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Nitrogen Fixation by Selected Native Legumes of South Dakota

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NITROGEN FIXATION BY SELECTED NATIVE LEGUMES OF SOUTH DAKOTA

BY

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NATIVE LEGUMES OF SOUTH DAKOTA

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

Thesis Advisor / Date

Head, Microbiology Department / Date

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INTRODUCTION AND LITERATURE REVIEW

Molecular nitrogen (N_2), a colorless, tasteless, odorless gaseous element constitutes approximately 79% of our earth's atmosphere. Protein nitrogen is a component of all living entities, yet none of the atmospheric N_2 is available for incorporation into living tissue until it has been changed or "fixed" from its elemental form to a form utilizable by living organisms. This fixation of nitrogen may be accomplished in a number of diverse ways.

Natural gas, a limited, non-renewable resource, is used as an energy source to convert N_2 to ammonia by the Haber process (21). Electrical storms may convert atmospheric N_2 to oxides of nitrogen which are then deposited on the soil with precipitation.

Nitrogen fixation occurs in the soil through a symbiotic relationship between leguminous plants and bacteria of the genus Rhizobium (1). In this symbiosis the bacteria cause growths (nodules) on the rootlets of the plants and this is the locus of fixation. Other bacteria, both aerobic and anaerobic, are also capable of fixing nitrogen in a variety of habitats. Agronomists have long realized that plants of the family Fabaceae (13) tend to enrich, with nitrogenous compounds, the soils in which they are cultivated. Early agrarian cultures relied heavily on these members of the bean family for their food supply

though they did not recognize these beans as being rich in protein (40).

Nitrogen availability may be the limiting factor in primary production of grasslands if one assumes adequate available moisture (7, 31). Biological nitrogen fixation has always been of agronomic importance, although only a few short years ago it was easy and inexpensive to fertilize with commercial nitrogen. Today, with world-wide food shortages and widespread famine in underdeveloped countries, the availability of adequate protein supplies has taken on added importance. More must be known about nitrogen fixation and forage value of the various legume plants under varied climatic and agricultural conditions.

Most species are recognized as producing good to excellent forage. Native legumes are grazed by wildlife and domestic stock (16, 33, 34). Some genera, Oxytropis and Lathyrus as examples, contain species which produce alkaloids toxic to animals. Others such as Astragalus bisulcatus (Hook) Gray are known to be toxic through the accumulation of heavy metals.

One of the more important genera of native legumes is the genus Astragalus, distributed throughout the temperate regions of the world. Species differentiation in this genus is extensive. Barneby (4) recognized more than 500 species native to North America. Komarov (22) recognized 849

species in the U.S.S.R. and described 575 of them. Chamberlain and Matthews (10) recorded 370 species of Astragalus from Turkey.

Davis (11) reports that 46 accessions obtained from the U.S.S.R. and Turkey were grown at Wawawai, Washington to evaluate general forage value and determine whether any of these species might be useful in the western range forage complex. He concludes from this study that:

> quality forages may be developed from *INTRODUCED Astragalus species. The characteristics of this genus are little understood and have been investigated only superficially because their potential may have been underestimated. Areas needing investigation are: species adaptation and establishment in adverse or harsh environments, seed production and harvest, dry matter yields, grazing tolerance, stand persistence, *FERTILITY REQUIREMENTS, interspecies compatibility, etc. Six of the accessions equaled or exceeded alfalfa averages for protein and crude fiber levels.

One of the six, Astragalus cicer Linn is being distributed throughout the western states by commercial seed companies to revegetate "overgrazed" rangelands. This introduced species was used in this investigation as a standard by which to gauge nitrogen fixation of a selected native legume.

All of the cultivated forage legumes grown in South Dakota are introduced plants obtained primarily from Asia

*Emphasis has been added.

and the Russian Steppes. There are, however, many leguminous plants indigenous to South Dakota, growing wild and not currently cultivated.

Aldo Leopold (24), a pioneer ecologist, raised a number of pertinent questions.

> Clean farming, to be sure, aspires to rebuild the soil, but it employs to this end imported plants, animals and fertilizers. It sees no need for the native flora and fauna that built the soil in the first place. Can stability be synthesized out of imported plants and animals? Is fertilizer that comes in sacks sufficient? These are the questions at issue.

The purpose of this study has been to advance knowledge of the nitrogen fixation potential of selected native legumes. With this objective in mind the investigation has been broken down into three segments.

1. Specimens of native legumes were sought on a statewide basis, collected, identified and preserved in a permanent herbarium.

2. Native legumes which showed good nodulation were evaluated for nitrogen fixing potential.

3. The microsymbiont was isolated from the nodules of selected native legumes and cultured.

Several methods exist for measuring nitrogen fixation. The Kjeldahl analysis (8, 39), ^{15}N-enrichment assayed by mass spectrometry and ^{13}N-incorporation assayed by radioactive counting (25) have all been used in the laboratory to quantitatively study nitrogen fixation. These methods

are quite insensitive except for the ^{13}N method, which is limited in its use by the 10 minute half-life of the isotope. Expensive and bulky equipment limit these approaches to studying nitrogen fixation to fixed laboratory situations.

The enzyme systems responsible for the reduction of atmospheric N_2 to ammonia (29) are, appropriately enough, named nitrogenase. Nitrogenase also reduces acetylene to ethylene (35). This knowledge has given rise to an innovative new technique for measuring nitrogen fixation. Hardy and Knight (17) stated:

> Utilization of the reduction of HCN to CH_4 or of C_2H_2 to C_2H_4 and detection of CH_4 and C_2H_4 by hydrogen flame ionization after gas chromatography may provide a sensitive new assay for detection of the N_2-fixing system.

Hardy et al. (18) extensively studied the relationships of acetylene reduction to nitrogen fixation. The acetylene reduction assay has since become widely used as a simple index of nitrogen fixing activity.

The reduction of N_2 to the amino level requires the transfer of 6 electrons, while the reduction of acetylene to ethylene involves the transfer of 2 electrons (36).

One might expect from these studies that the energy to form 3 moles of ethylene from acetylene would be the same as that required for each mole of nitrogen fixed. This seems to be the case in experimental conditions where cell-free

nitrogenase is extracted from nitrogen fixing organisms (35). However, studies conducted by Bergersen (5), in Australia, with excised soybean nodules, gave a ratio of C_2H_4:N_2 of 5.4-8.4:1 with a mean ratio of 6.6:1.

Since the acetylene reduction technique is an indirect assay of N_2 fixation and since relatively few correlation studies between acetylene reduction and N_2 isotope incorporation by intact living systems have been done, Stewart et al. (36) caution that the technique cannot be used as an absolute quantitative assay.

STUDY AREA

Most legumes indigenous to South Dakota are more readily found west of the Missouri River though originally many were found throughout the state. The topography of the state and rainfall patterns make this area less suitable for cropping. The native plant cover, though influenced by grazing, is relatively intact. Two areas of this "West River" country were selected as representative.

Cottonwood Range Research Station

The Cottonwood Range Research Station, Cottonwood, South Dakota is located 19 kilometers west of Philip on United States Highway 14. The Cottonwood study site is representative of midgrass prairie (14, 26). The major grasses are western wheatgrass (*Agropyron smithii* Rydb), green needlegrass (*Stipa viridula* Trin), blue grama (*Bouteloua gracilis* Lag), and buffalograss (*Buchloe dactyloides* Englm). Forbs, especially scarlet globemallow (*Sphaeralcea cocinea* Rydb), fringed sagewart (*Artemisia frigida* Weild), and dandelion (*Taraxacum officinale* Weber) are very common. Of the legumes present, vetch (*Vicia* spp.) and milkvetch (*Astragalus* spp.) are most common. Grazing intensity varies the composition of the cover with heavy grazing encouraging the short grasses.

Climate is of a continental type with large variations

of temperature from summer to winter. Temperatures may fluctuate rapidly from day to day, and even in the span of several hours. The average annual temperature is 11.0 C. Summer highs reach 38.0 C or higher. Winter lows may drop below -30.0 C.

Precipitation averages 38.4 centimeters per year, 30.33 centimeters (79% of the total) fall during the growing season consisting of the months April - September. Soil moisture averages 15.1% throughout the growing season. Thunderstorms produce most of the precipitation during the growing season. Cottonwood receives about 2/3 of possible sunshine during the year. July and August receive the most sunshine. Because of the warm temperatures, high prevalance of sunshine and northwest winds averaging 17.7 kilometers/hour evaporation and transpiration are high (23).

Soil textures are predominantly silty clay. The topography is rather gentle, with long, sloping hills.

Black Hills

The Black Hills rise gradually from the northern Great Plains. Their area encompasses altitudes of 763-2209 meters at Harney Peak.

The climate of the Black Hills is distinct from the surrounding semi-arid Great Plains and thus the transition from one to the other embraces differing vegetation types and growing seasons. The mean temperature averages higher

in winter and lower in summer in the Hills than on the plains. For the state the average temperature for the winter period is 12.5 C while for the Black Hills it is 15.5 C; the average for the summer period for the plains is 21.3 C and for the Black Hills is 19.0 C. As elevation increases, precipitation increases to approximately 61 centimeters per year on the higher peaks (28).

Three broad vegetational formations characterize the Black Hills, namely, the coniferous forest, the deciduous woodlands, and the prairie grassland.

Black Hills-Coniferous Forest

The most extensive and conspicuous formation is the coniferous forest. Over most of the Black Hills this is pine forest, dominated by the ponderosa pine (Pinus ponderosa Laws).

The pine forest varies markedly with respect to height and density. In the foothills and especially at lower elevations it consists of stunted, sparsely distributed stands with some intrusion of rocky mountain juniper (Juniperus scopulorum Sarg). It resembles in general aspect the pinyon-juniper or scrub forests on the lower slopes of the Rocky Mountains in Colorado and New Mexico. Most of the pine forest, however, is comprised of open, park-like stands of tall, straight, highly-branched trees which are far enough apart to permit a forest-floor growth of such

plants as bearberry (Arctostaphylos uva-ursi L) and common juniper (Juniperus communis L). Only where the pine forest is young or growing in sheltered situations are the stands crowded together or "closed" and is the ground below so heavily shaded as to be practically devoid of vegetation.

On a few northern slopes at higher elevations, in the cooler parts of the canyons, and in the upper reaches of Spearfish Canyon in the northern Hills, the coniferous forest includes, and is sometimes dominated by, white spruce (Picea glauca Moesch-Voss), with paper birch (Betula papyrifera Marsh) and quaking aspen (Populus tremuloides Michx) growing in close association. Although often impressive in height and richness of foliage, the stands of spruce are rarely "pure" or extensive enough to warrent the term spruce forest. As a rule they are narrow belts in close proximity to, if not intermixed with, ponderosa pines. Where the spruce forest is relatively pure, it is said by some to represent a relict boreal forest.

Black Hills-Deciduous Woodlands

The deciduous woodlands formation, compared to the coniferous forest, is notably discontinuous, occurring on the floors of wider valleys and canyons, on the alluvial bottomlands adjacent to the streams that course through the lower valleys, and on occasional slopes. The woodlands in wide valleys and canyons consist typically of American

elm (Ulmus americana L), green ash (Fraxinus pennsylvanica Marsh) and box elder (Acer negundo L), with an understory of numerous shrubs such as Hawthorn (Crataegus rotundifolia Moench), chokecherry (Prunus virginiana L), and hop hornbeam (Ostrya virginiana Koch). Along the streams of the lower elevations the woodlands also include cottonwoods (Populus spp.) and willows (Salix spp.).

Black Hills-Prairie Grassland

The prairie grassland formation represents an extension into the Black Hills of the same formation from the surrounding plains. Dominant among the grass species are those of both the short-grass and tall-grass prairies. Typical segments of prairie grasslands within the Black Hills appear on the more arid slopes of the foothills, on rolling uplands - hills, valleys and meadows - where they are locally called "prairies" or "balds".

Gillette Prairie-Black Hills-Alpine Midgrass Prairie

Gillette Prairie, a "bald" located in the central Black Hills, is at an elevation approaching 1990 meters above sea level.

The growing season usually extends from late May to mid August, though freezing may occur during any month of the year. Thus, the effective growing season may be retarded, shortened or periodically interrupted.

Predominant grasses are buffalograss (Buchloe dactyloides Englm), brome grass (Bromis inermis Leyss), timothy (Phleum pratense L), and Kentucky bluegrass (Poa pratensis L). Native legumes include species of Astragalus, Thermopsis, Oxytropis and Vicia. Among the cultivated legumes are alsike clover (Trifolium hybridum L), red clover (Trifolium pratense L), and cicer vetch (Astragalus cicer). Some of the lower valley meadows are cultivated in oats, alfalfa and cicer vetch, while most of the higher meadows are cut for wild hay. In some sheltered areas aspen have become established together with bearberry and other shrubby plants. The total area of the prairie approximates 200 hectares surrounded by coniferous forest.

Soils are generally shallow and rocky, varying in depth from 8 cm to 100 cm. Shallow soils are common on hillsides with deeper soils in the valley bottoms. Soil fertility is good with an average of 3.36 ppm total NO_3-N throughout the growing season, 3.66 kilograms/hectare phosphorus and 5.96% organic matter.

Lengkeek (23) has noted:

> native leguminous plants are very prevalent in the prairie and come into anthesis at various times during the growing season being grazed by domestic livestock as well as deer and grouse.

MATERIALS AND METHODS

1971 Growing Season - Plant Collection

Legume plant specimens were collected throughout South Dakota from the spring of 1971 through the summer of 1973. In 1971 the study took the form of a survey to become familiar with the native legumes, their normal habitats, growth habits and nodulation potential. An attempt was made to collect representative specimens of the same species from the same area several times throughout the growing season to include buds, flowers, seed pods (legumes) and root systems. A record was kept by accession number to indicate, where possible, the county, section number, road direction from a reference point, date and altitude. These records made it possible to return to the original plant collection spot in an attempt to collect seeds.

Plants were pressed and then poisoned with a solution of 0.1% mercuric chloride dissolved in 70% ethyl alcohol to prevent destruction by insects. The specimens were mounted on standard botanist's white mounting paper, identified and stored in the permanent herbarium of the Microbiology Department of South Dakota State University.

Coincidental with the collection of plants, roots were examined for nodulation (Fig. 1). In the 1971 growing season relative degrees of nodulation were recorded in field notes as heavily, moderately, or sparsely nodulated.

Fig. 1. A nodulated root system of Oxytropis campestris L illustrating a portion of the extensive tap root is shown. Note the nodules on the rootlets extending from the lateral roots.

Absence of nodular material was also noted. No attempts were made to quantify the nodules by weighing or counting due to the nature of the study at this time and the difficulty of removing intact root systems from the soil with the assurance that all the nodules remained attached to the roots. Noted, too, was the quality of nodulation to include size, interior color and moisture content, since it is now well accepted that the amount of leghemoglobin present in a nodule, indicated by the "redness" of the nodular tissue, is directly related to nitrogen fixation potential. Thus the more red the nodular tissue the more fixation one could expect (1).

Seed Collection and Testing

Diverse growing seasons and isolated, sporadic rainfall events complicate seed collection since many of these native plants are adapted to emerge, flower, set and ripen seeds within a 6 week period. It is characteristic that the legumes, or seed pods, of these plants dehisce and discharge their seeds when ripe. Plants of the same species growing 80 kilometers apart at approximately the same altitude and latitude were found to produce ripe seed as much as 2 weeks apart. Dried seed was stored in aluminum 35 mm film canisters.

The winter of 1971 and early spring of 1972 were spent investigating germination procedures and rates. Seeds were

scarified by mortar and pestle, using two methods. Washed white aquarium sand was added to small seeds in the mortar to equal approximately 10% of the seed volume. The pestle was used sparingly to avoid rupturing the seed. Larger seeds were scarified in the mortar with a pestle wrapped in number 220-A Norton, Adalox finishing sandpaper.

Germination tests were accomplished by surface sterilizing the seed to minimize microbial contaminants. The seeds were placed in a 0.1% solution of mercuric chloride ($HgCl_2$) for a few seconds, followed by two rinses in sterile water. Seeds were placed in sterile petri dishes containing a carpet of Whatman No. 1 filter paper, dampened with sterile water to maintain a high humidity, and germinated in a dark box at room temperature. After 10 days the number of germinated seeds was counted and germination percentages calculated.

1972 Growing Season - Evaluation of Nitrogen Fixation - Cottonwood

The Cottonwood Range Experiment Station was selected as a base from which to conduct the field investigations. During the months of March and April, 1972, an 8 by 50 foot mobile home was rebuilt into a mobile laboratory facility. The living-dining area became a chemistry laboratory. The smallest bedroom, which could be isolated from the rest of the trailer, became a microbiology laboratory. Air

conditioning was added to ensure proper incubation temperature for soil microbes.

Two native legumes, Astragalus missouriensis Nutt and Astragalus striatus Nutt were selected for the most stringent evaluation, though not to the exclusion of other legumes.

These 2 species were selected for a number of reasons. A. missouriensis is bountiful in the environs of Cottonwood and could, therefore, be collected with a minimum expenditure of time. A. striatus is less plentiful at Cottonwood but is found throughout South Dakota and appears adapted to diverse soils and climatic conditions. The herbarium catalogues specimens of A. striatus from extreme eastern South Dakota as well as from elevations in excess of 1800 meters in the Black Hills. Welch (37) notes that A. striatus is found from Alaska and the Northwest Territories to Colorado, and postulates that it is a variant of A. adsurgens Palas, a European species. If this hypothesis is borne out, the species will prove to have an hitherto unsuspected range. Both species were usually found to be well nodulated with moist, pink nodules throughout the growing season. Seeds from A. striatus germinated well and seedlings were easily grown in the greenhouse. These findings were supported by Soil Conservation Service data on cultivation of native legumes of the Great Plains (3) and by work

done in North Dakota (Warren C. Whitman, personal communication) (30, 38).

The acetylene reduction assay was the method chosen to evaluate nitrogen fixation. Some type of closed system to contain root nodules plus an artificial atmosphere containing acetylene from which periodic gas samples could be drawn was necessary. The best possible device for this assay would be one in which an intact plant growing in its natural environment could be used. This ideal "in situ" situation was impossible to achieve. We had no knowledge of how well nodulated a given specimen might be if, indeed, it was nodulated at all, without first disturbing the root system. These root systems overlap and intertwine roots of numerous other plants, legumes and non-legumes, nodulated and non-nodulated, effective and ineffective, making individual plants impossible to study.

Great difficulty is encountered when trying to expose a complete root system without destroying, damaging or losing the attached nodules. Tedious, time consuming washing processes must be used to remove all but the lightest sandy soil. Therefore no attempt was made to quantify the amount of nitrogen fixed per plant or per unit nodule weight. The study concerned itself with the question "Do they or do they not fix nitrogen and, if so, how well in relation to other legumes?"

The procedure used by R.H. Burris at the University of Wisconsin for exposure of roots and nodules to acetylene without excising the above ground portion of the plant seemed to best lend itself to our objectives (R.H. Burris, personal communication).

The leguminous plant was removed from the soil, where it naturally occurred, by estimating the diameter of the root system, drawing on past experience and familiarity with the species in such a way that there was minimal disruption of roots and nodules. After determining if a plant was nodulated it was not necessary to completely expose the roots, though fracturing the soil core facilitated a more rapid gas exchange between atmosphere and roots. If the stem was wet, it was dried near the crown and immediately wrapped with modeling clay in which a gasing port had been embedded. Refer to Fig. 2 for details of the gasing port. After sealing the modeling clay against the stem the root system was placed in a 12.7 x 30.5 centimeter saran bag. Saran bags were chosen because they are less permeable to gases than other plastics. The bag was then smoothly rolled around the modeling clay. Care was taken that there were no large wrinkles to cause leaks, and the bags were secured with 2 plastic "Twistems" of the type used to seal plastic food wrappers. Fig. 3 shows details of a plant with the root system enclosed in a saran bag. It was thought to be

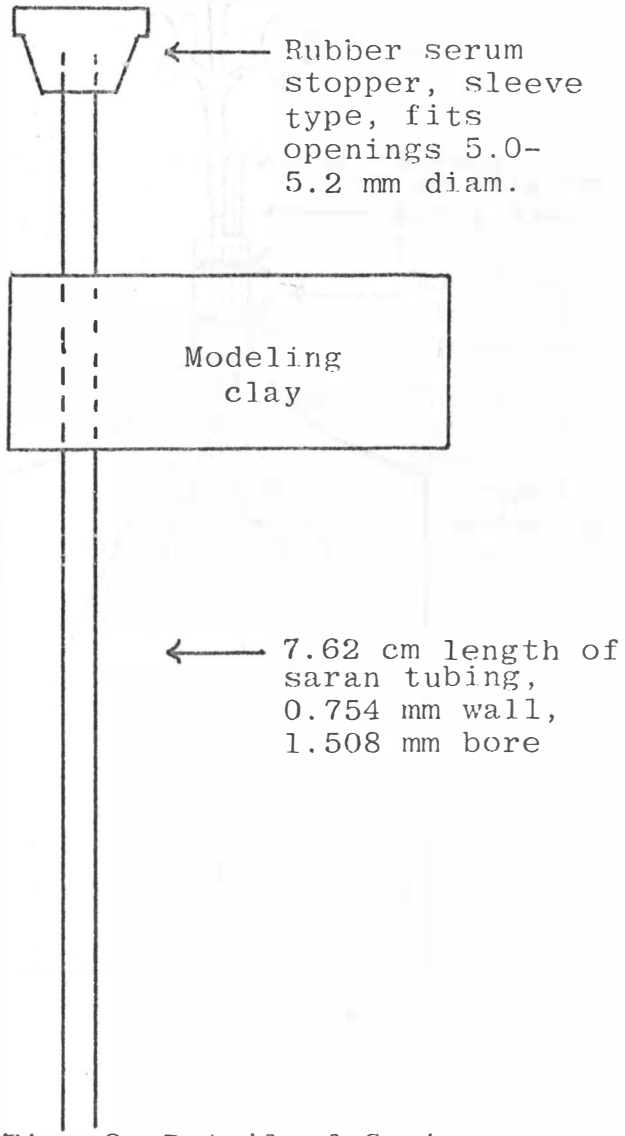

Fig. 2. Detail of Gasing Port.

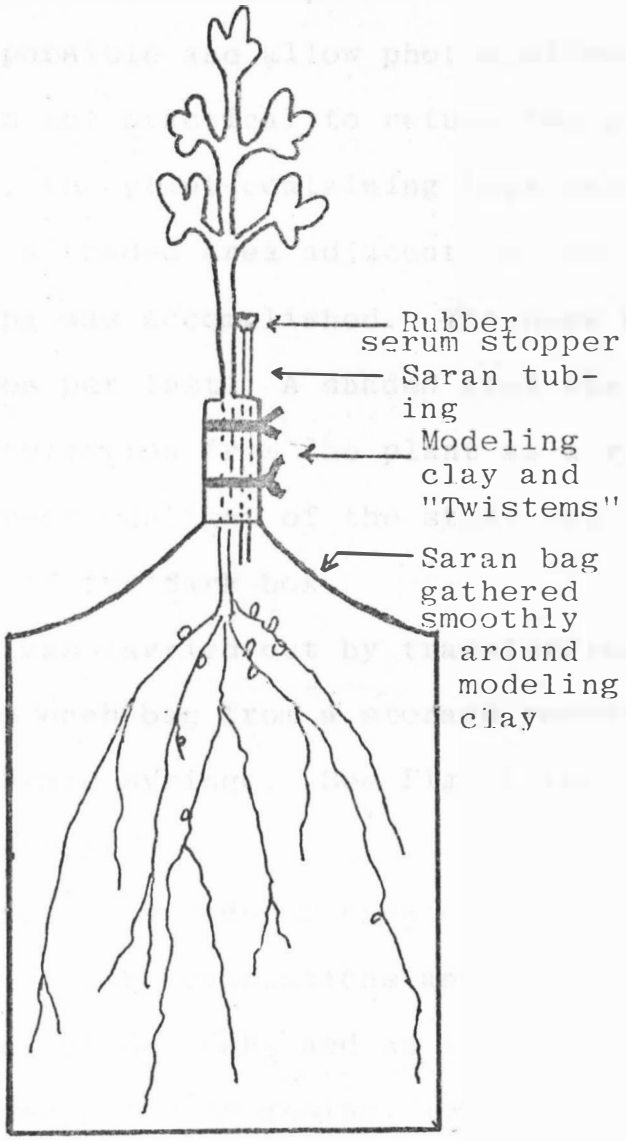

Fig. 3. Plant with root system enclosed in Saran bag.

desirable to simulate the plant's natural environment as closely as possible and allow photosynthesis to proceed. Since it was not practical to return the plants to the soil for testing, the plant-containing bags were suspended in a dark box in a shaded area adjacent to the mobile laboratory before gasing was accomplished. The dark box accommodated 10 replicates per test. A shaded area was desirable to reduce transpiration from the plant as a result of the high heat and direct sunlight of the study area. See Fig. 4 for details of the dark box.

Gasing was carried out by transferring 100 cc of pure acetylene to each bag from a storage reservoir with a 50 cc glass hypodermic syringe. See Fig. 5 for details of the storage reservoir.

No attempt was made to create a nitrogen free atmosphere by successive evacuations and refilling of the bags with mixtures of O_2, C_2H_2 and an inert gas. If the bags were distended prior to gasing, 100 cc of air was removed through the gasing port prior to addition of acetylene. The syringe was made "gas tight" by wetting the plunger with water to create a seal between plunger and barrel.

Gas samples were removed with a 5 cc glass syringe at 30 minutes, 2 hours, 5 hours and 24 hours from initial gasing. The syringe was pumped 3 times, after the needle had been inserted into the bag, before withdrawing to ensure a

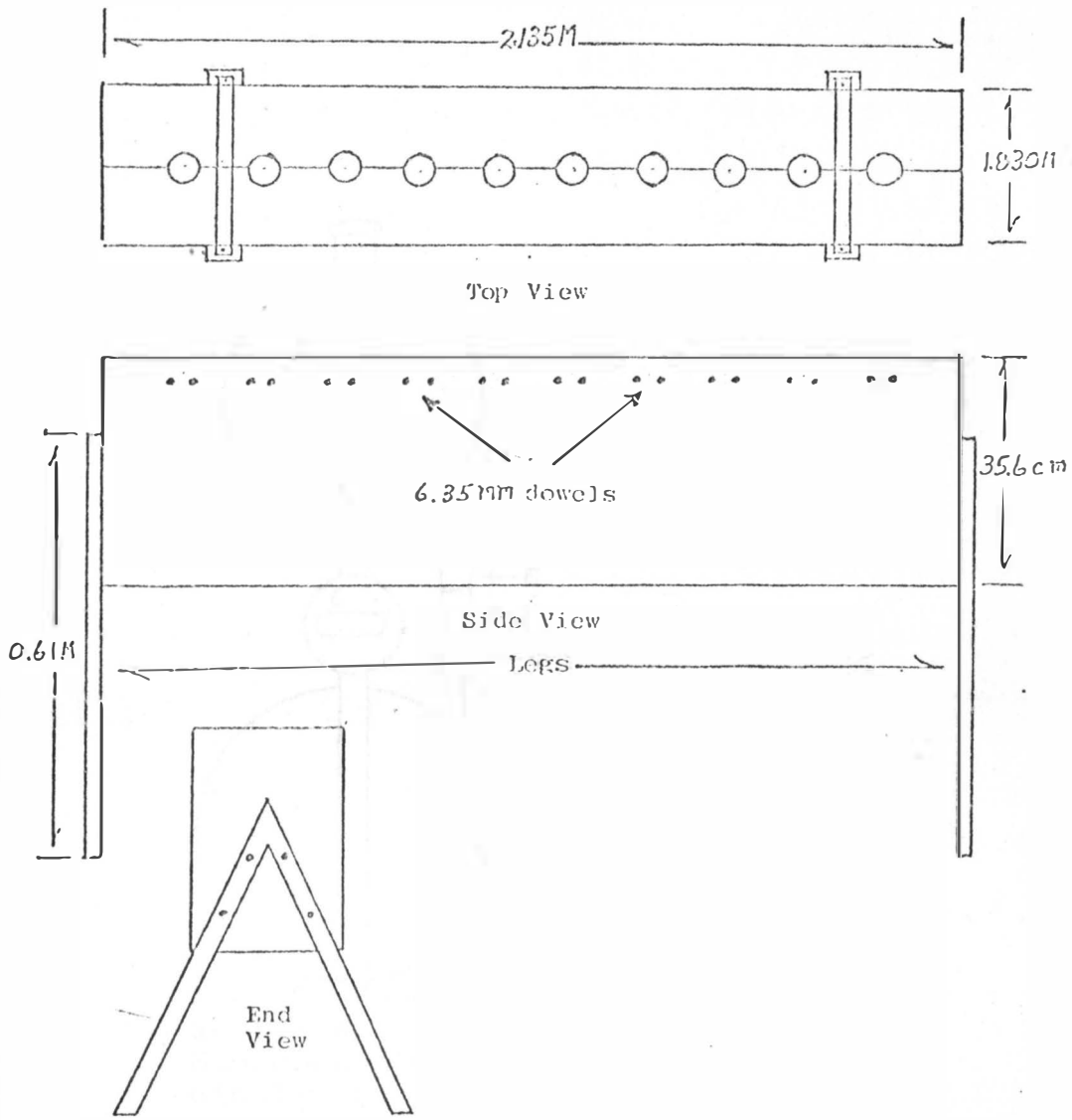

Fig. 4. Dark Box

Constructed of 6.35 mm plywood. The top is two pieces of plywood with half circles cut in each half. Each half is held in place with bars bolted across the top. Bolt on legs are 2.54 x 5.08 cm white pine. The Saran bags are suspended inside the box by strings secured to 6.35 mm dowels. Plants from crown up protrude from dark box so plants may photosynthesize.

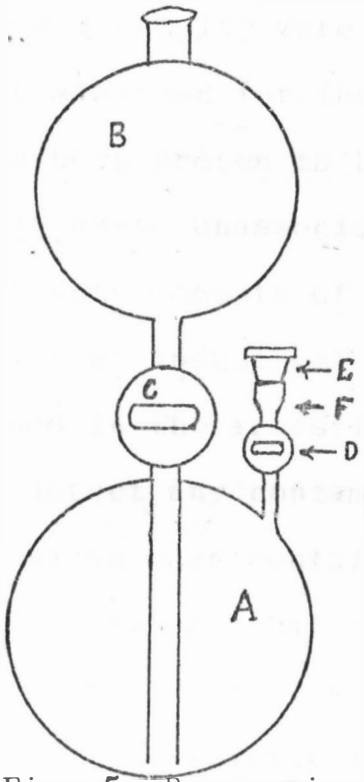

Fig. 5. Reservoir for storing acetylene.
Stopcock C controls entry of displacing fluid and D the exit of the gas mixture. After serum stopper E is seated, the space F can be evacuated through a hypodermic needle. With C and D open, the gas mixture can be removed from space F and A with a hypodermic needle and syringe.

representative sample. These gas samples were injected for storage into small 2 cc test tubes plugged with serum bottle stoppers. The test tubes had been previously evacuated in the laboratory with a Welch high-vacuum pump.

The stored samples were transported to Brookings bi-weekly to be analyzed for the presence of ethylene (C_2H_4). Ethylene has been proven to be a natural product of plants (32) normally quite unassociated with biological nitrogen fixation. Minute amounts of ethylene are often detected in the atmosphere of industrial areas. Control samples of acetylene used in the investigations were periodically analyzed to detect any contaminating ethylene. Plant controls in saran bags containing no acetylene were included in each test to assure that no detectable levels of ethylene were being evolved naturally by the plants. None was ever detected at the sensitivity levels used.

The gas-liquid chromatograph used was a dual column Varian Aerograph, Series 1520. Pure nitrogen at 12 PSI was the carrier gas. A 185.5 centimeter aluminum column, 3.17 millimeters in diameter, was utilized. The column was packed with fully activated alcoa, type F_1, chromatograph grade, wet screen classified alumina. Oven temperature was 150 C. The recorder was a Sargent, series SRG.

1972 - Rhizobium Culture

The media used for cultivation of nodule bacteria were

Yeast Extract Mannitol Agar (2) and Yeast Extract Mannitol Antibiotic Agar (15).

Yeast Extract Mannitol Agar

Mannitol	10.0 g
Dipotassium phosphate (K_2HPO_4)	0.5 g
Magnesium sulphate ($MgSO_4 \cdot 7H_2O$)	0.2 g
Sodium chloride (NaCl)	0.1 g
Calcium carbonate ($CaCO_3$)	3.0 g
Yeast Extract	10.0 g
Agar agar	15.0 g
Distilled water	1000.0 ml

Yeast Extract Mannitol Antibiotic Agar

Mannitol	5.0 g
Lactose	5.0 g
Dipotassium phosphate (K_2HPO_4)	0.5 g
Sodium chloride (NaCl)	0.2 g
Calcium chloride ($CaCl_2 \cdot 2H_2O$)	0.2 g
Magnesium sulfate ($MgSO_4 \cdot 7H_2O$)	0.1 g
Ferric chloride ($FeCl_3 \cdot 6H_2O$)	0.1 g
Yeast Extract	0.5 g
Agar agar	20.0 g
Distilled water	1000.0 ml

Autoclave for 15 minutes at 15 PSI. Then add the following antibiotics after allowing the medium to cool:

Pentachloronitrobenzine	100.0 mg
Na benzylpenicillin (penicillin G)	25.0 mg
Chloromycetin	10.0 mg
Sulfathiazole	25.0 mg
Neomycin	2.5 mg

Adjust pH to 7.0 and pour.

The Yeast Extract Mannitol Agar was the medium chosen since it was less expensive and time consuming to make. The antibiotic medium was used to purify cultures inasmuch as it was highly selective for rhizobia, although some slow,

poorly growing Rhizobium species were excluded.

Nodules were collected in the field from plants as they were removed from the ground for acetylene reduction assay. Nodules from each plant were placed in separate, sterile 15 ml serum bottles and capped with serum stoppers. One drop of sterile water was injected through the rubber stopper with a syringe to prevent dessication of the nodular material before the bottles were refrigerated for transportation to the microbiology laboratory at Cottonwood. Representative specimens of the nodulated legumes were collected for preservation in the herbarium, as documentation for species.

In the laboratory a culture technique was established through trial and error. Various rinses were used on the nodules to reduce surface contamination. Among the chemical antiseptics used were 70% ethyl alcohol, a 0.1% aqueous solution of mercuric chloride and a 0.0018% aqueous solution of sodium hypochlorite (household bleach). The sodium hypochlorite and mercuric chloride seemed to reduce viability of the rhizobia within the smaller nodules collected, so a 70% ethyl alcohol rinse was utilized. Two rinses of sterile water followed by 10 seconds in alcohol, then 2 additional rinses of water seemed to best reduce surface contamination without sterilizing the nodule contents. Transfers from one rinse to another were made with separate

sterile forceps.

Individual nodules were removed from the final rinse and transferred aseptically to separate culture plates where they were squeezed with forceps, to extrude the contents, streaked, and incubated at 26 C.

It was essential to rinse only one nodule in each series of rinse solutions to avoid cross contamination. One plant may be nodulated by more than one strain of Rhizobium. Some nodules may effectively fix nitrogen while others may not. Thus, to isolate effective strains of the symbiont it was essential to treat each nodule separately.

Cultures were initially screened by Gram's staining for Gram's negative bacilli. All other cultures were sterilized and discarded. Bergey's Manual of Determinative Bacteriology, 7th Edition (6) is vague about physiological traits of the genus Rhizobium though definite about morphology and Gram's staining characteristics. Only 6 species of this genus are recognized by Bergey's Manual. These 6 are all named for the leguminous plant which they effectively nodulate. Studies in Wisconsin have shown that this classification scheme is inadequate because of the presence of cross inoculation groups. (9).

Litmus milk and a series of phenol red fermentation broths including glucose, galactose, mannose, lactose and maltose were inoculated. Any cultures which produced gas

or rapid proteolysis were discarded. Cultures were kept in the physiological media for 4 weeks and reactions were recorded periodically. Isolates which form heavy mucoid colonies are most typical rhizobia but other growth habits have been recorded. Some are slow growing while others grow quite rapidly. Refer to Fig. 6 for typical litmus milk reactions.

The other 2 genera in the family Rhizobiaceae share many traits with their sister genus and therefore the only certain method of identifying isolates as Rhizobium spp. is by utilizing Koch's Postulates. The supposed Rhizobium must be isolated from a nodule, cultured, reinoculated into a plant of the same species and recultured.

Isolates which were assumed to be Rhizobium spp. are currently being maintained on Yeast Extract Mannitol Agar.

1973 Growing Season - Evaluation of Nitrogen Fixation - Gillette Prairie

In mid-May of 1973 the mobile laboratory was moved to the Gillette Prairie site south of Deerfield Lake in the Black Hills. It was decided to concentrate our efforts this growing season on Astragalus striatus and attempt to evaluate it for nitrogen fixation potential. A unique opportunity was presented for a comparative study, this year, between A. striatus and A. cicer cultivar Lutana. The former grows in abundance on the prairie as may be seen in

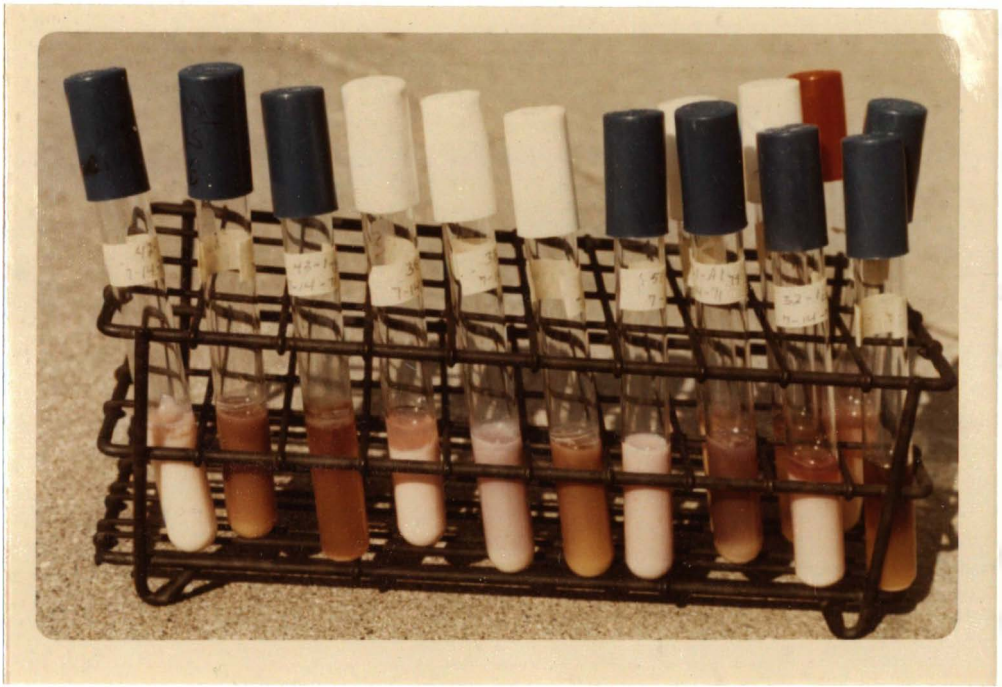

Fig. 6 Litmus Milk Reactions
Note the reactions in this rack of litmus milk medium one month after inoculation with *Rhizobium* spp. In some tubes there is a total lack of reaction. Some show complete proteolysis while others have only a slight serum zone.

Fig. 7, and the latter was planted as a forage crop directly adjacent to the native A. striatus.

We had experienced difficulty in the 1972 studies in transporting stored gas samples from the Cottonwood site to the Brookings laboratory for the acetylene reduction assay. For some undetermined reason the gas samples were not uniform after storage and transporation. They gave erratic data on the chromatograph. Accordingly, the mobile laboratory was equipped with the Varian Aerograph gas chromatograph obviating the need to store gas samples and greatly facilitating the assay work. Samples could be drawn directly from the incubation containers and injected into the GLC column.

This year (1973) since quantitation was still not an objective of the study, and the incubation of intact plants had proven to be cumbersome, we altered the acetylene atmosphere incubation technique. Excised nodules were placed in 15 cc serum bottles capped with rubber serum stoppers. As in the prior year acetylene was added to the containers with syringes and needles through the rubber stoppers. Periodic samples were withdrawn and analyzed. Astragalus cicer cultivar Lutana and Astragalus striatus samples were analyzed concurrently (Fig. 8). After the samples were subjected to the acetylene reduction assay the nodules were removed from the serum bottles and weighed.

Fig. 7. A native stand of Astragalus striatus on Gillette Prairie in the Black Hills of South Dakota. The investigators are collecting root nodules for nitrogen fixation experiments. Note the profuse growth and number of seed heads of A. striatus.

Fig. 8. In the mobile laboratory a gas sample is being removed from an incubation bottle with a micro-syringe for analysis on the chromatograph. The bottle is enveloped in the investigator's hand.

Various biotic and abiotic factors influence the growth, nodulation and nitrogen fixation potentials of leguminous plants. The following parameters were measured in this investigation.

Weather Data

Weather data were accumulated in 1972 and 1973 by various means and devices. Air temperature and rainfall data were obtained from a weather station maintained at Cottonwood. Surface and subsurface ground temperatures were obtained by using probe type thermometers.

At Gillette Prairie an automatic temperature recording device was tried but when humidity made it inoperative after heavy dew or rainfall events it was discarded in favor of probe type thermometers and rain gauges.

Organic Matter and Soil Water Data

Another investigation was being conducted on Gillette Prairie at this same time. The investigators worked together to provide data on soil water. Percent organic matter and soil chemistry data were determined by the Soils Testing Laboratory at South Dakota State University at Brookings, South Dakota from composite soil samples collected by the investigators.

Soil water data were derived by weighing composite samples in crucibles and then subtracting the dried crucible

weight to give weight of soil plus water. The soil samples were then oven-dried for 24 hours at 100 C and reweighed. Dry soil weight was subtracted from wet soil weight to give the weight of water driven off by heating. Calculation of percent soil water was determined by the following formula:

$$\% \text{ soil } H_2O = \frac{\text{wet soil weight - dry soil weight}}{\text{wet soil weight}} \times 100$$

RESULTS AND DISCUSSION

Plant Collection and Identification

The results of the collection and identification of native legumes commencing in 1971 and continuing through the present time are summarized in the following paper accepted for publication in the 1974 Proceedings of the South Dakota Academy of Science.

HERBARIUM OF NATIVE LEGUMES AND OTHER SYMBIOTIC NITROGEN-FIXING PLANTS OF SOUTH DAKOTA[1]

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The importance of biological nitrogen fixation in establishment and maintenance of soil nitrogen is often underestimated or neglected. Studies of cultivated legumes such as alfalfa and the clovers, all introduced species, have shown them to be valuable in replenishing soil nitrogen when properly nodulated and used as a green manure crop. Virtually no work has been done in measuring the value of native legumes in grasslands.

Studies in the Soil Microbiology Laboratory of the South Dakota Agricultural Experiment Station are underway to determine the value of these plants in the prairie grasslands of eastern, central, and western South Dakota and the alpine meadows of the Black Hills. Part of these studies involves determining the occurrence and prevalence of native leguminous species in the state.

The botanical specimens of the region have been collected and classified according to many schemes, beginning

[1]Approved for publication by the Director of the South Dakota Agricultural Experiment Station as paper 1270 of the Journal Series.

with the Lewis and Clark exploration of 1804-1806 which yielded a specimen and description of Astragalus tenellus Pursh. Among the early botanists studying the flora of the plains were Thomas Nuttall and John Bradbury in the United States and John Richardson and David Douglas in Canada. Around 1900, workers began to accumulate specimens of local plants in regional herbaria. O. H. Stevens has extensively studied the flora of North Dakota, and his specimens are in the herbarium of the North Dakota State University. Theodore Van Bruggen of the University of South Dakota and C. A. Taylor of South Dakota State University have contributed to the herbaria of these schools.

An herbarium collection devoted chiefly to the native legumes of South Dakota is maintained by the authors in the Soil Microbiology Laboratory of the Microbiology Department of the South Dakota State University, Brookings, South Dakota 57006. This collection is preparatory to an attempt to relate economic importance to naturally-occurring legumes and other nitrogen-fixing plants in South Dakota.

Variations in soil type, precipitation, latitude, elevation, and land use practice in South Dakota provide a wide range of habitats for plant growth. A diverse group of native legumes exists here. Many of these are indigenous to the whole state but are found most frequently west of the Missouri River where the native prairie is least

disturbed by agricultural practices. Therefore, most of our accessions are from western South Dakota where collecting has been most profitable. There are notable exceptions, such as the slopes of the "Coteau de Prairie" in northeastern South Dakota, railroad rights-of-way, Missouri River lowlands, and small undisturbed prairie areas which have confirmed the occurrence of certain species throughout the state. Examples of these are: Astragalus spp., Psoralea spp., Oxytropis spp. and Petalostemon spp.

The herbarium contains specimens from 3 families, 18 genera, and 38 species of legumes and 2 families, 3 genera, and 3 species of non-legumes native to South Dakota as noted in the following table. Numerous others exist and will be added as time and financing permit.

HERBARIUM COLLECTION, SOIL MICROBIOLOGY LABORATORY, SDSU

Native Legumes

Families - Genera - Species - Authority

Caesalpiniaceae		
	Cassia	fasciculata Michx.
Fabaceae		
	Amorpha	canescens Pursh.
	Amphicarpa	bracteata (L.) Fern.
	Apios	americana Medic.
	Astragalus	aboriginum Rich.
		agrestis Dougl.
		alpinus L.
		bisulcatus (Hook.) Gray
		canadensis L.
		crassicarpus Nutt.
		flexuosus Dougl.
		gilviflorus Sheld.
		gracilus Nutt.
		missouriensis Nutt.
		shortianus Nutt.
		spatulatus Sheld.
		striatus Nutt.
	Desmodium	canadense L.
	Glycyrhiza	lepidota (Nutt.) Pursh.
	Hedysarum	alpinum L.
	Lathyrus	ochroleucus Hook.
	Lotus	purshianus (Benth.) Clem. and Clem.
	Lupinus	argenteus Pursh.
		sericeus Pursh.
	Oxytropis	campestris (L.) D.C.
		lambertii Pursh.
		sericea Nutt.
	Petalostemon	candidum (Willd.) Michx.
		occidentale (Heller) Fernald
		purpureum (Vent.) Rydb.
	Psoralea	argophylla Pursh.
		cuspidata Pursh.
		esculenta Pursh.
		tenuiflora Pursh.
	Strophostyles	helvola (L.) Ell.
	Thermopsis	rhombifolia Nutt.
	Vicia	americana Muhl.

Native Legumes (Cont.)

Mimosaceae		
	Shrankia	*nuttallii* (D.C.) Standl.

Other Plants That Fix Nitrogen

Elaeagnaceae		
	Shepherdia	*argentea* (L.) Nutt.
	Elaeagnus	*angustifolia* L.
Compositae		
	Artemisia	*ludoviciana* Nutt.

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Germination Tests

Seeds of Astragalus missouriensis and Astragalus striatus were selected for germination tests as detailed in the methods section. Results varied markedly with the seed stock tested. Some seeds had apparently been collected too soon and were not thoroughly matured. Others were stored in 35 mm film canisters before they were completely dried. Still others became infested with insects which decimated them. Through experience good seed could be visually selected. No tetrazolium chloride viability tests were conducted.

Germination tests conducted with seed of uniform size selected for dry, dark, glossy seed coats properly scarified gave good germination results. A. striatus consistently yielded about 90% germination while A. missouriensis fell slightly behind with germination of 83% of seed tested.

One of the larger seeded native legumes, Lupinus argenteus, was also tested for germination. Results of less than 40% were disappointing.

Seeds of 15 additional native legumes have been collected and will be tested as time permits.

1972 Cottonwood

Tables 1 and 2 are representative of data collected at Cottonwood in 1972. Fig. 9 shows the relationship of soil

	Astragalus striatus plant number									
	1	2	3	4	5	6	7	8	9	10
Time -0-	-	-	-	-	-	-	-	-	-	-
1/2 hr.	-	-	-	-	-	-	-	-	-	-
2 hrs.	-	-	-	+	-	-	-	-	+	-
5 hrs.	+	+	-	-	-	+	-	-	+	-
24 hrs.	+	+	+	+	-	-	+	+	+	-

Table #1 *Astragalus striatus* Acetylene Reduction

\+ indicates reduction of acetylene to ethylene
\- indicates no reduction of acetylene
Data are derived from gas chromatograph samples run on June 22, 1972. All plants were nodulated with pink nodules.

	Astragalus missouriensis plant number									
	1	2	3	4	5	6	7	8	9	10
Time -0-	-	-	-	-	-	-	-	-	-	-
1/2 hr.	-	+	-	-	-	-	-	-	+	-
2 hrs.	-	+	-	+	-	-	-	-	-	-
5 hrs.	+	-	+	+	-	+	+	+	-	+
24 hrs.	+	+	+	+	+	+	-	+	+	-

Table #2 *Astragalus missouriensis* Acetylene Reduction

\+ indicates reduction of acetylene to ethylene
\- indicates no reduction of acetylene
Data are derived from gas chromatograph samples run on June 21, 1972. All plants were nodulated with pink nodules.

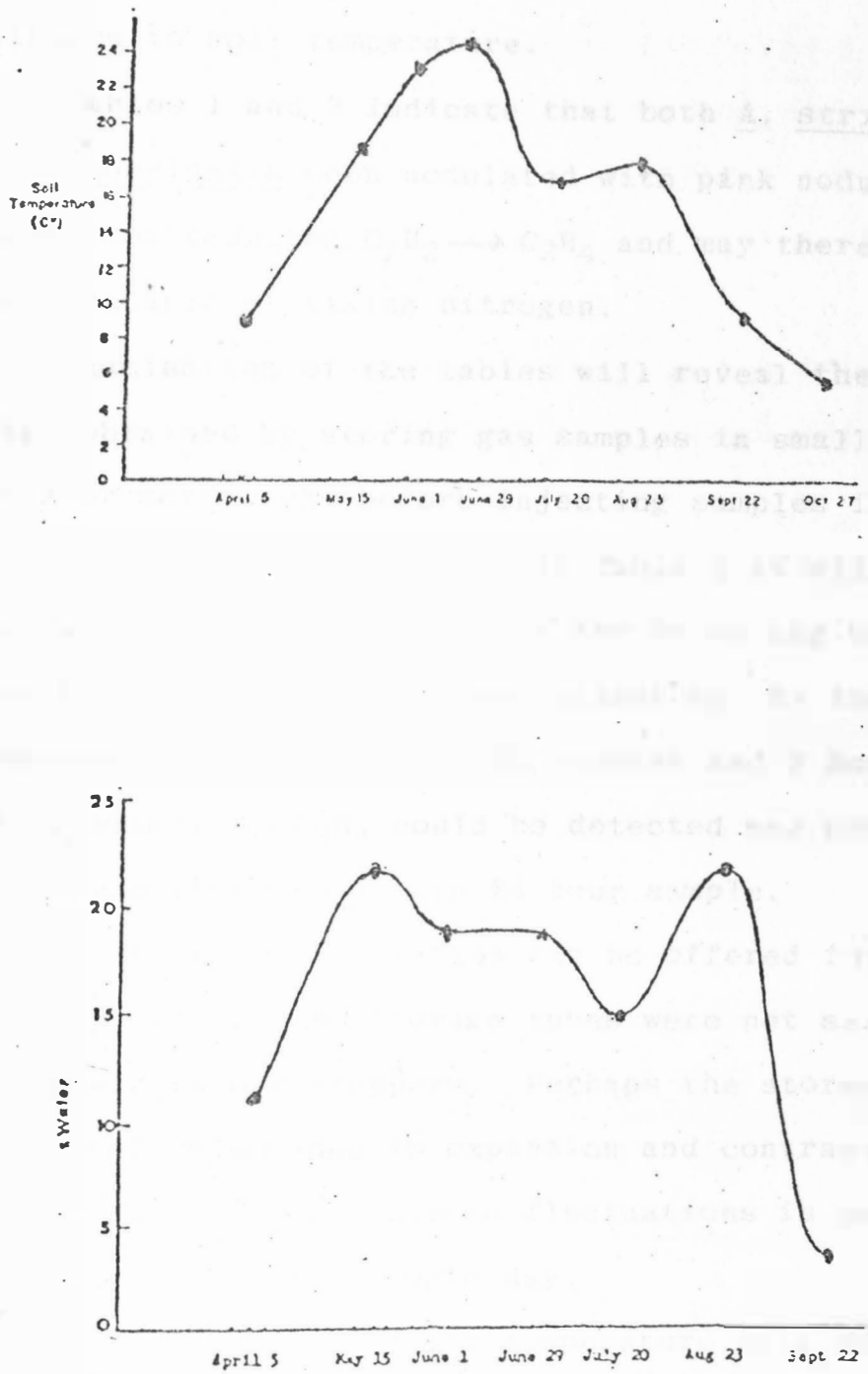

Fig. 9. Showing relationship of soil temperature to percent soil water at Cottonwood for the 1972 growing season. Points on the graphs indicate known data. The lines have been added in an attempt to interpolate between points and establish the trends throughout the season.

moisture to soil temperature.

Tables 1 and 2 indicate that both A. striatus and A. missouriensis when nodulated with pink nodules are capable of reducing $C_2H_2 \longrightarrow C_2H_4$ and may therefore be presumed capable of fixing nitrogen.

Examination of the tables will reveal the erratic results obtained by storing gas samples in small test tubes for 2 or more weeks before injecting samples into the gas chromatograph for analysis. In Table 2 it will be noted that a gas sample removed from the saran bag containing an intact A. missouriensis plant (plant no. 2) indicated the presence of ethylene after 30 minutes and 2 hours. In the 5 hour sample no C_2H_4 could be detected and positive results were again observed in the 24 hour sample.

No adequate explanation can be offered for these data. Possibly some of the storage tubes were not sealed properly with their rubber stoppers. Perhaps the stored samples were of unequal volume due to expansion and contraction of the gases as a result of extreme fluctuations in ambient temperature throughout the sample day.

The soil water and air temperature data were available and are included only to show the conditions on the range at the time these fixation data were obtained.

The saran bag technique is a useful tool in nitrogen fixation experiments. The technique is time consuming. One

must be certain that there are no gas leaks around the plant stem where it emerges from the modeling clay. It is easy to rip the bag while placing a plant inside of it for incubation of the roots and nodules in an acetylene atmosphere.

The technique would have been more useful as a field procedure if a gas chromatograph had been available to analyze samples immediately.

Nodules from several other native legumes were analyzed for nitrogen fixation potential at Cottonwood. Petalostemon purpureum and Vicia americana of the family Fabaceae and Shrankia nuttallii (sensitive plant) of the family Mimosaceae all demonstrated acetylene reduction.

Numerous plant specimens were added to the herbarium. Of the plants listed as native legumes in the herbarium only Astragalus spatulatus could not be found in a nodulated condition.

1973 Gillette Prairie-Black Hills

The two areas on Gillette Prairie selected for intense study, though less than 500 meters apart, are dissimilar. The study area seen in Fig. 7 selected for it's abundance of A. striatus is approximately 45 meters higher than the area containing the cultivated A. cicer. The soil in the A. striatus plot is shallow, rocky and the entire area of slightly more than 1 hectare is exposed to the wind. It

has never been cultivated. The soil in which A. cicer is cultivated is at the lower end of a hay meadow where the rich loam has accumulated to a depth of over 45 centimeters. This 1/4 hectare area through its protected location and the nature of its soil tends to retain a more constant humidity although temperature inversions sometimes cause it to be colder at night. Heavier frost was observed here than on the A. striatus and it persisted later in the day as the sun's rays were later reaching the meadow bottom.

Figs. 10-13 give an idea of the relationships that existed through June, July and August of 1973 between soil temperature, ambient air temperature and soil water, while table 3 shows total recorded precipitation for the growing season. Data are known for the period June 6 through June 25 and from then on only for the dates indicated on the graphs. The dots have been connected in an attempt to establish the trends which existed, but the lines must not be interpreted to give reliable data for particular dates. By studying these figures one may get an idea of the climatic differences between the two adjacent areas.

About 150 meters north of the A. striatus study area is an ecotone of aspen separating the prairie from a second growth pine forest. As early as May 31, 1973, while snow

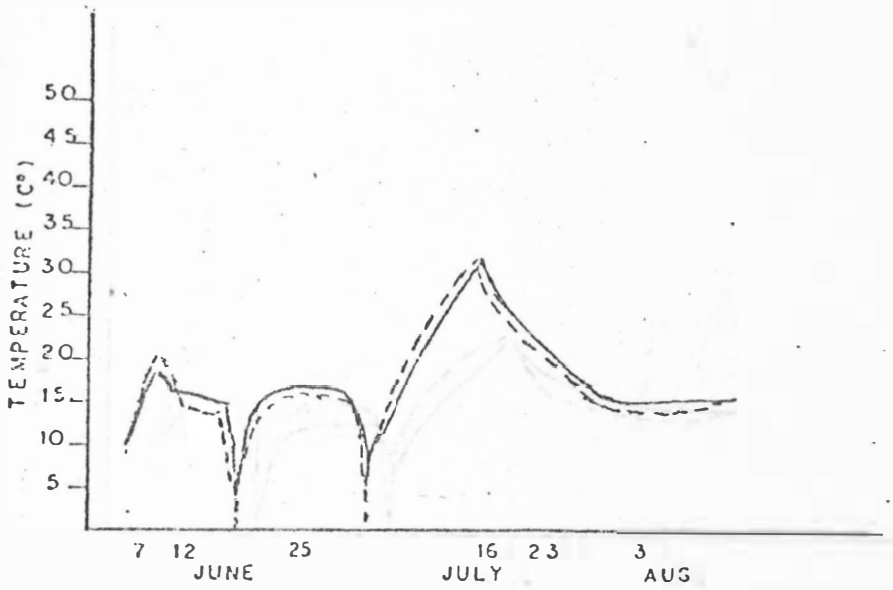

Fig. 10. Astragalus cicer study area, 1973
—— Average daily soil temperature at 10 centimeters
--- Average daily ambient air temperature at 2 meters

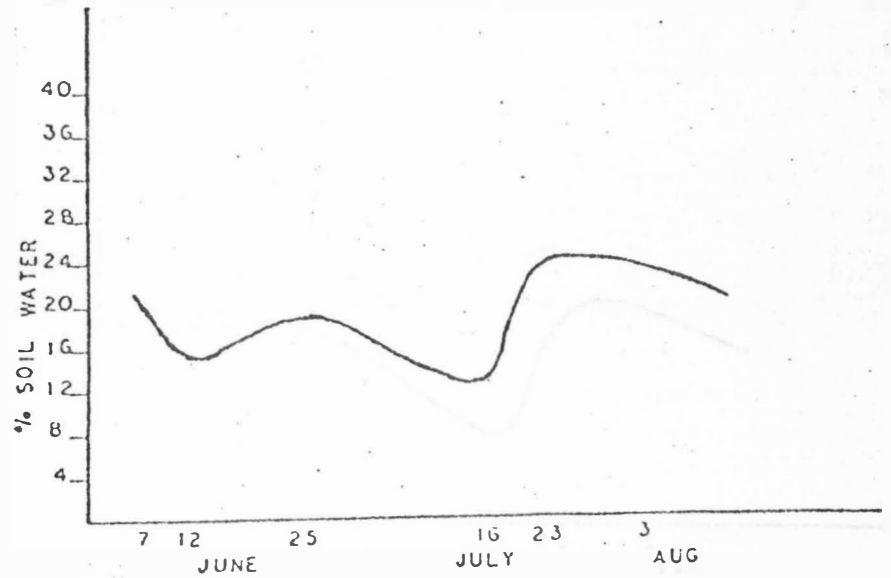

Fig. 11 Astragalus cicer study area, 1973
Soil water (Average 18.5%)

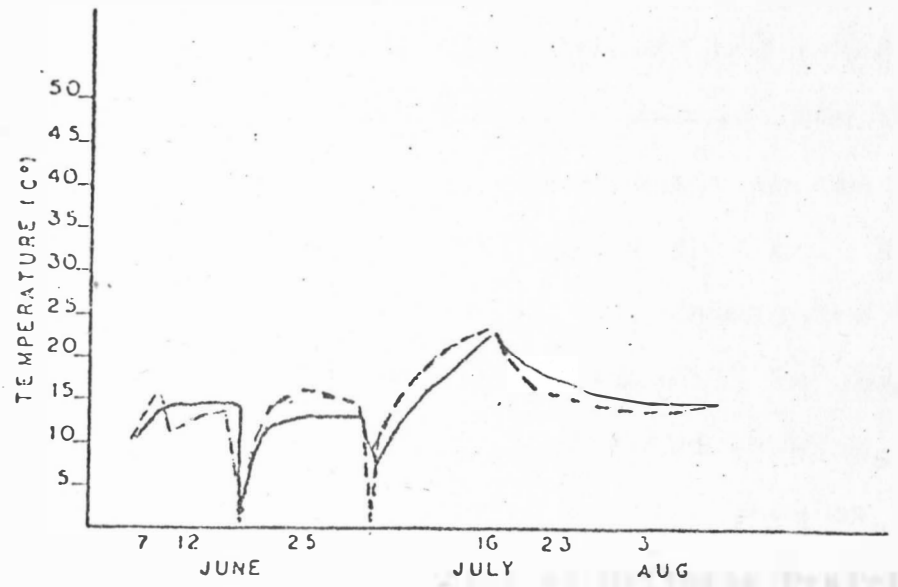

Fig. 12 Astragalus striatus study area, 1973
—— Average daily soil temperature at 10 centimeters
--- Average daily ambient air temperature at 2 meters

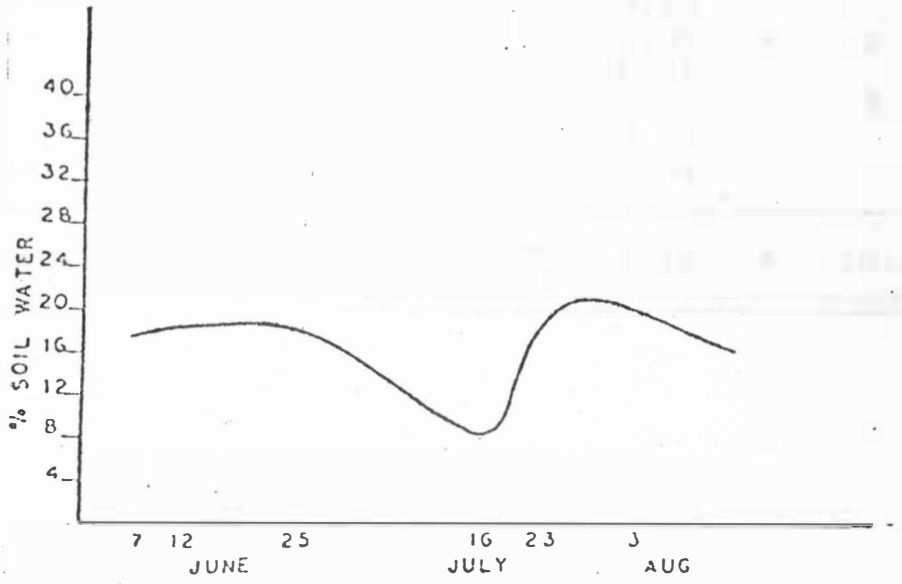

Fig. 13 Astragalus striatus study area, 1973
Soil water (Average 16%)

Table 3 Recorded precipitation at Gillette Prairie in centimeters

Date		Rain		Snow
June 12		0.89		
June 13		0.13		
June 14		2.29		
June 18		1.91	+	2.59
June 21-22		0.51		
July 5				7.75
July 19-23		0.51		
July 25-August 2		2.54		
1973 Growing Season	TOTAL	8.78	+	10.34

still lay in the draws, A. striatus was nodulated and giving positive acetylene reduction assays from the shelter provided by the pine forest. Immediately to the south no pink nodules could be found in the study area. However, since A. striatus is a perennial, old dessicated nodules from prior years could be found. The soil in open areas of the forest where these plants were found growing along with A. alpinus was warmer by several degrees than on the open prairie. One might suppose that the presence of much litter on the ground provided a more favorable C-N ratio. These factors may account for earlier nodulation. The gas chromatograph was again used for these assays.

Note in Fig. 14 the difference in attenuation (sensitivity setting) necessary to record the reduction of $C_2H_2 \longrightarrow C_2H_4$. The most sensitive setting is necessary to record ethylene while a setting 320 times less sensitive is necessary to keep the pen on the chart when the C_2H_2 comes through the column. No nodule weight was recorded for this assay since the nodules had very little pink color and much digging was necessary to produce enough nodules for several tests of each species. A. striatus and A. alpinus were used early in the season since the "cicer vetch" in the meadow situation was not far enough advanced to be easily recognized and was not sufficiently nodulated to permit collecting nodules for assays.

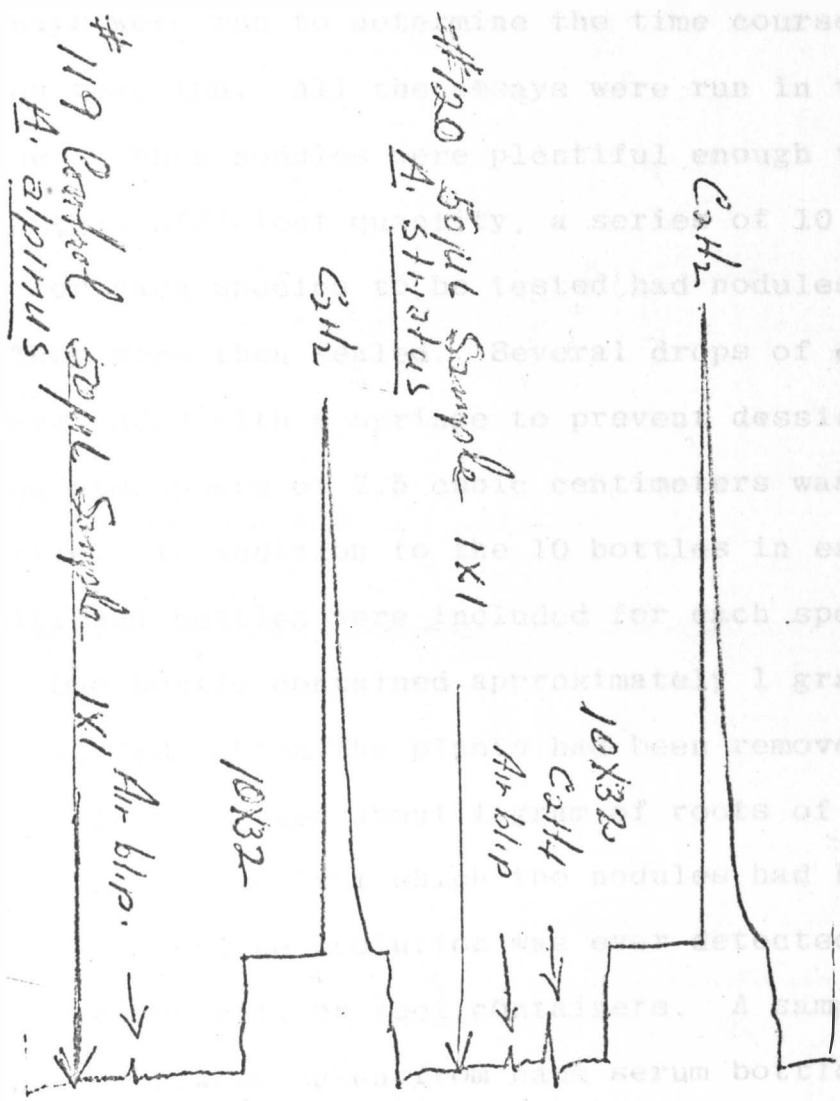

Fig. 14 Gas chromatograph chart showing the results of an assay run on Astragalus alpinus and Astragalus striatus nodules collected from plants dug in pine forest. This section of chart is from gas samples taken after nodules had been exposed to acetylene for 4 hours. Note the absence of an ethylene (C_2H_4) peak after injection of A. alpinus sample and the C_2H_4 peak indicating a positive assay for A. striatus. Note, too, the difference in attenuation of the machine: 1X1 versus 10X32.

As the 1973 growing season advanced, acetylene reduction assays were run to determine the time course of the reduction reaction. All the assays were run in the following manner. When nodules were plentiful enough to permit collecting a sufficient quantity, a series of 10 serum bottles for each species to be tested had nodules added to them. They were then sealed. Several drops of deionized water were added with a syringe to prevent dessication. An acetylene atmosphere of 7.5 cubic centimeters was added to each bottle. In addition to the 10 bottles in each series two additional bottles were included for each species being tested. One bottle contained approximately 1 gram of sifted soil from which the plants had been removed and the second bottle contained about 1 gram of roots of the species being tested from which the nodules had been removed. No ethylene evolution was ever detected from the acetylene in the soil or root containers. A sample of acetylene was always taken from each serum bottle immediately after it had been added to assure that the acetylene was not contaminated with ethylene.

Time intervals between withdrawal of the 50 microliter samples were varied to determine the best time for sampling. Samples were taken from the serum bottles and injected directly into the gas chromatograph. Fig. 15 will show that 1/2, 2, 5, and 24 hour samples were decided upon.

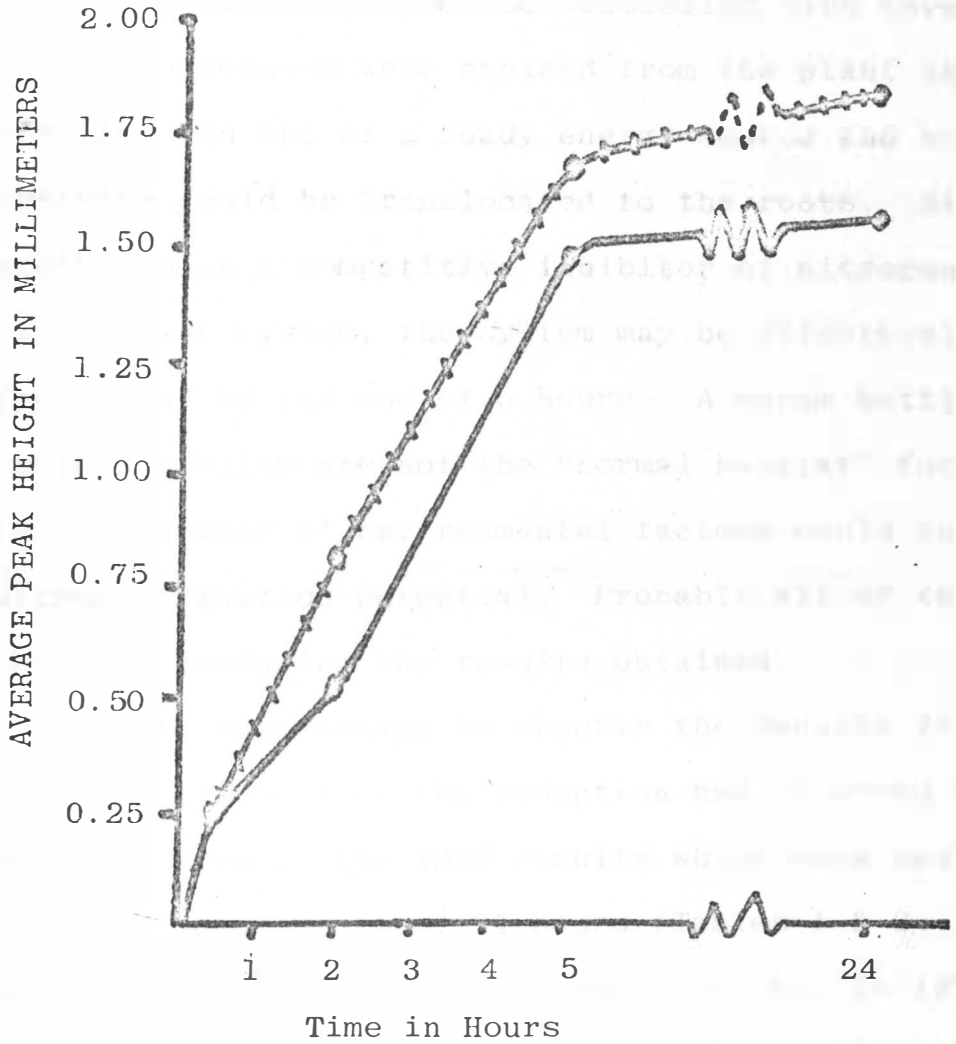

Fig. 15 Plot of ethylene peak in millimeters per 10 milligrams of nodule weight against time in hours from addition of acetylene to excised nodules in serum bottles.

—— Astragalus cicer

~~~ Astragalus striatus
~~~

Most of the ethylene was evolved at the end of 5 hours with very little more being produced between 5 and 24 hours.

These results could be reconciled with several ideas. Since the nodules were excised from the plant the system may have run out of a ready energy source and no new photosynthate could be translocated to the roots. Since the acetylene is a competitive inhibitor of nitrogen for the nitrogenase system, the system may be effectively "poisoned" by the end of 5 hours. A serum bottle and excised nodules are not the "normal habitat" for the system and any number of environmental factors could influence its nitrogen fixation potential. Probably all of these things share in producing the results obtained.

It is interesting to observe the results from 1973 showing that most of the reduction had occurred in 5 hours when compared to the 1972 results which show maximum reduction had occurred after 24 hours (Tables 1 & 2). This is most readily explained by remembering that in 1973 we were working with excised nodules and in 1972 intact plants with root systems encased in soil were used. Gas exchange could more readily occur between excised nodules and the atmosphere.

From the middle of June to the end of July the plants in both principal study areas were well nodulated with pink nodules. The "cicer vetch" roots had much larger nodules

and were more easily excavated since the soil was more friable and contained few stones. The root systems of the native legume Astragalus striatus were much more extensive and had massive tap roots. Lateral roots extended to a length of 1/2 meter or more.

Astragalus cicer tends to be an erect plant growing to a height of 1/2 meter in the study area. The plant was in intense competition with an infestation of quackgrass (Agropyron repens (L.) Beauv) and may not normally grow this tall. Astragalus striatus possesses a decumbent growth habit with the flower heads growing erect to a maximum height of 36 centimeters. This researcher feels confident that the native legume was growing in a habitat completely unsuitable for the introduced "cicer vetch".

Fig. 16 illustrates the results of four comparative acetylene reduction assays on A. cicer and A. striatus covering the period June 27 to July 25, 1973. During this time killing frosts occurred on July 4 and 5 and snow fell on Gillette Prairie. Both species were frozen back and flowering did not take place until July 25 for A. striatus and August 3 for A. cicer.

The results illustrated in Fig. 16 are average peak heights in millimeters for the test run of 10 samples corrected for 10 milligrams of nodule weight. This correction was necessary because the A. cicer nodules were

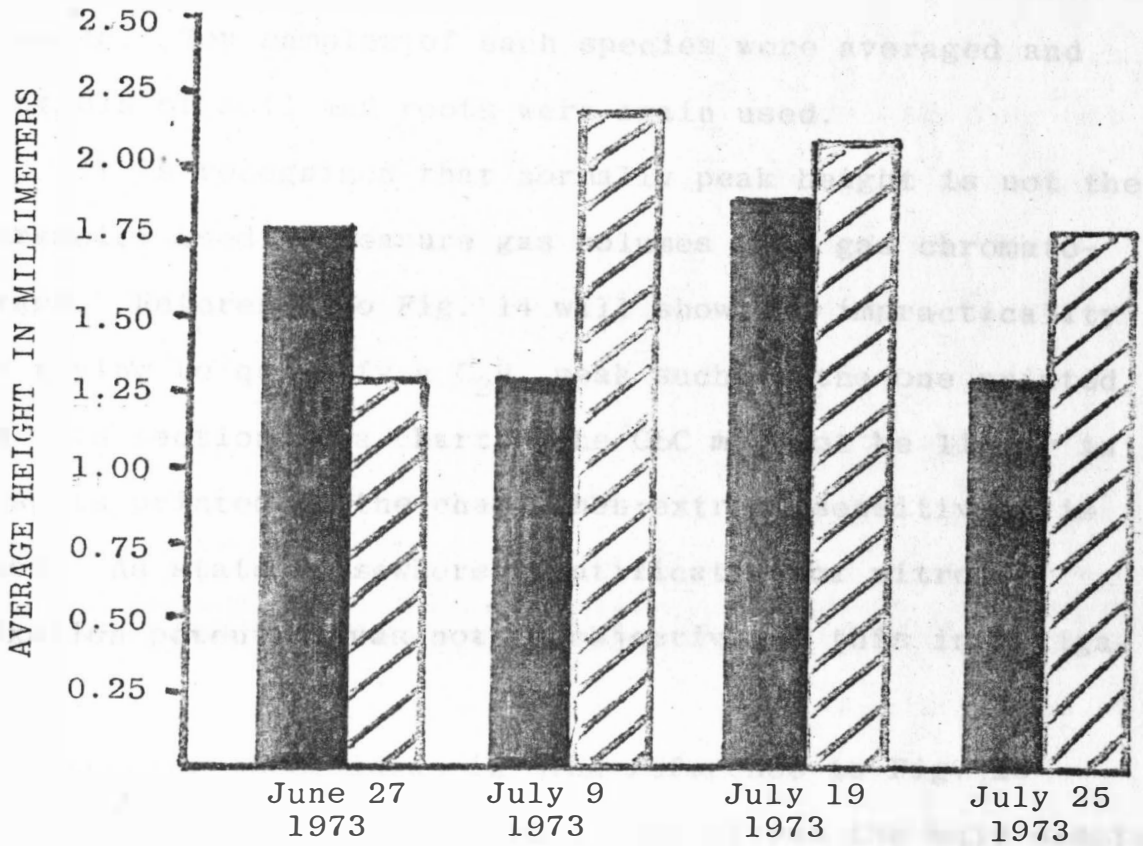

Fig. 16 Ethylene peak height in millimeters per 10 milligrams of nodule weight after 24 hours of exposure to an acetylene atmosphere in the series of 10 replicate samples.
Astragalus cicer
Astragalus striatus

larger and more readily collected. These nodules tended to give a greater weight in the bottles in which they were assayed. Ten samples of each species were averaged and controls of soil and roots were again used.

It is recognized that normally peak height is not the parameter used to measure gas volumes on a gas chromatograph. Reference to Fig. 14 will show the impracticality of trying to quantify a C_2H_4 peak such as the one printed on this section of a chart. The GLC may not be linear in results printed on the chart when extreme sensitivity is used. As stated elsewhere quantification of nitrogen fixation potential was not an objective of this investigation.

Keeping these ideas in mind reference to Fig. 16 reveals some interesting data. June 27 was the only sample date when A. cicer gave greater acetylene reduction than A. striatus, the native legume. On July 9 one of the ten A. striatus sample bottles gave no evidence of ethylene evolution at any of the 4 sample times (1/2, 2, 5, or 24 hours). Yet, after frosts on July 4 and 5, 4 days prior to the sample date, 10 samples of A. striatus, one of which gave no results, gave nearly twice the average peak height as 10 samples of A. cicer, all giving positive results. To this researcher this points up the fact that this native legume is better adapted to the severe weather conditions

experienced in South Dakota than the introduced species.

As anthesis approached, the ethylene evolution remained fairly constant as indicated by the data from July 19 and 25. Within a week of anthesis, seeds had been set and the nodules were visibly less pink and were becoming senescent. This agrees with the data obtained by Hardy, et al. (21). Anthropomorphically one could say that the plants recognized a lessened need for nitrogen and therefore cut down the production. There is, doubtless, a relationship here that governs the nitrogen fixation carried on by the plant and its symbiont at the time of flowering and seed setting when greater amounts of fixed nitrogen are required.

From the data obtained in 1973 probably no truly significant difference in nitrogen fixation potential exists between these two plants as they existed in the study area. Each did an adequate job of nitrogen fixation in the habitat where they were found. It would be interesting to attempt to establish A. cicer in the area where the native A. striatus was naturally growing. This researcher predicts that if it could be established it would not flourish in the more xeric, rocky soil. The comparative studies would give different results clearly indicating a niche better filled by a native legume.

Rhizobium Culture

Many hundreds of cultures have been screened as possible Rhizobium spp. from the inception of this investigation to the present. Many hundreds of cultures have been discarded after being identified as microorganisms other than rhizobia. The only positive identification of a Rhizobium sp. as stated before, is the ability to effectively nodulate a legume plant. This testing demands legumes grown in sterile soil where they cannot be inoculated by "wild" rhizobia and careful inoculation of pure cultures of suspected Rhizobium spp. into the sterile soil. This testing is time consuming and extremely frustrating. Many cultures carried on maintenance media in the laboratory have a tendency to die out after repeated transfers and never are identified. These are "wild" bacteria and many strains have never been successfully isolated and cultured. No one knows how many strains there may be or how host specific they are.

Some limited success in this area was attained in this investigation. One culture obtained from A. striatus in 1973 was inoculated into soil containing healthy A. striatus and A. cicer plants grown under controlled conditions. Successful nodulation was obtained with both plant species from the one culture.

A conversation with the Pennington County, South

Dakota Agricultural Extension Agent indicated that the "cicer vetch" planted on Gillette Prairie had not been inoculated with any commercial inoculum, although a commercial inoculum is now available for this species. The successful inoculation of A. cicer with a Rhizobium sp. isolated from A. striatus shows that a native Rhizobium sp. successfully nodulated the Gillette Prairie "cicer vetch".

Conclusions

This investigation has shown the need for further study of native legumes and their nodule bacteria. One native legume, Astragalus striatus has been shown to be adapted to grow under various climatic conditions in South Dakota, from the plains to the higher elevations of the Black Hills and to do an adequate job of nitrogen fixation under varied weather conditions.

Further studies should include the following:

1. The stock cultures of Rhizobium spp. maintained in the Soil Microbiology Laboratory of South Dakota State University should be identified by inoculation into known native legumes.
2. Serological identification of Rhizobium strains should be accomplished.
3. Media should be devised to better maintain these "wild" Rhizobium species.
4. Native legumes which show promise in their ability to fix nitrogen should be evaluated for their forage quality if they can be successfully cultivated in test plots.
5. The most effective inocula for these promising native legumes should be determined.

This researcher envisions the possibility of inter-seeding adapted native legumes into the existing ranges of

South Dakota to improve their productivity by adding available nitrogen to the soil and by providing acceptable forage for herbivorous livestock.

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