



An evaluation of factors influenced by nitrogen fixation in sainfoin (*Onobrychis viciifolia* Scop.)
by Nicholas Scott Hill

A thesis submitted in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE
in Agronomy

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Abstract:

Nitrogen fixation in the legume plant depends on the legume genotype, the Rhizobium strain, the interaction of the legume plant and the Rhizobium strain, and the interaction of microorganisms in the field with the legume plant and the Rhizobium inoculum. This study was conducted to 1) evaluate the performance of single strains and composites of strains of Rhizobium on Remont sainfoin and identify indices of nitrogen fixation affected by Rhizobium strains; 2) determine if an inhibitory factor of nitrogen fixation exists in the soil; and 3) characterize sainfoin as to its nitrogen fixing potential.

Seven commercial strains of Rhizobium specific for sainfoin, two cross inoculant strains, four strains from Russia, and one field selected strain of Rhizobium were used to inoculate greenhouse grown sainfoin plants and evaluated for indices of nitrogen fixation. The Rhizobium differed significantly for all indices of nitrogen fixation except mg root/plant and mean nodule weight. Milligrams nodule/plant and %N forage were indices most closely associated with acetylene reduction. Based on the indices of nitrogen fixation, the strains of Rhizobium were clearly separated into two groups: effective and non-effective. The four best strains were then tested individually and in all possible combinations. Based on acetylene reduction rates, the general effect of adding strains of Rhizobium to other strains of Rhizobium was additive. However, the degree of additivity was altered, presumably by toxins produced spontaneously by the Rhizobium. Two individual strains of Rhizobium, *O. viciifolia* 116A8 and *O. viciifolia* 116A27, expressed overall beneficial effects when averaged across all treatments. A strain combination of *O. viciifolia* 116A8 + *O. viciifolia* 116A14 + *Petalostemum* 124A1 was also beneficial. Strain combination *O. viciifolia* 116A27 + *Petalostemum* 124Z1 had a detrimental effect when averaged across all treatments.

Based on indices of nitrogen fixation, soil microflora and soil nutrients had no effect on the performance of Rhizobium on greenhouse grown sainfoin with exception of mg dry forage/plant. Significant differences may have been detected for other indices of nitrogen fixation with increased replications.

There was a significant positive correlation between fibrous rootedness and nodule number in greenhouse grown Melrose and Remont sainfoin. Both cultivars expressed normal distributions for root and nodule scores. This suggests that increased nitrogen fixation in sainfoin can be obtained by selecting for increased root fibrosity.

When screened for nitrogen fixation, five replicates in time of Remont sainfoin did not express normal distributions for acetylene reduction rates. Rates were' skewed to the lower reduction rates.

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AN EVALUATION OF FACTORS INFLUENCED
BY NITROGEN FIXATION IN SAINFOIN
(Onobrychis viciifolia Scop.)

by

NICHOLAS SCOTT HILL

A thesis submitted in partial fulfillment
of the requirements for the degree

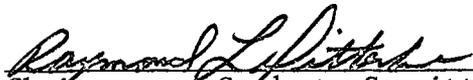
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ABSTRACT

Nitrogen fixation in the legume plant depends on the legume genotype, the Rhizobium strain, the interaction of the legume plant and the Rhizobium strain, and the interaction of microorganisms in the field with the legume plant and the Rhizobium inoculum. This study was conducted to 1) evaluate the performance of single strains and composites of strains of Rhizobium on Remont sainfoin and identify indices of nitrogen fixation affected by Rhizobium strains; 2) determine if an inhibitory factor of nitrogen fixation exists in the soil; and 3) characterize sainfoin as to its nitrogen fixing potential.

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There was a significant positive correlation between fibrous root-ness and nodule number in greenhouse grown Melrose and Remont sainfoin. Both cultivars expressed normal distributions for root and nodule scores. This suggests that increased nitrogen fixation in sainfoin can be obtained by selecting for increased root fibrosity.

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INTRODUCTION

Nitrogen is the major plant nutrient restricting crop production. Nitrogen fertilizers are made by the Haber-Bosh process in which compressed nitrogen is reduced by methane and heated by natural gas in conversion to ammonia. Fossil fuels comprise 50% of the cost of producing nitrogen fertilizers (93). As our dependence for fossil fuels increases on foreign sources, there is an increasing probability that fuel prices will rise as supply declines. Recently emphasis has been placed on becoming more energy efficient in our farming techniques. As a result, maximum utilization of nitrogen fixation by legume crops has become a focal point of research by U.S. agronomists.

Sainfoin (Onobrychis viciifolia Scop.) is a non-bloating legume with high forage quality. Its use is for hay and pasture in irrigated and dryland areas of Montana. Although alfalfa (Medicago sativa L.) is the major hay crop in Montana, its production is periodically limited by alfalfa weevil infestations. This has forced growers to use other legumes, such as sainfoin, as alternative hay crops (20).

In sainfoin, visual observations indicate that there is variability from field to field and plant to plant in its nitrogen fixing capabilities (69,93). This suggests that soil factors are present which inhibit nitrogen fixation. Soil conditions such as pH, temperature, moisture, and fertility influence the effectiveness of the Rhizobium bacteria when in association with legumes. Other strains of

Rhizobium as well as fungi can limit the growth of a particular strain of Rhizobium (85, 86, 87). Until recently no attempt has been made to characterize this inhibitory effect in sainfoin.

The purposes of this study were: 1) to evaluate strains of Rhizobium individually and in combinations for their nitrogen fixing performance on sainfoin under nil-nitrate greenhouse conditions; 2) to characterize the origin of the inhibition of nitrogen fixation in sainfoin; 3) to determine variability of nitrogen fixation in sainfoin; and 4) to characterize some indices of nitrogen fixation for possible use as rapid screening methods to circumvent expensive lab techniques used in nitrogen fixation research.

LITERATURE REVIEW

History of Sainfoin

Sainfoin (Onobrychis viciifolia Scop.) has been grown in the Azerbaidzhan, Armenian, and Georgian areas of Russia for over 1,000 years (32). It was introduced into Western Europe where it was first cultivated in France in 1852, however its culture not described until 1692. It was grown in Germany in the 17th century and in Italy in the 18th century (75). Common and Giant sainfoin were introduced from France into Great Britain in the middle 1700's and 1800's, respectively (107). The spread of sainfoin throughout Europe is credited to its profitable cultivation on calcareous lands (75).

Sainfoin did not gain early popularity in the United States. Eslick (36) attributed this to: 1) early tests being conducted on soils where it was not adapted; 2) sainfoin was tested under high moisture conditions which it does not tolerate; 3) visual notes on coarseness, leafiness, and probable palatability may have misled investigators; and 4) its non-bloating characteristic was overlooked.

Sainfoin was first grown in Montana around 1896 in the Hall, Townsend, Augusta, and Harrison areas of the state (36). The origin of these plants is unknown (30).

'Eski' is a cultivar developed from an introduction from Eskisehire, Turkey, brought to the United States in 1952. It was

re-selected for winter hardiness and released as a variety in 1964 by the Montana Agricultural Experiment Station (36). It rapidly gained popularity in Montana but in recent years its production has been limited by root and crown rot (4, 67).

Description of Sainfoin

Sainfoin, in French, means "wholesome hay". Its botanical name Onobrychis means "that for which asses bray". It is also known as Esparcette (64).

Sainfoin is a long lived, deep rooted, perennial legume crop. Its roots grow to a diameter of 5 cm and as long as 5.7 cm (75). It may have twice as many lateral roots as alfalfa (Medicago sativa L.) (66). Sainfoin has a branched crown from which numerous stems arise. The stems grow to a length of 26-52 cm. Each may produce 5-40 tillers with 3-5 inflorescences per tiller (21). The inflorescence is a rose-colored raceme which reaches a length of 5-13 cm (75), consisting of 5-80 florets. Fruits are one-seeded pods which are brown, indehiscent, lenticular, and reticulated on the surface (21).

Three types of sainfoin are grown in Russia - Common (O. sativa Lam = O. viciifolia Scop.), Sand (O. arenaria Kit D.C.), Transcaucus (O. transcaucasica Grossh). Common sainfoin has medium winterhardiness and drought tolerance. Sand sainfoin is relatively new to Russia (grown 60 years) and is the most winterhardy and drought

tolerant. Both are single cut species. Transcaucus sainfoin is more drought resistant and winterhardy than Common and has the highest yield of the Russian species. It is a two cut species (3). In milder climates, Common sainfoin has the greatest longevity and best yield. The forms of Common sainfoin, f. bifera, f. communis, and f. persica, flower earlier and yield more than the Transcaucus and Sand species. Forma communis, however, does not have the ability to flower during the first year of growth. O. arenaria dies three years after planting (5).

In parts of the Middle East, O. viciifolia f. persica is grown. In contrast to the European form (f. europæana), f. persica is superior in yield. It is a multi-cut type (3-4 cuttings/yr), has modest soil requirements, and good resistance to drought, freezing, disease, and insects (59).

In Great Britain, Giant and Common sainfoin (Onobrychis viciifolia Scop.) are grown. Common sainfoin is much smaller and longer lived than Giant sainfoin. It is a one-cut type used primarily in temporary and permanent pastures. With good management, stands last for 5-10 years. Giant sainfoin is shorter lived but has higher yields than Common sainfoin. It is a multi-cut type which gives two cuts per year. Giant sainfoin is usually grown for short periods only (107).

In the United States and Canada, all sainfoin grown is O. viciifolia Scop. Eski, 'Remont', and 'Melrose' are the primary cultivars. In western Canada, sainfoin is used primarily for hay, pasture, and seed production. Melrose is the preferred cultivar (31).

In Montana, Eski is used for hay production on dryland and land with limited irrigation. It is a one-cut type. Remont is recommended for pastures or areas where two or three cuttings of hay are expected (31). The average yield of these two cultivars is the same but the distribution of growth differs. Eski yields most of its seasonal growth the first cutting. Remont yields less than Eski on first cut, and more than Eski on the subsequent cuttings (14).

Uses of Sainfoin

Hanna and Smoliak (43) stated "It is inevitable that sainfoin has to have its hay yield performance compared with alfalfa". Depending upon environmental conditions, sainfoin yields as much as, more than, or less than alfalfa (19, 20, 26, 31, 43, 60, 80). Sainfoin contributes more to total yield when planted with other legumes than when planted with grasses. This is due to the capability of other legumes to supply sainfoin with nitrogen (24). In addition, sainfoin yields may increase 1T/ha when nitrogen is applied in excess of 112 kg/ha (52).

Sainfoin contains 14-15% crude protein (7, 31) compared to alfalfa which has 16-17% crude protein (31). The nitrogen free extract content and total digestible nutrients are higher than alfalfa (19, 31, 53). Sainfoin should be equal to alfalfa in forage quality (19, 53).

Yearling steers gain more on irrigated pastures of sainfoin than on pastures of Ladino clover (Trifolium repens L.) mixed with either Kenmont tall fescue (Festuca arundinaceae Schreb), brome grass (Bromus inermis Leyso) or Orchardgrass (Dactylis glomerata L.) mixtures. However, because of poor regrowth, lower stocking rates are necessary (60). Swine gain equally well on alfalfa and sainfoin diets (71).

Honey produced from sainfoin nectar is excellent in quality (2, 33, 64, 74). Sainfoin plants yield high amounts of nectar (2, 61, 71) and bees prefer pollinating sainfoin over alfalfa (33).

The Nature of Rhizobium and Plant Symbiosis

Responses of sainfoin to Rhizobium inoculation are inconsistent. In two studies, forage yields increased 33 and 28% respectively by the use of inoculant at Lethbridge, Canada, but in other trials plants developed nitrogen deficiency symptoms even though they were inoculated (43). In Bozeman, Montana, nitrogen deficiency symptoms usually appear in inoculated fields in mid-June and disappear by mid-July (15, 32, 94).

Rhizobium spp. bacteria are free living aerobic, flagellated rods approximately 1-3 microns in length. Although aerobic, partial pressures of oxygen as low as .01 atmospheres are necessary for nitrogen fixation to occur. They have a widespread occurrence in the soil, especially where legumes are grown. The absence of legumes reduces their numbers (98).

Rhizobium bacteria and legume plants live symbiotically (98). Legumes are adapted to soils with low nitrogen levels (98, 103) because effective Rhizobium can supply the plant with as much nitrogen as fertilizers (62). Properly inoculated alfalfa fixes up to 146 kg/ha of added nitrogen in the soil (98). Legumes are the major source of soil nitrogen in New Zealand and Australian pastures (23).

Grasses and other non-nitrogen fixing plants benefit when grown with effectively nodulated legumes because 1) after nodulation, legumes take up less nitrogen from the soil, 2) some fixed nitrogen in the nodules is excreted into the soil and becomes available to the non-legumes, and 3) short-lived nodules decay and provide the soil with nitrogen (103).

Non-nodulated legumes remove more nitrogen from the soil than corn (54). Johnson et al. (54) suggest following heavily nitrogen fertilized crops with legumes to utilize excess nitrates in the soil and prevent their leaching into streams.

Inoculation with infective and effective Rhizobium strains is necessary when a "new" legume crop is sown (44, 45, 95, 98), but may not be necessary when effectively nodulated plants of the same species have been grown previously (44). Soybeans do not require yearly inoculation to maintain yield and protein content (44, 45). However, annual inoculation of soybeans results in earlier nodulation, more extensive root systems, thicker stems, bigger leaves, and earlier maturation. "Wild" Rhizobium exert their effects later in the plant's life (77).

Anatomy, Physiology, and Biochemistry of Nitrogen Fixation

There is good general agreement on the theory of infection and nodulation although the exact mechanisms are poorly understood (48, 58, 72, 76, 83, 98, 106). The infection process is initiated when root exudates are excreted into the soil (72, 98). These exudates are not formed until the first true leaves of the plant have developed. They contain free amino acids, enzymes, sugars, and vitamins (98), and help provide the Rhizobium with an environment optimum for growth (72).

Infection usually occurs in the root hairs (48, 58, 76). The bacteria produce a substance, thought to be indole acetic acid, synthesized from tryptophan secreted from the root (58, 76, 98). This substance causes the root hair to curl (58). The plant then forms poly-galacturonase extracellularly which increases the plasticity of

the cell walls and loosens cross links in the root hair (58, 76, 98). Infection occurs at the root hair apex and a bulge develops (98).

An infection thread then develops (48, 98). The thread is non-septate and of plant origin (7, 83). The thread moves down the root hair in conjunction with the root hair nucleus (98). The nucleus remains at the tip of the infection thread (72). The infection thread penetrates the root cortex (72, 96), locates a tetraploid (4x) cell, releases the Rhizobium in membrane bound packages containing several bacteria (bacteroids), which stimulate meristematic activity of the 4x cell (73, 106).

The 4x cells are the focal point of infection, and together with surrounding diploid cells make up the initial nodule primordium (72). The 4x cells are present in uninfected plants (72, 106) and have different meristematic activity than their 2x neighbors. They often occur where lateral roots initiate (72).

The mature nodule has a cap of uninfected cortical cells, a layer of rapidly dividing cells, and an area of enlarged plant cells. These enlarged cells contain the Rhizobium bacteria in bonded and branched bacteroids in the resting stage (72). Leghaemoglobin is found in the nodule cells containing the bacteroids which are actively fixing nitrogen. The function of the leghaemoglobin is to provide low partial pressures of O_2 for optimum fixation to occur (48).

As the nodule ages, the leghaemoglobin content decreases. During decomposition, the nodule turns brown, becomes hollow, and the nodule content crowd around the periphery of the wall. The Rhizobium then digest the cell contents and cell wall liberating the bacteria back into the soil (106).

The biochemistry and physiology of nitrogen fixation is complex (10, 11, 13, 14, 22, 27, 37, 57, 58, 77, 83, 91, 93, 96, 98, 106). Symbiotic nitrogen fixation begins and ends at the shoot. A portion of the plant photosynthate is translocated from the leaves and stems to the nodules (86). This is a major limiting factor to nitrogen fixation in all legumes (80, 93). Photosynthesis is limiting because the lower leaves are responsible for providing photosynthate for the nodules (93). Of 100 units of photosynthate produced by the leaves, 33 units move to the nodule. Of these 33 units, 12 are lost in respiration, 6 are utilized in nodule growth and 15 are transported back to the shoot as amino acids (91, 93).

The nitrogenase enzyme is responsible for catalyzing the reduction reaction. Its production occurs in the Rhizobium bacteroid in the legume nodule. The nif plasmid of the bacteria contains 15 genes which direct the coding and sequencing of amino acids for the enzyme (93).

All nitrogenase enzymes (including those in other genera) consist of two components, neither which have nitrogenase activity individually

(29, 93). Component II is an azoferrodoxin (29) which contains 4 iron atoms and 4 acid labile sulfur atoms (29, 93). Its molecular weight is approximately 55,000 (93). Component I is a molybdoferrodoxin (29) which contains 24 iron atoms, 24 acid labile sulfur atoms, and 1 molybdenum atom (29, 93). Its molecular weight is approximately 22,000 (29).

Nitrogenase systems are dependent upon ATP, Mg^{++} , and an electron donor for activity. Pyruvate oxidation activates electrons which reduce ferrodoxin. The ferrodoxin then passes electrons to component II (azoferrodoxin) of the nitrogenase enzyme (29). Mg^{++} linked ATP then binds to component II resulting in a conformational change. This change shifts the redox potential of component II such that it reduces component I (molybdoferrodoxin). In doing so, the Mg^{++} linked ATP is oxidized to Mg^{++} linked ADP. Free electrons then displace the linked ADP from component II and permits another Mg^{++} linked ATP to bond to the protein. As long as ferrodoxin is present component II is capable of bonding the Mg^{++} linked ATP (29, 93).

The added electrons from component II pass to component I which passes them to the nitrogen molecule for reduction. There are no enzyme intermediaries in this process. Diazine ($HN=NH$) and hydrazine ($H_2N=NH_2$) are enzyme bound intermediaries and ammonia (NH_3) is the end product. A total of 12-15 ATP's and 6 electrons are required for the conversion of N_2 to $2NH_3$ (29, 93).

The ammonia produced is incorporated into glutamic acid, alpha-ketoglutarate, and other carbon skeletons to produce amino acids. These carbon skeletons are transferred from the phloem into the nodular bacteroid tissue via a transfer cell located on the periphery of the nodule. The amino acid is formed and transferred into the xylem via another transfer cell (29, 93).

The amino acid translocated to the shoot varies with the legumes but aspartate, asparagine, glutamate, and glutamine often predominate. As glutamine builds up, glutamine synthetase is manufactured at a relatively high rate. The level of glutamine synthetase reaches a threshold where it acts as a positive control for nitrogenase biosynthesis, i.e. - as glutamine synthetase builds up nitrogenase biosynthesis ceases (93).

Nitrate nitrogen acts as a nitrogenase repressor (29, 91, 93). Nodules exhibit a high rate of nitrate reducing activity because the Rhizobium bacteria also synthesize molybdenum containing nitrate reductase. When small amounts of molybdenum deficient nitrate reductase are present, the molybdenum atom becomes mobilized from component I of the nitrogenase enzyme and becomes incorporated into the nitrate reductase. However, nitrate nitrogen must be present in the nodule to induce the production of nitrate reductase (29, 93).

Molecular hydrogen is a gaseous by-product of the nitrogenase enzyme system (10, 11, 13, 84). Schubert and Evans (84) pioneered the

study of hydrogen evolution and its effect on the efficiency of the enzyme. In air as much as 50% of the electron flow of the system is lost as hydrogen. They showed that acetylene completely suppresses hydrogen evolution. This initiated the argument that there is considerable reason to doubt the precision of the acetylene reduction technique for measurement of nitrogen fixation. They hypothesized that the relative efficiency (R.E.) of the system, expressed as $R.E. = 1 - \frac{H^+ \text{ evolved}}{C_2H_2 \text{ reduced}}$, should be used in order to obtain valid estimations of nitrogen fixation. They also stated that a reduction of the R.E. of the system is comparable to a decrease in the photosynthetic efficiency resulting from photorespiration (84).

However, Bethlenfalvay and Phillips (10, 11) demonstrated that R.E. increases as the available energy to the nodule decreases. Ontogenetic interactions between photosynthesis and nitrogen fixation in peas (Pisum sativum L.) and beans (Phaseolus vulgaris L.) demonstrated that there is a linear increase in the R.E. from floral initiation to pod filling followed by a leveling of the R.E. after pod filling. Hydrogen evolution also coincides with CO₂ uptake and accelerates as the total dry matter of the plant accumulates (10).

Increasing light intensity increases H₂ evolution and C₂H₂ reduction. However, the R.E. of the system decreases with increasing light intensity. The net N₂ fixation rate (C₂H₂/3 x R.E.) shows the same trend as the net carbon exchange as light approaches saturation.

Therefore, the variation in photosynthetic efficiency and plant productivity produced by variations of light intensities are directly correlated with the rate of nitrogen fixation. As light intensities increase, the R.E. of the enzyme decreases but the net nitrogen fixation rate increases (11).

The decline in the R.E. is not an indicator of decreased nitrogenase activity. H^+ may be preferentially recycled under energy deficient systems resulting from lower light intensities. Therefore the production of H_2 evolving from the bacteria is reduced during energy deficiencies indicating that the lower R.E. may provide for greater nitrogenase activity (10, 11).

Nodule Volume and Nodule Number

Rhizobium strains differ in their ability to produce nodules on the host (8, 15, 17, 28, 34, 38, 45, 62, 80, 94, 96, 100, 101). Stewart (98) attributes this variation to an inhibitory substance produced in the nodule apex which prevents other nodules from developing in the immediate vicinity. Larger nodules produce more inhibitory substance which reduces the nodule number. Conversely, smaller nodules produce less inhibitory substance and more nodules form. Ineffective nodules do not produce the substance and large masses of small ineffective nodules are possible (98).

Nutman (72) imputes that limiting soil properties control the number and size of the nodules. A deficiency in nodule number is compensated for by nodule size. He suggests that a plant substrate controls the total nodule volume and the specific nodule volume at one particular time is directly related to the nitrogen requirements of the plant. Plants that depend solely on symbiotic fixation as a nitrogen source maintain an optimum nodule volume when inoculated with infective and effective strains of Rhizobium (72).

Factors Affecting Nodulation and Nitrogen Fixation

Rhizobium strains survive equally well at constant temperatures (49). However, Rhizobium strains specific for white clover (Trifolium repens L.) differ in their temperature requirement for nodulation to occur. Rhizobium strains selected from plants growing in cold climates nodulate better in cool soil temperatures than strains selected from plants growing in warm climates. High infectivity and rapid growth give cold temperature selected strains a competitive advantage (35).

Low temperature has a greater effect on decreasing root hair infection than on initiation of nodule growth or nitrogen assimilation (62, 98). The minimum temperature varies with plant species and is related to the plant's origin (40). Tropical plants have a higher

minimum temperature requirement for root hair infection than species from colder climates (62).

Nitrogen fixation is inhibited by temperature extremes. Greater inhibition occurs at high temperatures than at low temperature (98). High temperatures destroy bacterial tissues and hasten Rhizobium degeneration (40).

Light

Natural light inhibits infection of inoculated soybean roots. Inhibition increases as light intensity increases (42). Sunlight has antiseptic and germicidal properties which cause a reduction in inoculum potential (1). However, once root hair infection has occurred, inhibition of nodulation does not occur (42).

When inoculated seeds of alfalfa are exposed to 5 and 16 hours of direct sunlight, they produce chlorotic plants with reduced yields, indicating nitrogen deficiency. Birdsfoot trefoil (Lotus corniculatus L.) inoculated seed exposed to 3, 5, and 16 hours of sunlight also produced less effectively nodulated plants (1).

The photoreceptor system of the plant is controlled by red and farred light. Red light produces an effect similar to growth promoters which results in more abundantly developed nodules on plants (23). Farred light inhibits root growth (62), which indirectly reduces nodulation by decreasing the number of sites available for nodules to form (73).

Soybean plants receiving optimum light develop the most nodule weight and volume in the root zone just below the crown (23). One day or darkness results in a 50% reduction of nitrogen fixation, 75% decrease in available energy sources, 60% decrease in adenosine phosphates, 70% decrease in ATP, and a 55% decrease in ATP/ADP ratios (22).

Shading of the plant reduces nodule number and weight, and decreases root growth, nodule/root ratios, and the total nitrogen found in the nodules (23).

Moisture

Rhizobium can survive short periods of low soil moisture but long periods greatly decrease the population size (38). As the soil dries, the Rhizobium live in the water film surrounding the roots and soil colloids (92).

Alfalfa and birdsfoot trefoil express nitrogen deficiencies when under drought conditions for 3 to 4 weeks (1). Low surface soil water can reduce nodule number by 50% near the soil surface (105). Respiration and nitrogen fixation decrease when nodules decrease from 100% to 80% turgidity. Once below 80% turgidity the nodules suffer from water stress. They do not respire or fix nitrogen and irreversible structural changes occur (97).

Nodulation of peanuts (Arachis hypogea L.) occurs at a faster rate when they are watered moderately at weekly intervals. Rhizobium

multiplication occurs rapidly during these periods of high soil moisture (92),

Waterlogging reduces the rate of nitrogen fixation, but the bacteria survive and full activity resumes after drainage occurs (97).

Plant Maturity

During legume maturation, nodule activity increases until full flowering (10, 61). Total nodule activity then decreases, although the relative efficiency of the nodule is high (10, 11) at 10-30% pod filling. Stored assimilates immobilize during pod filling and move to the pod leaving less nutrition for the bacteria in the nodule (61). In general, nodule activity increases during acceleration of plant growth and decreases during deceleration of plant growth (10).

pH

Nitrogen fixation varies with pH. Rhizobium are capable of fixing nitrogen from a soil pH of 3-9, but pH 6.5-7.6 is optimum (41, 98). During seed germination, soil pH is the most critical factor for Rhizobium survival. In acid soils, seed pelleted with lime offers the Rhizobium a more neutral pH and increases survival (6).

Diseases

Root and crown rot is the most important disease in sainfoin. Sears (88) and Auld (4) identified Fusarium solani (Mart) Appel & Wr.

as being the causal organisms. It is a soil borne organism and cannot be controlled by crop rotation (67). Recent investigations by Gaudet (39) demonstrate that Erwinia amylovora, Pseudomonas syringe and Pseudomonas marginalis are involved with the rotting complex. The disease reduces stand, longevity, and forage yield by interfering with the vascular system (39, 67).

Similarly there is no control for crown rot in alfalfa (Fusarium oxysporum f. vasinfectum Atk Snyder & Haus). It causes a decrease in total nitrogen fixation due to a decrease in nodule number and volume. A nitrogen stress results in the plant and further rot occurs (56).

In alfalfa, if the Fusarium infection occurs prior to Rhizobium infection, a decrease in nodulation occurs. However, if the Rhizobium infection occurs first, a reduction in cortical rot is caused by the plant's resistance (56).

Fusarium oxysporum and Rhizobium compete for plant substrates. However on agar, Rhizobium outcompetes Fusarium and suppresses the growth of Fusarium oxysporum (56).

Nutrients

Mineral nutrition is important for optimum nitrogen fixation by legumes (51). Several elements are constituents of the fixation scheme. Cobalt is directly related to coenzyme B₁₂ which is found in both the nodules and the Rhizobium (98). Molybdenum, sulfur, and iron

are constituents of nitrogenase (6, 51, 98). Active ferredoxin contains seven iron atoms, all in the ferric state. These atoms form an electronegative compound which in turn carries hydrogen ions to the nitrogenase enzyme (29, 98).

Maximum nodulation requires high levels of phosphorous and potassium. Maximum nodule activity requires even higher phosphorous levels than for nodulation. High phosphorous levels compensate for low pH. Calcium increases both nodule number and nodule weight (68).

Molecular nitrogen is not a limiting factor of nitrogen fixation when present at normal atmospheric levels (98). However, when combined nitrogen is present in the inorganic form, nitrogen fixation is inhibited (13, 14, 29, 37, 94, 98).

Plant Conditioners for Nodulation and Fixation

Root exudates supply the Rhizobium with enough essential materials to maintain specific populations in high numbers. Concentrations of various root exudates are stimulatory to the growth of some Rhizobium strains while toxic to others (75). In some non-nodulated legume plants roots exudates inhibit Rhizobium growth (72).

After infection, nodulation is related to the plant's ability to form lateral roots. Selection for high nodule number results in plants with many lateral roots, but not necessarily larger root

systems. Inoculation with Rhizobium causes a slight reduction of lateral roots within plant clones (72).

Plant breeders have demonstrated that selection for high nodule number is practical (70, 72, 73). Red clover (Trifolium pratense L.) plants differ in the number of nodules on their roots. Plants selected for high yield have more nodules than plants selected for low yield. Progeny of the high yielders have more nodule mass than progeny of the low yielders (73).

Selected progenies from white clover show increased variation of nodule numbers and nodule efficiency. Selection for high nodule numbers increased nodule number on most of the progeny, but after the first generation of selection no increase in growth accompanied the increase in nodule tissue. As the nodule number increased, the average nodule size decreased (70). Mytton et al. (70) attribute this to either a reduction of the requirement of nitrogen from each nodule or that symbiotic nitrogen fixation is not a major limiting factor of plant growth.

A series of gene conditioners controls effective nodulation of soybeans. The recessive gene rj_1 controls the non-nodulating condition of the plant (102). The dominant gene Rj_2 controls the effective condition of the nodule (16, 102). Vest et al. (102) suggest that another gene, Rj_3 , is a dominant gene involved with the selection of bacteria to infect the plant.

Effectively nodulated Rj₂ soybean lines do not respond consistently to nitrogen fertilizers while non-nodulated lines do (103). Therefore since the nitrogen levels of the non-nodulated lines can be maintained, Liu et al. (63) suggested using this trait to screen for superior plants. High yielding and high protein plants can be selected by this method with the assumption that they utilize nitrogen most efficiently. These plants could then be crossed with nodulated plants with expectations of finding superior clones (63).

Rhizobium Characteristics Important for Effective Nodulation

Under natural conditions, the presence of moderately effective and parasitic strains of Rhizobium poses a demand on the plant (62). Desirable characteristics of a Rhizobium inoculum are; 1) symbiotic effectiveness; 2) ability to form nodules promptly; 3) ability to compete with other Rhizobium and; 4) ability to persist in the soils concerned (8).

Specialized inoculants selected for host species, sub species, or even cultivars are important to get the maximum productivity from nitrogen fixation (8). Different strains of Rhizobium cause different sizes and numbers of nodules to form and result in different protein contents of soybean seed. Strain differences also result in different grain weights and volumes of soybean plants (17). Sainfoin may similarly be affected by strains of Rhizobium which are specific for

crownvetch (Coronilla varia L.), sweet vetch (Hedysarum boreale Nutt.), and white prairie clover (Petalostemum candidum Michx) which will also effectively nodulate sainfoin (15).

Ineffectivity of Rhizobium strains may be due to other soil microorganisms. Viomycin and Kanamycin, antibiotics produced by Streptomyces spp., cause destruction of the nif plasmids (28, 34). These non-functional plasmids then can be transferred from bacteria to bacteria by conjugation and transduction. In this manner, the specific infectivity and effectivity of the Rhizobium populations can be altered (28). The growth rates of effective and ineffective Rhizobium mutants are similar and therefore compete with one another for plant substrate (28, 34).

Sainfoin is a poor nitrogen fixer (14, 86). Nodulation following inoculation with Rhizobium is often erratic and abundantly nodulated plants may be chlorotic in early spring (15, 52, 98). These chlorotic plants represent low nitrogen levels in the soil and plant suggesting ineffective nodulation. This chlorosis disappears in 6-8 weeks due to soil nitrification (98).

Small young nodules are generally the most effective in sainfoin. These nodules are scarce in early stages of growth in the field (15). It may be possible to increase the effective nodulation of sainfoin through plant breeding (6, 16, 57, 70, 73, 78, 96, 98, 102, 103, 104).

and Rhizobium selection (8, 15, 17, 29, 34, 38, 45, 62, 81, 94, 96, 98, 100, 101).

CHAPTER I

General:

The purpose of these experiments was to determine: 1) if the strains of Rhizobium used in the commercial inoculum for sainfoin express the highest rates of acetylene reduction and, if not, select strains which do; 2) if superior strain combinations could be developed from the screened Rhizobium strains; and 3) if indices of nitrogen fixation can be used to replace expensive and time consuming lab techniques in a Rhizobium screening program.

MATERIALS AND METHODS CHAPTER I

Unless specified, the following procedures, when used, were standard.

Greenhouse: Seed pods were removed from Remont seed with a belt thrasher. The depodded seed was then sized through a 3.12 mm sieve followed by a 1.95 mm sieve. Only seeds remaining on the 1.95 cm sieve were used in the experiments. Seeds were surface sterilized by soaking for 3 minutes in 0.5% NaOCl.

Plants were grown in the greenhouse with a 16-hour photoperiod provided by supplemental lighting from incandescent lights. A vermiculite growth medium was used with fertility maintained with twice weekly applications of Hoagland's (nil-nitrate) nutrient solution (Appendix 1).

Black plastic pots, 15 cm X 12.5 cm, were surface sterilized in 0.5% NaOCl for five minutes, filled with vermiculite, and watered thoroughly with tap water. Fifteen sterile sized seeds were planted 1.25 cm deep and equidistant from each other. The pots were placed on greenhouse benches which had received a 2.5% NaOCl spray.

Rhizobium: Sainfoin Rhizobium cultures were obtained from Dr. J. C. Burton (Nitragin Co., Milwaukee, Wis.) and Dr. D. D. Brezhnev (Leningrad, USSR via H. L. Hyland, Plant Germplasm Quarantine Center, Beltsville, MD). These stock cultures were transferred to Yeast Extract Mannitol agar (YEM) (Appendix 2). They were then incubated at 25C for three days before refrigerating at 2C.

All bacterial cultures screened were inoculated into YEM broth (Appendix 2), incubated for 7 days with shaking at 25C and diluted to equal concentrations prior to inoculation on the sainfoin plants. Cells were counted on a Levy-Hauser haemocytometer. Inoculations were made 7 days after planting.

Nitrogen Fixation: Indices of nitrogen fixation on an individual plant basis included: $\mu\text{l C}_2\text{H}_2$ reduced/hr, $\mu\text{l C}_2\text{H}_2$ reduced/mg nodule, %N forage, %N roots, %N total, mg N in the forage, mg N in the roots, mg N total, mg dry forage, mg dry root, nodule number, mg dry nodule, and mean dry weight nodule (mg).

Eight weeks after inoculation the forage was removed at the crown and dried at 85C for 24 hours. The roots were carefully washed free

of vermiculite in tap water and placed in an air tight 1000 ml erlenmeyer flask, capped with a septum, and assayed for acetylene reduction as described by Hardy et al. (45). Ten percent of the air was evacuated from each flask and replaced with purified acetylene (Linde Specialty Gases, Los Angeles, Calif.) Reduction of acetylene to ethylene was measured after a 4 hour incubation period with a Beckman GC 2-A hydrogen flame detecting gas chromatograph. Gas separations were performed with a 2 m x 8 mm stainless steel column packed with 80/100 mesh F1 activated alumina packing. Gas pressures entering the system were 7, 15, and 20 psi for air, hydrogen, and helium carrier gas, respectively. Column temperature was 130 C. A 1 ml sample size was injected into the gas chromatograph with attenuation set at 1×10^2 .

Following the acetylene reduction assay, the nodules were removed from the roots, counted, and roots and nodules dried separately at 85 C for 24 hours. Dry weights of the forage, roots, and nodules were recorded. Total and percent N of the forage and roots were determined by microkjeldahl analysis (Appendix 3).

Data were analyzed by analysis of variance and means were separated using T test or by Least Significant Differences. Correlation coefficients were determined among indices within experiments.

Experiment I: Single Strain Rhizobium Screening Study

Fourteen Rhizobium strains (Table 1-1) were tested in a randomized complete block design with four replications. They were grown in YEM broth for seven days and diluted to equal concentrations of 1×10^9 cells per ml. For inoculation, ten ml of each Rhizobium suspension were diluted to 100 ml and poured into the pots. A positive control of commercial inoculum (100 ml of 1×10^8 cells/ml) and a negative control of no Rhizobium were included in the treatments.

Experiment II: Rhizobium Strain Composite Study

The four best nitrogen fixing strains of Rhizobium from Experiment I, based on the acetylene reduction assay, were tested individually and in all possible combinations (Table 1-2) in the greenhouse using a randomized complete block design with four replications. Each strain was grown in YEM broth for 7 days and diluted to concentrations of 1.6×10^9 cells/ml. Inoculation treatments were made such that equal concentrations of each strain in the combination were present with a total count of 1.6×10^{10} cells inoculated per treatment. A positive control of the 5 strains of the commercial inoculum and a negative control of no Rhizobium were used. All previously listed indices of N fixation were tested after 8 weeks.

Table 1-1 Rhizobium strains tested for acetylene reduction.

	<u>Rhizobium Strain</u>	<u>Source</u>
1.	<u>Onobrychis viciifolia</u> 116A8	Nitragin Company
2.	" " 116A12	"
3.	" " 116A14	"
4.	" " 116A15	"
5.	" " 116A17	"
6.	" " 116A27	"
7.	" " 116A28	"
8.	<u>Petalostemum</u> 124B1	"
9.	" 124Z1	"
10.	Russian strain 5856	Dr. D.D. Brezhnev U.S.S.R.
11.	" 5857	"
12.	" 5859	"
13.	" 5860	"
14.	FSEB 1	Bozeman, Mt.
15.	Commercial Composite <u>O. viciifolia</u> 116A8, 116A12, 116A14, 116A15, 116A17	Nitragin Company
16.	No <u>Rhizobium</u>	

Table 1-2. Individual Rhizobium strains and composites of strains tested for acetylene reduction on sainfoin.

Strains	
1.	<u>O. viciifolia</u> 116A8
2.	<u>O. viciifolia</u> 116A14
3.	<u>O. viciifolia</u> 116A27
4.	<u>Petalostemum</u> 124Z1
5.	1 + 2
6.	1 + 2 + 3
7.	1 + 2 + 4
8.	1 + 3
9.	1 + 3 + 4
10.	1 + 4
11.	2 + 3
12.	2 + 3 + 4
13.	2 + 4
14.	3 + 4
15.	1 + 2 + 3 + 4
16.	No <u>Rhizobium</u>
17.	Commercial composite

RESULTS AND DISCUSSION CHAPTER I

Experiment I: Single Strain Rhizobium Screening Study

Rhizobium strains differed significantly for all indices of nitrogen fixation except mg root/plant and mean nodule weight (Table 1-3). In general the strains of Rhizobium tested can be broken down into effective and non-effective nitrogen fixing groups based upon the indices measured. Effective strains include O. viciifolia 116A27, O. viciifolia 116A14, O. viciifolia 116A8, Petalostemum 124Z1, and Petalostemum 124B1. All other strains were non-effective including three of the five strains found in the commercial inoculum - O. viciifolia 116A12, O. viciifolia 116A15, and O. viciifolia 116A17.

Among the effective strains of Rhizobium, strain O. viciifolia 116A27 significantly reduced more acetylene/plant/hr than strains O. viciifolia 116A14, O. viciifolia 116A8, and Petalostemum 124B1 but not significantly better than strain Petalostemum 124Z1. Strains Petalostemum 124Z1, O. viciifolia 116A14 and O. viciifolia 116A8 reduced acetylene/plant/hr at the same rate but of those three only strain Petalostemum 124Z1 reduced acetylene/plant/hr significantly more than strain Petalostemum 124B1. Strains O. viciifolia 116A27, O. viciifolia 116A14, and Petalostemum 124Z1 reduced acetylene/plant/hr at the same rate as the commercial composite inoculum.

Table 1-3. The effect of Rhizobium strains on indices of N fixation in sainfoin.

Treatment	C ₂ H ₂ Reduction		Nitrogen						Weight				Nodule	
	Per plant per hour	per mg nod. per hour	Forage	Root %	Plant	Forage	Root mg	Plant	Nodule	Forage mg	Roots	Total	Number/ plant	% weight mg
<u>O. viciifolia</u> 116A27	.2725	.0385	4.42	2.06	3.92	2.04	0.35	2.39	7.25	46.00	17.15	71.95	14.40	0.58
Composite	.2563	.0455	3.86	2.08	3.25	1.30	0.35	1.65	5.72	33.80	17.22	56.75	8.78	0.66
<u>Petalostemum</u> 124Z1	.2386	.0428	3.38	2.08	3.25	1.06	0.40	1.46	5.55	29.25	20.15	54.95	12.60	0.45
<u>O. viciifolia</u> 116A14	.2340	.0388	3.37	1.85	2.74	1.04	0.30	1.34	5.58	39.75	16.37	61.67	10.50	0.53
<u>O. viciifolia</u> 116A8	.2016	.0545	3.88	2.03	3.32	1.35	0.31	1.66	3.93	34.63	15.17	53.72	6.70	0.57
<u>Petalostemum</u> 124B1	.1977	.0558	3.73	2.07	2.88	1.31	0.31	1.62	3.58	36.45	17.90	57.93	7.20	0.51
<u>O. viciifolia</u> 116A15	.0873	.0445	2.58	1.49	2.28	0.76	0.25	1.01	2.03	27.65	17.10	46.77	1.78	1.31
Russian 5857	.0460	.0173	2.19	1.34	1.81	0.60	0.30	0.90	1.23	27.35	22.35	50.92	1.03	0.58
Russian 5859	.0380	.0188	2.44	1.46	2.03	0.59	0.23	0.82	1.05	23.85	16.17	41.07	1.30	0.45
<u>O. viciifolia</u> 116A12	.0222	.0166	2.28	1.43	1.93	0.53	0.22	0.74	1.08	23.25	16.00	39.97	0.58	1.58
Russian 5860	.0038	.0043	2.03	1.42	1.77	0.47	0.28	0.75	0.80	23.25	20.50	44.55	0.98	0.76
Russian 5856	.0031	.0051	1.94	1.26	1.64	0.46	0.25	0.71	0.53	23.70	19.85	44.07	0.98	1.60
Control	.0028	.0023	2.01	1.62	1.74	0.52	0.22	0.74	0.30	25.95	17.32	43.57	0.28	0.28
FSEB 1	.0000	.0000	1.94	1.35	1.79	0.45	0.25	0.70	0.25	21.25	17.85	39.35	0.20	0.70
<u>O. viciifolia</u> 116A28	.0000	.0000	1.97	1.52	1.84	0.43	0.29	0.72	0.03	24.95	19.30	43.57	0.03	0.25
<u>O. viciifolia</u> 116A17	.0000	.0000	1.91	1.28	1.67	0.47	0.25	0.72	0.00	23.67	19.52	43.20	0.00	0.00
LSD*	.0389	.0092	0.20	0.11	0.15	0.09	0.02	0.10	0.51	1.87	NS	2.71	0.89	NS
CV	80.39	79.61	15.83	14.49	22.58	21.70	15.67	17.12	43.96	13.41	20.31	11.34	44.14	132.83

*LSD (0.05)

Among the non-effective strains of Rhizobium, only strains O. viciifolia 116A15 and Russian 5857 reduced acetylene/plant/hr significantly better than the no Rhizobium control. Strain O. viciifolia 116A15 reduced acetylene/plant/hr significantly better than Russian strain 5857.

All nitrogen fixation indices measured were highly significant and positively correlated (Table 1-4) except mean nodule weight, which was not correlated with any of the indices, and root weight, which was negatively correlated with %N root ($p = .05$) and %N plant ($p = .05$).

Milligram nodule/plant and %N forage were the indices most closely associated with $\mu\text{l C}_2\text{H}_2$ reduced plant/hr. The C.V. for %N forage is much lower than that of mg nodule/plant or C_2H_2 reduced/plant/hr indicating that it may be a better measurement for Rhizobium strain performance than acetylene reduction measurements. With the use of infra-red analysis (i.e. Infraalyzer) many more plant samples with different Rhizobium strain treatments can be run at a lower expense than acetylene reduction assays. This is an attractive index for a procedure to be used in a screening program because it is non-destructive, quick, and easy.

When ranked based upon %N forage, treatment O. viciifolia 116A27 is the best N fixer followed by O. viciifolia 116A8, the commercial composite, Petalostemum 124B1, Petalostemum 124Z1, and O. viciifolia

Table 1-4. Correlations among indices of N fixation measured in single strain screening study

	C ₂ H ₂ Reduction		Nitrogen						Weight			Nodule		
	Per plant per hour	Per mg. nod. per hour	Forage	Root %	Plant	Forage	Root mg	Plant	Nodule	Forage	Root mg	Total	Number	% nodule Weight mg
C ₂ H ₂ red/ plant/hr.	1.00	.82**	.88**	.66**	.79**	.79**	.70**	.82**	.89**	.70**	.10	.75**	.84**	-.03
C ₂ H ₂ red/ mg. nod/hr.		1.00	.73**	.58**	.66**	.62**	.49**	.64**	.63**	.53**	-.10	.52**	.60**	.04
% N forage			1.00	.82**	.95**	.91**	.64**	.92**	.90**	.72**	-.19	.70**	.86**	-.02
% N root				1.00	.84**	.72**	.53**	.74**	.72**	.55**	-.49**	.43**	.69**	-.07
% N plant					1.00	.92**	.55**	.92**	.87**	.71**	-.31*	.64**	.84**	-.03
Mg N forage						1.00	.55**	.99**	.88**	.87**	-.15	.83**	.84**	-.04
Mg N root							1.00	.64**	.69**	.43**	.40**	.63**	.73**	-.15
Mg N plant								1.00	.90**	.86**	-.09	.84**	.87**	-.06
Mg nodule/ plant									1.00	.78**	-.08	.80**	.94**	.02
Mg forage/ plant										1.00	1.00	.28*	-.02	-.10
Mg root/ plant												.93**	.70**	-.04
Mg total/ plant												1.00	.75**	-.06
Nodule #/ plant													1.00	-.09
% nodule weight(mg)														1.00

* r(.05) = .25

** r(.01) = .33

116A14 (Table 1-3). These strains can be considered effective strains based upon this index.

When based upon C_2H_2 reduction/mg nodule/hr, the effective strains of Rhizobium would include the five mentioned and strain O. viciifolia 116A15 (Table 1-3). Although C_2H_2 reduction/mg nodule/hr accounts for the efficiency of the nodule, it does not account for the capability of the Rhizobium to infect the plant. Infectivity of the bacteria, when taken into account based on nodule number per plant, eliminates strain O. viciifolia 116A15 from the effective group of Rhizobium strains. Therefore the measurement of C_2H_2 reduction/mg nodule/hr is deceptive. Nodule number of strain O. viciifolia 116A15 is highly significantly lower than other treatments classified as effective, but not significantly different than those classified as non-effective.

Experiment II: Rhizobium Strain Composite Study

Significant differences among Rhizobium composites were not detected for five nitrogen fixation indices; mg N/plant root, mg root/plant, mg total/plant, mean nodule weight, and C_2H_2 reduced/mg nodule/hr (Table 1-5). Significant differences among Rhizobium composites were detected for the other indices. Composites expressing the most acetylene reduction/plant/hr were composites of strains 1) O. viciifolia 116A8 + O. viciifolia 116A27, 2) Petalostemum 124Z1 + O.

Table 1-5. The effect of Rhizobium strains and composites of strains on indices of N fixation in sainfoin.

Treatment #	C ₂ H ₄ Reduction		Forage Root Plant			Forage Root		Nodule	Weight			Number/ plant	x weight mg
	Per plant per hour	per mg nod. per hour	Forage	Root %	Plant	Forage mg	Root		Forage mg	Root	Total		
1 + 3	1.1440	.1519	4.82	2.35	3.48	3.04	1.24	8.28	62.57	52.38	123.2	17.28	0.50
1 + 2 + 4	1.0370	.2258	4.53	2.01	3.03	2.64	1.02	6.98	52.17	49.05	108.2	14.60	0.51
Composite	0.9675	.1097	3.69	2.26	3.41	3.06	1.30	8.76	64.84	57.93	131.5	18.26	0.48
2 + 3	0.9244	.1214	4.69	2.40	3.40	2.50	1.17	7.84	50.70	49.33	107.9	11.86	0.67
1 + 4	0.7702	.0900	5.00	2.12	3.31	3.24	1.34	8.56	62.00	64.35	134.9	14.28	0.60
3 + 4	0.7387	.1066	4.19	2.20	3.16	2.25	1.29	6.05	48.82	55.07	104.9	10.73	0.66
1 + 2 + 3	0.7041	.1044	4.91	2.02	3.22	2.40	1.02	7.10	47.99	51.30	106.4	8.61	0.96
1 + 2 + 3 + 4	0.6907	.0708	4.62	2.15	3.21	2.73	1.17	6.48	53.67	53.79	113.9	9.97	0.50
1 + 3 + 4	0.6753	.0805	4.16	2.21	3.03	2.40	1.22	6.30	54.87	52.99	114.2	10.45	0.49
2 + 3 + 4	0.6743	.0847	4.16	2.15	2.95	2.25	1.19	5.96	49.03	55.11	110.1	9.21	0.54
3	0.6273	.0996	3.60	1.96	2.45	1.19	0.86	6.35	32.52	44.13	83.0	10.11	0.64
4	0.5932	.0962	4.02	1.97	2.80	1.79	0.92	4.78	42.74	47.83	95.4	9.19	0.56
1 + 2	0.4387	.1686	3.92	2.09	2.82	1.75	0.85	5.70	40.71	39.74	86.3	6.33	0.83
2	0.3890	.0936	3.59	1.88	2.54	1.24	0.73	4.15	34.67	38.72	77.5	10.96	0.38
1	0.3880	.0988	3.47	1.75	2.42	1.25	0.71	3.94	35.62	40.67	80.2	11.03	0.36
2 + 4	0.3014	.1504	3.58	1.90	2.55	1.03	0.70	2.30	26.83	36.56	65.7	1.75	2.61
Control	0.0000	.0000	2.33	1.68	1.90	0.53	0.66	0.00	23.08	39.27	62.4	0.00	0.00
LSD*	0.1760	NS	0.32	0.12	0.20	0.43	NS	1.13	7.60	NS	NS	2.13	NS
CV	56.25	15.85	15.85	12.14	13.86	44.42	35.24	40.31	34.31	31.68	31.20	43.20	109.69

*LSD (0.05)

viciifolia 116A8 + O. viciifolia 116A14, and 3) O. viciifolia 116A14 + viciifolia 116A27, which were not significantly different than the commercial inoculum. All other strains and composites of strains reduced less acetylene than the commercial inoculum and more than the no-Rhizobium control. Although acetylene reduction of the composite of strains Petalostemum 124Z1 + O. viciifolia 116A8 + O. viciifolia 116A14 was the same as the commercial inoculum, %N root, %N plant, mg nodule/plant, mg dry forage/plant, and nodule number/plant were significantly lower than the commercial inoculum. The composite of strains O. viciifolia 116A14 + O. viciifolia 116A27 had significantly higher %N forage, %N root, and mg N/plant, but lower forage dry weight and nodule number. The composite of strains O. viciifolia 116A8 + O. viciifolia 116A27 had significantly higher %N forage than the commercial inoculum.

The general effect of the particular treatments averaged across all treatments based on acetylene reduction, were calculated using orthogonal comparisons. The effects of four strain and strain combination treatments, 1) O. viciifolia 116A27, 2) Petalostemum 124Z1 + O. viciifolia 116A27, 3) O. viciifolia 116A8, and 4) Petalostemum 124Z1 + O. viciifolia 116A14, were significantly different when averaged across all treatments (Table 1-6). Three of these composites 1) O. viciifolia 116A27, 2) O. viciifolia 116A8, and 3) Petalostemum 124Z1 + O. viciifolia 116A8 + O. viciifolia 116A14, expressed beneficial

Table 1-6. The effect of individual strains and strain combinations on acetylene reduction when averaged across all treatments.

Treatment	M.S.	t
<u>O. viciifolia</u> 116A8	0.6402*	2.1618*
<u>O. viciifolia</u> 116A14	0.0124	0.3010
<u>O. viciifolia</u> 116A27	1.2790**	3.0560**
<u>Petalostemum</u> 124Z1	0.1871	1.1690
1 + 2	0.0478	-0.5907
1 + 2 + 3	0.1928	-1.1860
1 + 2 + 4	0.7348*	2.3160*
1 + 3	0.3030	-1.4880
1 + 3 + 4	0.1676	-1.1060
1 + 4	0.0043	0.1777
2 + 3	0.0921	-0.8193
2 + 3 + 4	0.0780	0.7547
2 + 4	0.0343	-0.5009
3 + 4	1.1100**	-2.8470**
1 + 2 + 3 + 4	0.0016	-0.1085

** p = 0.01

* p = 0.05

effects. Composite Petalostemum 124Z1 + O. viciifolia 124A27 had a detrimental effect on acetylene reduction.

In general, the effect of combining strains of bacteria on the rate of acetylene reduction was additive (Table 1-5). For example, when the acetylene reduction rates for strains O. viciifolia 116A8 and O. viciifolia 116A27 are added together (.3880 + .6273 ul C₂H₂ reduced/plant/hr = 1.0153 ul C₂H₂ reduced/plant/hr) the sum was not significantly different than the treatment combination of the two strains of Rhizobium (1.1440 ul C₂H₂ reduced/plant/hr). This was also true for strains O. viciifolia 116A14 and O. viciifolia 116A27 (.3890 + .6273 ul C₂H₂ reduced/plant/hr = 1.0163 ul C₂H₂ reduced/plant/hour).

Strains Petalostemum 124Z1 and O. viciifolia 116A8 and O. viciifolia 116A14 express the additivity in a different manner when in combination. When strains O. viciifolia 116A8 and O. viciifolia 116A14 were in combination the amount of acetylene reduction was somewhat less than if the reduction rates for the individual strains were added together (.3880 + .3890 ul C₂H₂ reduced/plant/hr = .7770 ul C₂H₂ reduced/plant/hour; Reduction rate for composite = .4387 ul C₂H₂ reduced/plant/hr). But if the reduction rate for strain Petalostemum 124Z1 is added to the reduction rate for the O. viciifolia 116A8 + O. viciifolia 116A14 treatment combination (.5392 + .4387 ul C₂H₂ reduced/plant/hr = 1.0319 ul C₂H₂ reduced/plant/hr) the sum is not significantly different than the treatment combination of the three strains

together (1.0370 ul C₂H₂ reduced/plant/hr). The effects of Petalostemum 124Z1 + O. viciifolia 116A8 were additive when they were in combination. If the rate of acetylene reduction for strain O. viciifolia 116A14 is added to the rate of acetylene reduction for the Petalostemum 124Z1 + O. viciifolia treatment combination (.3890 + .7702 ul C₂H₂ reduced/plant/hr = 1.0592 ul C₂H₂ reduced/plant/hr) the sum is not significantly different than the reduction rate for the treatment combination of the three (1.0370 ul C₂H₂ reduced/plant/hr).

It appears that the degree of the additivity of the treatment combination can be altered. Recent evidence suggests that Rhizobium can produce bacteriocins, antibiotics, and phages which have the ability of slowing growth or killing other Rhizobium of the same species (85, 86, 87). The bactericidal activity expressed is generally mild but it exhibits a wide host range (85). Rhizobium produce bacteriocins in the early to mid-exponential phase in broth culture (86). Production of bacteriocins is spontaneous (85, 86).

The bacteriocins produced by Rhizobium are phage like particles of both high and low molecular weight. They contain protein and DNA or RNA (85, 86, 87). The activity of the bacteriocin is dependent upon the pH of the environment and the type of growth medium in which the Rhizobium are grown.

Since these bactericidal agents are produced abundantly in the field (86) researchers studying Rhizobium should isolate and examine

the effects of these agents on specific strains of Rhizobium. Rhizobium species indigenous to the area should be evaluated for their competitiveness with the Rhizobium species to be introduced.

Rhizobium strains also vary in their competitiveness for food sources provided in the soil and by the plant (10). Therefore the growth rates of the populations of species in the soil may shift so that the most competitive Rhizobium build in numbers quickly. Subsequently, the majority of the bacteroids found in the nodules would develop from more competitive Rhizobium strains.

Physiological interaction between Rhizobium and plant may cause a depression of additivity of acetylene reduction rates when strain combinations are used as inoculum. The strains of Rhizobium may be competing for the same specific amino acid or carbon skeleton which transfer the synthesized ammonia to the plant's vascular system. If the compound is not provided to the bacteroids in sufficient quantity by the plant, feedback inhibition could result due to a buildup of ammonia.

All indices of nitrogen fixation were positively correlated with one another except mean nodule weight and C_2H_2 reduction/mg nodule (Table 1-7). The variable most closely associated with $ul C_2H_2$ reduction was mg nodule/plant. Milligrams N/plant forage, mg forage/

Table 1-7. Correlations among indices of N fixation using strains and strain composites on sainfoin.

	C ₂ H ₂ Reduction		Nitrogen					Weight per plant			Nodule		
	Per plant per hour	Per mg. nod. per hour	Forage	Root %	Plant	Forage mg	Root	Nodules	Forage mg	Root	Total	Number/ plant	% Weight mg
C ₂ H ₂ red/ plant/hr.	1.00	.36**	.70**	.66**	.71**	.75**	.70**	.77**	.75**	.58**	.72**	.74**	-.05
C ₂ H ₂ red/ plant/hr.		1.00	.10	.19	.10	.08	.12	.05	.08	.03	.06	.09	.26*
% N Forage			1.00	.66**	.95**	.86**	.61**	.88**	.77**	.51**	.74**	.77**	-.05
% N Root				1.00	.79**	.64**	.63**	.64**	.63**	.31*	.53**	.61**	-.05
% N Plant					1.00	.88**	.64**	.86**	.81**	.47**	.71**	.79**	-.06
Mg N forage						1.00	.82**	.88**	.98**	.75**	.93**	.76**	-.06
Mg N root							1.00	.68**	.88**	.73**	.94**	.55**	-.09
Mg nodule/ plant								1.00	.85**	.60**	.81**	.87**	-.06
Mg forage/ plant									1.00	.81**	.97**	.75**	-.08
Mg root/ plant										1.00	.93**	.44**	-.09
Mg total/ plant											1.00	.67**	-.09
Nodule #												1.00	-.19
% nodule wt (mg)													1.00

*r(.05) = .25
**r(.01) = .33

plant, and %N forage were also highly correlated with $\mu\text{l C}_2\text{H}_2$ reduction/plant/hour.

CONCLUSIONS CHAPTER I

Three of the five Rhizobium strains used in the commercial inoculum are non-effective strains. Strain O. viciifolia 116A27 has a general beneficial effect when combined with other strains of Rhizobium. Petalostemum strains of Rhizobium are cross inoculant groups which allow significant rates of acetylene reduction on sainfoin. Further investigations of other Petalostemum strains should be conducted.

The effect of combining strains of bacteria on acetylene reduction is additive. However, bacteriocins and antibiotics may alter the degree of expressivity of the additive effect. Rhizobium strains should be screened for acetylene reduction on plants grown in soil containing natural microflora in order to obtain estimates of performance in the field.

A superior strain combination of Rhizobium, when compared to the commercial inoculum, was not found.

Milligrams nodule/plant was the indice of nitrogen fixation most closely associated with acetylene reduction when testing single strains and strain composites of Rhizobium. Percent nitrogen of the forage was also closely associated with acetylene reduction. Percent

nitrogen of the forage could be used as a screening index for nitrogen fixation when screening single strains and strain composites of Rhizobium.

CHAPTER II

General:

The purpose of these experiments was to determine 1) if any inhibiting factor of nitrogen fixation exists in the soil; and 2) if the inhibiting factor is biotic or abiotic.

MATERIALS AND METHODS CHAPTER II

Experiment I: The effect of soil extract on indices of N fixation.

Greenhouse bench, plastic pots, seed and Rhizobium preparations were the same as in Chapter I. Pots were arranged on two benches in a split plot randomized complete block design with six replications. Rhizobium inoculated vs. non-inoculated pots were main plots and soil extract, sterile soil extract, and no soil extract were sub plots.

Pure cultures of each strain of Rhizobium in the commercial inoculum were diluted to equal concentrations of 1.8×10^9 cell/ml. Equal proportions of each strain were mixed and 10 ml of the suspension was diluted with 90 ml of H₂O and poured into each Rhizobium inoculation treatment.

A soil paste of Bozeman silt loam soil (argic pachic cryoboral, fine silty, mixed) was made and allowed to stand for one hour. Two Whatman number 1 filter papers (W. and R. Balston Co., England) were placed on the bottom of a pressure membrane extractor (Soil Moisture Co., Santa Barbara, Calif.). The soil paste was placed on the filter paper, the chamber sealed, and 20 psi of gaseous nitrogen applied for 10 minutes. This process was repeated until three liters of solution were collected. One and one-half liters of extract were autoclaved for 30 minutes at 121C and 20 psi in an Amsco General Purpose autoclave (American Sterilizer Co., Erie, Penn.).

Seventy-five ml of soil extract, sterile soil extract, or tap water were applied to the appropriate pots 7 and 14 days after planting. Fertility levels were maintained with twice weekly applications of Hoagland's (nil nitrate) nutrient solution (Appendix 1). After eight weeks plants were harvested and assayed for nitrogen fixation as in Chapter I. All indices of nitrogen fixation were measured except nodule number and weight. Nodule scores were measured on a 1-4 basis (1 = no nodules, 4 = abundantly nodulated).

Data were analyzed by analysis of variance and means separated by Duncan's New Multiple Range test. Correlation coefficients among indices were calculated.

Experiment II: The effect of various nutrient chelates on forage yield and late spring-early summer chlorosis of sainfoin.

This experiment was conducted in the middle of a field of three year old sainfoin established from bulked W-40 seed. The experimental range was divided into 1.55 x 3.1 meter plots. Treatments were 1.12 kg/ha Zn, 1.12 kg/ha Mn, 1.12 kg/ha Cu, and 5.6 kg/ha Fe chelates with a no chelate control. Treatments were sprayed on the foliage June 2, 1978. A randomized complete block design with four replications was used.

On June 29 a 0.61 x 3.1 meter swath was harvested from each plot with a Rem Forage Plot Harvester (Rem Mfg., Swift Current, Canada). Wet forage weights were recorded and a sample of each plot was weighed, dried at 40C for 48 hours, and reweighed to determine percent moisture. Yield is reported as kilos/ha at 12% moisture.

Data were analyzed by analysis of variance and the means separated by Duncan's New Multiple Range test.

RESULTS AND DISCUSSION CHAPTER II

Experiment I: The effect of soil extract on indices of nitrogen fixation

With the assumption that the soil microflora is capable of withstanding short periods of waterlogging and the pressure which was applied to the paste in the pressure membrane extractor, we would

expect to find a representative sample of the microflora from the Bozeman silt loam soil to be present in the soil extract solution. Inoculations made with the soil extract should therefore express differences for the indices of N fixation if a biotic factor is present.

All indices of N fixation were significantly higher when Rhizobium was present than when Rhizobium was absent except root weight (Table 2-1). Significant differences among soil extract, sterile soil extract, and no soil extract treatments were not detected with any of the N fixation indices except mg dry forage/plant. This suggests that the Rhizobium inoculum used had a positive effect on nitrogen content of the plant and plant growth in all ways except root growth. Also, the soil extract did not have any effect on N fixation but reduced forage growth.

Although not significantly different, most indices of N fixation were lower when non-sterile soil extract was applied. This suggests a possibility that biotic factors present in the soil may adversely affect N fixation of sainfoin. It appears that with increased replications in our experimental procedures we could easily detect significances in four other classes - %N root, %N plant, mg N/plant, and nodule score. With the assumption that these differences are real, we can assume that there is some biotic factor in the soil which is inhibiting the nodulation of the plant. Orser (unpublished data) has found that Pseudomonas isolated from sainfoin plants kills the Rhizobium

Table 2-1. The effect of Rhizobium and soil extract on indices of N fixation in sainfoin.

Treatment	Levels	ul C ₂ H ₄ red per plant/hr	Nitrogen						Nodule score	Weight/plant		
			%			mg				forage	root	total
Rhizobia			forage	root	total	forage	root	total				
	Present	0.41 ¹⁾	2.68 ^a	1.24 ^a	1.78 ^a	1.72 ^a	1.25 ^a	2.97 ^a	4.11 ^a	63.69 ^a	100.51	165.32 ^a
	Absent	0.01 ^b	1.27 ^b	0.61 ^b	0.81 ^b	0.39 ^b	0.50 ^b	0.89 ^b	1.00 ^b	30.65 ^b	83.68	112.11 ^b
Soil Extract	Present	0.17	1.99	0.86	1.21	0.92	0.81	1.72	2.42	39.01 ^a	90.64	130.49
	Present Sterilized	0.23	1.97	0.92	1.31	1.08	0.86	1.94	2.50	49.02 ^{ab}	92.87	141.89
	Absent	0.22	1.96	0.99	1.36	1.17	0.95	2.12	2.75	53.49 ^b	92.67	143.67
Difference (Abs-Pres)		0.05	0.03	0.13	0.15	0.15	0.14	0.40	0.33	14.48	2.12	13.27
Diff need for signif. Reps req. for signif		0.13	--	0.16	0.20	0.46	0.37	0.77	0.55	10.39	--	45.13
		46	--	11	12	57	43	15	17	6	--	70

1) Values followed by different letters within a column are significantly different at the P = 0.05 level.

2) Nodules Scored based on 1 = no nodules; 5 = abundantly nodulated

strains which infect sainfoin when in pure culture. Investigations concerning the effect of Pseudomonas on the infection, nodulation, and acetylene reduction of sainfoin by Rhizobium are now in progress.

All indices of N fixation were highly correlated with one another except mg root/plant which was not correlated with %N plant root, %N plant, or nodule score. Milligrams N forage, mg N/plant and mg dry forage were the indices most closely related to acetylene reduction (Table 2-2). This indicates that mg N forage is an index which can be used as a quick screen in place of C_2H_2 reduction.

Experiment II: The effect of various nutrient chelates on forage yield and late spring-early summer chlorosis of sainfoin.

There was no evidence of late spring-early summer chlorosis during the 1978 growing season. The growing season was unusually cool and wet which may have altered growing conditions such that factors causing chlorotic conditions were not expressed.

Yield data suggests that Zn, Cu, and Mn are toxic to sainfoin when applied at rates as high as 1.12 kg/ha (Table 2-3).

Table 2-3. The effect of various micronutrient chelates on yield of sainfoin.

Treatment	Yield T/ha (1)
Zn	6.660 a
Cu	7.119 a
Mn	7.119 a
Control	7.926 b
Fe	7.939 b

(1) Values followed by different letters are significantly different at the $p = 0.05$ level.

Table 2-2: Correlation matrix for the effect of Rhizobia and soil extract on indices of N fixation.

	ul C ₂ H ₂ red per plant/hr	Nitrogen						Weight			Nodule Score
		Forage	Root %	Plant	Forage	Root mg	Plant	Forage	Root mg	Plant	
ul C ₂ H ₂ red. per plant/hr	1.00	.73**	.75**	.78**	.90**	.83**	.89**	.86**	.49**	.71**	.68**
% N Forage		1.00	.77**	.92**	.90**	.73**	.86**	.75**	.39**	.62**	.82**
% N Root			1.00	.93**	.83**	.83**	.85**	.82**	.21	.54**	.82**
% N Plant				1.00	.91**	.78**	.88**	.82**	.24	.54**	.86**
mg N Forage					1.00	.90**	.99**	.95**	.56**	.81**	.83**
mg N Root						1.00	.96**	.92**	.69**	.88**	.72**
mg N Plant							1.00	.96**	.63**	.85**	.81**
mg Forage								1.00	.62**	.85**	.80**
mg Root									1.00	.92**	.30
mg Plant										1.00	.59**
Nodule Score											1.00

*r(0.05) = .36

**r(0.05) = .46

1) Nodules scored on a basis of 1 = no nodules; 5 = abundantly nodulated

Excessive amounts of Cu depress the activity of iron and may cause iron deficiencies which could explain why chlorosis appears in the spring. However, Cu availability decreases with increased pH (97) and the problem of Cu toxicity would not be expected in the Bozeman area. Likewise, Mn and Zn become more available at lower soil pH's (97) and toxicity problems would not be expected in Bozeman.

CONCLUSIONS CHAPTER II

Although the observed differences do not suggest that there is a significant difference among extract treatments, the lower values of the indices of N fixation may have been caused by a biotic factor. Additional experiments with increased replications should be conducted in order to determine the origin of these effects.

Zinc, Mn, and Cu applications cause yield reductions of sainfoin. These may have been due to a toxic effect. The source of these micronutrients and reason for them being present in relatively high concentrations at neutral to above neutral pH's is not understood.

CHAPTER III

General:

The purposes of the following experiments were to 1) determine if rooting habit of sainfoin has any significant effect on nodule number; 2) select plants for rooting habit to be used in a breeding program to

increase the N fixing potential of sainfoin; 3) develop a non-destructive technique for assaying individual sainfoin plants for acetylene reduction capabilities; and 4) select plants on the basis of acetylene reduction to be used in a breeding program to increase the N fixing potential of sainfoin.

MATERIALS AND METHODS CHAPTER III

Root studies:

Greenhouse benches were sprayed with a 2.5% NaOCl spray. The benches were then filled with plaster grade vermiculite, wetted thoroughly with tap water, and leveled. Sterile, depodded, sized Remont seeds were prepared as in Chapter I and planted 2.5 cm deep and 2.5 cm apart in the vermiculite in furrows spaced 9 cm apart.

Conetainer studies:

Conetainers (Ray Leach Co., Camby, Oregon), 2.5 cm x 15 cm, and racks were surface sterilized in 0.5% NaOCl for 15 minutes. The conetainers were then filled with vermiculite. Sized, depodded, sterile Remont sainfoin seeds were prepared as in Chapter I and planted 2 cm deep in each conetainer. One seed was planted in each conetainer.

Pure strains of the commercial composite Rhizobium inoculum (Nitragin Co.) were grown for one week in YEM broth (Appendix 2) and diluted to equal concentrations. Equal portions of each strain were

mixed and 0.5 ml of this suspension added to each container 7 days after planting.

Plants were grown in a growth chamber with a 16 hour photoperiod at a light intensity of 100 watts/m² (16,000 lux) provided by fluorescent and incandescent lights. Alternating day-night temperatures were 25 and 20C respectively. Fertility levels were maintained with Hoagland's (nil-nitrate) nutrient solution (Appendix 1) applied twice weekly.

Containers with intact plants were placed in 1000 ml erlenmeyer flasks and capped with an air tight septum. Acetylene reduction assays were performed on these plants as described by Hardy et al (45) (Chapter 1).

Experiment I: The effect of rooting habit on nodule number.

Pure cultures of each of the commercial inoculum Rhizobium strains (Nitragin Co.) were grown in YEM broth (Appendix 2) for 7 days and diluted to equal concentrations of 2×10^8 cells/ml. Equal portions of these suspensions were mixed. One -iter of the final suspension was used to inoculate one greenhouse bench each of Melrose and Remont sainfoin 7 days after planting. Fertility levels were maintained with Hoagland's (nil-nitrate) nutrient solution (Appendix 1).

After four months, the plants were harvested and the roots gently washed free of vermiculite. The roots were then scored for fibrosity

(1=tap, 4=fibrous) and degree of nodulation (1=few, 4=abundant).

Correlation coefficients for the degree of root fibrosity and degree of nodulation were determined for each cultivar.

To determine if sufficient root variability exists in Remont sainfoin to warrant selection for different rooting habits, two replicates in time of Remont sainfoin were grown in a surface sterilized greenhouse bench. No Rhizobium was applied and nutrient levels were maintained with daily applications of Peter's Greenhouse Special nutrient solution. The plants were harvested four months after planting and roots scored for fibrosity (1=tap, 5=fibrous)(Figure 3-1).

Experiment II: Pilot study to determine if conetainers can be used in a non-destructive acetylene reduction assay screening technique for sainfoin plants.

Two layers of cheesecloth were placed around the bottom of ten conetainers and held in place with rubber bands. The conetainers were filled with 1.25 mm screened vermiculite. Seeds were planted and conetainers inoculated with 0.5 ml of the composite of the commercial inoculum (Nitragin Co.) at a concentration of 5×10^8 cells/ml.

Eight weeks later, at harvest, acetylene reductions were performed at 4, 8, 12, and 24 hour incubation periods. Attenuation of the gas chromatograph was set at 1×10^2 . Tops were then cut at the crown, the roots washed free of vermiculite, and the roots and tops dried separately at 85C for 24 hours. Dry weights were recorded.

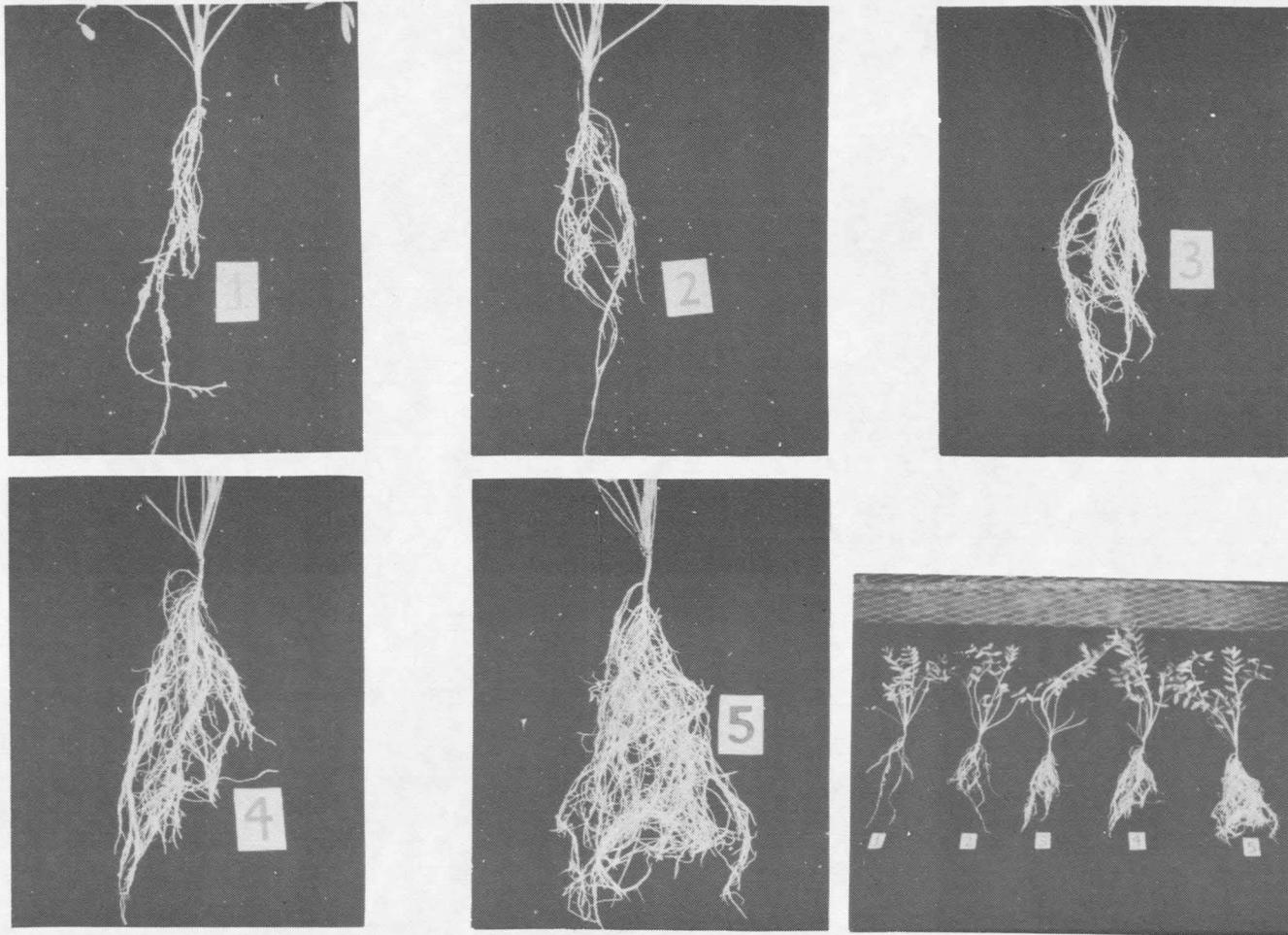


Figure 3-1. Variability among rooting habit in sainfoin.

Correlation coefficients between forage dry weight, root weight, and acetylene reduction at the 4 hour incubation period were calculated. Correlation coefficients were also calculated for acetylene reduction at 4 hours vs. 8, 12, and 24 hours.

Experiment III: Breeding program to select sainfoin for nitrogen fixation using conetainers.

Five hundred conetainers were filled with unscreened vermiculite and planted as previously described. One half ml of a 1×10^9 cells/ml concentration of commercial inoculum Rhizobium suspension (Nitrogen Co.) was added to each conetainer.

Eight weeks after inoculation the plants were assayed for acetylene reduction in blocks of 100 plants. A four hour incubation period was used with an attenuation of 50 x 1 on the chromatograph.

The top 5% of each block and the bottom 5% of each block of 100 plants were transplanted into pots and saved for future polycrosses.

Histograms of the distributions of the acetylene reduction rates for the plants of each block were made.

RESULTS AND DISCUSSION CHAPTER III

Experiment I: The effect of rooting habit on nodule number.

Fibrous rootedness and high nodule number were significantly correlated in Melrose ($r = 0.44^{**}$) and Remont ($r = 0.56^{**}$) sainfoin (Figure 3-2). Mean values for root habit of Melrose (2.17) and Remont

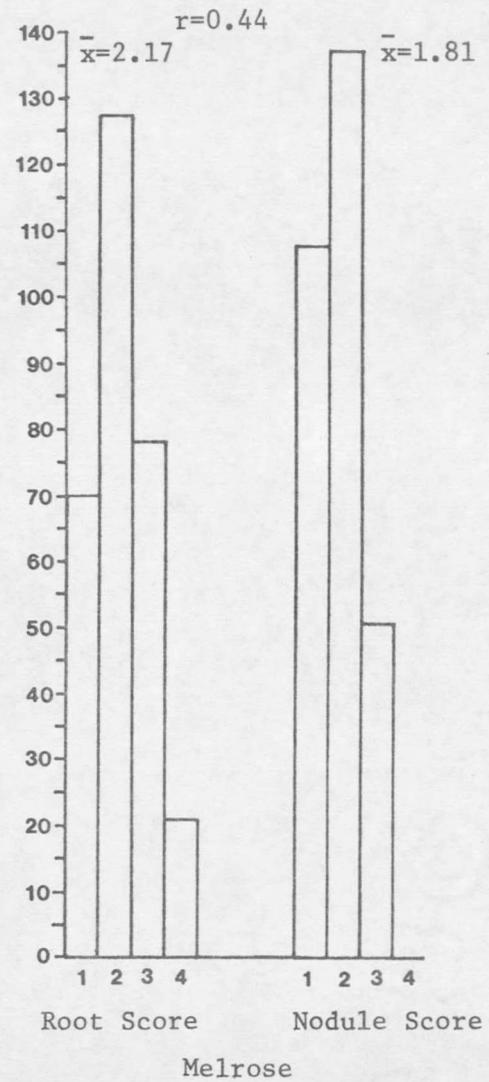
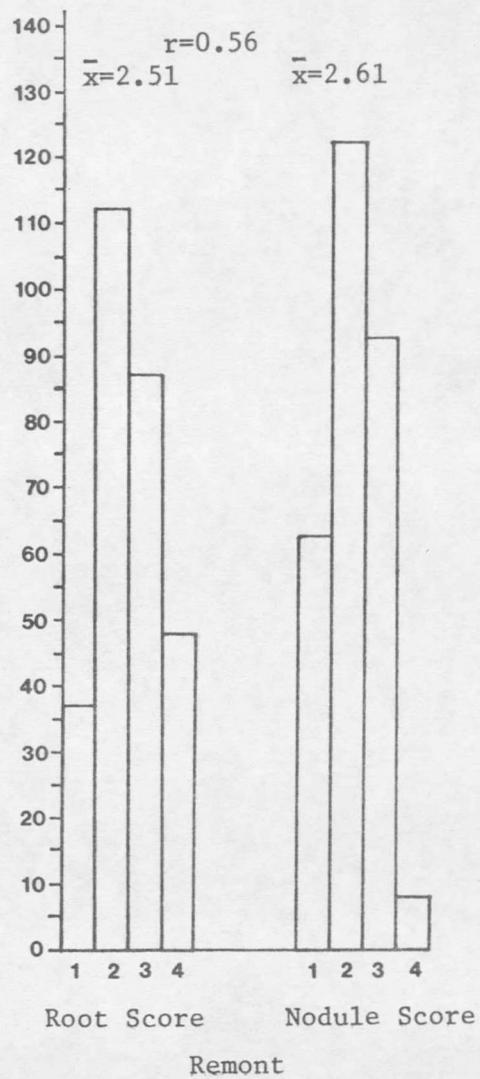


Figure 3-2. Frequency distribution of root and nodule habit of sainfoin.

(2.51) and nodule habit of Melrose (1.81) and Remont (2.61) were not significantly different.

Although the relationship between fibrous rootedness and increased nodule number is not extremely close, the relationship is close enough to warrant divergent selections for rooting habit and evaluation of the population developed for indices of N fixation. Breeding for increased root fibrosity has increased N fixation significantly in alfalfa (80).

Both replications of sainfoin express normal distributions. Mean root scores and variances among replicates in time were not significantly different (Figure 3-3). There is enough variability in rooting habit to use divergent selection to develop fibrous and tap rooted populations. These populations will be tested to see if rooting habit has an effect on N fixation. Initial plant selections for these characteristics have been made.

Experiment II: Pilot study to determine if conetainers can be used in a non-destructive acetylene reduction assay screening technique for sainfoin plants.

As time increased, the acetylene reduction rate, expressed per plant per hour, decreased (Table 3-1). This can be attributed to one or more of the following five factors: 1) acetylene is toxic to the plant at the concentration present in the flask; 2) ethylene was produced at a high enough rate to cause toxic effects on the plants;

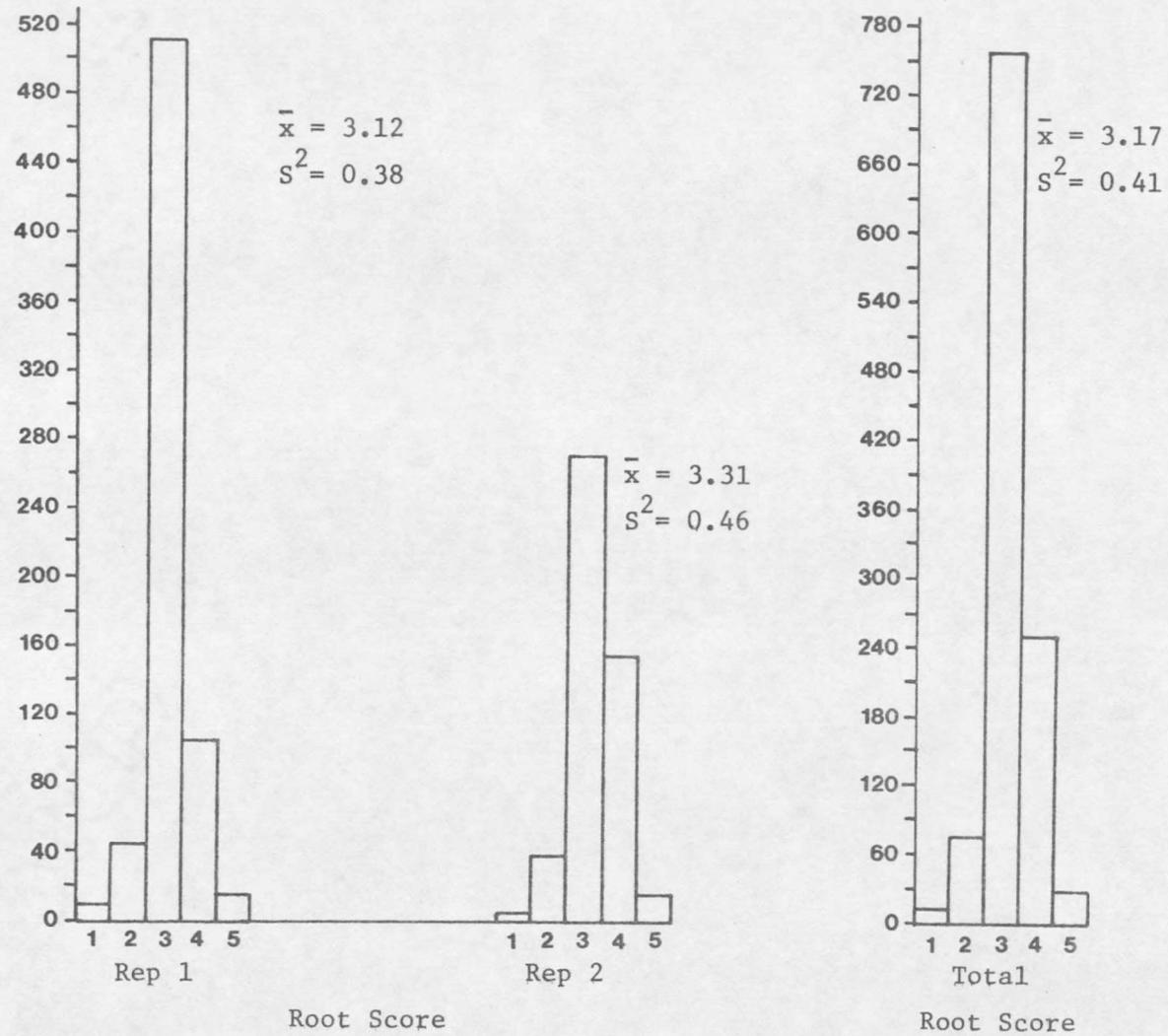


Figure 3-3. Frequency distribution of roots of Remont sainfoin.

Table 3-1. Top weight, root weight, and acetylene reduction rates for four sampling periods on intact six week old sainfoin plants in conetainers.

Plant #	Top wt (mg)	Root wt (mg)	Acetylene reduction/plant/hr			
			4 hrs (Rank)	8 hrs (Rank)	12 hrs (Rank)	24 hrs (Rank)
1	44.8	82.8	2.4871- (5)	2.8437- (5)	1.7383- (5)	1.0727- (5)
2	34.8	43.9	1.1232- (8)	0.5393- (9)	0.3187- (8)	0.1835- (8)
3	77.8	190.5	1.5243- (7)	1.5199- (7)	0.8931- (7)	0.5928- (7)
4	99.6	225.7	8.5042- (2)	8.3839- (2)	4.1429- (2)	2.9529- (2)
5	54.4	96.1	3.6103- (4)	3.6281- (4)	2.3467- (4)	1.6436- (4)
6	69.9	139.5	0.5616- (10)	0.4413- (10)	0.2897- (9)	0.1393- (10)
7	96.3	134.6	8.0229- (3)	6.9130- (3)	3.8822- (3)	2.0893- (3)
8	101.6	102.7	10.3495- (1)	9.8057- (1)	5.4466- (1)	2.9529- (1)
9	62.5	123.3	2.3266- (6)	2.5005- (6)	1.1009- (6)	0.6407- (6)
10	73.5	117.7	0.6418- (9)	0.5883- (8)	0.2028- (10)	0.1671- (9)

r value
to 4 hr
sampling

.69**

.12

1.00

.99**

.99**

.97**

r value for top wt. to root wt.

.68**

3) CO₂ was not present in ample quantity and limited production of photosynthate; 4) light was not present in ample quantity and limited production of photosynthate; and/or 5) O₂ was not present in ample quantity and respiration was limited. Wilting appeared after 12 hours which indicates that ethylene or acetylene toxicity probably caused decreased acetylene reduction rates. Plants recovering from this stress were obviously more susceptible to mites than healthy plants during recovery.

Acetylene reduction/plant/hr ranged from 10.3495 ul/plant/hr to 0.5616 ul/plant/hr. The correlation coefficients of acetylene reduction rates respectively were .99**, .99**, and .96** for the 8, 12, and 24 hr reduction rates vs. the 4 hr reduction rate respectively. This indicates that the 4 hour incubation period is sufficient to determine acetylene reduction potentials of the plants. Rankings of plants based upon acetylene reduction were consistent over all sampling periods.

Top weight was correlated with acetylene reduction ($r = .69^{**}$) and root weight ($r = .68^{**}$). Root weight was not correlated with acetylene reduction ($r = .12$).

Experiment III: Breeding program to select sainfoin
for nitrogen fixation using conetainers.

The five replications of sainfoin did not have normal distributions for acetylene reduction assays. The acetylene reduction rates

were skewed towards lower fixation rates. Variances for these populations ranged from .4625 to 1.1691. The total variances of all populations was .6428 (Figure 3-4).

A total of twenty high nitrogen fixers and twenty-four low nitrogen fixers (based upon acetylene reduction values) were selected. These plants will be placed in a polycross breeding program and the heritability of nitrogen fixation calculated.

It is not surprising that acetylene reduction values were not distributed normally. First, the genetics of the symbiotic relationship is not controlled by one organism. With the sainfoin program being in its infancy, the probability is low that we have found the strain of Rhizobium which has the maximum fixation potential. If the maximum fixation potential by Rhizobium can be reached, it is possible that a normal distribution for acetylene reduction rates would be exhibited. Second, mite damage was evident and as a result of infestation, alternate applications of Pentac (Bis Pentachlore - 2, 4 - Cyclopentadien - 1 - yl) (Hooker Chemical Co., Niagra Falls, NY) and Dibrom (Naled) (Wilbur-Ellis Co., Fresno, Calif.) miticides were made weekly. While Pentac seemed to have no effect on the plant or Rhizobium, use of Dibrom gave plant damage in the form of tip burn on the leaves. The phytotoxicity of Dibrom to the plant may have caused a decrease in acetylene reduction.

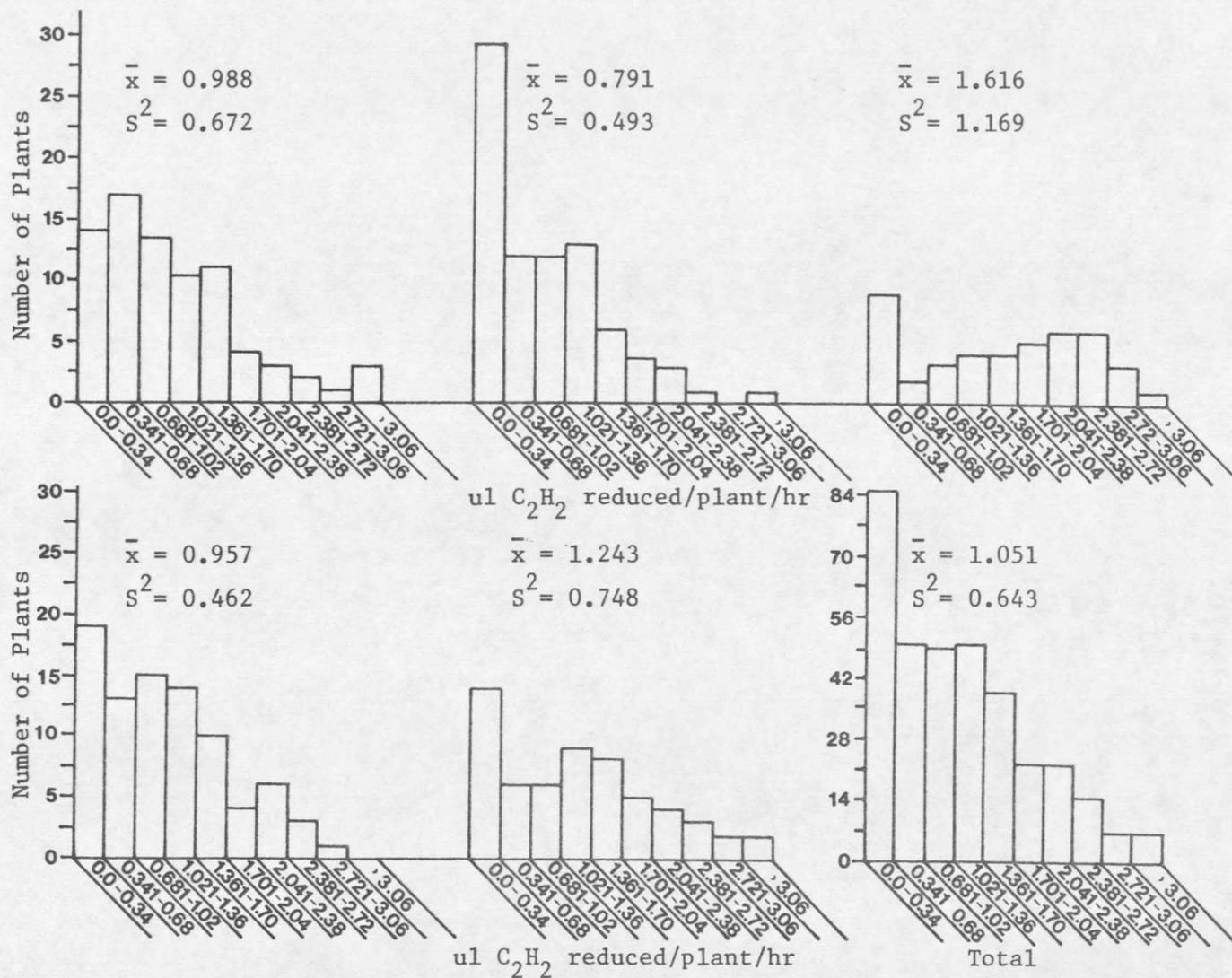


Figure 3-4. Frequency distribution of acetylene reduction on five replicates in time of Remont sainfoin.

Nitrogen fixation is a heritable characteristic in soybeans (103), alfalfa, red clover (71), and white clover (69). It is probably a heritable characteristic in sainfoin and can be transferred in a breeding program.

CONCLUSIONS CHAPTER III

Root fibrosity is positively correlated with nodule number. Although the correlation coefficient is not high, breeding for root fibrosity should significantly increase nodule number and therefore nitrogen fixation.

A non-destructive technique for screening plants for acetylene reduction was developed. Post assay care must be taken to prevent spider mite infestation. Pre-assay treatment of the plants is important to keep spider mite infestations low without causing damage to the sainfoin plant or the Rhizobium.

The nitrogen fixing symbiotic relationship between sainfoin and Rhizobium is not distributed normally. This may be an indication that the proper Rhizobium strain for maximum nitrogen fixing potential has not been found.

SUMMARY AND CONCLUSIONS

Nitrogen fixation in the legume plant depends on the legume genotype, the Rhizobium strain, the interaction of the legume plant and the Rhizobium strain, and the interaction of field organisms with the legume plant and the Rhizobium inoculum. This study was conducted to: 1) evaluate the performance of single strains and composites of Rhizobium strains on Remont sainfoin and identify indices of nitrogen fixation affected by the Rhizobium strains and composites; 2) determine if any inhibitory factor of nitrogen fixation in sainfoin exists in the soil; and 3) characterize sainfoin as to its nitrogen fixing potential.

Seven commercial strains of Rhizobium specific for sainfoin, two cross inoculant (promiscuous) strains, four strains from Russia, and one field selected strain of Rhizobium were inoculated on greenhouse grown sainfoin plants and evaluated for indices of nitrogen fixation. The four best strains, based upon acetylene reduction were tested individually and in all possible combinations for indices of nitrogen fixation.

Individual Rhizobium strains differed significantly for all indices of nitrogen fixation except mg dry root/plant and mean nodule weight. Based on the indices of nitrogen fixation, the strains of Rhizobium can be broken down into two classes: effective and

non-effective. Three of the five strains of Rhizobium present in the commercial inoculum were non-effective. Two cross inoculant strains were effective. This suggests that an improper inoculum is being used for sainfoin.

Milligram dry nodule/plant and %N forage were indices most closely associated with acetylene reduction/plant/hr. The coefficient of variability for %N forage was lower than mg dry nodule/plant. This suggests that %N forage can be used to select individual strains of Rhizobium in place of acetylene reduction.

Significant differences were not detected in five indices of nitrogen fixation when Rhizobium strains were mixed. This suggests that there is some Rhizobium x Rhizobium interaction causing increased variation in the indices measured.

The general effect of the particular strain combinations when averaged across all treatments was additive based on acetylene reduction values. Rhizobium strains O. viciifolia 116A27 and O. viciifolia 116A8 and strain composite Petalostemum 124Z1 + O. viciifolia 116A8 + O. viciifolia 116A14 were beneficial and strain combination Petalostemum 124Z1 + O. viciifolia 116A27 was detrimental.

Saturated soil extract and sterile soil extract were tested for their effect on the performance of Rhizobium by measuring indices of nitrogen fixation. There were no significant differences within the indices of nitrogen fixation except mg dry forage/plant. Although not

significantly different, most indices of nitrogen fixation were lower when non-sterile extract was applied. Increased replication of the experiment may have detected significant differences with four other indices. This suggests that although not detected, biotic factors may exist which inhibit nitrogen fixation in sainfoin.

Studies were conducted to determine if rooting habit had any significant effect on nodule number, to characterize the variability of the rooting habit within Remont sainfoin, and to determine the variability of nitrogen fixation found in Remont sainfoin. Results of these were:

Fibrous rootedness and high nodule number were significantly and positively correlated in Remont and Melrose sainfoin. Root and nodule scores did not differ significantly between the two cultivars.

In developing a screening program to assay individual plants for acetylene reduction, a simple non-destructive, space conserving technique was developed.

Acetylene reduction rates were not normally distributed. This may be an indication that the proper Rhizobium inoculum for maximum fixation has not been found.

APPENDIX 1

HOAGLAND'S NITROGEN FREE NUTRIENT SOLUTION

<u>Nutrient</u>	<u>Quantity</u>
Potassium (0.5m KCl)	5ml/L
Magnesium (1m MgSO ₄)	2ml/L
Phosphorus (0.05m Ca(H ₂ PO ₄) ₂)	10ml/L
Calcium (0.01m CaSO ₄)	200ml?1
Boron (H ₃ BO ₃)	0.5ppm
Manganese (MnCl=4H ₂ O)	0.5ppm
Zinc (ZnSO ₄ 7H ₂ O)	0.05ppm
Copper (CuSO ₄ 5H ₂ O)	0.02ppm
Molybdenum (MO ₃)	0.01ppm
Iron (Fe(H ₂ PO ₄) ₃)	6.00ppm
Cobalt (CaCl ₂ 6H ₂ O)	0.01ppm

Bring volume to 1000 ml.

APPENDIX 2

YEAST EXTRACT MANNITOL AGAR (BROTH)

<u>Ingredient</u>	<u>Quantity</u>
.1N K_2HPO_4	8 ml
.1N KH_2PO_4	2 ml
.1N NaCl + .1N CaCl	1 ml
.1N $MgSO_4$	1 ml
YEAST EXTRACT	1 g
D-Mannitol	10 g
Bacto Agar	15 g

Bring volume to 1000 ml w/ H_2O

Sterilize 20 min. at 121C, 15 psi.

APPENDIX 3

DETERMINATION OF NITROGEN BY THE MICRO-KJELDAHL METHOD

This method involves the conversion of the nitrogen of the sample with concentrated H_2SO_4 in the presence of appropriate catalysts, subsequent liberation of the nitrogen as NH_3 , and collection of the ammonia in standard acid, which can then be back-titrated with standard acid.

Reagents

Concentrated Sulfuric Acid

Catalyst; powdered selenium, pulverized mixture of 1 part Na_2SO_4 and 3 parts $CuSO_4$

Mixed indicator; 0.125g methyl red

0.0825g methylene blue

Prepare each in 50 ml EtOH. Mix together 2% boric acid solution; 30% sodium hydroxide; 0.01N hydrochloric acid.

Procedure

Weigh 30 mg of sample and transfer to the bottom of a digestion flask. Add 3 mg selenium and 5 mg $CuSO_4-Na_2SO_4$ mixture. Add 1 ml concentrated H_2SO_4 . Place digestion flasks on heat (370C) for 24 hours, cool and add 2 ml H_2O .

Steam out distillation apparatus for five minutes. Place 8.00 ml boric acid solution and 3 drops indicator solution in a 50 ml

ernelmeyer flask, transfer digested sample into distillation apparatus with an additional 2 ml H_2O). Place boric acid under condenser of apparatus. Introduce 10 ml 30% NaOH into distillation apparatus with digested sample. Distill to a final volume of 35ml in boric acid solution. Titrate boric acid solution back to its original color with .01N HCl.

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