NITROGEN FIXATION IN SELECTED PRAIRIE LEGUMES AS RELATED TO SUCCESSION

By

S. MARGUERITE LOFTON

Bachelor of Science in Education
University of Tulsa
Tulsa, Oklahoma
1968

Master of Science
Oklahoma State University
Stillwater, Oklahoma
1972

Submitted to the Faculty of the Graduate College
of the Oklahoma State University
in partial fulfillment of the requirements
for the Degree of
DOCTOR OF PHILOSOPHY
July, 1976

NITROGEN FIXATION IN SELECTED PRAIRIE

LEGUMES AS RELATED TO SUCCESSION

Thesis Approved:

J. J. Crockett
Thesis Adviser

John E. Thomas

Lester W. Reed

Eddie Basler

Glenn W. Todd

Norman N. Durham
Dean of the Graduate College

ACKNOWLEDGMENTS

The author wishes to express her appreciation to Dr. Jerry J. Crockett for his guidance and advice during the course of this investigation and for helpful criticism in the preparation of this manuscript.

Appreciation is also extended to Drs. Eddie Basler, Glenn W. Todd, John E. Thomas and Lester Reed for their high standards, assistance and encouragement throughout this study.

Special thanks is due Dr. Harry Gearhart for the advice, materials and much time spent in assistance with the gas chromatography used for analysis of nitrogen fixation.

Drs. Robert Ahring and J. Q. Lynd were very helpful in explaining various laboratory techniques in connection with growth of plants and bacteria used in this study.

Dr. J. K. McPherson and other members of the OSU faculty were patient and helpful in offering advice and moral support at various times during this investigation, and their kindness is deeply appreciated.

Finally, most sincere and heartfelt appreciation is due husband, Ray, and children, Adam and Laura, for their patience, assistance, encouragement, sacrifices and active support throughout a most rewarding three years. Without them and their contributions, this study would never have succeeded.

TABLE OF CONTENTS

Chapter Page

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Early stages of succession are characterized by an excess of potential energy and a relatively high energy flow per unit of biomass (Smith, 1971). All ecosystems are said to conserve and concentrate essential elements as mechanisms evolve that promote recycling within the system. Succession is described as a process through which populations accumulate enough nutrients to make possible the rise of succeeding populations (Odum, 1968).

Areas of secondary succession occupied by pioneer species have certain general characteristics. First, the soil surface is usually modified, being loose and unstable or hard and compacted. An absence of shading often results in temperature extremes, relatively rapid evaporation rates and high light intensities. Precipitation may fail to penetrate the soil and runoff will be high. Phases of nutrient deficiency are common due to leaching or nutrient immobilization by reaction with the soil. Often the nutrients have been depleted before abandonment of agricultural practices. Daniel and Langham (1936) found an average decrease of 15 percent in total nitrogen due to cultivation in the top 12 inches of soil in the Oklahoma Panhandle as compared to virgin soils. Chaffin (no date) stated that soils in Oklahoma have lost 30-35 percent of their nitrogen in less than a half-century of cultivation and Swanson (1915) in a study of Kansas soils found that from 22.6 percent to 43.5 percent of the nitrogen, based on the virgin soil content, was lost through cultivation.

Cultivation seems, then, to bring about a decrease in the total nitrogen over a period of time and may also result in decreases in the amounts of other nutrients (Rice, Penfound and Rohrbaugh, 1960).

In order to invade a disturbed area, pioneer plants must be able to tolerate the rigorous conditions found there and a variety of adaptations are used to accomplish colonization.

Many pioneer plants generally produce great numbers of seeds and possess the capacity for variable seed dormancy which reduces the risk of total loss if conditions for establishment are unfavorable following germination. The ability to grow and reproduce early in the spring or late in the fall may give a pioneer species an advantage which will facilitate its persistence in an extreme environment (Bunting, 1960).

In the more stable environment, fewer or less severe challenges will be presented to the organisms characteristic of that ecosystem. Less regulatory activity will be required and energy will be channeled into productivity; i.e., growth and production of offspring (Connell and Orias, 1964). Therefore, in a mature ecosystem there is less waste and less accumulation of energy because the energy flows through more diverse channels. The least amount of energy is needed to maintain one unit of the structure in a mature community (Smith, 1971). A climax state is the final, stable community in a successional series. It is self-perpetuating and is in equilibrium with the physical habitat (Odum, 1953).

In a three-year study of paririe vegetation by Bokhari and Singh (1975) it was found that the major portion of the nitrogen in the system was retained in the soil. A short grass prairie, the Pawnee National Grassland in Colorado; a mixed grass area at the Cottonwood Site in South Dakota; and a tallgrass prairie at the Osage Site in Oklahoma were examined. All were under long term protection. Only 4-8 percent of the nitrogen was found to reside in the biological material with over 90 percent retained in the soil. The amount in the soil remained constant over the three-year term of the study and it was concluded that most of the nitrogen available to plants must come from the biological turnover.

In grassland communities where there is no harvested output such as meat products or hay, a very low rate of nitrogen fixation would be adequate to maintain the nitrogen status. This rate might be on the order of one gram per meter square per year of fixed nitrogen. In a study of a climax prairie near Columbia, Missouri, the majority of the nitrogen content of the system was located in the soil with only a small fraction (less than one percent) in the biological components. In this case, it was suggested that most of the nitrogen required for production of biological materials came from the rapid biological turnover rate which was said to be weekly for microorganisms and four years for roots. These estimates were based on an evaluation of annual increments of below ground plant parts, total organic matter and the time factor for the humus level to reach equilibrium under existing conditions of primary production and mineralization (Dahlman, Olson and Doxtader, 1969).

It may be possible to discover a trend in efficiency of nitrogen fixation as related to successional status of various prairie legumes and to gather evidence concerning whether pioneer plants are able to survive and thrive in areas of nutrient deficiency because they are relatively efficient in fixing atmospheric nitrogen. Species nearer the climax condition may be less efficient due to the conservation and gradual buildup of nutrients, specifically nitrogen, in the mature ecosystem and the consequent depression of the symbiotic nitrogen fixing complex as described by Hardy et al. (1973). This investigation will seek to determine if there is indeed any measurable difference in nitrogen fixation between various species and if there are any discernable relationships between efficiency and successional status.

CHAPTER II

LITERATURE REVIEW

In succession on glacial moraines it has been shown that the sequence from bare morainic detritus to mature spruce forest takes approximately 250 years. During this period the most significant reaction of the vegetation appears to be the buildup of soil nitrogen (Kershaw, 1973). During succession on sand dunes, the entry of the oak climax vegetation is presumably controlled by the accumulation of soil nitrogen and organic carbon which occurs as a result of modification of the environment by vegetation (Kershaw, 1973).

An investigation of soil development relative to vegetational succession in such diverse systems as mudflows, coastal dunes and glacial till showed a rapid buildup of total nitrogen in each system. This increase occurred during the pioneer and early stages of succession and was attributed to biological nitrogen fixation by such pioneer plants as *Dryas*, *Shepherdia*, and *Alnus* and it was suggested that rapid nitrogen buildup may be characteristic of pioneer communities (Crocker, 1967).

While studying plant succession in areas of glacial retreat in Alaska, W. S. Cooper (1931) noted that among the mosses and horsetails which are first to be established in this harsh environment the species considered most important, *Dryas drummondii*, Rich is found. This species is described as a mat-forming plant which produces seeds that germinate readily. It forms almost circular mats and spreads rapidly to cover the surrounding area beneath a green mantle. It is intolerant of shade and is quickly eliminated by the shrubby stage of succession.

The early pioneers are followed by several species of willow and alder, Alnus spp., which becomes the most characteristic dominant plant of the second, shrub thicket successional stage. Alder spreads locally rather than in a wide-spread way due to the relative immobility of its disseminules and is able to increase its dominance at the expense of the willows in the association because of its taller, denser growth. Sitka spruce, Picea stichensis Carr., finally replaces the shrubs and is joined by two hemlocks, Tsuga heterophylla. Sarg., and T. mertensiana Carr (Cooper, 1931).

Further studies by Cooper in 1939 enforced the early observations of the importance of Dryas drummondii and Alnus sp. to the successional progress in deglaciated areas (Cooper, 1939).

In a paper written in 1927, Elmer Campbell described a study of five locations in various stages of succession in the south and the middle west. On the sites studied, he established that the percentage of native legumes is highest on impoverished soil such as abandoned cotton fields and on sites adjacent to gravel pits. As revegetation progressed, the relative percentage of wild legumes decreased proportionally to the increase of total nitrogen in the soil. Melilotus alba, a wild sweet clover, was said to be the leading revegetation legume in the middle western states and various species of Lespedeza were the leaders in the southern states (Campbell, 1927).

When a study was made of the revegetation of coal stripped areas in Illinois, Melilotus alba was shown to be dominant in areas where the pH was at or near neutral. Growth of this species usually began in the first two years of revegation and dominance was achieved within five years. Several other legumes were among abundant species on the more acid soils, (pH 5.0-6.0). They included *Cassia sp*., and *Amorpha fruticosa* (Croxton, 1928).

Bayberry, *Myrica pennsylvanica*, is a pioneer successional plant of eastern United States coastal communities and postagricultural sites and it is suggested that the success of *M. pennsylvanica* in nitrogen deficient coastal communities and postagricultural sites is due to its ability to fix nitrogen (Morris et al., 1974).

CHAPTER III

SOURCES OF NITROGEN GAIN AND LOSS

Nitrogen differs from other plant nutrients in that almost the whole of the nitrogen reservoir in a soil is in the organic form with only a small proportion of inorganic nitrogen present as ammonium ions and a somewhat larger amount of nitrate ions in soils that are not too acid. The rate of supply of ammonium ions to the soil, which in turn controls the rate of production of nitrates, thus depends both on the rate of decomposition of plant debris and soil humus and on the rate of supply of soluble nitrogen compounds produced by the nitrogen-fixing systems (Russell, 1969).

Sources of nitrogen gain in an abandoned field to which fertilizer is not added include the fixation of dinitrogen which results from man's technological activities; fixation by lightning; by free-living bacteria; by blue-green algae and symbiotic nitrogen fixation. The amount of available nitrogen is regulated by the rate of nitrogen fixation which in turn regulates the rate of increase of organic nitrogenous compounds in soils low in total nitrogen (Rice, 1974).

The biological nitrogen cycle accounts for a turnover of 10^8-10^9 tons of nitrogen annually. The local productivity of soils is dependent upon the turnover rate of the cycle and this depends on the rate of nitrogen fixation in all but the most advanced agricultural communities. In addition to lightning and ultraviolet radiation in the atmosphere, the fixation step is aided by atmospheric pollution by electrical equipment and the internal combustion engine, both of which produce oxides of nitrogen which reach the soil as nitrates in rainfall. While this process may account for as much as .5% of the global nitrogen turnover, biological nitrogen fixation must account for more than 90% of the terrestrial nitrogen in the cycle (Postgate, 1974).

The total contribution to the earth's nitrogen balance by nonleguminous angiosperms has been estimated to be about 5 x 10^6 metric tons/yr by Hardy and Holsten (1972) as cited in Quispel (1974). Akkermans (1973) used the acetylene reduction technique to determine fixation rates ranging from 2 kg/ha/yr for Hippophaë to 56 kg/ha/yr for Alnus glutinosa, both non-leguminous angiosperms. Blue-green algae, probably significant only in rice culture, have been shown to fix an estimated 30 kg/ha/yr of nitrogen by Burns and Hardy (1975). These sources along with all other free living and nonleguminous symbiotic dinitrogen fixing systems are postulated to contribute from 4 x 10^6 metric tons/yr to as much as 95 x 10^6 metric tons/yr to the earth's total nitrogen budget (Quispel, 1974).

Legumes, which have a world-wide distribution, may be responsible for the biological fixation of over 90% (Donald, 1960) of the estimated 175 x 10^6 metric tons of atmospheric nitrogen returned to the soil each year or approximately 140 kg/ha/yr (Burns and Hardy, 1975).

Most of the information concerning fixation rates of legumes is based on studies done on domestic species and similar information concerning native grassland legumes is lacking (Coaldrake, 1962). The total contribution to the pasture nitrogen in soils under subterranean clover in southern Australia is estimated to be between 67-115 kg/ha/yr by Williams (1970). Soybeans have been postulated to fix 94 kg/ha/yr; clovers 104-140 kg/ha/yr; and lucerne 158 kg/ha/yr by Stewart (1966). Erdman (1968) obtained rates of 57 kg/ha/yr for soybeans; 105-220 kg/ha/yr for clovers and 208 kg/ha/yr for lucerne.

Four legumes were tested to measure the amount of nitrogen fixation in a dry-monsoonal area of northwestern Australia over a period of three years. During the second and third years of establishment, guar, *Cyamopsis tetragonoloba*, averaged a fixation rate of 80 kg/ha/hr; Townsville lucerne, *Stylosanthes humilis*, averaged 83 kg/ha/hr; cowpea, *Vigna sinensis*, fixed 60 kg/ha/yr on the average and peanuts, *Arachis hypogaea* cv. Natal averaged 24 kg/ha/yr (Wetselaar, 1967).

Native pasture legumes were estimated to return between 8 and 10 kg/ha/yr to virgin prairie soils in Nebraska. Alway and Pinckney (1909) based this estimate on Kjeldahl analysis of the total nitrogen in harvested plant tops. Becker and Crockett (1976) studied the growth rates, extent of nodulation and nitrogen fixing capacity of five native prairie legumes. The acetylene reduction method and Kjeldahl analysis were used on soil cores from nodulated plants to establish a rate of approximately 2 kg/ha/yr nitrogen fixed by the total grassland flora of a Canadian mixed prairie (Vlassak et al., 1973). Paul et al. (1971) measured nitrogen fixation in grassland in Southern Saskatchewan, Canada and determined the rates to be low; .6-1.8 kg/ha in 28 days in the laboratory and 1 kg/ha/season in the field. They analyzed soil cores and small surface soil samples for nitrogen fixation, using the N^{15} incorporation method and acetylene reduction technique. There were 16 nodulated legume species present and the three most common of these contributed 10 percent of the total nitrogen fixed. These three species, whose abundance is approximately one plant/m^2 are *Vicia americana*, *Thermopsis rhombifolia*, and *Oxytropis sericea*. The remainder of the nitrogen contribution was attributed to blue-green algae and non-leguminous angiosperms.

CHAPTER IV

SOME ENVIRONMENTAL EFFECTS ON NITROGEN FIXATION

According to Lie (1971), symbiotic nitrogen fixation depends on both inherent characteristics of the host plant and on the particular *Rhizobium* associated with it. It is the symbiotic system rather than the plant which is most affected by the environment if combined nitrogen is not limiting.

In order to describe how the environment affects the symbiotic system, it is necessary to discuss briefly some of what is known about the biotic partners and the establishment of the symbiosis. While present day legumes are believed to be tropical in origin and are most abundant in the tropics, they have a world-wide distribution (Stewart, 1966). The *Leguminosae* rank second or third among the flowering plants in number of species contained and of the species examined to date, around 89% have been found to be nodulated (Vincent, 1974). According to Bergerson (1974), *Rhizobium*, the bacterial partner, though not believed to be present in large numbers, is widespread in the soil where it lives as an aerobic saprophyte and, like other rhizosphere-stimulated soil bacteria, is present in greater numbers in the plant root vicinity than in the soil away from the roots. Russell (1961) stated that nodule bacteria can live for as long as 10 years in the absence of a legume host once they have become established.

To initiate the infection process, rhizobia move toward the legume root in response to an attractant produced by the plant. The attractant is as yet unidentified, but is possibly a polysaccharide. The bacteria commonly enter the root by invading root hairs where the plant cell walls are weakened by polygalacturonase produced by the host apparently in response to the bacterial presence on the root surface (Burns and Hardy, 1975). According to Vincent (1974), the infection process is very specific and is affected by the genetic constitution of both partners. Some species including Medicago laciniata and Mimosa caesalpiniaefoliae have quite specific rhizobial requirements while others such as Phaseolus atropurpureus are able to form a symbiosis with a wide range of Rhizobium strains.

Upon entry into the root hair, the bacteria are walled off by invagination of the cell wall, forming a structure called an infection thread which grows down the root hair along with its enclosed bacteria until it reaches the cortex where the rhizobia are generally deposited in polyploid host cells. A cellulose-free membrane which possibly originates on the host cell nucleus or which may be of endoplasmic reticulum origin is synthesized by the host cell. The bacteria are enclosed in the membrane whereupon they increase in number until they completely fill the cell, pushing the host cytoplasm to the outer edges of the cell, in nodules on soybeans and cowpeas. In other plants such as lucerne and clover, the bacterium enlarges to many times its original volume, often almost completely filling the membrane envelope. Nodules are formed when the infection thread stimulates cortical cell division as it grows through the cortex and passes close to the nuclei of the individual cells (Burns and Hardy, 1975).

After the bacteria are deposited in the host cells and proliferation has occurred, they undergo physiological and biochemical changes which enable them to reduce nitrogen. Free-living Rhizobium does not contain the nitrogenase enzyme which catalyzes the reduction of atmospheric nitrogen and, thus, requires combined nitrogen. One of the changes they undergo upon establishment within the host cells is the de novo synthesis of nitrogenase. The production of leghaemoglobin is stimulated by the symbiosis, the heme portion of the molecule being produced by the bacteria while the protein component is evidently a product of the host plant (Dilworth, 1969).

In addition to specificity in the infection process, a complex pattern of specificity related to nitrogen fixation is evident, as discussed in a review by Vincent (1974). While there are probably no naturally occurring rhizobia which cannot fix nitrogen with an appropriate host, their relative efficiency with every host they are able to nodulate varies. Also, no legume host is fully compatible with every species and strain of Rhizobium which forms nodules on its roots. Out of a group of 24 Rhizobium strains which commonly form nodules on species of Pisum, Vicia, Lathyrus and Lens only one strain was effective on all seven hosts tested, effectiveness being defined as the ability of the legume/rhizobium combination to fix nitrogen. It was also shown that seven were effective on only one host and six were effective on none. Evans and Russell (1971) pointed out that further complications arise due to the environmental factors which influence the effectiveness of the Rhizobium-legume association.

One environmental factor, soil pH, has a profound effect on bacterial survival and ability to establish a symbiosis with legumes.

It has been shown that nodule initiation is generally inhibited in an acid medium. While one strain of Rhizobium was shown by Lie (1971) to cause abundant nodulation on pea plants at pH 4.6, the optimum pH for effective nodulation by most of the species and strains of Rhizobium investigated is from 6.5-7.5 (Virtanen, 1963). However, Dart (1974) reviewed the effect of soil factors on the infection and nodulation of legumes and mentioned that some clovers and medics will nodulate at a pH up to 9 or 10.

Lie (1974) reviewed the effects of combined nitrogen on the legume symbiosis. Both nodulation and nitrogen fixation are strongly depressed in the presence of moderate to high levels of combined nitrogen. When Oghoghorie and Pate (1971) examined nitrate effects on field peas, they found that any level of nitrate appears to supress nitrogen fixation. The effect of combined nitrogen can be lessened by increasing photosynthesis or by supplying sugar to the leaves. While there is evidence that the inhibitory effect is due to the C:N ratio established by the addition of combined nitrogen, there may be a more direct effect. Nitrate, nitrite, ammonium and urea all reduced the number and weight of nodules formed and delayed the time of appearance of nodules and nitrate specifically has been shown to reduce the number of root hairs and impair the growth of the infection thread. Although there is generally a reduction in nitrogen fixation when nitrogenous compounds are supplied to the legume plant; the amount of inhibition is dependent upon the concentration and form of the nitrogen compound, the time of application, the specific host and bacterial strain and the growing conditions. Many factors are involved and the complexity of the effects requires much more investigation (Lie, 1974).

Another environmental influence, soil temperature has a complex effect on the legume-Rhizobium system and commonly affects every stage of the formation and function of nodules. The lower limit for nodulation is about 7°C for temperate legumes and tropical species are inhibited below 20°C. Subterranean clover exhibits a marked delay in root hair infection at 7°C but once the infection has occurred, nodulation is accelerated beyond the rate which will occur if the whole process takes place at 19°C, the optimum temperature for the symbiosis in this species. The rate of fixation is low in plants growing at low temperatures but if nodules are formed at favorable temperatures, they will retain their activity when transferred to lower temperatures. There is a slight reduction in activity but it appears that nodule formation is more sensitive to low temperature than nodule efficiency (Lie, 1974).

Both nodule formation and nitrogen fixation are reduced at high temperatures, with the magnitude of the effect again depending on the plant species being investigated. An upper limit of 36°C was established by Gibson (1971), beyond which no nodulation occurred in those species examined. Lie (1974) reviewed high temperature effects and cited several workers who demonstrated that bean nodulation was strongly reduced when the plants were held at 30°C for three days and then returned to the 25°C, the optimum temperature for the symbiosis. In another investigation, beans and peas were found to be completely devoid of nodules when kept at 30°C throughout the growing period. Hardy et al. (1968) found that the temperature of growth had a marked effect on nitrogen fixation. Soybeans grown from one to fourteen days at 30°C had only 10-20% of the nitrogen fixing activity of plants grown at 20°C.

Both the number of nodules formed and their longevity are affected by soil moisture. An optimum moisture content of 60-75% of the water holding capacity of a soil had been determined by Fred et al. (1932) for nodulation of lucerne and soybean and by Habish (1970) for nodulation of Acacia. The amount of nitrogen fixation within the nodules is also strongly affected by soil moisture. Sprent (1971), using the acetylene reduction technique, showed that soybeans immersed in water had a significant reduction in nitrogen fixation efficiency and Minchen and Pate (1975) discovered a similar significant reduction in the ability of peas to fix nitrogen when waterlogging occurred. They attributed this effect to an oxygen deficit. In addition to too much water, a water deficiency also has marked effects on nitrogen fixation. When soybean nodules were dehydrated to 80% of maximum moisture content, the amount of nitrogen reduced, based on acetylene to ethylene conversion, was less than 10 percent of that at maximum water content. Sprent (1971) attributed this to the reduction in the respiratory rate of the plants with a consequent reduction in carbohydrate availability for the symbiosis and to gross structural changes within the nodules. According to Russell (1961), the first effect of drought on field legumes is the shedding of nodules. In the Stillwater area, prairie legumes growing in the field will shed the nodules growing on their roots in the upper levels of the soil as drying occurs with the progression of summer. It is very difficult to find nodulated specimens after the end of June except where they can be found growing in very moist areas such as the edges of woods and around lakes and other bodies of water.

All of the major and minor inorganic elements commonly needed for plant growth are necessary for the legume-Rhizobium symbiosis and certain ones are apparently required in greater amounts for nitrogen fixation than for normal growth of either partner alone. Evans and Russell (1971) reviewed the specific requirements for several nutrients needed for both the initiation of nodulation and for the nitrogen fixation process. The calcium requirement for symbiosis is higher than for either the growth of the plant or of the bacterium alone. It seems to be specifically required for infection of root hairs. Dart (1974) stated that molybdenum is an essential constituent of the nitrate reductase of both legumes and non-legumes and the additional molybdenum needed for the nitrogen fixing process is a part of the component I protein of nitrogenase. This element also exerts an effect on nodulation. More nodules form in molybdenum-deficient conditions but they are less efficient at nitrogen fixation and their structure resembles that of ineffective nodules. According to Evans and Russell (1971) iron is part of both protein components of nitrogenase and is also included in the leghemoglobin molecule which is a necessary constituent of the nitrogen fixation system. A specific requirement for copper has been established but its function is unknown as yet. Another necessary element, cobalt, is essential for the growth of legumes which depend on atmospheric nitrogen and it is believed to be a requirement in several phases of Rhizobium metabolism (Evans and Russell, 1971).

CHAPTER V

SPECIES AND HABITAT DESCRIPTION

Soils and Topography

Payne County is part of the Redbed Plains physiographic region which is an area of gently rolling plains with hills seldom exceeding 100 feet in height (Bruner, 1931). The Stillwater area, with an elevation of approximately 900 feet, is a region dominated by the Renfrow-Zaneis-Vernon soil association underlain by interbedded sandstones and clay beds.

Renfrow soils are brown to reddish-brown silt loam surface soils with very firm, reddish, blocky clay subsoils which are slowly permeable to water. The Vernon (Typic Ustochrepts) soils are limy red clay soils of steep slopes which are very little changed from the red clays themselves and the Zaneis (Udic Argiustolls) soils are brown loam soils with granular, reddish heavy clay loam subsoils (Gray and Galloway, 1959).

Climate

Payne County has a continental climate with pronounced seasonal temperature ranges. The mean date of the last freezing temperature in the spring is April 4, and the first freezing temperature of the fall occurs around October 28 (Curry, 1970).

The summers are characterized by hot, dry winds from the south and west and are preceded by a long, balmy spring and followed by several weeks of cool autumn weather. The high summer temperatures and windy conditions result in rapid evapotranspiration of soil moisture. Although the normal average summer temperature (June-August) is 83^{o}F, the average for the period of this study (1973-1975) was slightly lower being 78.2^{o}F (Climatological Data, 1973, 1974, 1975). The normal winter average is 42.5^{o}F (Curry, 1970).

Annual precipitation averages 33.42 inches in the Stillwater area and around 75% of this falls during the growing season. May is generally the wettest month and January is the driest. The amount of precipitation normally decreases from around 4.24 inches in June to 3.21 inches in August, averaging 3.66 inches for the three summer months (June-August) (Curry, 1970). During the period of this study, the summer averages for 1973, 1974, 1975 were 2.89 inches, 3.26 inches, and 3.32 inches, respectively, all slightly lower than normal (Climatological Data, 1973, 1974, 1975).

Vegetation

The Stillwater area lies in a transition zone between eastern deciduous forest and the tall grass prairie. The dominant prairie species in the region include the grasses *Andropogon gerardii*, *A. scoparius*, *Sorghastrum nutans* and *Panicum virgatum*. The families *Compositae* and *Leguminosae* are abundantly represented in the area along with many other forbs. Several of the legumes will be discussed in detail in another section. The two principal tree dominants in the region are *Quercus stellata* and *Q. marylandica* (Bruner, 1931).

Species Description[1]

Cassia fasciculata is a tall annual which reproduces by seed and commonly inhabits sandy loam soils. Rather leafy, this species is a warm season plant with showy yellow flowers which generally blooms from late June to September. It forms dense stands in old fields and disturbed areas (Pasture and Range Plants, 1955).

This species is generally well nodulated with large, dark red nodules most often attached to the primary root. Nodules disappear during the hot dry season, but new ones are often produced following soaking rains if the soil remains wet for several days. The nodules can often be found at ground level in heavily mulched areas and are seldom found deeper than 30 cm.

Desmanthus illinoensis is a native legume which is a deep-rooted perennial which reproduces from seed. It is adapted to a wide range of soils and habitats, but grows particularly profusely in moist depressions and ditches where it may attain a height of 2-4 feet (Pasture and Range Plants, 1955).

This species blooms from May to August in the Stillwater area. Plants growing in moist areas or in well-mulched areas such as the edge of woods are heavily nodulated even during the driest part of the summer. Nodules are large, numerous and coralloid in form and are spread widely through-out the soil, attached to secondary roots.

Amorpha canescens is a deep rooted, warm-season perennial which reproduces from seed. It reaches a height of 2-4 feet and may consist of one to several basal stems. This plant is found throughout most of

[1]Nomenclature follows that of Waterfall (1969).

the upland prairies, associated with the bluestem grasses. It is common in roadsides and in protected areas and is an important climax species (Pasture and Range Plants, 1955).

This species is always found in well-drained soils in the Stillwater area and is never seen in moist, poorly drained areas. When found in climax prairie areas, it is generally on the higher parts of slopes and hills. It is extremely deeply rooted, the woody roots sometimes reaching a depth of 18 feet according to Weaver (1968).

A. canescens begins to leaf out in Payne County in the latter part of April and flowering occurs generally in June. Nodules are round and have a deep red color internally. Never numerous on any plant found growing in the field, the nodules have been observed as deep as 3-4 feet in the present study, although they have been reported at depths of 10-12 feet by Weaver and Albertson (1956). Nodules on field specimens were always found attached to the main root.

Petalostemum purpureum is a deep-rooted warm-season perennial native species which reproduces from underground stems and from seed. The plant consists of from one to ten slender stems and attains a height of 1-3 feet (Pasture and Range Plants, 1955).

Described as a postclimax species, P. purpureum is ranked third among the most important legumes. It is abundant in upland prairies, being present in some 75% of North American prairies (Weaver, 1968).

In the Stillwater area, this species occurs in well-drained climax areas. Nodules are large and ovate in form but are seldom found on field grown specimens and are never numerous even when present. The roots are very woody and nodules occur only on fine secondary roots which are easily detached in excavation. Blooming begins in mid-June and continues into August.

Neptunia lutea is a yellow flowered perennial with a prostrate growth habit. It occurs in all types of habitats from disturbed areas to climax prairie areas. It blooms profusely all summer from mid-June to September in the Stillwater area and produces a large number of relatively large shiny seeds with extremely hard seed coats. This species has a deep, woody root system and, while only one field specimen was found with nodules it readily nodulates when grown in pots. The nodules are ovate in form.

Desmanthus leptolobus is a small, prostrate perennial which is found in disturbed areas, particularly in very dry localities. It blooms from May through July and produces abundant seed with very hard seed coats. It bears large round to ovate nodules and is well nodulated early in the summer before the ground becomes dry. No nodules can be found after the soil dries in midsummer and no new nodules were observed following soaking rains.

This species is abundant in certain grassy areas surrounding Boomer Lake which is located in northeast Stillwater and it easily escapes mowing due to its extreme prostrate habit.

Desmodium paniculatum is a woody perennial which attains a height of 2-4 feet. It occurs in disturbed areas, along roadsides and in the edges of woods. Tolerant of moderate shade, this species blooms from late June to August. It produces numerous small round nodules and nodulated field specimens can be found until late in the summer in more moist wooded areas.

Melilotus alba is a white flowered introduced annual or biennial which grows profusely in disturbed areas and generally blooms in early May (Pasture and Range Plants, 1955). A vigorous species, *M. alba*, grows from 2-6 feet tall and produces a great number of small seeds with impermeable seed coats. This species has a woody, deep tap root and produces large numbers of ovate to coralloid shaped nodules on delicate lateral roots. It can be found to be nodulated under most conditions until flowering and fruit set occurs at which time nodulation diminishes or disappears. Young field seedlings are almost always nodulated until the soil becomes completely dry to a depth of approximately 12 inches.

Melilotus officinalis is a yellow-flowered annual or biennial which blooms somewhat earlier than *M. alba* and has a more slender, delicate growth form. Attaining a height of 2-4 feet, *M. officinalis* is an introduced, pioneer plant which grows in great numbers in disturbed areas, but, like *M. alba*, does not invade areas of climax stability. This species also produces a deep tap root and nodulates readily and abundantly under almost all conditions except extremely dry soil conditions. Large clusters of coralloid shaped nodules are borne on lateral roots and ramify throughout the soil surrounding the plant.

CHAPTER VI

SEED GERMINATION

Methodology

After dry storage at $0^{o}C$-$4^{o}C$ for a period of four months, legume seeds were treated in various ways according to techniques suggested by Ahring (1975). Several methods were needed because of different mechanical and/or physiological factors which control germination in these plants. All seeds were collected from areas around Stillwater in the summer of 1974. Each treatment consisted of four replications.

Seeds of all species were surface disinifected in a 10 percent Clorox solution for five minutes and rinsed five times in sterile water. They were then placed on filter paper moistened with 2 ml of distilled water and incubated in sterile petri dishes at $28^{o}C$. A 12/12 hour light-dark cycle was used and light intensity was approximately 21,520 lux. Twenty seeds per dish were used and incubation time was 7-28 days.

The second treatment consisted of surface sterilization of the seeds, mechanical scarification and incubation as previously mentioned.

A third treatment consisted of surface sterilization and exposure to different temperature regimes during incubation. Fifty seeds aged 9 to 10 months were placed in 6.35 cm x 6.35 cm x 2.54 cm covered plastic boxes lined with Kimpar tissue and moistened with 8 ml of distilled water. The seeds were then placed in germinators under a photoperiod of eight hours and a sixteen hour dark period. One germinator was maintained at a constant 20°C and the other was held at 30°C during the photoperiod and at 20°C during the dark period.

Due to the extreme hardness of the seed coats and the failure to effect germination by any of the previous methods, seeds of two species, (Neptunia lutea and Desmanthus leptolobus) were soaked in undiluted sulfuric acid for varying periods from 0-8 hours. The seeds were rinsed in running water for a minimum of 10 minutes and then treated as previously described and placed in the germinators at the same temperatures used in the third treatment.

Another type of treatment consisted of stratification of seeds using 50 seeds of each species and storing them in plastic boxes on moistened Kimpar tissue at 4°C for periods ranging from one to four weeks.

The final treatment consisted of mechanical scarification of seeds aged nine to ten months. Neptunia lutea seeds were lightly rubbed with sandpaper due to their relatively large size and ease of handling, and the other, smaller seeds were lightly scratched with a sharp engraving tool. It was determined that only a small break in the seed coat was necessary to bring about germination. Seeds were then incubated in plastic boxes on Kimpar tissue as previously described or placed on washed sand moistened with 8 ml of distilled water at a 30°C/20°C temperature schedule with a 12/12 hour photoperiod.

Results and Discussion

The only two species which germinated readily without any treatment other than cold storage were Amorpha canescens and Desmodium paniculatum.

These two species consistently germinated at rates greater than 75 percent and no further treatment was given them. All other species required some additional type of treatment to induce germination.

During the first scarification series, the percentage of germination for all species improved but none reached 75 percent and only two, Psoralea tenuiflora and Desmanthus illinoensis reached 50 percent (Table I).

In the treatment using different temperature regimes, only one species showed any response. Cassia fasciculata increased from one percent germination at a constant 20°C to 38 percent germination when the temperature was varied from 30°C during the photoperiod to 20°C in the dark. Improvement in germination rates of the other species was only slight or non-existent (Table II).

The two species which were acid scarified, Desmanthus leptolobus and Neptunia lutea, both attained 100 percent germination after being soaked for one hour in sulfuric acid. The rate of germination dropped rapidly after one hour of treatment for Desmanthus leptolobus, but remained high for Neptunia lutea through four hours of acid soaking due to its extremely hard and impervious seed coat (Table III).

The final series of scarification treatments resulted in large increases in germination rates of each species (Table IV). The change in response to scarification between the first treatment and the final one was probably due to the increased age of the seeds. Ballard (1971) also found that germination in legumes increased with age. One effect of age is fracturing of the strophiole by diurnal temperature variations, the strophiole being the only place of permeability on the seeds. After refrigeration for four to six months at an average temperature of 4°C the seeds were kept at room temperature, 20-24^{o}C, for 18 months and all species subsequently responded favorably to scarification.

TABLE I

GERMINATION RESPONSE OF LEGUME SEEDS TO MECHANICAL SCARIFICATION BY SCRATCHING THE SEED COAT WITH A SHARP TOOL, AFTER DRY STORAGE AT 0-4^{o}C FOR FOUR MONTHS

Species	% Germination	
	Untreated	Scarified
Melilotus alba	0	30
Melilotus officinalis	20	20
Psoralea tenuiflora	0	50
Desmanthus illinoensis	0	70
Neptunia lutea	0	40
Petalostemum purpureum	10	40
Desmanthus leptolobus	0	20
Cassia fasciculata	16	32
Lespedeza virginica	1	15
Lespedeza capitata	0.5	19

TABLE II

GERMINATION RESPONSE OF SEEDS TO EXPOSURE TO TWO DIFFERENT TEMPERATURE REGIMES WITH A 12/12 HOUR PHOTOPERIOD

Species	% Germination	
	$20^{o}C$ Constant	$30^{o}C/20^{o}C$
Melilotus alba	0.5	5
Melilotus officinalis	0.5	4
Psoralea tenuiflora	4	4
Desmanthus illinoensis	6	10
Neptunia lutea	0	0
Petalostemum purpureum	5	4
Desmanthus leptolobus	0	5
Cassia fasciculata	1	38
Lespedeza virginica	0	3
Lespedeza capitata	1.5	3

TABLE III

GERMINATION RESPONSE OF SEEDS OF TWO SPECIES OF LEGUMES TREATED WITH 36 N SULFURIC ACID FOR VARIOUS PERIODS OF TIME

Time (Hours)	% Germination	
	Desmanthus leptolobus	*Neptunia lutea*
0	0	0
1	100	100
2	28	100
4	0	72
8	0	0

TABLE IV

GERMINATION RESPONSE OF SCARIFIED LEGUME SEEDS STORED
18 MONTHS AT ROOM TEMPERATURE AFTER DRY STORAGE
FOR FOUR-SIX MONTHS AT $0^{o}C-4^{o}C$

Species	% Germination Unscarified	Scarified
Melilotus alba	0.5	100
Melilotus officinalis	0.5	100
Psoralea tenuiflora	4	57
Desmanthus illinoensis	6	100
Neptunia lutea	0	100
Petalostemum purpureum	5	87
Desmanthus leptolobus	0	100
Cassia fasciculata	1	80
Lespedeza virginica	0	90
Lespedeza capitata	1.5	75

(N. lutea and D. leptolobus were scarified with sand paper and seeds of the other species were scratched with a sharp engraving tool.)

CHAPTER VII

GROWTH MEDIUM

Methodology

A variety of materials was tested to determine a medium in which seedlings would grow and from which roots with attached nodules could be easily extricated.

Five different types of medium were tested. Ten cm sterilized clay pots or 284 gm sterile styrofoam cups were filled with each of the following materials:

a. Perlite

b. washed sand

c. coarse vermiculite #1

d. coarse vermiculite #1 plus washed sand, 50% of each

e. fine vermiculite #3 plus washed sand, 50% of each.

All seedlings were watered with a nitrogen-free nutrient solution according to techniques described by Becker and Crockett (1976).

Results and Discussion

The material which proved to be most satisfactory was the fine vermiculite-sand mixture. It provided favorable water relations, was easily removed from the roots and did not cause loss of nodules during its removal.

The extremely small size of the seedlings and their slow growth rate seemed to be factors in the poor survival rates in Perlite, coarse vermiculite and coarse vermiculite-sand mixture as these materials apparently provided less surface area for water absorption by the tiny roots. The roots also tended to penetrate individual large vermiculite particles, making it impossible to adequately extricate roots and intact nodules from the medium.

The washed sand provided favorable water relations and adequate growth but the sand tended to pack tightly around the roots causing some loss of nodules.

CHAPTER VIII

SEEDLING DEVELOPMENT

Methodology

Potted seedlings were placed on metal trays, root irrigated to saturation with a nitrogen-free nutrient solution and periodically flushed with distilled water to prevent any excess salt accumulation as described by Becker and Crockett (1976).

One group of seedlings was placed in a growth chamber maintained at $20^{o}C$ with a 12-hour photoperiod and the other group was grown at $25\text{-}28^{o}C$ in a greenhouse in a 12-hour photoperiod according to techniques discussed by Hardy et al. (1968).

Results and Discussion

Seedlings in the growth chamber grew extremely slowly or not at all. The constant $20^{o}C$ was apparently a powerful deterrent to growth of all species as all other factors were the same for both groups. The seedlings grown in the greenhouse were used exclusively for testing. Some specimens of field grown, *C. fasciculata*, *M. officinalis* and *M. alba* were analyzed also.

Neptunia lutea, *Desmanthus illinoensis* and *Amorpha canescens* showed some chlorosis at the tips of older leaves after six weeks but seemed to recover and were dark green by 10 weeks.

Desmanthus illinoensis and *Desmodium paniculatum* were badly infested with mealy bugs, first discovered after seven weeks. Plants in the greenhouse were sprayed one week later but those of *D. illinoensis* were never completely free of the pests and were not as vigorous or healthy looking as field specimens. Both of these species were extremely susceptible to insects. An infestation of white flies was found at age seven weeks and was not completely controlled until about four weeks later. Although the damage caused by these insects was not apparent, there probably was an effect on overall plant vigor. *D. paniculatum* attained the largest size of all species examined, becoming as large and woody as field specimens of similar age. All plants were somewhat depauperate in appearance due to the insect activity early in the development of the seedlings.

Both *Neptunia lutea* and *Desmanthus leptolobus* appeared to be free from insect attack but were very slow growing. Some *Neptunia* plants bloomed sparsely at about sixteen weeks of age but no seeds ever formed.

Only one species, *Cassia fasciculata*, bloomed extensively. All seedlings were bearing buds at age nine weeks and most had several buds, open blooms and seed pods at age 12 weeks. The plants of this species grew rapidly, were apparently free from insect invasion, were vigorous and dark green in appearance and attained a size similar to field specimens of the same age.

Relative to all other species examined, *Amorpha canescens* and *Petalostemum purpureum* were the slowest growing and were ultimately smallest in size. Although these plants apparently were not bothered by insects, the *Petalostemum purpureum* plants were never vigorous and none survived beyond 15 weeks. *Amorpha* plants, though small, were dark green and healthy looking and were growing throughout the study period.

Melilotus alba was much more vigorous and rapid growing than *M. officinalis* during the first weeks of growth but by the time both species had reached 12 weeks of age, there were no differences in size. Neither of these species was as vigorous or large as field specimens and no branching of the shoots of any seedlings occurred. *M. alba* plants did not survive beyond 14 weeks although no specific cause of death could be established.

CHAPTER IX

BACTERIAL CULTURE AND INOCULATION

Methodology

Field specimens of each species used were collected and the nodules detached. Only plump, healthy looking nodules with pink or red interiors were selected to insure the presence of leghemoglobin, the amount of which is positively correlated with the amount of nodular nitrogen fixed according to Chopra and Subba-Rao (1967). Using a technique provided by Lynd (1974) the nodules were immersed for 2-5 minutes in a 10 percent Clorox solution for surface sterilization and then washed five times in sterile water. They were then crushed in 1 ml of sterile water and a drop of the mixture was placed on a nutrient agar-yeast medium in petri dishes. Transfers were made until pure colonies were obtained. A final transfer of each bacterial colony was made to mannitol agar slants in test tubes. After 7-14 days growth of the colonies, the test tube cultures were freeze-dried and permanent inoculum sources established.

At the time of transfer of seedlings from germination vessels to pots, a slurry was prepared using some of each bacterial type collected. One cubic centimeter sized pieces of all the permanent cultures were ground together and mixed with 1,000 ml of water. Ten ml of the mixture was added to each pot as the seedling was transplanted.

Results and Discussion

Nodule development occurred 100% of the time and most nodules were healthy looking with pink or red interiors. Nodules first appeared from two through six weeks after inoculation and continued to develop in size and number until the time of assay.

CHAPTER X

ASSAY TECHNIQUE

Methodology

Assays for acetylene reduction were made at intervals from 9 to 24 weeks of plant growth. Plants were removed from pots and the root systems of intact plants were suspended in 125 ml glass filter flasks. The plant stems were inserted in rubber stoppers and all openings around the stoppers were sealed with an inert modeling clay, Plastolene. The opening at the arm of the flask was covered with a gas-tight rubber serum cap and used as the gassing port.

The flasks were flushed with air and then evacuated by hand with 50 cc gas-tight syringes until no more air could be removed. A 12.5 cc oxygen - 50 cc acetylene mixture was then injected into each flask. The acetylene mixture used was 0.1 atm C_2H_2: 0.9 atm He. Plants were incubated for one hour at 22-26^{o}C under a 40-watt Agro-lite and samples were then removed and stored in 13 ml hand evacuated serum bottles. The samples were analyzed within four hours in a Hewlett-Packard gas chromatograph with a hydrogen flame-ionization detector using 25 μl injections. A 3.175 mm, 1.8 meter stainless steel column containing 80/120 mesh Porapak N held at 50^{o}C was used to separate the acetylene and ethylene (Hardy et al., 1973). Four samples per plant were analyzed and the nodules were then removed from the roots and weighed.

CHAPTER XI

RESULTS

Nodular Weights

At age nine weeks all plants were effectively nodulated and there were significant differences in the nodule weights of some species (Table V).

TABLE V

MEAN NODULE WEIGHTS OF THE LEGUME SPECIES AT VARIOUS AGES EXPRESSED IN MILLIGRAMS, $n \geqq 10$

Species	Age in Weeks		
	9	12	16-21
Amorpha canescens	18.6[a]	12.4	106.8[b]
Cassia fasciculata	60.2[c]	84.3	
Petalostemum purpureum	15.9[a]	26.6	
Desmanthus illinoensis	80.0[c]	13.3[b]	
Desmanthus leptolobus	31.5	24.2	79.2[b]
Desmodium paniculatum	23.1	111.8[b]	104.8
Melilotus alba	35.7	59.7	
Melilotus officinalis	30.8	22.1	355.3[b]
Neptunia lutea	34.4	123.3[b]	118.8

[a]Significantly different at P<.05 from species designated with (c) calculated using Duncan's multiple Range Test.

[b]Significantly different at P<.05 from plants of the same species at different ages calculated using Duncan's Multiple Range Test (Steel and Torrie, 1960).

Petalostemum purpureum and Amorpha canescens had nodule weights significantly lower than both Cassia fasciculata and Desmanthus illinoensis at age nine weeks as determined using Duncan's Multiple Range Test for detecting differences between treatment means (Steel and Torrie, 1960). There were no significant differences in nodule weight among any of the other species and by the time the plants had reached 12 weeks of age, no significant differences were detected in nodule weight among any of the different species examined at the same age. However, there were significant differences between plants of the same species as age changed. Nodule weight increased significantly from age nine weeks to 12 for Desmodium paniculatum and Neptunia lutea (Table V). Desmanthus illinoensis showed a significant decrease in nodule weight from age nine weeks to age 12 weeks.

Beyond the age of 12 weeks there was a significant increase in nodule weight of Amorpha canescens, Desmanthus leptolobus and Melilotus officinalis (Table V). An insufficient number of plants of the other species was available for analysis beyond the age of 12 weeks.

Nodular Efficiency

The nodular efficiency of Desmanthus illinoensis was significantly lower than Amorpha canescens, Petalostemum purpureum and Desmodium paniculatum. There was also a significant difference between Desmodium paniculatum and Neptunia lutea but there were no apparent differences which were significant among any of the other species at age nine weeks (Table VI).

At age 12 weeks, there were no significant differences in nodular efficiency although the ranking from most efficient to least efficient changed (Table VI).

TABLE VI

NODULAR EFFICIENCY OF THE LEGUME SPECIES AT VARIOUS AGES

Species	Age in Weeks		
	9	12	16-21
Amorpha canescens	3.4[bc]	4.2[a]	1.4[a]
Cassia fasciculata	2.3[abc]	1.1[a]	
Petalostemum purpureum	4.0[bc]	3.2[a]	2.1[a]
Desmanthus illinoensis	1.2[c]	4.4[a]	
Desmanthus leptolobus	2.5[abc]	1.8[a]	0.8[a]
Desmodium paniculatum	5.3[bc]	0.7[a]	1.7[a]
Melilotus alba	2.7[abc]	1.9[a]	
Melilotus officinalis	2.6[abc]	6.2[a]	0.7[a]
Neptunia lutea	2.1[ab]	0.7[a]	0.8[a]

(Expressed in moles of C_2H_2 reduced per milligram of nodule weight per day, four readings per sample, $n \geq 10$)

[a]Significance calculated at $P<.05$. Values for one age group followed by the same letter are not significantly different.

Fixation Rate per Species According to Age

In terms of total fixation capacity per day, per plant, Cassia fasciculata had an apparent rate significantly greater than Melilotus alba, Petalostemum purpureum and Amorpha canescens at age nine weeks. Desmanthus illinoensis had a rate significantly higher than Melilotus alba and Petalostemum purpureum at age nine weeks. No significant differences were apparent among any of the other species at this age (Table VII).

TABLE VII

AVERAGE REDUCTION CAPACITY OF THE LEGUME SPECIES AT AGE NINE WEEKS AND AGE TWELVE WEEKS

Species	Age in Weeks	
	9[a]	12[b]
Cassia fasciculata	85.2[c]	55.8[ab]
Desmanthus illinoensis	83.2[bc]	52.7[ab]
Desmodium paniculatum	70.2[abc]	126.2[c]
Melilotus officinalis	62.8[abc]	55.3[ab]
Desmanthus leptolobus	59.1[abc]	45.1[a]
Neptunia lutea	55.9[abc]	87.6[b]
Amorpha canescens	55.1[ab]	47.8[a]
Petalostemum purpureum	49.4[a]	56.0[ab]
Melilotus alba	47.6[a]	70.0[ab]

(Expressed as μ moles of C_2H_2 reduced per species per day, four readings per sample, n≥10.)

[a]Significance calculated at $P<.05$.

[b]Significance calculated at $P<.10$.

(Values followed by the same letter are not significantly different.)

At age 12 weeks the rank in order of decreasing total fixation capacity per day changed and Desmodium paniculatum had an apparent rate which was slightly higher than all other species and Neptunia lutea had a somewhat higher rate than Desmanthus leptolobus and Amorpha canescens. Each of these was significant at the $P<.10$ level (Table VII).

Average Fixation Capacity of Plants of All Ages

By giving the assay results of plants of both ages (nine weeks and 12 weeks) equal weight and by obtaining a mean fixation rate according to species for plants of all ages, still another pattern emerged (Table VIII). It should be remembered, however, that this estimate was based on unequal sample sizes of plants at each age so they may or may not have equal value though they were assigned equal value for purposes of comparison. It was felt that this was an approximation of something like a seasonal average which may have merit in this discussion.

TABLE VIII

AVERAGE REDUCTION CAPACITY OF THE LEGUME SPECIES OF ALL AGES

Species	Mean μ moles C_2H_2
Desmodium paniculatum	98.2
Neptunia lutea	71.8
Cassia fasciculata	70.5
Desmanthus illinoensis	67.9
Melilotus officinalis	59.0
Melilotus alba	58.8
Petalostemum purpureum	52.7
Desmanthus leptolobus	52.1
Amorpha canescens	51.5

(Expressed as mean μ moles of C_2H_2 reduced per day for plants of all ages.)

CHAPTER XII

DISCUSSION

At both age nine weeks and in terms of average fixation rate for plants of all ages (Fig. I), Cassia fasciculata and Desmodium paniculatum were among the three top species, with Cassia fasciculata having the highest fixation rate at age nine weeks while Desmodium paniculatum had the highest average for plants of all ages and also showed the highest fixation potential of any species at age 12 weeks. Both of these species occupy disturbed areas and Cassia fasciculata has wide ecological amplitude, being present from pioneer to late seral stages of succession. In a study of five prairie legumes, Becker and Crockett (1976) found Cassia fasciculata highest in total fixation capacity being significantly ($P < .001$) higher in potential than the other four species. Amorpha canescens, the only other species common to both investigations, was shown to have a relatively low fixation rate as substantiated in the present study. Another species with wide ecological amplitude, Neptunia lutea, was among the three species leading in fixation capacity. This species was second in fixation potential at age 12 weeks and also second in terms of average rate for plants of all ages. Neptunia lutea can be found in all types of habitat from disturbed areas to climax prairie sites. At age nine weeks, Desmanthus illinoensis, which occupies a wide range of habitats including ditches, creek banks, road shoulders and low fields showed the second greatest fixation

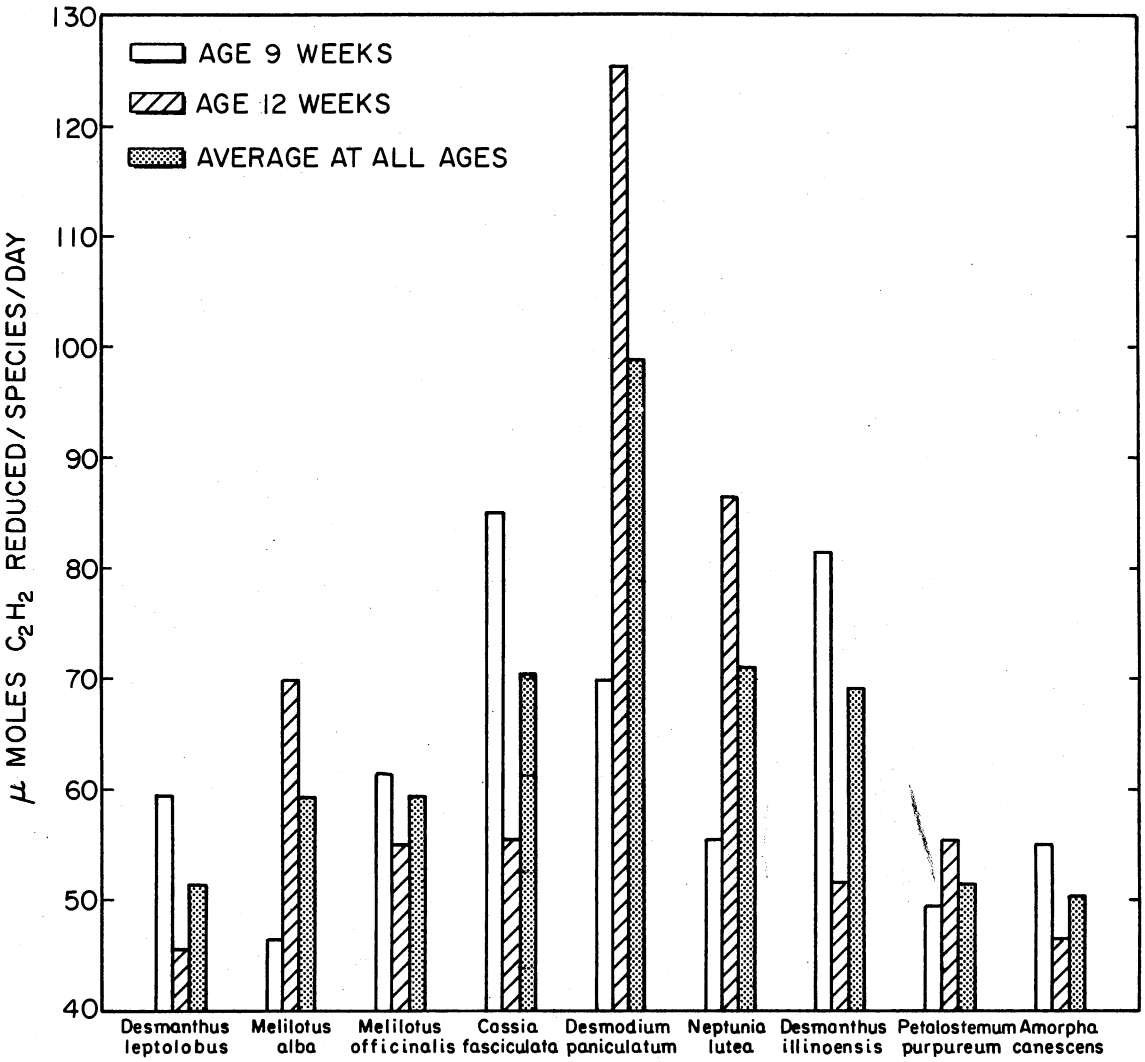

Figure 1. Average μ Moles C_2H_2 Reduced per Species per Day at Different Ages and the Average for all Ages

rate. However, this species declined with age and fell to fourth place in average fixation rate for plants of all ages. The performance of this plant was probably greatly affected by the insect damage it suffered from about age nine weeks on. The significant decrease in nodule weight between nine and 12 weeks is further evidence of the damage due to insects. At age 12 weeks, *Melilotus alba*, a pioneer species was third in total fixation capacity, after having the lowest rate of any species at age nine weeks. Its relatively low fixation rate at the beginning of the observation period reduced its average for plants of all ages, so that in these terms, *Melilotus alba* had one of the lowest rates. *Melilotus officinalis*, another pioneer species, was relatively high in fixation rate at age nine weeks, being fourth in fixation potential, but like *M. alba*, it was low in average rate when plants of all ages were considered. *Amorpha canescens*, a species commonly found in climax prairies was consistently low in total fixation rate, and in terms of the rate averaged for plants of all ages, it was the lowest of all species. Another species primarily limited to a near-climax habitat, *Petalostemum purpureum*, while being somewhat intermediate in ranking at age 12 weeks, was among species having the lowest fixation rate at age nine weeks. This species was also one of the lowest in terms of fixation rate averaged for plants of all ages. *Desmanthus leptolobus* was also fairly consistent in its relatively low fixation rate. While *D. leptolobus* has been found in disturbed areas, it is described as an inhabitant of climax prairie by Stemen and Myers (1937). The authors' observation refute their conclusion.

In terms of nodular efficiency (Table VI), the pattern was quite different from that of total nitrogen fixed per plant. Desmodium paniculatum, a species common to pioneer habitats such as sandy woods and creek banks, is also said to be an inhabitant of climax prairie by Steyermark (1963). This plant was apparently most efficient at age nine weeks. The two climax or near-climax species, Amorpha canescens and Petalostemum purpureum, were also ranked among those species with a significantly higher ($P < .05$) apparent efficiency than the other plants tested. Becker and Crockett (1976) also found a high nodular efficiency in A. canescens while in the present investigation the inverse relationship was observed. A. canescens nodules were shown to be significantly ($P < .05$) more efficient, in terms of μ moles of C_2H_2 reduced per milligram of nodule weight, than Cassia fasciculata. This pattern persisted through age 12 weeks except in the case of Desmodium paniculatum, which decreased significantly in efficiency and was replaced by Melilotus officinalis in order. M. officinalis and Desmanthus illinoensis had significantly higher ($P < .05$) apparent nodular efficiencies than Neptunia and Cassia but none of the other species differences were significant ($P < .05$) at this age. The decrease in efficiency of Desmodium paniculatum may have been due to the presence of larger numbers of older, less effective nodules whose weight was included along with younger effective nodules. A modification of leghaemoglobin to bile pigment due to senescense often causes a green color at the base of nodules according to Vincent (1974) and this effect could be observed in some of the D. paniculatum nodules although they remained firm and healthy looking in appearance. Other species which showed a slight but not significant decrease in apparent efficiency were C. fasciculata, P. purpureum, Desmanthus leptolobus, Melilotus alba and Neptunia lutea. This decline also could have been due to the increase in damaged or senescent nodules which were included in total nodule weight. A growth curve phenomenon may also be a factor in these observations, since the carbon:nitrogen ratio is so influential to nitrogen fixation as discussed by Lie (1974). A low carbon:nitrogen ratio is inhibitory to nitrogen fixation as is a carbon:nitrogen ratio which is too high, although the specific effect is not understood. It may be that the plants which showed a decline in efficiency had entered a growth phase in which available carbohydrate was being used primarily for plant respiration and the supply available to nitrogen-fixing bacteria was sub-optimal for their own growth activities. Alternatively, the plants may have had a decreased growth rate during this phase, causing a buildup of nitrogen reserves and a carbon:nitrogen ratio which was too low for efficient fixation.

It appears that certain climax species or near-climax species such as Amorpha canescens and Petalostemum purpureum have very efficient nodules, at least in early stages of their development, and a relatively small number of nodules. Field specimens several years old were also found to be very sparsely nodulated or not nodulated at all during this study. The efficiency of the nodules of field specimens was not measured due to the extensive, woody root system present on these plants and the difficulty of excavating the plants with intact nodules. In contrast, species such as D. illinoensis at age nine weeks and C. fasciculata, Desmodium paniculatum, N. lutea and M. alba at age 12 weeks had large numbers of nodules which were relatively less efficient than those of the climax species. These species, with the exception of

N. lutea were also found to be more frequently and more heavily nodulated under field conditions, lacking nodules only when the weather became quite dry in mid-summer. Field specimens of C. fasciculata, Melilotus alba and M. officinalis were analyzed for ability to reduce C_2H_2 and there were no significant differences in performance between greenhouse and field grown specimens.

Pioneer species may exert their modifying influence on disturbed areas by fixing large amounts of nitrogen at a specific period, such as at anthesis, which was not manifested in this study. Climax or near climax species may have the potential for efficient fixation as shown in this study, but may be inhibited by the relatively larger amount of combined nitrogen present in the mature habitat. Plants with wide ecological amplitude may be able to tolerate the higher levels of nitrogen in the climax state, by establishing a favorable carbon:nitrogen ratio, perhaps by some modification of growth rate.

Certainly the ability to retain nodules under stress conditions as exhibited by Cassia, Desmodium, Demanthus illinoensis, Melilotus alba, and M. officinalis is another factor in the potential nitrogen fixing status of a plant. Climax species have the combined nitrogen conserved in the mature habitat to fall back on when nodules are shed in response to drought. Species such as Cassia and D. illinoensis may be able to inhabit a broad spectrum of sites by having the ability to produce more nodules and to retain more of them for a longer period of time after stress sets in. Pioneer species may survive in disturbed areas by this same mechanism, rather than by the ability to fix large amounts of nitrogen.

Another factor which might contribute to the higher concentration of combined nitrogen in a mature ecosystem is the larger number of free-living diazotrophs which inhabit this type of area. According to Burns and Hardy (1975) bacterial diazotrophs are very sparse in soils lacking organic matter and many are partial to the rhizosphere and phylloplane of higher plants. These habitat preferences are due to the necessity for an available carbon source as well as favorable moisture and temperature conditions for optimum nitrogen-fixing activity. In the climax habitat these organisms may be responsible for contributing combined nitrogen to the reserve already present and climax legume species may utilize this nitrogen source. Pioneer species, inhabitants of areas where conditions are more unfavorable for the free-living diazotrophs, may have competitive advantage since they need not depend on a supply of combined nitrogen.

In terms of nodular efficiency (Table VI), several of these legumes compare favorably with certain other nitrogen fixing species as described by Hardy et al. (1968). At ages nine and 12 weeks, Amorpha canescens and Petalostemum purpureum have nodular efficiencies similar to those of Medicago sativa, as does Desmanthus illinoensis at age 12 weeks. Melilotus alba, M. officinalis and Cassia fasciculata, at age nine weeks, are comparable to Phaseolus vulgaris and at 12 weeks, M. officinalis has an efficiency similar to Glycine max. Desmodium paniculatum is also comparable to Glycine max in apparent nodular efficiency at age nine weeks.

While certain trends are suggested in the relationship between the nitrogen-fixing capacity of a legume and its successional status, much more work must be done before clear cut correlations, if any, can be established. In the present study, Desmanthus illinoensis almost certainly reacted subnormally due to insect infestation. Desmodium paniculatum was also somewhat affected by insects although the infestation was overcome. The disappointing performance of the two Melilotus species could have been due to sub-optimal growing conditions. By keeping all environmental factors, including light, temperature and nutrients, constant for all species, it is doubtful that optimum conditions were provided for all species. Certainly, quite different habitat preferences prevail among these species in natural environments. Amorpha canescens, Petalostemum purpureum, Neptunia lutea and Desmanthus leptolobus prefer well drained soil while D. illinoensis and Desmodium paniculatum grow well in such relatively moist environments as ditches, edges of woods and creek banks. Cassia fasciculata seems to thrive in either type of moisture situation. While certain nutrients are necessary for all species of legumes, different ratios and amounts of some nutrients are required by different species. Hewitt (1958) mentioned a higher copper requirement by lucerne than for subterranean clover. Lucerne had marked chlorosis and abnormal leaf development under chlorine deficient conditions while beans showed no response except an increase in epinasty in the evenings.

Another question regarding results of this study might be whether optimally effective symbioses were established for each species. Although the Rhizobium were cultured from nodules collected from field grown plants it is possible that other species and strains may be found which will form a more effective symbiosis with some or all of the species examined.

Finally, nitrogen fixing capacity as related to phenology needs to be investigated. The annual species in this study had a significantly ($P < .002$) higher fixation rate than the perennial species. The stage of development of many legumes had been correlated with ability to fix nitrogen. Hardy et al. (1968) found that C_2H_2 reducing activity closely paralleled development in soybeans, increasing through flowering, pod formation and filling whereupon there was a rapid decline in activity. Peanuts have been shown by Hardy et al. (1971) to have a very low activity during the vegetative growth phase and to increase rapidly during fruit formation and maturation. However, Ploughley and Dart (1969) suggested that subterranean clover had maximal nodular efficiency early in its vegetative growth phase and declined thereafter. The failure of most of the species in this study to reach anthesis probably is a significant factor which must be contended with in future investigations.

Considering these variables and many others not discussed, the results of this study are inconclusive. It may suggest, however, that species such as Cassia and Neptunia, which have wide ecological amplitude have a relatively high nitrogen-fixing potential while species closer to either extreme in the successional pattern, have a relatively lower potential. Further studies are certainly necessary to discover if this relationship holds true.

A SELECTED BIBLIOGRAPHY

Ahring, R. M. Agronomy Department, Oklahoma State University, Stillwater, Oklahoma. Personal Communication (1975).

Akkermans, A. D. L. (Unpublished Ph.D. Thesis, University of Leiden) 1971.

Alway, F. J. and R. M. Pickney. "On the Relation of Native Legumes to the Soil Nitrogen of Nebraska Prairies." The Jour. of Ind. and Eng. Chem. 1:271-2 (1909).

Ballard, L. A. T. "Physical Barriers to Germination." Seed Quality Research Symposium. V. K. O'Toole and L. W. Woodstock (Eds.), International Seed Testing Association, Norway, 1432 As-NLH, 1971, 285-303.

Becker, D. A. and J. J. Crockett. "Nitrogen Fixation in Some Prairie Legumes." Amer. Nat. (In Press) 1976.

Bergerson, F. J. "Biochemistry of Symbiotic Nitrogen Fixation in Legumes." Ann. Rev. Pl. Physiol. 22:121-140, (1971).

Bokhari, U. G. and J. S. Singh. "Standing State and Cycling of Nitrogen in Soil-Vegetation Components of Prairie Ecosystems." Ann. Bot. 39:273-285, (1975).

Bruner, W. E. "The Vegetation of Oklahoma." Ecol. Mono. 1:101-189 (1931).

Bunting, A. H. "Some Reflections on the Ecology of Weeds." The Biology of Weeds. J. L. Harper (Ed.), Oxford: Blackwell Scientific Publications, 11-26 (1960).

Burns, R. C. and R. W. F. Hardy. Nitrogen Fixation in Bacteria and Higher Plants. New York: Springer Verlag (1975).

Campbell, E. "Wild Legumes and Soil Fertility." Ecology. 8:440-483 (1927).

Chaffin, W. "A Soil Improvement Program for Oklahoma. Stillwater, Oklahoma: Extension Service, Oklahoma State University, Circular 412 (no date).

Chopra, C. L. and N. S. Subba-Rao. "Mutual Relationships Among Bacteriods, Leghaemoglobin and Nitrogen Content of Egyptian Clover (Trifolium alexandrinum) and Gram (Cicer arietinum)." Arch. Mikrobiol. 58:71-76 (1971).

Climatological Data, Oklahoma 1973, 1974, 1975. Silver Springs, Md.: U.S. Dept. of Commerce, Volumes 82, 83, 84.

Coaldrake, J. E. "The Nitrogen of Natural Plant Communities." (C.S.I. R.O. staff ed.), A Review of Nitrogen in the Tropics With Particular References to Pastures. Berkshire, England: Commonwealth Bureau of Pastures and Field Crops, Hurley, Bulletin 46, 35-42, (1962).

Connell, J. H. and E. Orias. "The Ecological Regulation of Species Diversity." Am. Naturalist. 98:399-414 (1964).

Cooper, W. S. "A Third Expedition to Glacier Bay, Alaska." Ecology. 12:61-95 (1931).

Cooper, W. S. "A Fourth Expedition to Glacier Bay, Alaska." Ecology. 20:130-155 (1939).

Crocker, R. L. "The Plant Factor in Soil Formation." Selected Papers in Soil Formation and Classification. J. V. Drew (Ed.), Madison, Wisconsin: The Soil Sciences Society of America, Inc., 179-190 (1967).

Croxton, W. C. "Revegetation of Illinois Coal Stripped Lands." Ecology. 9:155-175 (1928).

Curry, B. R. "Climates of the States." Climatography of the United States. No. 60-34. Silver Springs, MD.: U. S. Department of Commerce/Environmental Services Administration, Environmental Data Service, Vols. 82, 83, 84 (1970).

Dahlman, R. C. and C. L. Kucera. "Root Productivity and Turnover in Native Prairie." Ecology. 46:84-89 (1965).

Dahlman, R. C., J. S. Olson, and K. Doxtader. "The Nitrogen Ecology of Grassland and Dune Soils." Biology and Ecology of Nitrogen, Proceedings of a Conference. Washington, D. C.: National Academy of Sciences, 54-82 (1969).

Daniel, H. A. and W. H. Langham. "The Effect of Wind Erosion and Cultivation on the Total Nitrogen and Organic Content of Soils in the Southern High Plains." Jour. Amer. Soc. Agron. 28:587-596 (1936).

Dart, P. J. "Development of Root-Nodule Symbioses." The Biology of Nitrogen Fixation. A. Quispel (Ed.), New York: American Elsevier Publishing Co., Inc. (1974).

Dilworth, M. J. "Dinitrogen Fixation." Ann. Rev. Pl. Physiol. 25:81-114 (1974).

Donald, C. M. "The Impact of Cheap Nitrogen." J. Aust. Inst. Agri. Sci. 26:319-338 (1960).

Erdman, L. W. World Farming. 20-23 (1968).

Evans, H. J. and S. A. Russell. "Physiological Chemistry of Symbiotic Nitrogen Fixation by Legumes." The Chemistry and Biochemistry of Nitrogen Fixation. J. R. Postgate (Ed.), London-New York: Plenum Press (1971).

Fred, E. B., I. L. Baldwin, and E. McCoy. "Root Nodule Bacteria and Leguminous Plants." Univ. of Wisc., Stud. in Sci. 5 (1932).

Gibson, A. H. "Factors in the Physical and Biological Environment Affecting Nodulation and Nitrogen Fixation by Legumes." Plant and Soil Spec. Vol. T. A. Lie and E. G. Mulder (Eds.), Prague and Wageningen (1971).

Gray, F. and H. M. Galloway. "Soils of Oklahoma." MP-56. Stillwater, Oklahoma: Oklahoma State University Experimental Station (1959).

Habish, H. A. "Effect of Certain Soil Conditions on Nodulation of Acacia, spp." Plant Soil. 33:1-6 (1970).

Hardy, R. W., R. D. Holsten, E. K. Jackson, and R. C. Burns. "The Acetylene-Ethylene Assay for Nitrogen Fixation: Laboratory and Field Evaluation." Pl. Physiol. 43:1185-1207 (1968).

Hardy, R. W. and R. D.Holsten. "Global Nitrogen Cycling: Pools, Evolution, Transformations, Transfer, Quantitation and Research Needs." Symposium Proceedings Environmental Protection Agency on 'The Aquatic Environment: Microbial Transformations and Water Quality Management Implications.' R. K. Ballantine and L. J. Guarraia (Eds.), Washington, D. C. (1972).

Hardy, R. W. F., R. C. Burns, and R. D. Holsten. "Applications of the Acetylene-Ethylene Assay for Measurement of Nitrogen Fixation." Soil Biol. Biochem. 5:48-81 (1973).

Hewitt, E. J. "Some Aspects of Mineral Nutrition in Legumes." Nutrition of the Legumes. E. G. Hallsworth (Ed.), New York: Academic Press, Inc. (1958).

Kershaw, K. A. Quantitative and Dynamic Plant Ecology. New York: American Elsevier Publishing Co., Inc. (1973).

Lawrence, D. B., R. E. Schoenike, A. Quispel, and G. Bond. "The Role of Dryas drummondii in Vegetation Development Following Ice Recession at Glacier Bay, Alaska, With Special Reference to Its Nitrogen Fixation by Root Nodules." Journal of Ecology. 55:793-813 (1967).

Lie, T. A. "Symbiotic Nitrogen Fixation Under Stress Condition." Plant and Soil Spec. Volume. T. A. Lie and E. G. Mulder (Eds.), 117-127 (1971).

Lie, T. A. "Environmental Effects on Nodulation and Symbiotic Nitrogen Fixation." The Biology of Nitrogen Fixation. A. Quispel (Ed.), New York: American Elsevier Pub. Co., Inc. (1974).

Lynd, J. Q. Department of Agronomy, Oklahoma State University. Personal Communication. (1974).

Minchin, F. R. and J. S. Pate. "Effects of Water, Aeration and Salt Regime on Nitrogen Fixation in a Nodulated Legume - Definition of an Optimum Root Environment." Journ. of Exp. Bot. 26:60-69 (1975).

Morris, M., D. E. Eveleigh, S. C. Riggs, and W. N. Tiffney, Jr. "Nitrogen Fixation in the Bayberry (Myrica pennsylvanica) and Its Role in Coastal Succession." Amer. Jour. Bot. 61:867-870 (1974).

Odum, E. P. Fundamentals of Ecology. Philadelphia: W. B. Saunders (1953).

Odum, H. T. Symposium Primary Prod. Miner, Cycling in a Mature Ecosystem. H. E. Young (Ed.), 81-138 (1968).

Oghoghorie, C. G. and J. S. Pate. "The Nitrate Stress Syndrome of the Nodulated Field Pea (Pisum arvense L.)." Plant and Soil Spec. Vol. T. A. Lie and E. Mulder (Eds.), Prague and Wageningen (1971).

Paul, E. A., R. J. Myers, and W. A. Rice. "Nitrogen Fixation in Grass-Land and Associated Cultivated Ecosystems." Plant and Soil Spec. Vol. T. A. Lie and E. G. Mulder (Eds.), Prague and Wageningen (1971).

Ploughley, R. J. and P. J. Dart. "Reduction of Acetylene by Nodules of Trifolium subterraneum as Affected by Root Temperature, Rhizobium Strain and Host Cultivar." Arch. Mikrobiol. 69:171-179 (1969).

Postgate, J. R. "New Advances and Future Potential in Biological Nitrogen Fixation." Jour. Appl. Bact. 37:185-202 (1974).

Quispel, A. (Ed.). "The Biology of Nitrogen Fixation." Frontiers of Biology. Amsterdam-Oxford: North Holland Pub. Co., Vol. 33 (1974).

Rice, E. L., W. T. Penfound, and L. M. Rohrbaugh. "Seed Dispersal and Mineral Nutrition in Succession in Abandoned Fields in Central Oklahoma." Ecology. 41:224-228 (1960).

Rice E. L. Allelopathy. San Francisco and London: Academic Press (1974).

Russell, E. W. Soil Conditions and Plant Growth. Norwich: Jarrold and Sons Ltd. (1961).

Russell, E. W. "A Soil Scientists Viewpoint." Ecological Aspects of the Mineral Nutrition of Plants. I. H. Rorison (Ed.), Oxford and Edinburgh: Blackwell Scientific Publications (1969).

Smith, R. L. Ecology and Field Biology. New York: Harper and Row (1971)

Sprent, J. I. "Effects of Water Stress on Nitrogen Fixation in Root Nodules," Plant and Soil Spec. Vol. 225-228 (1971).

Steel, R. G. and J. H. Torrie. Principles and Procedures of Statistics. New York: McGraw-Hill Book Co., Inc. (1960).

Stemen, T. R. and W. S. Myers. Oklahoma Flora. Oklahoma City, Oklahoma: Harlow Publishing Co. (1937).

Stewart, W. D. P. Nitrogen Fixation in Plants. The Athlone Press, Univ. of London (1966).

Steyermark, J. A. Flora of Missouri. Ames, Iowa: Iowa State Univ. Press (1963).

Swanson, C. O. "The Loss of Nitrogen and Organic Matter in Cultivated Kansas Soils and the Effect of This Loss on the Crop Producing Power of the Soil." Jour. Ind., and Eng. Chem. 7:529-532 (1915).

Vincent, J. M. "Root Nodule Symbioses With Rhizobium." The Biology of Nitrogen Fixation. A. Quispel (Ed.), (1974).

Virtanen, A. I. "Biological Nitrogen Fixation." Plant Phys. III. F. C. Steward (Ed.), (1963).

Vlassak, K. E., E. A. Paul, and R. E. Harris. "Assessment of Biological Nitrogen Fixation in Grassland and Associated Sites." Plant and Soil. 38:637-649 (1973).

Waterfall, U. T. Keys to the Flora of Oklahoma. Published by the author. (1969).

Weaver, J. E. and F. W. Albertson. Grasslands of the Great Plains. Lincoln, Nebraska: Johnsen Pub. Co. (1956).

Weaver, J. E. Prairie Plants and Their Environment. Lincoln, Nebraska: Univ. of Nebraska Press (1968).

Wetselaar, R. "Estimation of Nitrogen Fixation by Four Legumes in a Dry Monsoonal Area of North-Western Australia." Aust. J. Exp. Agri. and An. Husb. 7:518-522 (1967).

Williams, C. H. "Pasture Nitrogen in Australia." J. Aust. Inst. Agri. Sci. 36:199-205 (1970).

VITA

S. Marguerite Lofton

Candidate for the Degree of

Doctor of Philosophy

Thesis: NITROGEN FIXATION IN SELECTED PRAIRIE LEGUMES AS RELATED TO SUCCESSION

Major Field: Botany

Biographical:

Personal Data: Born in Tahlequah, Oklahoma, the daughter of Mr. and Mrs. H. V. Brewster; married Ray H. Lofton at Tulsa, Oklahoma, June, 1956; son, Adam, born 12-28-60 and daughter, Laura, born 10-3-64.

Education: Received Bachelor of Science in Education degree from University of Tulsa, Tulsa, Oklahoma, June, 1968; received the Master of Science degree from Oklahoma State University, Stillwater, Oklahoma, May, 1972; fulfilled the requirements for Doctor of Philosophy degree in Botany in July, 1976.

Experience: Science teacher at Jenks High School, Jenks, Oklahoma, 1968-1973; Graduate teaching assistant, Department of Biological Sciences, Oklahoma State University, Stillwater, Oklahoma, 1973-1976.

Professional Organizations: Oklahoma Academy of Science, American Institute of Biological Science.

Honorary Organizations: Phi Gamma Kappa, Kappa Delta Pi.