth of heterotrophs in the area.
It is probable that more extensive production of legumes

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could result in less demand for nitrogenous fertilizers, and therefore less eutrophication.

While we know that legume species occupy a diversity of habitats and thus probably have wide-ranging nutrient requirements, the effect of specific nutrients on the capability of legumes to fix nitrogen needs further study (Lie, 1974).

The age at which leguminous plants initiate the nitrogen-fixing process and the age at which the plants are most efficient as nitrogen fixers varies with the species (Lofton, 1976). The temperature at which plants are cultured may also affect their potential as nitrogen fixers (Hardy et al., 1968). More research is needed concerning the effect of phenophase and temperature on the nitrogen-fixing properties of the prairie legumes.

Statement of the Problem

The purpose of this study was to investigate the effect of nutrients, phenophase, and temperature on the nitrogen-fixing potential of the following prairie legume species:

   I.   _Psoralea tenuiflora_ Pursh

  II.   _Cassia fasciculata_ Michx.

III.   _Desmodium sessilifolium_ (Torr.) T.&G.

Hypotheses

   I. Nutrient deficiencies in the plant species investigated have a significant effect on their nitrogen-fixing potential.

  II. The phenophase of plant species investigated has a significant effect on their nitrogen-fixing potential.

III. Changes in temperature have a significant effect on the nitrogen-fixing potential of the plants investigated.

CHAPTER II

REVIEW OF LITERATURE

Nitrogen is the major plant nutrient which limits pro-
duction of food and fiber in our population (Evans, 1976).
Owens (1976) states that nitrogen appears to be the primary
major nutrient which limits plant production in the world
oceans, as well as certain fresh water systems.

Biological nitrogen fixation is accomplished primarily
in those plants which belong to the family Leguminosae in
association with nitrogen-fixing bacteria. According to
Vincent (1974), legumes have worldwide distribution and rank
second or third among flowering plants in the number of spe-
cies which they contain.

Burns and Hardy (1975) have estimated the total annual
rate of biological nitrogen fixation to be in the area of
175×10^6 metric tons per year. With the increasing demand
for food, the amount of nitrogen which is fixed industrially
in the form of nitrogen fertilizers has been increasing
yearly. The amount of nitrogen fixed by the Haber-Bosch
method for the year 1975 was estimated by Burns and Hardy to
be 44×10^6 metric tons.

Large amounts of energy are required in the industrial
production of nitrogen fertilizers. In 1972, 11.4 million

tons of anhydrous ammonia were produced in the United States requiring 456 billion cubic feet of natural gas. This represented two percent of all of the natural gas consumed during the year (Evans, 1975). With increasing demands for energy, it is apparent that more research in the area of biological nitrogen fixation is necessary. Postgate (1972) states that research in biological nitrogen fixation made enormous progress in the decade 1960-1970, with the most impressive advances in the enzymological and chemical aspects, but far-reaching developments also occurred in the more biological and ecological aspects. According to Quispel (1974), a renewed interest in biological nitrogen fixation is developing.

Yates (1974) and Postgate (1971) report that nitrogen fixation has been studied more intensively in Russia than in most countries during this century. Most of their work has been done with emphasis on the agricultural aspects of nitrogen fixation. There has been little contact between the East and West concerning problems in nitrogen fixation.

It has been found that the addition of nitrogen to soil surrounding the roots of legumes results in a decrease in biological nitrogen fixation by the legumes. Pate (1976) found that in field peas, addition of 315 parts per million nitrogen caused a drastic curtailment of nitrogenase activity within 48 hours. After the removal of nitrogen, it took an additional 48 hours to re-establish nitrogenase activity. Dilworth (1974) reported that the addition of high levels of

fixed nitrogen to legumes is known to inhibit nodule forma-
tion and accelerate nodule destruction. Hardy and Havelka
(1976), and Lie et al. (1976) both report drastic decreases
in nitrogen fixation with the addition of fertilizer nitro-
gen.

It is unfortunate that so little work has been done in
nitrogen fixation using native prairie legume species. Pate
(1976) states that virtually no work has been done on the
response of naturally occurring species of legumes to added
nitrogen in their native habitats. Similarly, Nutman (1971)
reports that little work has been done on the amounts of
nitrogen fixed by naturally occurring legumes. He assumes
that the amounts are quite large since naturally occurring
legumes usually contain more nitrogen than associated non-
legumes, and are widely distributed as herbs in grasslands,
bushes, and trees in savannas. Frequently legumes are a
major constituent of the flora. Stewart (1966) assumes that
wild legumes are effectively nodulated as they rapidly colo-
nize nitrogen-deficient habitats such as nutritionally
exhausted arable lands, gravel wastes, and newly cleared
areas.

Hewitt and Smith (1974) state that the amount of fer-
tilizer applied to crop land doubles every 10 years. Some
of the disadvantages to fertilizer application have been
discussed previously. It is known that several minerals are
necessary if nitrogen fixation in legumes is to occur.
Epstein (1972) and others have recognized the importance of

iron, molybdenum, and cobalt in the nitrogen-fixing systems of legumes. Postgate (1974b) indicates that molybdenum seems to be the strongest candidate for involvement in the N_2 binding site, but presents no direct evidence for this view. He also states that limitation of phosphates affect the ATP-ADP ratio, and therefore inhibits the nitrogen fixation mechanism.

Barber (1968) found that accumulations of phosphate in the roots of tomato and clover plants, and its transfer to the shoots were increased in the presence of microorganisms. If phosphate concentration is low, little phosphate is transferred to the shoots as microorganisms apparently absorb phosphate at the expense of the plant. Barber suggests that microorganisms may release phosphate into the soil and promote an increase in crop yields through mineral phosphate accumulation in the soil. This phenomenon has reportedly received much attention in the Soviet Union. At present, our knowledge of the role of microorganisms in the inorganic nutrition of plants is very incomplete.

Van Overbeek (1976) has reviewed the possibility of eventually producing wheat and rice plants with the aid of ammonia from biological nitrogen fixation rather than from synthetic fertilizers. The primary objective is to produce nutritious crops without the high cost in energy now required in the manufacture and transport of fertilizers.

Some work has been done in determining the effect of phenophase on the ability of plants to fix nitrogen. Sprent

(1976) determined that nitrogen fixation in peas and field beans decreased when pods were developing. Ham, Lawn, and Brun (1976) state that the acetylene-reducing capacity of field grown soybeans (Glycine max) increased during flowering, reached a maximum near the end of the flowering period, and declined sharply during early pod filling. They state that the decrease during pod filling was due to an inadequate supply of photosynthate transported to the nodules. The addition of nitrogen fertilizer after flowering resulted in an increased seed yield and protein content. Hardy and Havelka found that in Glycine max more than 90 percent of the total nitrogen fixed by the plant occurred during the last half of the growth cycle which was represented as the period of reproductive growth. They believe that the amount of photosynthate available to nodules may be a most significant factor limiting nitrogen fixation. As plants mature, the reproductive sinks appear to compete with nodules for available photosynthate. Lofton (1976) working with nine different prairie legume species found wide variation in the capacity of individual species to reduce acetylene to ethylene at age nine weeks and twelve weeks. It appears that plant phenology has a great effect on the capacity of plants to fix atmospheric nitrogen.

The effect of temperature on nitrogen fixation in field legumes has been reported by several investigators. Lie, et al. (1976) found that no nodulation occurred in peas at 30°C but did occur at 26°C. Stewart (1966) reports that in

Phaesolus vulgaris nodulation is reduced or entirely inhibited by high temperatures, but the higher temperatures do not affect root growth. Low temperatures do not affect the fixation process so markedly. An increase of 4°C above optimum for fixation inhibited fixation by 50 percent, while a decrease of 5°C decreased fixation by about four percent. Gibson (1971) states that lower root temperatures retard root hair infection more than they affect nodule initiation, and that higher root temperatures upset the formation of bacteroid tissue and hasten its degeneration. Hardy, et al. (1968) found that in soybeans (Glycine max) the optimum temperature for nitrogen fixation was from 20-30°C and possibly at 35°C. Higher temperatures may result in a decrease in fixation rate because of the adverse effect on bacteroid formation. Dart and Day (1971) indicate that the temperature at which plants are cultured can greatly affect legume-Rhizobium symbiosis by decreasing nodule formation and development, and consequent nitrogen fixation. They found that the optimum temperature varied with the species. Of five species which were investigated the optimum temperature was between 20 and 35°C except for cowpea (Vigna sinensis) which had an optimum of 40°C.

Lie (1971) states that the effect of temperature on the symbiotic system is complex. In peas, nodulation occurred at 26°C but not at 20°C. This requirement for the higher temperature was only confined to the second or third day after inoculation. He found that bean and pea plants are

devoid of nodules when kept at 30°C. High temperatures result in reduction of both nodule formation and nitrogen fixation. Masterson and Murphy (1976) conclude that soil temperature is the environmental factor having the greatest single influence on nitrogen fixation and growth in white clover (<u>Trifolium repens</u>). The highest rate of fixation occurred at 21°C and decreased as temperature was increased to 27°C. There appears to be little doubt that temperature plays an important part in the nitrogen-fixing activity of legume plants.

CHAPTER III

DESCRIPTION OF SPECIES

Psoralea tenuiflora

Better known as few-flowered scurfpea, Psoralea tenui-
flora is a perennial legume which is usually found on dry
prairies, open woods, and rocky banks. It is a drought-
resistant species, and occurs on plains and prairies through-
out the United States. It grows to a height of one meter,
produces small purple flowers in June, and palmately trifoli-
ate leaves with linear to oblong-oblanceolate leaflets. It
begins growth in early spring. During late summer an abscis-
sion layer forms at the base of the stem and the upper por-
tion of the plant detaches from the roots, and is blown about
by the wind. It produces abundant seeds with extremely
resistant seed coats. It is not considered as a major type
of forage for livestock, but is eaten in the early stages of
development (Pasture and Range Plants, 1956; Gray's Manual
of Botany, 1950).

Cassia fasciculata

This plant is known as the showy partridgepea. It is a
native, warm season annual legume which produces abundant
yellow flowers on short branches from July to September. It
grows to a height of 1.5-9 dm and is found on sandy loam

soils of central and eastern United States. It is a common
plant on old fields or disturbed areas. Cassia is readily
eaten by livestock and is reported to be very nutritious.
It produces seed with resistant seed coats. It nodulates
abundantly with the bulk of the nodules attached to the pri-
mary root. It appears to offer possibilities for cropland
improvement, and food and cover for wildlife (Pasture and
Range Plants, 1956; Gray's Manual of Botany, 1950).

Desmodium sessilifolium

Commonly referred to as sessile tickclover, this plant
is a warm season, deep rooted perennial legume. It produces
sessile leaves or leaves with petioles from 2-3 mm in length.
It produces small whitish-purple flowers and hairy seed pods
which stick to clothing and animals. The plant usually grows
to heights of 1-1.5 meters, and is found with tall grasses in
the central and eastern parts of the United States.
Desmodium is abundant on sandy loam soils. It is often
observed along roadsides. This species is nutritious and is
readily eaten by livestock. It produces abundant nodules
(Pasture and Range Plants, 1956; Gray's Manual of Botany,
1950).

CHAPTER IV

METHODOLOGY

Seed Collection and Germination

All seeds used in this investigation were collected during the summer and fall of 1976. Adequate quantities of seed from <u>Psoralea</u> <u>tenuiflora</u>, <u>Cassia</u> <u>fasciculata</u>, and <u>Desmodium</u> <u>sessilifolium</u> were gathered locally in the Lake Carl Blackwell area approximately 10 miles west of Stillwater, Oklahoma.

Seeds of the <u>Leguminosae</u> characteristically possess seed coats which are somewhat impervious to water (Ballard, 1971). Scarification was therefore necessary before seeds were placed into the growth medium. Seeds were scarified individually using a number 3 square jewelers file. Magnification was provided by use of a Bausch and Lomb 7 power jewelers loop. In each case the testa was penetrated to permit absorption of water by the seed and therefore enhance the germination process. Trial germination tests were conducted to determine seed viability. These tests also indicated that the scarification technique employed resulted in a decrease in the time required for germination. Seed which normally require weeks or months to germinate using other methods of scarification were found to germinate readily within a period of 1 to 5 days.

Fine, white, washed river sand was placed in 100 ml petri dishes and moistened with distilled water. These were autoclaved for 20 minutes at a pressure of 15 p.s.i. Scarified seeds were placed into the moist sand in the petri dishes. Twenty seeds were placed in each petri dish and incubated in an illuminated growth chamber with a light intensity of approximately 10,000 lux using a 12 hour photoperiod. The temperature was maintained at 27°C. _Desmodium_ seed germinated in 1-3 days. _Psoralea_ and _Cassia_ seed germinated in 1-5 days.

Transplanting and Seedling Development

Styrofoam pots with a capacity of 250 ml were used throughout this study. The base of each pot was pierced for drainage. The potting medium consisted of equal parts of white, washed river sand (washed 5 times) and number 3 vermiculite (Lofton, 1976). The medium was mixed thoroughly, sterilized, and placed into the styrofoam pots. Germinated seeds in the petri dishes were transplanted into the styrofoam pots using one seedling per pot.

At the time of transplanting, each seedling was inoculated with _Rhizobium_ spp. The inoculum was prepared by isolating _Rhizobium_ spp. from nodules of each of the three plant species using the streak-plate technique. Nodules were detached from the roots and placed in sterile petri dishes. Surface sterilization of nodules was accomplished by use of a 10 percent Clorox solution. The nodules were removed after

3-5 minutes and washed 5 times in sterile distilled water. Each nodule was then dissected, and crushed in 2 ml of sterile distilled water using a sterile 1 cm diameter glass rod. The resulting suspension was used to streak petri dishes containing a nutrient agar-yeast extract medium. The organisms were then incubated for 1-2 weeks at $29^{\circ}C$. _Rhizobium_ cultures from each of the plant species were obtained by subsequent sub-culturing.

Prior to inoculation, a _Rhizobium_ bacterial suspension was prepared from each of the three plant species. The three suspensions were then combined. The resulting slurry was used in inoculating all of the seedlings. Ten ml of slurry were added to each pot into which the seedlings had been transplanted. The pots were placed on 65 cm x 45 cm x 2.5 cm aluminum trays, each tray containing 35 pots. The trays were placed in the greenhouse. The temperature was maintained at approximately $27^{\circ}C$. All plants were illuminated using overhead agro-lites with an intensity of approximately 20,000 lux. A 12 hour photoperiod was maintained throughout the investigation.

Three different nutrient combinations were used in this study.

1. One group of seedlings received a complete nutrient solution (Arnon and Hoagland, 1940).

2. A second group of seedlings received a complete nutrient solution except for being nitrogen free.

3. A third group received a complete nutrient solution except for being phosphorus and nitrogen free.

All seedlings were root-irrigated to saturation by placing the appropriate nutrient solution into the aluminum trays and watering periodically as necessary with the solution. The technique used for watering is described by Becker and Crockett (1976). All pots were leached with distilled water at 10 day intervals to prevent salt accumulation in the potting medium.

In order to determine if plant phenophase affects their nitrogen-fixing capabilities, three groups of plants were assayed, each group at a different age and phenophase. The plants were randomly assigned to groups.

Group 1--age 4 weeks

Group 2--age 8 weeks

Group 3--age 12 weeks

The acetylene-ethylene reduction technique was employed to provide an index of the nitrogen-fixing capacity of the plants used.

Assay Technique

At the proper phenophase level each plant was removed from its pot, the roots suspended in a 125 ml filter flask, and the stem inserted into a number 4 rubber stopper to provide support (Lofton, 1976). Stoppers were sealed with plasticine modeling clay (Burris, 1974) to eliminate entry of outside air into the flasks. The side-arm of each flask

was fitted with a size 6 serum cap. Air in each flask was
evacuated through the side-arm by use of a Phillips-Drucker
Model M-803 suction surgical pump which developed a vacuum
of 18-20 inches of mercury. A specially designed apparatus
consisting of a 3 ml disposable hypodermic syringe fitted
into one end of a 90 cm length of vacuum tubing was used to
connect the flasks to the pump. A hypodermic needle attached
to the syringe was inserted into the serum cap covering the
side-arm of the flask, and the opposite end of the vacuum
tubing was attached to the vacuum pump. This provided a very
efficient, effective and uniform method of evacuation of air
from the flasks.

A 22.5 ml oxygen-90 ml acetylene mixture was injected
into each evacuated flask using a 50 ml air tight disposable
syringe. The acetylene mixture was composed of 0.1 atm acet-
ylene and 0.9 atm helium.

The flasks containing the plants were randomly placed
into three groups, each group containing an equal number of
the three plant species to be investigated. Each group was
placed into a separate illuminated growth chamber for 60
minutes at a given temperature and at a light intensity of
approximately 10,000 lux. The temperatures used were as
follows:

Group 1--15°C.

Group 2--22°C.

Group 3--30°C.

At the end of the 60 minute exposure period, a gas sample was collected from each flask and each sample was stored in a 13 mm evacuated serum bottle which was previously fitted with a number 6 serum cap.

Gas Chromatographic Analysis

The gas in each serum bottle was analyzed to determine the amount of ethylene produced from the acetylene which had been reduced by the Rhizobium spp (Wacek and Brill, 1976). The gas analyses were performed by use of a Hewlett-Packard gas chromatograph with a hydrogen flame-ionization detector (Hardy, Burns, and Holsten, 1973). Nitrogen was used as the carrier gas. Nitrogen gas flow was adjusted to 14 p.s.i., oxygen to 20 p.s.i., and hydrogen to 7.5 p.s.i.

Twenty-five μl gas samples were injected into the gas chromatograph using a Hamilton 50 μl gas-tight syringe. A 3.175 mm x 1.8 m stainless steel column containing 80/100 mesh Porapak N held at 50°C was employed to separate the ethylene from the acetylene. The quantity of ethylene produced was determined by use of the hydrogen flame-ionization detector. This provided a measure of the nitrogen-fixing potential of the plants which were investigated (Roughley and Dart, 1969). Each treatment consisted of five replications.

Statistical Design

Analysis of variance was employed to determine the relationship between nutrients, phenophase, and temperature as they affect acetylene reduction in the legumes studied.

CHAPTER V

RESULTS

The results are divided into two sections since two
separate analyses were employed in the statistical design.

Temperature and Age Effects

Section one deals with the relationship between tempera-
ture and age as they influence acetylene reduction in the
three species of legumes studied. The mean number of μ moles
of acetylene reduced per day by each of the three legume
species at ages four, eight, and twelve weeks, and at temper-
atures of $15^{\circ}C$, $22^{\circ}C$, and $30^{\circ}C$ was determined (Appendix A).

The statistically significant three-way interaction
(Appendix B) indicated that the combined effects of any two
variables were different at each increment of the third vari-
able. For example, age and temperature interact differently
for each plant species. It is, therefore, impossible to
draw general conclusions concerning the effects of one or
two variables over all increments of the third variable.
Based on the presence of the statistically significant three-
way interaction, a two-way analysis of variance was done
(Table I) which examined the effects of age and temperature
for each plant species.

TABLE I

ANALYSIS OF VARIANCE SUMMARY TABLE FOR AGE
AND TEMPERATURE FOR EACH PLANT SPECIES

| Plant | Effect | | | | | | Error |
| | Temperature | | Age | | Temp. x Age | | |
	Mean Square	F Value[a]	Mean Square	F Value	Mean Square	F Value	Mean Square
Psoralea tenuiflora	135.89	33.21*	11.70	2.86	7.47	1.86	4.09
Cassia fasciculata	2130.09	132.62*	1027.64	63.98*	305.45	19.02*	16.06
Desmodium sessilifolium	1034.36	128.75*	1719.74	210.01*	121.66	14.86*	8.19

[a]Appropriate degrees of freedom for all computed F values are: $df_{num}=2$, $df_{den}=36$.

*$p < .01$

In _Psoralea_ _tenuiflora_, the only statistically signifi-
cant effect observed in the two-way analysis of variance was
that concerning temperature (Table I). The effect of tem-
perature on the ability of _Psoralea_ to reduce acetylene was
further analyzed by use of the Scheffé test (H. Scheffé,
1959) which indicated that the mean at $22^{o}C$ (8.13) was sig-
nificantly different (α = .01) from the means at $15^{o}C$ (4.47)
and $30^{o}C$ (2.20). The means at $15^{o}C$ and $30^{o}C$, however, were
not significantly different (Figure 1). Since the interac-
tion effect was not significant for _Psoralea_, one-way
analyses were unnecessary.

For both _Cassia_ _fasciculata_ and _Desmodium_ _sessilifolium_
the interaction in the two-way analysis of variance was sig-
nificant (Table I, and Figures 2 and 3). It was therefore
necessary to perform a one-way analysis of variance on the
data from both species.

In Table II, the effect of age at each increment of tem-
perature is presented for both _Cassia_ and _Desmodium_. The
Scheffé test indicated that at $15^{o}C$ the amount of acetylene
reduced by Cassia was significantly greater at eight weeks
than at four weeks, but was not significantly greater at
twelve weeks than at eight weeks. At $22^{o}C$ Cassia reduced
significantly more acetylene at eight weeks than at four or
twelve weeks. At $30^{o}C$ there was no significant difference
in the amounts of acetylene reduced by _Cassia_ at four, eight,
or twelve weeks. At each of the temperature regimes ($15^{o}C$,
$22^{o}C$, and $30^{o}C$) the amount of acetylene reduced by _Desmodium_
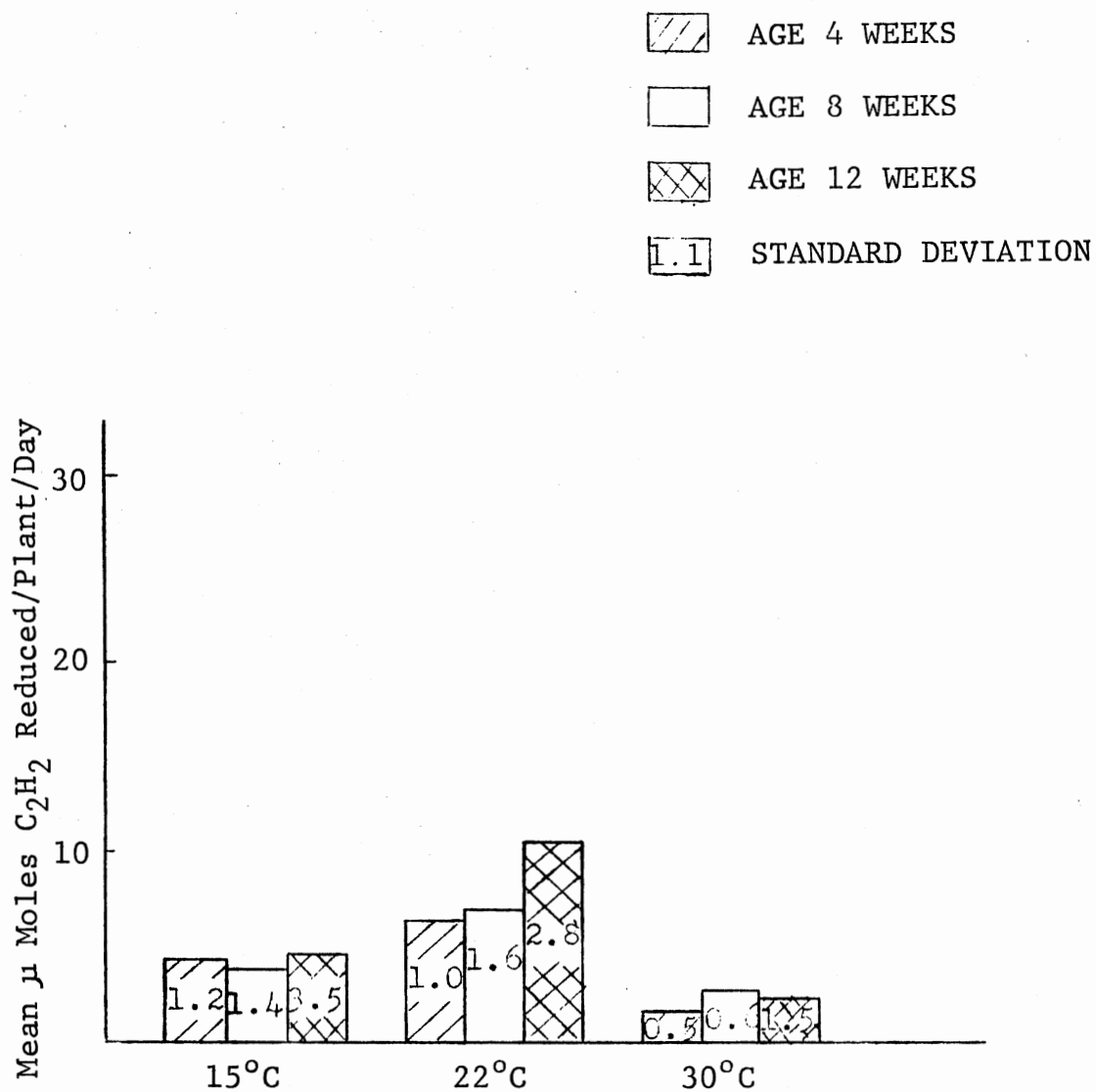
Figure 1. C_2H_2 Reduced by <u>Psoralea</u> <u>tenuiflora</u>
in μ Moles per Plant per Day at
Varying Age and Temperature
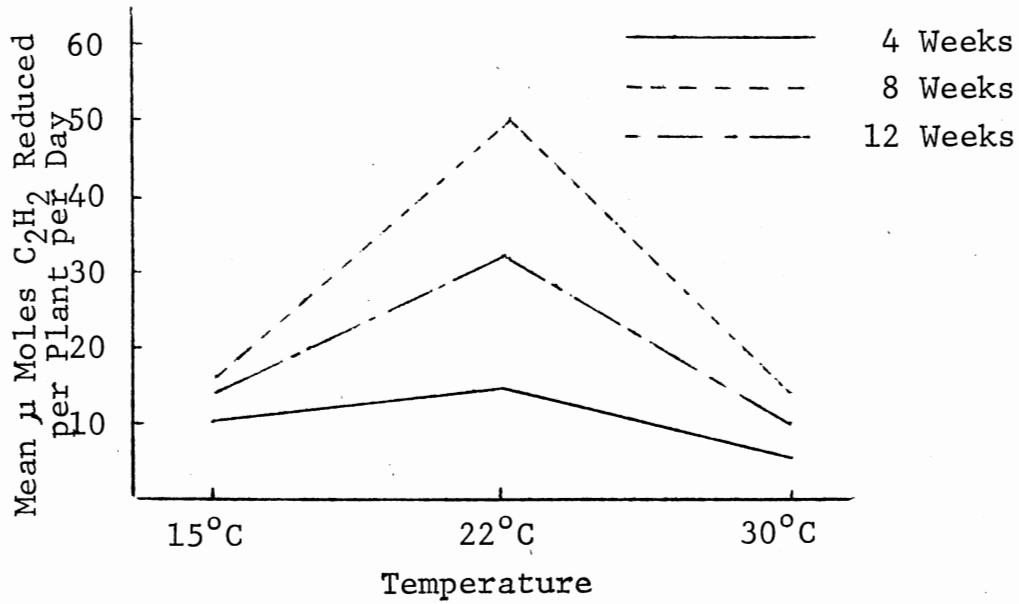
Figure 2. _Cassia faciculata_. Time x Temperature Interaction
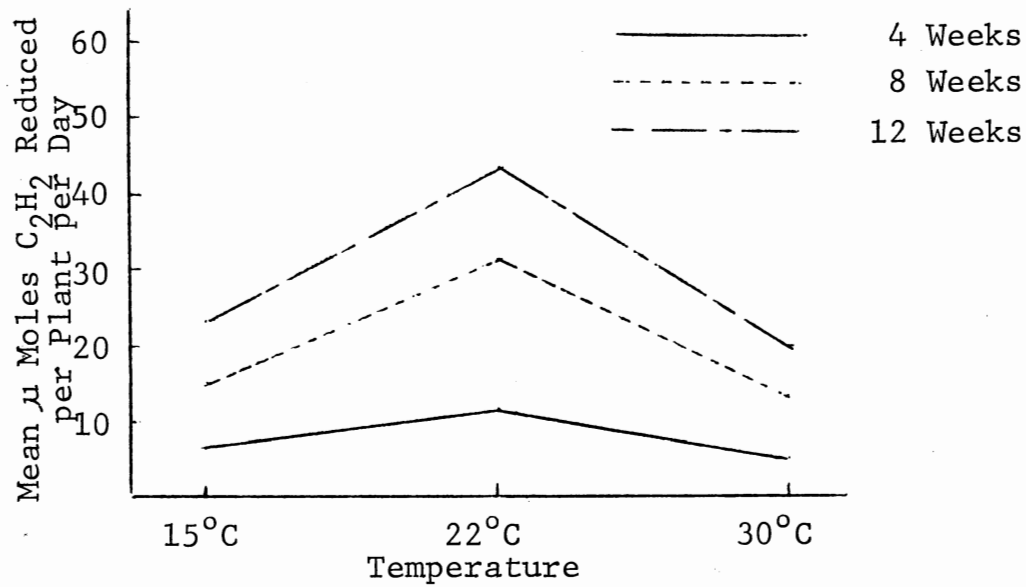
Figure 3. _Desmodium sessilifolium_. Time x Temperature Interaction

TABLE II

SUMMARY TABLE FOR EFFECT OF AGE AT EACH INCREMENT OF TEMPERATURE
FOR CASSIA FASCICULATA AND DESMODIUM SESSILIFOLIUM WHEN
WATERED WITH A NITROGEN-FREE NUTRIENT SOLUTION

Plant	Temperature	Mean Square (Age)	Mean Square (Error)	F Value[b]	Mean C_2H_2 Reduction[a]
Cassia fasciculata	15°C	53.32	4.05	13.16*	10.2 - 4 14.0 -12 16.7 - 8
	22°C	1492.30	29.65	50.33*	15.5 - 4 50.0 - 8 32.0 -12
	30°C	92.93	14.48	6.42	6.2 - 4 14.8 - 8 10.3 -12
Desmodium sessilifolium	15°C	320.52	6.77	47.32*	7.3 - 4 16.2 - 8 23.3 -12
	22°C	1339.76	7.12	188.10*	11.5 - 4 31.4 - 8 44.0 -12
	30°C	302.79	10.67	28.37*	5.0 - 4 15.3 - 8 20.3 -12

[a]Means are rank-ordered with number following dash indicating age in weeks. Vertical lines indicate means for which no pairwise difference exceeded the appropriate Scheffé critical difference.

[b]In all cases above, $df_{num}=2$, $df_{den}=12$

*$p < .01$

was significantly greater at eight weeks than at four weeks, and at twelve weeks than at eight weeks.

Based on the results of Table III and Figures 1, 4, and 5, all three species reduced more acetylene at 22°C than at 15°C or 30°C. For both Cassia and Desmodium there was no significant difference in the amount of acetylene reduced at 15°C and 30°C when assayed at either age eight or twelve weeks.

Nutrient and Age Effects

Section two deals with the relationship between nutrients and age as they affect acetylene reduction in the three species. The mean number of μ moles of acetylene reduced per day for each species at ages four, eight, and twelve weeks, and when supplied with no phosphorus as compared to a complete nutrient solution was determined (Appendix C).

The three-way interaction (Appendix D) again indicated that the combined effects of any two variables were different at each increment of the third variable. By use of the three-way analysis alone, again it was not possible to draw general conclusions concerning the effects of one or two variables over all increments of the third variable. Since the three-way interaction was significant which indicated that time and nutrient status was different for each plant species, a two-way analysis of variance was conducted (Table IV). The results of the two-way analysis of variance

TABLE III

SUMMARY TABLE FOR EFFECT OF TEMPERATURE AT EACH AGE INCREMENT FOR
CASSIA FASCICULATA AND DESMODIUM SESSILIFOLIUM WHEN WATERED
WITH A NITROGEN-FREE NUTRIENT SOLUTION

Plant	Age	Mean Square (Temp.)	(Error)	F Value	Mean C_2H_2 Reduction
Cassia fasciculata	4 Weeks	108.77	1.81	60.18[*b]	15.5 -22° [a] 10.2 -15° 6.2 -30°
	8 Weeks	1961.90	22.83	85.93[*]	50.0 -22° 16.7 -15° 14.8 -30°
	12 Weeks	670.33	23.55	28.47[*]	32.0 -22° 14.0 -15° 10.3 -30°
Desmodium sessilifolium	4 Weeks	54.32	6.23	81.14[*]	11.5 -22° 7.3 -15° 5.0 -30°
	8 Weeks	411.31	9.11	45.15[*]	31.4 -22° 16.2 -15° 15.3 -30°
	12 Weeks	832.06	14.84	56.08[*]	44.0 -22° 23.3 -15° 20.3 -30°

[a]Means are rank-ordered with number following dash indicating temperature in degrees C. Vertical lines indicate means for which no pairwise differences exceeded the appropriate Scheffe' critical difference.

[b]In all cases above, $df_{num}=2$, $df_{den}=12$.

[*]$p < .01$
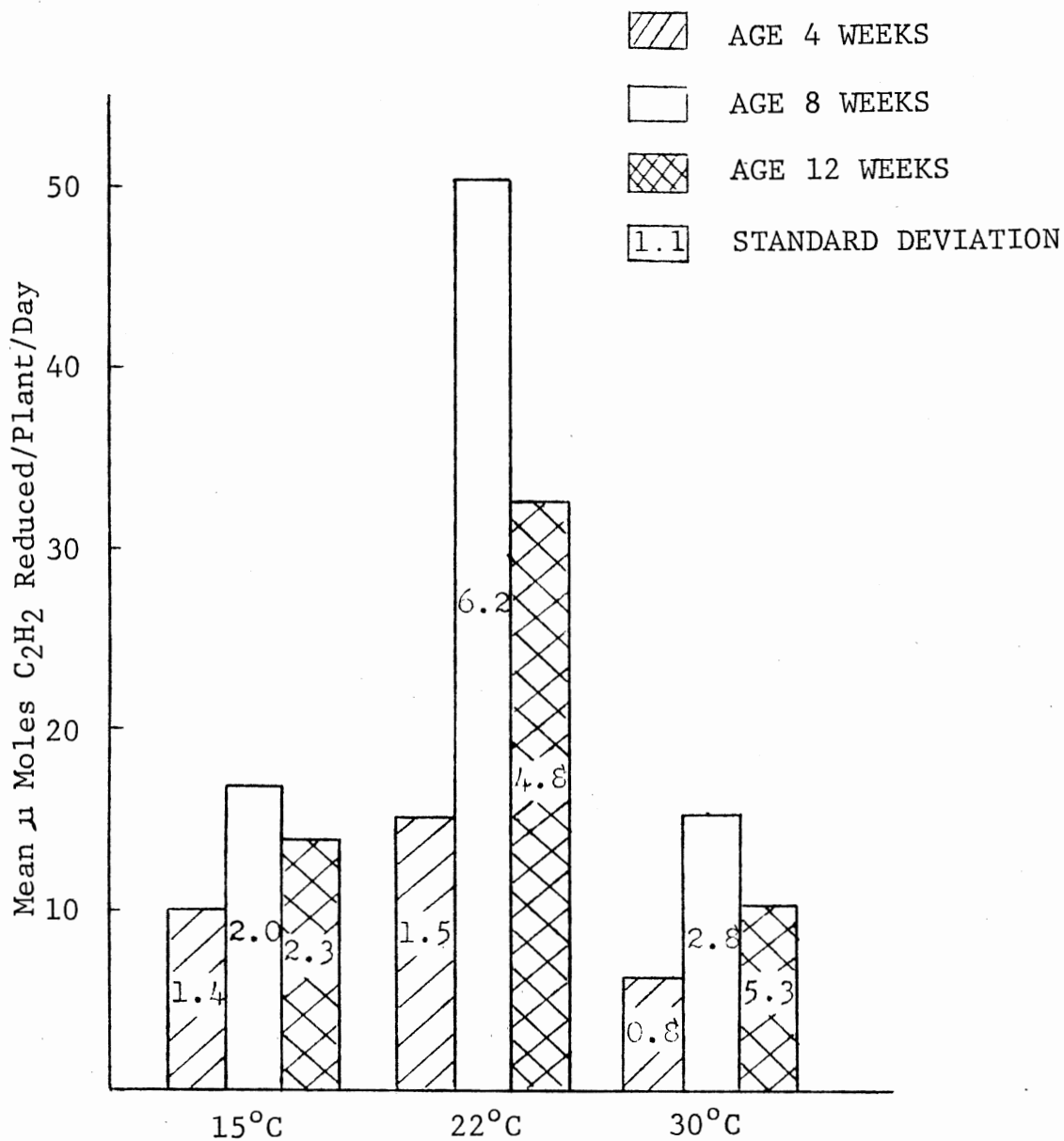
Figure 4. C_2H_2 Reduced by <u>Cassia</u> <u>fasciculata</u>
in μ Moles per <u>Plant</u> per <u>Day</u> at
Varying Age and Temperature
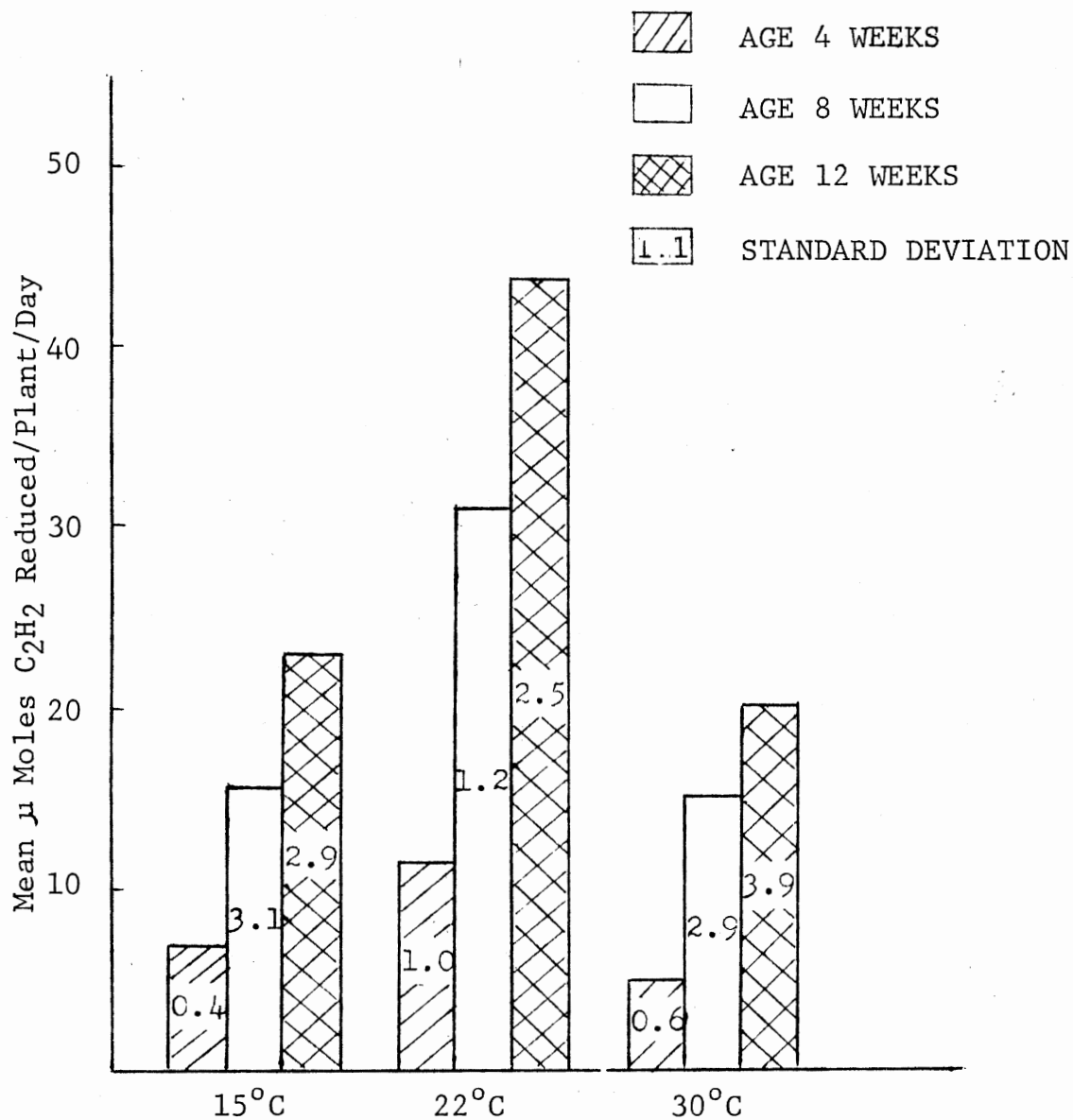
Figure 5.   C₂H₂ Reduced by Desmodium sessilifolium
            in μ Moles per Plant per Day at Vary-
            ing Age and Temperature

TABLE IV

ANALYSIS OF VARIANCE SUMMARY TABLE FOR NUTRIENT
MAKE-UP AND AGE FOR EACH PLANT SPECIES

	Effect						
Plant	Nutrient		Age		Nut. x Age		Error
	Mean Square	F Value[a]	Mean Square	F Value	Mean Square	F Value	Mean Square
Psoralea tenuiflora	179.42	99.34*	15.91	8.81*	9.18	5.69*	1.81
Cassia fasciculata	3897.53	324.86*	748.59	62.40*	395.27	32.95*	12.00
Desmodium sessilifolium	3088.31	864.28*	632.64	177.05*	372.99	104.38*	3.57

[a]Appropriate degrees of freedom for all computed F values are: $df_{num}=2$, $df_{den}=36$.

*p < .01

indicated that the age x nutrient interaction was statistically significant for all three of the plant species investigated.

A one-way analysis of variance was done to examine the effects of age at each nutrient matrix, and the effects of nutrients at each increment of time. As can be observed in Table V, the Scheffe' test revealed that no significant differences existed between the capacity of the three plant species to reduce acetylene when the plants were provided with a complete nutrient solution, or a phosphorus-free nutrient solution. The results were the same for plants at all ages (four, eight, and twelve weeks). Table V verifies that in every case, regardless of plant species or phenophase, a statistically significant increase in acetylene reduction occurred in plants which received a nitrogen-free nutrient solution as compared to plants which received a complete nutrient solution, or a phosphorus-free solution. This is also illustrated in Figures 6 and 7.

An examination of Table VI indicates that when Psoralea was watered with a nitrogen-free nutrient solution and assayed at the three age increments, based on the Scheffe' test no statistically significant differences in acetylene reduction occurred. Similar results were obtained when Psoralea was watered with a phosphorus-free nutrient solution. When watered with a complete nutrient solution, Psoralea reduced significantly more acetylene at twelve weeks

TABLE V

SUMMARY TABLE FOR EFFECT OF NUTRIENT MATRIX AT EACH AGE
INCREMENT FOR PSORALEA TENUIFLORA, CASSIA FASCICULATA,
AND DESMODIUM SESSILIFOLIUM, AT A TEMP. OF 22°C

Plant	Age (Weeks)	Mean Square (Nut.)	(Error)	F Value[b]	Mean C_2H_2 Reduction
Psoralea tenuiflora	4	44.66	0.37	121.58*	6.7 -N[a] 1.7 C 1.4 -P
	8	32.37	1.35	24.00*	7.0 -N 3.4 C 2.1 -P
	12	120.75	3.70	32.62*	10.7 -N 4.2 C 1.1 -P
Cassia fasciculata	4	255.63	1.00	256.64*	15.5 -N 3.8 C 2.5 -P
	8	3089.32	22.25	138.85*	50.0 -N 7.6 C 6.4 -P
	12	1343.12	12.75	105.35	32.0 -N 5.5 C 2.0 -P
Desmodium sessilifolium	4	140.84	1.26	111.74*	11.5 -N 2.9 C 1.8 -P
	8	1173.24	3.83	305.97*	31.4 -N 5.4 C 4.4 -P
	12	2520.21	5.63	447.93*	44.0 -N 7.8 C 2.8 -P

[a]Means are rank-ordered with number following dash indicating
nutrient (N=no nitrogen; C=complete nutrients; P=no phos-
phorus). Vertical lines indicate means for which no pair-
wise difference exceeded the appropriate Scheffé critical
difference.

[b]In all cases above, $df_{num}=2$, $df_{den}=12$

*$p < .01$
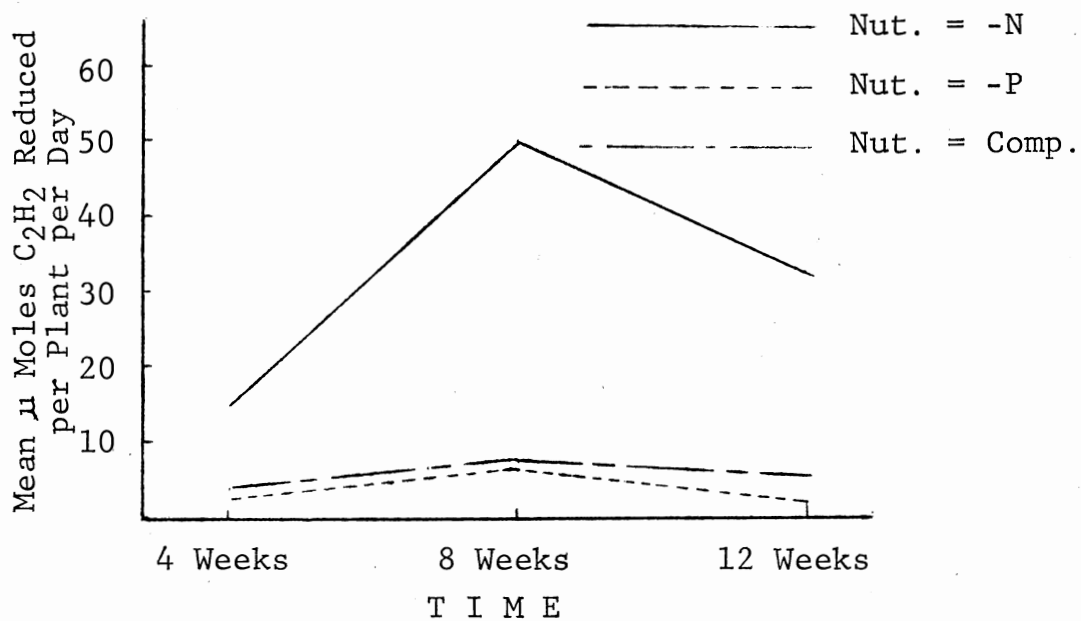
Figure 6. <u>Cassia</u> <u>fasciculata</u>. Time x
Nutrient Interaction
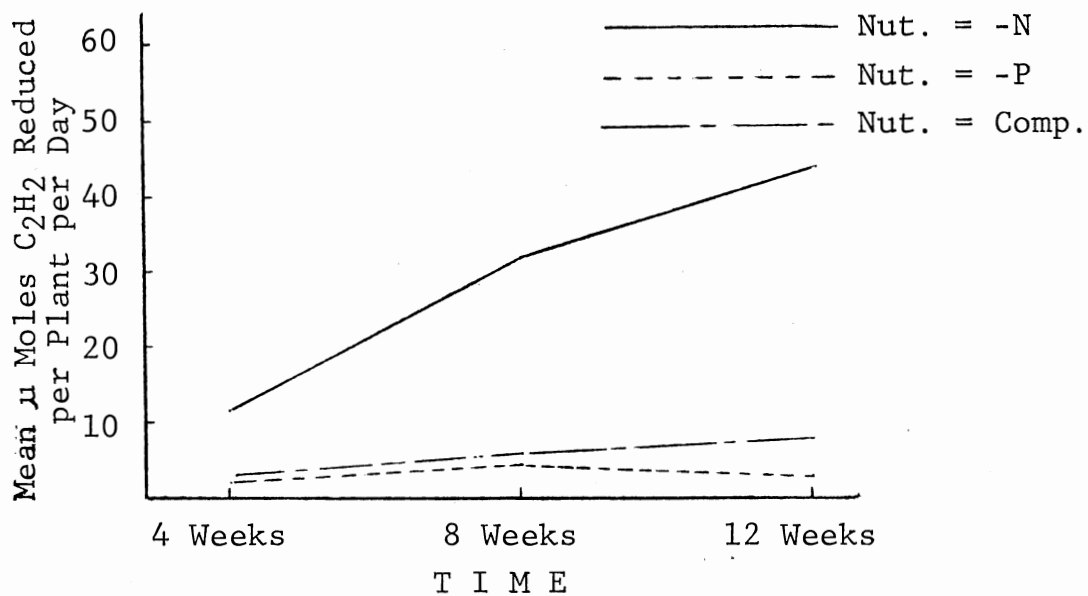
Figure 7. <u>Desmodium</u> <u>sessilifolium</u>. Time
x Nutrient Interaction.

TABLE VI

SUMMARY TABLE FOR EFFECT OF AGE AT EACH NUTRIENT MATRIX
FOR PSORALEA TENUIFLORA, CASSIA FASCICULATA, AND
DESMODIUM SESSILIFOLIUM AT A TEMP. OF 22°C

Plant	Nutrient	Mean Square (Age)	(Error)	F Value[b]	Mean C_2H_2 Reduction
Psoralea tenuiflora	-N	24.67	4.14	5.97	6.7 – 4[a] 7.0 – 8 10.7 –12
	-P	1.41	0.65	2.15	1.4 – 4 2.1 – 8 1.1 –12
	C	8.20	0.63	13.04*	1.7 – 4 3.4 – 8 4.2 –12
Cassia fasciculata	-N	1492.30	29.65	50.33*	15.5 – 4 50.0 – 8 32.0 –12
	-P	28.90	1.91	15.15*	6.4 – 8 2.5 – 4 2.0 –12
	C	17.92	4.43	4.04	3.8 – 4 7.6 – 8 5.5 –12
Desmodium sessilifolium	-N	1339.76	7.12	188.10*	11.5 – 4 31.4 – 8 44.0 –12
	-P	8.60	1.51	5.70	1.8 – 4 4.4 – 8 2.8 –12
	C	30.26	2.09	14.48*	2.9 – 4 5.4 – 8 7.8 –12

[a]Means are rank-ordered with number following dash indicating age in weeks. Vertical lines indicate means for which no pairwise difference exceeded the appropriate Scheffé critical difference.

[b]In all cases above, $df_{num}=2$, $df_{den}=12$

*$p < .01$

than at four weeks, but not more at twelve weeks than at
eight weeks.

When watered with a nitrogen-free nutrient solution,
Cassia reduced significantly more acetylene at eight weeks
than at four weeks, and also more at eight weeks than at
twelve weeks (Table VI). When Cassia was watered with a
phosphorus-free nutrient solution, significantly more acety-
lene was reduced at eight weeks than at four weeks, but no
statistically significant difference at twelve weeks from
four weeks. No statistically significant differences were
found when a complete nutrient solution was added to Cassia
and assayed at four, eight, and twelve weeks.

When exposed to a nitrogen-free solution, Desmodium
reduced significantly more acetylene at eight weeks than at
four weeks, and more at twelve weeks than at eight weeks
(Table VI). There were no statistically significant differ-
ences in acetylene reduction at any of the age increments
when Desmodium was watered with a phosphorus-free nutrient
solution. When a complete nutrient solution was employed,
Desmodium reduced significantly more acetylene at twelve
weeks than at four weeks, but not more at twelve weeks than
at eight weeks.

An examination of Figures 8 and 9 indicates that both
Cassia and Desmodium reduce more acetylene at all three age
increments when provided with a nutrient solution lacking
nitrogen than when provided with a complete nutrient solu-
tion, or one which lacks phosphorus. Figure 10 reveals that
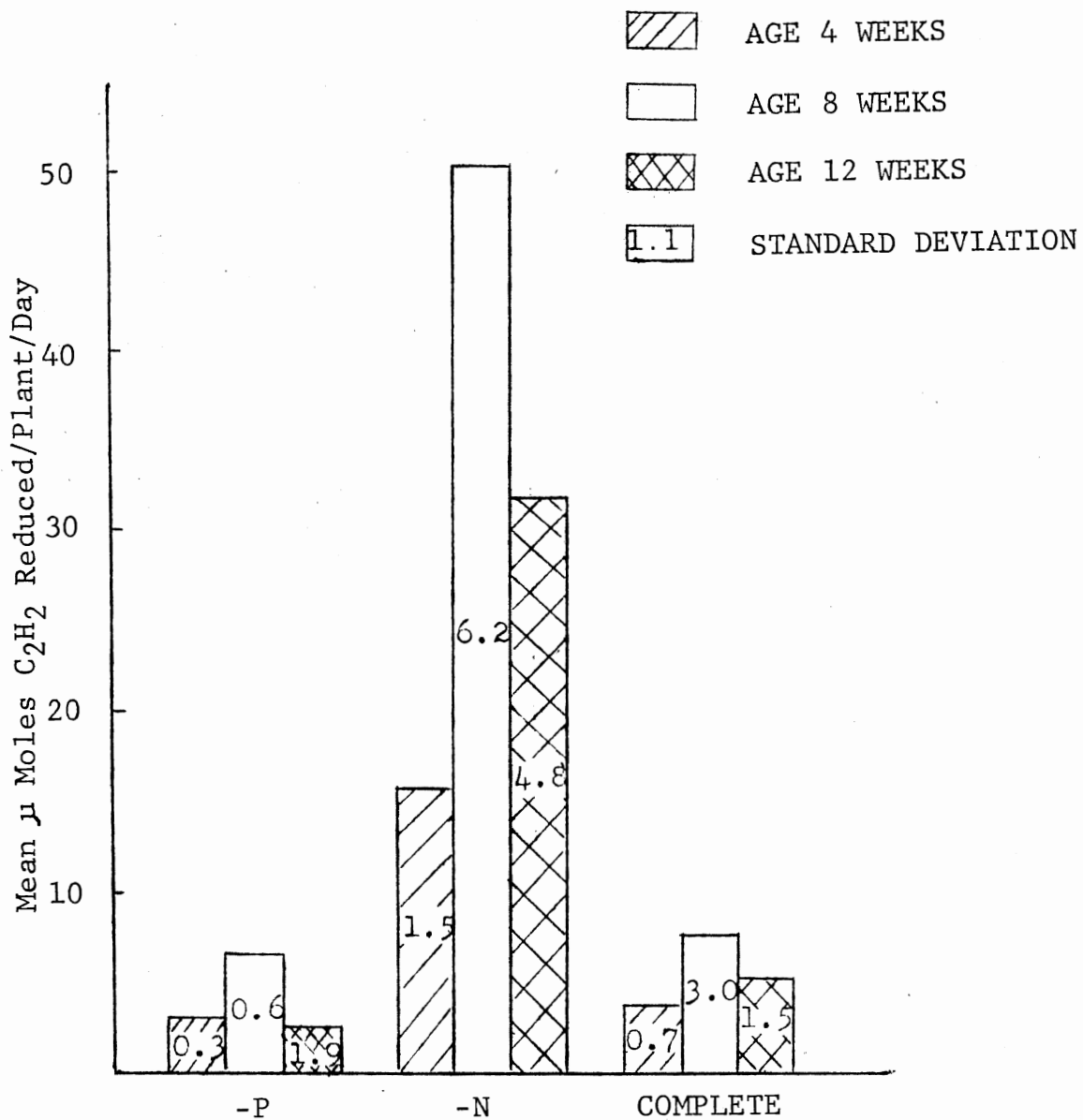
Figure 8. C_2H_2 Reduced by <u>Cassia</u> <u>fasciculata</u> in μ Moles per <u>Plant</u> per Day at Varying Age and Nutrient Make-up
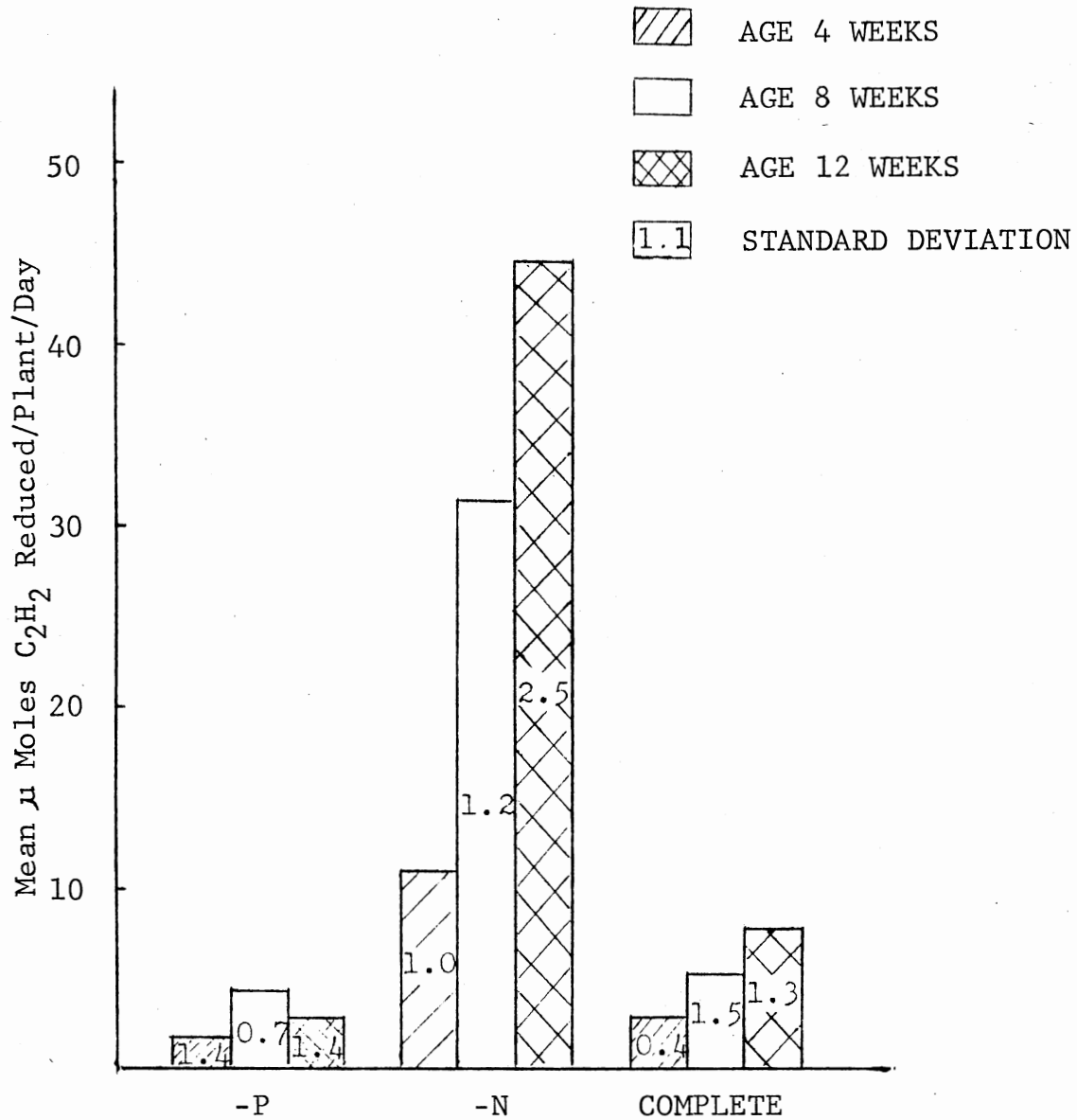
Figure 9. C_2H_2 Reduced by <u>Desmodium sessilifolium</u> in μ Moles per Plant per Day at Varying Age and Nutrient Make-up
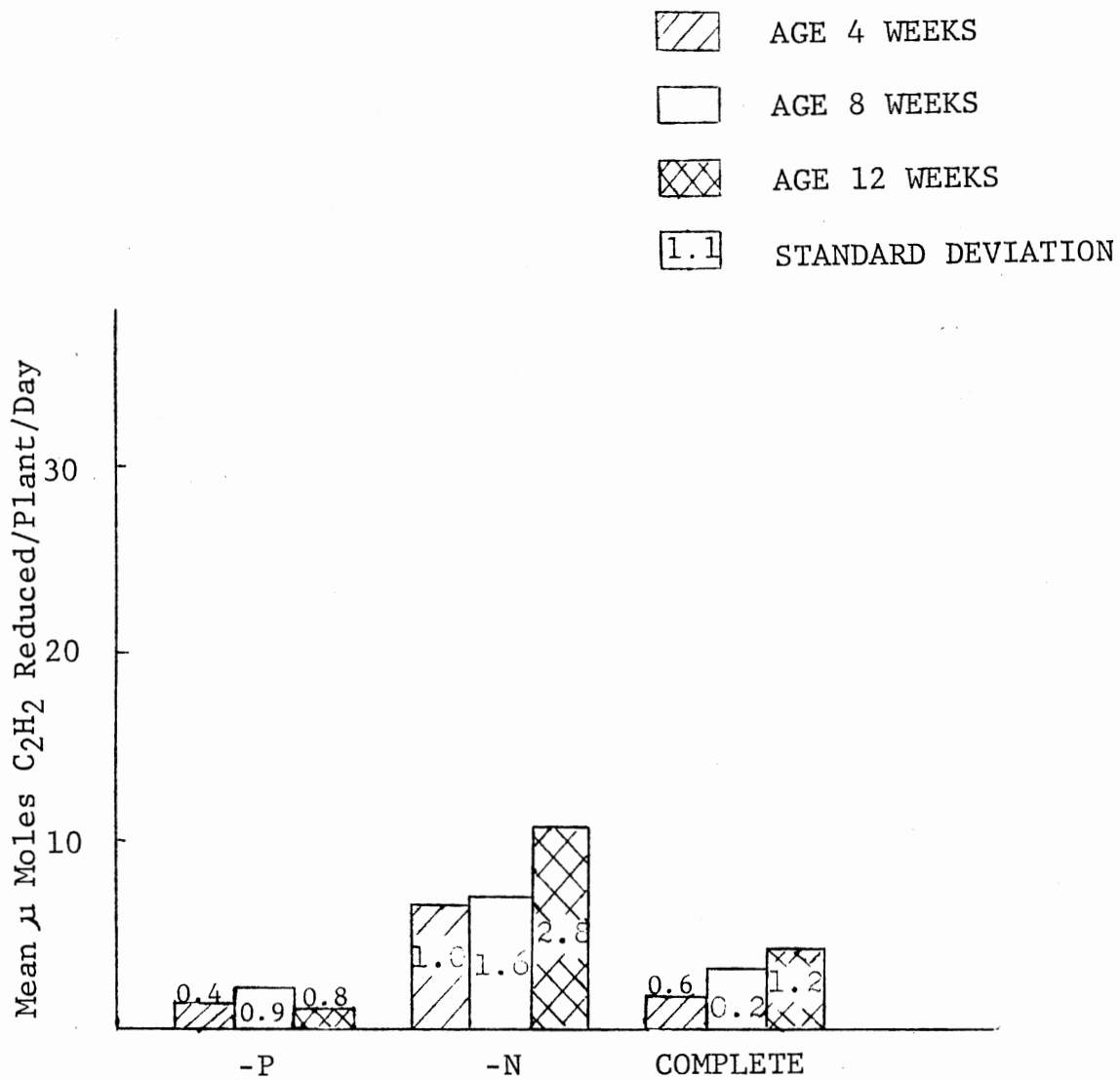
Figure 10. C_2H_2 Reduced by <u>Psoralea tenuiflora</u> in μ Moles per Plant per Day at Varying Age and Nutrient Make-up

essentially the same pattern occurs in _Psoralea_ but the dif-
ferences are much less pronounced.

CHAPTER VI

DISCUSSION

Of the legumes studied (<u>Psoralea</u> <u>tenuiflora</u>, <u>Cassia</u> <u>fasciculata</u>, and <u>Desmodium</u> <u>sessilifolium</u>), <u>Psoralea</u> reduced the least amount of acetylene at all ages (four, eight, and twelve weeks), at all nutrient regimes (complete nutrient matrix, phosphorus-free matrix, and nitrogen-free matrix), and at all temperatures (15°C, 22°C, and 30°C) investigated. An inspection of the root systems revealed that <u>Psoralea</u> produced fewer nodules, and that the nodules were generally small compared to those which were produced by either <u>Cassia</u> or <u>Desmodium</u>. It seems quite probable that <u>Psoralea</u> did not become effectively nodulated. After formation of the sixth trifoliate leaf, <u>Psoralea</u> appeared to cease, or drastically reduce growth, but the plants did persist throughout the twelve week period.

Lofton (1976) reported that <u>Psoralea</u> <u>tenuiflora</u> when growing in the greenhouse did not survive beyond the sixth or seventh week. Becker and Crockett (1976) state that growth of <u>Psoralea</u> <u>agrophylla</u> stopped after formation of the third or fourth trifoliate leaves. In its natural habitat, <u>Psoralea</u> <u>tenuiflora</u> grows luxuriantly and usually reaches anthesis in early summer. It seems that more research is

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necessary before a satisfactory explanation for the rather poor growth pattern of Psoralea under controlled conditions can be established.

When assayed at age four weeks, Cassia reduced relatively small quantities of acetylene. At four weeks the plants were small, but tremendous growth took place between four and eight weeks. At age eight weeks, Cassia reduced more acetylene than either Psoralea or Desmodium. At twelve weeks Cassia exhibited a marked decrease in the amount of acetylene reduced (Figure 6). Flowering and fruiting took place in Cassia at age eight to twelve weeks. Hardy and Havelka (1976) found that in some legumes the reproductive sinks compete with nodules for available photosynthate. Sprent (1976) reported a decrease in nitrogen fixation in peas and beans during pod development. It is possible that the decrease in acetylene reduction by Cassia at age twelve weeks was due to flowering and fruiting prior to the twelve-week assay.

While Desmodium reduced less acetylene at age eight weeks than did Cassia, at age twelve weeks Desmodium reduced significantly more acetylene than Cassia. There was, however, a slight decrease in the accelerated rate of acetylene reduction by Cassia from eight weeks to twelve weeks as can be observed in Figure 7. This could have been due to the approaching flowering and fruiting periods, or perhaps due to injury suffered from insect infestation at age seven to twelve weeks.

In all three species, significantly more acetylene was reduced at a temperature of 22°C than at temperatures of 15°C or 30°C. Since only the three above temperatures were investigated, it cannot be stated that 22°C is the optimum for acetylene reduction in the three species. It appears, however, that the optimum temperature lies in the area of 22°C rather than at 15°C or 30°C. During the growing season the soil surrounding the roots of the three legumes is usually nearer a temperature of 22°C than at 15°C or 30°C. Soil temperature may well provide a partial explanation for the distribution patterns found in the three species.

In this study, the effect of minerals on the ability of Cassia and Desmodium to reduce acetylene has been shown to be quite pronounced. The largest quantity of acetylene was reduced when plants were provided with a nitrogen-free nutrient solution. When ample quantities of available nitrogen were added, acetylene reduction by Cassia and Desmodium decreased significantly. The complete explanation for this phenomenon is not known. If adequate quantities of nitrogen are added, the plant has no need for the presence of Rhizobium, but in the absence of available nitrogen the presence of Rhizobium in the nodules on the roots is the only means of survival for the plant.

When watered with a phosphorus-free nutrient solution both Cassia and Desmodium reduced significantly less acetylene than when watered with a nitrogen-free nutrient solution. In the absence of phosphorus, nucleic acid synthesis

no doubt was reduced as well as synthesis of ATP (Postgate, 1974b). With decreasing amounts of these and other organic constituents containing phosphorus, photosynthesis would be expected to decrease and therefore less carbohydrate would be translocated to the roots. Nodular development, and Rhizobium activity in nodules which had developed would decrease (Pate, 1976).

During the study it was extremely difficult to control some of the variables. The greenhouse temperature, for example, varied somewhat because of the unusually cool winter experienced here in Oklahoma. What effect this had on the outcomes of the study is not known. At age seven weeks the plants became mildly infested with Drosophila spp. Eggs were laid by the Drosophila on the soil surface in the pots and the larvae presumably burrowed into the potting medium. Insecticide (Diazinon 50W) had to be applied to the soil in order to control the insects. What effect the larvae had on the roots of the plants, and the effect of the insecticide on Rhizobium in the nodules on the roots of the plants is also not known.

It appears that there are several areas which should be investigated in future studies. An attempt should be made to determine if the patterns which were established using the three legume species in this study occur in other legumes. An extension of the phenophase before assaying could prove to be of value. For example, it would be interesting to learn what effect a sixteen, twenty, or twenty-four week

growth period would have on the capacity of certain legumes to reduce acetylene. The effects of other minerals on the nitrogen-fixing capabilities of legumes could prove to be of great value. Perhaps other strains of _Rhizobium_ could establish a more effective symbiotic relationship with the legume plants.

CHAPTER VII

SUMMARY

The ability of Psoralea tenuiflora, Cassia fasciculata, and Desmodium sessilifolium to reduce acetylene has been shown to be dependent on the make-up of the nutrient solution with which they were watered. When provided with a nitrogen-free nutrient solution, all three species of legumes reduced significantly more acetylene than when watered with a complete nutrient solution, or a phosphorus-free nutrient solution.

The acetylene-reducing capacity of Desmodium was found to increase progressively with age: more acetylene was reduced at age eight weeks than at four weeks, and more at twelve weeks than at eight weeks. Cassia reduced more acetylene at eight weeks than at four weeks, but not more at twelve weeks than at eight weeks. With Psoralea, age had no significant effect on the ability to reduce acetylene when watered with a nitrogen-free or a phosphorus-free nutrient solution. Based on this investigation, it appears that the age of plants usually has a significant effect on their capacity to reduce acetylene.

The effect of temperature on the ability of the three species to reduce acetylene has been shown to be a

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significant factor. More acetylene was reduced when plants were subjected to a temperature of $22^{o}C$ than at $15^{o}C$ or $30^{o}C$.

As with most investigations, it appears that as many or more problems arose as were solved during the progress of the study. This investigation supports the premise that nitrogen fixation in some leguminous plants is severely affected by temperature changes, by nutrient availability, and by the phenophase of the plants themselves.

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APPENDICES

APPENDIX A

MEAN NUMBER OF μ MOLES OF C_2H_2 REDUCED PER SPECIES
PER DAY AT AGES 4, 8, AND 12 WEEKS, AT
TEMPERATURES OF 15°C, 22°C, AND 30°C
WHEN SUPPLIED WITH A NITROGEN-FREE
NUTRIENT SOLUTION

Species	Age in Weeks	15°C	22°C	30°C
Psoralea tenuiflora	4	4.5[*]	6.7[*]	1.6[*]
	8	4.1	7.0	2.6
	12	4.8	10.7	2.4
Cassia fasciculata	4	10.2	15.2	6.2
	8	16.7	50.1	14.8
	12	14.0	32.0	10.3
Desmodium sessilifolium	4	7.3	11.5	5.0
	8	16.1	31.5	15.3
	12	23.3	44.0	20.3

[*]All values based on five replications.

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APPENDIX B

ANALYSIS OF VARIANCE SUMMARY TABLE FOR PLANT x TEMPERATURE x AGE

Source	Degrees of Freedom	Mean Square	F Value
Plant	2	3017.28	319.41[*]
Temperature	2	2697.44	285.56[*]
Age	2	1547.56	163.87[*]
Plant x Temperature	4	311.42	32.97[*]
Plant x Age	4	605.54	64.10[*]
Temperature x Age	4	233.64	24.73[*]
Plant x Temp. x Age	8	100.46	10.64[*]
Error	108	9.44	

[*]$p < .01$

APPENDIX C

MEAN NUMBER OF μ MOLES OF C_2H_2 REDUCED PER SPECIES
PER DAY AT AGES 4, 8, AND 12 WEEKS WHEN PLANTS
WERE SUPPLIED WITH A COMPLETE Vs A
PHOSPHORUS-DEFICIENT SOLUTION

Species	Age in Weeks	No Phosphorus	Complete Nutrients
Psoralea tenuiflora	4	1.4[*]	1.7[*]
	8	2.1	3.4
	12	1.1	4.2
Cassia fasciculata	4	2.5	3.8
	8	6.4	7.6
	12	2.0	5.5
Desmodium sessilifolium	4	1.8	2.9
	8	4.4	5.4
	12	2.8	7.8

[*]All values based on five replications.

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APPENDIX D

ANALYSIS OF VARIANCE SUMMARY TABLE FOR
PLANT x NUTRIENT x AGE

Source	Degrees of Freedom	Mean Square	F Value
Plant	2	1217.47	210.21*
Nutrient	2	5741.61	991.37*
Age	2	855.55	140.82*
Plant x Nutrient	4	711.83	122.91*
Plant x Age	4	290.80	50.21*
Nutrient x Age	4	432.43	74.67*
Plant x Nut. x Age	8	172.51	29.79*
Error	108	5.79	

*$p < .01$

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