



Variability for traits associated with N_2 fixation of juvenile sainfoin (*Onobrychis viciifolia* Scop.)

by Steven Dennis Cash

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in
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Abstract:

Poor symbiotic nitrogen (N_2) fixation may restrict the productivity and stand longevity of sainfoin (*Onobrychis viciifolia* Scop.). The objectives of this research were to evaluate potential sources of variability for N_2 fixation by juvenile sainfoin and to determine if improvement could be made through selection.

Growth and N_2 fixation patterns of 'Remont' seedlings evaluated at 14 d intervals to 84 d were closely related to initial seed size (ISS). The N_2 fixation rates of five sainfoin populations known to differ in seed size was not differentially affected by ISS.

Five high yielding sainfoin populations had similar N_2 (C_2H_2) fixation rates when inoculated with locally indigenous *Rhizobium* strains or commercial inoculants. Indigenous rhizobia were less effective than the least effective commercially-available inoculants.

Considerable variability exists among and within the five sainfoin populations examined for growth and N_2 (C_2H_2) fixation. Remont was generally superior to other populations in growthroom and field experiments. Adequate variability is present within all populations for potential improvement by selection.

Total nitrogenase activity (TNA) was highly correlated with total N and dry matter accumulation. Heritability estimates of TNA from parent-offspring regression and variance components were 14 and 62%, respectively. Although TNA is variable, it appears that substantial progress could be made by breeding for improved TNA.

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ABSTRACT

Poor symbiotic nitrogen (N_2) fixation may restrict the productivity and stand longevity of sainfoin (*Onobrychis viciifolia* Scop.). The objectives of this research were to evaluate potential sources of variability for N_2 fixation by juvenile sainfoin and to determine if improvement could be made through selection.

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Total nitrogenase activity (TNA) was highly correlated with total N and dry matter accumulation. Heritability estimates of TNA from parent-offspring regression and variance components were 14 and 62%, respectively. Although TNA is variable, it appears that substantial progress could be made by breeding for improved TNA.

Chapter I

INTRODUCTION

Sainfoin (*Onobrychis viciifolia* Scop.) is a perennial forage legume that is recommended for pasture and hay in the western United States and Canada. One of the primary limitations to forage productivity and stand longevity of sainfoin appears to be poor nitrogen (N_2) fixation.

Efficient symbiotic N_2 fixation is dependent upon the legume genotype, the *Rhizobium* strain, and environmental conditions. Substantial improvement of N_2 fixation in several forage legumes has been accomplished through plant breeding.

The objectives of this research were to: (1) examine the sequence of growth, nodulation, and N_2 fixation in juvenile sainfoin, (2) evaluate the variability among and within diverse sainfoin populations for traits associated with N_2 fixation, (3) determine if N_2 fixation by diverse sainfoin populations is differentially affected by indigenous or commercially obtained *Rhizobium* strains, (4) establish the heritability of total nitrogenase activity and its applicability as a selection tool for improving N_2 fixation in sainfoin, and (5) initiate breeding populations from genotypes selected within adapted germplasms for improved N_2 fixation.

Chapter II

LITERATURE REVIEW

Sainfoin (*Onobrychis viciifolia* Scop.) is a recently reintroduced forage legume that has much potential in the western United States and Canada. Sainfoin was cultivated for forage over 1,000 years ago in Russia [93]. Six hundred years later, it was introduced into western Europe [86,93]. In Russia and Europe, sainfoin was grown primarily on dry, calcareous soils where other forage legumes did not thrive [2,86,93,99].

Sainfoin was introduced into North America prior to 1900 [40]. It was evaluated in the early 1900s as a potential forage crop. The early lack of acceptance of sainfoin may have been due to evaluations on soils where it was not adapted, failure to recognize its forage quality and the use of inferior strains [40,52,86].

Recent research indicates that sainfoin is equivalent to or exceeds the production and feeding value of many of the major forage legumes [18,19,29,36,54,55,67,69,87,98].

Botanical Description of Sainfoin

The common name "sainfoin" includes many *Onobrychis* spp. The three major sainfoin species utilized for forage production in Russia are Common (*O. viciifolia* Scop. = *O. sativa* Lam), Sand (*O. arenaria* Kit ex Willd. D.C.), and Transcaucaus (*O. transcaucasia* Grass H.) [2]. All sainfoin currently in production in North America is *O. viciifolia* [36]. It is a cross-pollinated tetraploid, with $2n = 28$ chromosomes [26,44].

Sainfoin is a long-lived perennial legume which has several distinctive morphological characteristics. Sainfoin has multiple, erect stems 90-150 cm tall arising from a branched,

prostrate crown. Stems are hollow and bear pinnately-compound leaves with 11-29 leaflets [86,99].

The raceme inflorescence is erect and determinate with 5-80 florets [22,86]. Flower color is predominantly pink, however, white or light intermediates may occur [2,86]. Each fertilized floret produces a single-seeded pod [22,86]. Pods are brown, indehiscent, lenticular, and reticular on the surface [86]. Kidney-shaped seed are olive, brown, or black in color, and are approximately 2.5 mm long, 2.0 to 3.5 mm wide, and 2.5 to 2.0 mm thick [99].

The extensive root system of sainfoin consists of a main tap root with numerous lateral roots. The tap root of a mature plant may be 5 cm in diameter and extent 1-10 m [2,86,99]. Sainfoin has twice as many lateral roots as alfalfa (*Medicago sativa* L.) grown under the same conditions [2,75,99]. Nodules are formed primarily on young tap roots and fine lateral roots [15,90].

Agronomic Characteristics of Sainfoin

Several assets merit sainfoin's recognition as a potential substitute for alfalfa. Sainfoin is resistant to the alfalfa weevil (*Hypera postica* Gyllenhal), relatively drought tolerant, winterhardy, and can be used for pasture or hay [29,36,40,54].

Sainfoin forage is similar in quality to alfalfa. The hay has less crude fiber, crude protein, and ash than alfalfa, but contains more total digestible nutrients and nitrogen-free extract [36]. In feeding trials with beef cattle, sainfoin was shown to be equivalent to alfalfa for average daily gains, feed consumption, and digestibility [67]. Palatability of sainfoin hay is also considered higher than that of alfalfa [98].

Hay yields of sainfoin are variable depending on location and environmental conditions. Sainfoin has yielded less than, more than, and the same as alfalfa grown under the same conditions [18,19,29,36,54,55,69,87]. Five major sainfoin cultivars in production are 'Eski' [41], 'Remont' [21], 'Melrose' [27], 'Nova' [53], and 'Renumex' [76]. Eski, Nova, and Melrose produce more hay than alfalfa in the first cutting, and less in subsequent cuttings [36]. Remont and Renumex are multicut varieties that have seasonal yield distributions similar to alfalfa [36,76].

Sainfoin pastures under irrigation are superior to most other forage legumes. Sainfoin does not cause bloat [36]. The feed value and broad range of adaptation enhance the role of sainfoin as a pasture legume. Cattle and sheep in pasture trials prefer sainfoin over alfalfa and other forage species [36]. Sainfoin can be pastured in pure stands or in mixtures with compatible bunchgrasses such as Russian wildrye (*Elymus junceus* Fisch.), crested wheatgrass (*Agropyron desertorum* Fisch. ex Link), and Regar bromegrass (*Bromus biebersteinii* Roem. and Schult) [36,54]. Beef cattle had higher weight gains on irrigated sainfoin pastures than on Ladino clover (*Trifolium repens* L.)-tall fescue (*Festuca arundinacea* Schreb.) or bromegrass (*B. inermis* Leyss.)-orchardgrass (*Dactylis glomerata* L.) mixtures [70]. Recent studies indicate that sainfoin could replace the grass component of alfalfa-grass mixtures, which are used to reduce incidence of bloat in grazing systems [28,36]. Total productivity and feed value of these mixtures are superior to alfalfa-grass mixtures.

Sainfoin seed has the potential of becoming an excellent protein supplement in animal diets. Sainfoin seed has approximately 36% crude protein and the essential amino acid composition is equivalent to that of soybean meal [36]. A major advantage of sainfoin

seed is that trypsin inhibitors do not decrease feeding values or cause excretion of sulfur-containing amino acids, which restricts the utilization of soybean meal in diets of monogastric animals. Seed yields as high as 1,300 kg ha⁻¹ have been reported in Montana [36].

A major problem of sainfoin is root and crown rot which causes stand reduction in irrigated and dryland sainfoin. This disease is caused by a complex of organisms including *Fusarium solani* (Mart) Appel & Wr. [4,90,91], and the bacterial pathogens *Pseudomonas syringae*, *P. marginalis*, and *Erwinia amylovora* [47,102].

A second major restriction of sainfoin is poor nitrogen (N₂) fixation. Sims et al. [95] attributed visual symptoms of N deficiency in inoculated sainfoin grown at several sites in Montana to ineffective nodulation. Nodulation following inoculation is often erratic, and abundantly nodulated plants may be chlorotic in early spring. Burton and Curley [15] reported that inoculation of Eski sainfoin with nine single strains of rhizobia increased dry matter yield by an average of 36% over uninoculated seedlings. Addition of inorganic N increased the dry weights of the inoculated treatments and control by 83 and 92%, respectively. These data indicated that the *Rhizobium* strains were not effective in supplying adequate amounts of N to the seedlings grown under nil-N conditions.

Cecil [25] reported that greenhouse-grown alfalfa had levels of N₂ fixation five times that of sainfoin when measured by the acetylene reduction technique. Under field conditions, sainfoin yield is increased by application of N fertilizer [66,95] suggesting that sainfoin is inefficient in N₂ fixation. Improvement of N₂ fixation by *Rhizobium* strain selection and plant breeding would be advantageous to the production and stand longevity of sainfoin in the western United States.

Methods of Estimating N₂ Fixation

Hardy and Holsten [58] characterized measurements of N₂ fixation into three categories: (1) growth, morphology, and leghemoglobin content; (2) various N analyses including ¹⁵N isotopic enrichment; and (3) reaction products of alternate substances.

Total dry matter accumulation and topgrowth yield inoculated legumes grown in N-free media are currently being used as indicators of N₂ fixation [5,12,14,23,24,25,37,38,49,62,63,64,84,92]. Appropriate controls such as uninoculated seedlings or non-nodulating isolines of the legume are utilized to determine differences in plant growth attributable to N₂ fixation.

Nodule number, mass, and weight have been used as indicators of N₂ fixation [5,12,23,37,48,49,62,63,64,77,92]. Nodule size and number appear to be due to complex interactions between the host genotype and *Rhizobium* strain [79,82]. Nodulation alone does not reflect effective N₂ fixation, therefore these criteria should only be used as qualitative indicators of N₂ fixation [5,14].

Hardy et al. [56] reported that pink coloration of nodules, attributable to presence of leghemoglobin, was associated with active N₂ fixation; whereas white, green, or brown nodules were ineffective. Visual estimates of nodule color have been used as qualitative evidence for N₂ fixation by several workers [23,37,63,64]. LaRue and Child [72] reported a fluorescent measurement of the porphyrin component of leghemoglobin which is 10³ times more sensitive than other estimates of nodule color. Although leghemoglobin concentration is related to active N₂ fixation, the relationship may not be constant throughout the complete growth cycle of the legume [58].

Other plant characteristics such as root fibrosity, mass, and degree of secondary branching of roots are associated with N_2 fixation [23,37,62,63,64,92]. Utilization of these traits has been limited, however, they do appear to be quantitatively associated with N_2 fixation.

Until recently, various N determinations were the most widely accepted measurements of N_2 fixation. Kjeldahl-N analyses have been used extensively to measure N_2 fixation by nodulated legumes [5,14,23,37,49,57,62,63,64,92]. Inoculated legumes grown under N-free conditions compared to uninoculated or non-nodulated controls reflects the amount of N derived from symbiosis. The major restrictions to estimates of total N by these analyses include the time required for analysis and the destruction of plant tissue.

The most definitive measurement of N_2 fixation is the use of ^{15}N isotopic enrichment as proposed by Burris and Wilson [13]. Mass spectrometry of ^{15}N -enriched plant tissue is about 10^3 times as sensitive as the Kjeldahl-N analysis [57]. This procedure is demanding in chemical manipulation and costly in terms of equipment and the isotope. Its use has therefore been limited to small scale field experiments or laboratory evaluations.

The versatility of nitrogenase as a catalyst has enabled the measurement of N_2 fixation by the use of alternate substrates. Dilworth [34] found the reduction of acetylene (C_2H_2) to ethylene (C_2H_4) to be analogous to the reduction of N_2 to ammonia (NH_3). The application of this alternate reaction to a sensitive assay procedure for measuring N_2 fixation was proposed by Hardy and Knight [59]. Since its introduction, C_2H_2 reduction and C_2H_4 detection by gas chromatography has become the most widely used measurement of total nitrogenase activity (TNA).

Acetylene reduction measurements have proven to be more precise, rapid, and less expensive than the ^{15}N isotopic enrichment [57]. Because of the nondestructive nature of the acetylene reduction assay, this technique is currently being utilized to increase N_2 fixation in sainfoin [24,25,62] and alfalfa [5,37,63,64,92]. Acetylene reduction rates are reported to be highly associated with other measurements of N_2 fixation in these forage legumes [25,37,58,59,62,92].

The reduction of N_2 to 2NH_3 requires the transfer of six electrons, whereas the reduction of C_2H_2 to C_2H_4 requires the transfer of two electrons. Therefore, the theoretical ratio of three moles of C_2H_2 reduced per mole N fixed is suggested [6,57,58]. Reported conversion factors for nodulated legumes are slightly higher than the three:one ratio, however [57,89].

Schubert and Evans [89] used H_2 evolution and C_2H_2 reduction measurements to calculate the relative efficiency (R.E.) of electron transfer to N via nitrogenase. The rate of H_2 evolution should represent the total electron flux to the nitrogenase systems, however in the presence of saturating amounts of C_2H_4 , no H_2 is formed. Consequently, the estimates of TNA determined by C_2H_2 reduction alone may exceed actual N_2 fixation because the total electron flow to nitrogenase is likely utilized in the reduction of C_2H_2 . The R.E. values of 11 forage legumes ranged from 0.20-0.69, indicating a 31-80% loss of energy through H_2 evolution [6].

ATP-dependent H_2 evolution has become an important assay for *in vitro* measurements of nitrogenase activity. Hydrogen evolution by legume nodules does not correspond directly with N_2 fixation determined by other methods [58]. Despite the precision in

estimation of N_2 fixation offered by R.E. determination, this technique has not been applied to large-scale selection programs.

Breeding for Increased N_2 Fixation in Forage Legumes

Symbiotic N_2 fixation is dependent on the host genotype, the *Rhizobium* strain, and the interaction of these symbionts with the environment. Most early attempts to increase N_2 fixation dealt primarily with *Rhizobium* strain selection [37,100]. However, when laboratory-selected *Rhizobium* strains are inoculated into soil inhabited by indigenous strains, plant performance has generally not been improved [16,23,51,68,100].

Specificity among *Rhizobium* strains and host genotypes for efficiency of N_2 fixation in several legumes [14,23,39,46,49,88] indicates that genetic control of traits associated with N_2 fixation is shared among both symbionts. Although investigations on the influence of the host genotype have been limited in forage legumes, several inheritance studies have shown that N_2 fixation can be improved by conventional plant breeding methods.

Wilson [103] found that the alfalfa genotype was an important factor in nodulation in the presence of two *Rhizobium* strains. Aughtry [3] inoculated lines of *M. sativa* L. and *M. falcata* L. with diverse *R. meliloti* strains. Single crosses were made between lines which nodulated and others failing to nodulate with individual *R. meliloti* strains. Resulting hybrids were evaluated, backcrossed to the nodulating parent, and also self-pollinated. Symbiosis with single *Rhizobium* strains was shown to be heritable, and the data suggested that quantitative factors are involved in the expression of symbiotic traits in alfalfa.

Gibson [50] isolated one line of subterranean clover (*T. subterraneum* L.) which was ineffectively nodulated by *R. trifolii* strain NA30. Single crosses were made between this cultivar and six other cultivars which were effectively nodulated by NA30. The F₂ exhibited a wide range of effectiveness with NA30 apparently due to the segregation of a single major gene and several modifying genes. Similarly, Gibson [49] found that 'Rambler', a Canadian alfalfa cultivar, was unable to form an effective symbiosis with 13 Australian *R. meliloti* strains. Two Australian cultivars were each crossed to Rambler. Resulting hybrids inoculated with the *R. meliloti* strains had dry matter yields midway between the performance of the parents, suggesting that symbiotic factors in alfalfa behave additively.

Gershon [48] conducted genetic experiments with interspecific crosses between *Lotus corniculatus* L. and *L. uliginosus* L. Two *Rhizobium* strains were isolated which were reciprocally effective and ineffective for each species. The *L. uliginosus* genome was found to contain a dominant expression when combined with the *L. corniculatus* genome in regard to nodulation specificity and effective N₂ fixation. Several genes located on different chromosomes were involved with symbiosis.

Nutman [78,82] described five simply inherited genes involved with N₂ fixation in red clover *T. pratense* L. These genes are recessive and appear to be non-allelic. One gene governs strain specificity; another gene depresses or prevents effective symbiosis with many *R. trifolii* strains; and three strain-dependent genes regulate varying degrees of symbiotic effectiveness.

Holl and LaRue [65] estimated that a minimum of 10 genes are responsible for symbiotic function in most legumes. Three genes are thought to be required for strain recognition, root hair invasion, and formation of infection threads. Another three loci may be

involved with cell differentiation, nodule structure, and formation of enzymes. Four genes are presumably necessary for the formation of leghemoglobin, metabolism, and energy supply.

Host-determined variability for factors associated with N_2 fixation is broader in cross-pollinating species than in self-pollinated species [81], probably due to heterozygosity of factors governing symbiotic N_2 fixation. Nutman [80] found that variability among subterranean clover genotypes (a self-pollinating species) was too low for effective selection.

Sharma et al. [94] compared Australian and Indian alfalfa cultivars for efficiency of N_2 fixation. Nitrogen fixation of the Indian cultivar exceeded that of the Australian variety by 14%. Hoffman and Melton [64] reported no significant differences for variability among or within diverse alfalfa cultivars for TNA. Considerable variability was present within all cultivars, therefore selection for increased N_2 fixation would be effective, utilizing any population as a germplasm source.

Mytton and Jones [77] increased N_2 fixation in white clover (*T. repens* L.) by two cycles of phenotypic recurrent selection for increased nodule tissue. Nutman et al. [84] slightly improved N_2 fixation in red clover by two cycles of phenotypic recurrent selection for increased dry matter yield under zero-N conditions. Nutman [83] later found that selection for increased N_2 fixation in red clover was effective and did not reduce variability among plants in the selected population.

Seetin and Barnes [92] conducted diallel crosses with three high and three low N_2 (C_2H_2)-fixing alfalfa clones. Progenies from the high $\times$ high crosses had acetylene reduction rates more than 200% higher than those of the low $\times$ low crosses. Progeny from the low $\times$ high crosses were intermediate between the progeny groups. The source cultivars

were similar to the low X low progeny, indicating that those existing varieties may be inefficient N_2 fixers.

Duhigg et al. [37] increased N fixation in 'Mesilla' alfalfa by 82% by one cycle of selection for high TNA. This was accompanied by a 60% increase in dry matter yield. A broad sense heritability estimate of 0.78 was obtained for rates of acetylene reduction. Hoffman and Melton [64] reported 60 and 34% increases in TNA of second cycle progeny over the original source population and the first cycle progeny, respectively. Nodule number and root mass were enhanced by selection for acetylene reduction activity, however topgrowth yield was only slightly increased in the second cycle. Topgrowth yield was closely associated with total N in topgrowth and C_2H_2 reduction activity. It was suggested that a program designed to handle large alfalfa populations would include initial screening for topgrowth yield, followed by subsequent selection based on TNA.

Viands et al. [100] recently summarized extensive investigations involving several alfalfa populations selected for individual or combined traits associated with N_2 fixation. Following two cycles of bidirectional phenotypic recurrent selection, significant responses to selection were obtained for shoot dry weight, nodule mass, root fibrosity, and TNA in two experimental populations. The linearity of response to selection for all traits indicated that substantial improvement could be made by further selection. Populations from first cycle selections for these traits obtained 43% of their total N in the year of seeding through symbiotic N_2 fixation as compared to 36% for the unselected check cultivar as determined by ^{15}N isotope dilution. Although this does not appear to be a drastic improvement in N_2 fixation, this study demonstrates that selection under controlled environmental conditions was effective in improving N_2 -fixing potential under field conditions.

Improvement of N₂ Fixation in Sainfoin

Sainfoin appears to differ from the other forage legumes which are being exploited for their high N₂-fixing capacities. Low N₂ fixation may be responsible for poor stand longevity in many soils [25,95] and may restrict the potential of this species in forage systems.

The approach to improving N₂ fixation of sainfoin at Montana State University will involve: (1) selection of highly effective *Rhizobium* strains or strain composites [62], (2) selection of highly efficient N₂-fixing sainfoin genotypes when inoculated with selected *Rhizobium* inoculants, and (3) presentation of an efficient *Rhizobium* inoculant X sainfoin population "package" to field conditions. Sainfoin rhizobia exhibit broad variability for their effects on N₂ fixation. Hill [62] evaluated 16 single *Rhizobium* strains, seven of which increased C₂H₂ reduction rates by an average of 76 times the rates of uninoculated controls. The most effective inoculants were composites, which were significantly superior to the individual strains comprising them.

No work has been done to increase N₂ fixation of sainfoin by host selection. Cecil [25] found considerable variability in C₂H₂ reduction rates among 26 half-sib families selected for root development. Major et al. [74] found similar levels of phenotypic variability among individual seedlings of sainfoin and alfalfa for TNA. Distribution for TNA in Remont sainfoin is skewed towards inefficient N₂ fixation [62]. Since the breeding behavior of sainfoin is similar to that of alfalfa [35], it is anticipated that adequate variability is present for improvement of N₂ fixation in sainfoin by host selection.

Chapter III

LINEAR PHASE OF NITROGENASE-DEPENDENT ACETYLENE REDUCTION IN SAINFOIN

An important factor in measuring enzymatic reaction rates is the linearity of product formation with time during the assay. This experiment was conducted to determine the linear phase of acetylene reduction by sainfoin. The primary objective was to obtain an incubation period which provided reliable estimates of N_2 fixation and was as short as possible to facilitate testing large numbers of seedlings in a breeding program.

Materials and Methods

Remont sainfoin seed were dehulled and sieve sized 1.98 to 3.12 mm. Remont was developed by the Montana Agricultural Experiment Station, and it performs well in multi-cut hay systems or pasture [36]. The seed lot utilized was commercially obtained, certified seed which had been extensively utilized for *Rhizobium* strain evaluations [62]. The seed were surface sterilized in 1% NaOCl for three minutes and rinsed with sterile, distilled water. Seed were planted 20 mm deep into 25 × 160 mm conetainers (Ray Leach Co.,¹ Camby, OR) containing plaster grade vermiculite. The vermiculite was inoculated at planting with a 1 mL suspension (6.3×10^8 cells mL⁻¹) of a Nitragin Co.¹ composite *Rhizobium* inoculum (equal quantities of strains 116A8, 116A12, 116A14, 116A15, and 116A17) previously shown by Hill [62] to be highly effective with Remont sainfoin. Forty plants were grown in a growth chamber with a 16 h photoperiod (100 Wm⁻²). The alternating

¹ Mention of a trademark, proprietary product, or vendor is included for the benefit of the reader, and does not imply endorsement by Montana State University or the Montana Agricultural Experiment Station to the exclusion of other suitable products.

day/night temperature regime was 28/20°C. Fertility levels were maintained with a modified Hoagland (zero-N) nutrient solution applied twice weekly. Remaining irrigations were with tap water.

When the seedlings were 56 d old, individual plants were subjected to the acetylene reduction assay as described by Hill [62]. Each container was placed intact into a 1000 mL Erlenmeyer flask. The flask was sealed with a stopper fitted with an air-tight tygon sleeve. One hundred cm³ of air were removed with a syringe and replaced with purified acetylene (C₂H₂). The plants were allowed to incubate in the C₂H₂, and sampled after 1, 2, 3, 4, 6, 8, 12, and 26 h. Gas samples were withdrawn into 1-cm³ disposable syringes. The needles on the sampling syringes were inserted into rubber stoppers to prevent leakage prior to gas analysis.

Gas samples (1 cm³) were injected into a Beckman¹ Model GC 2-A gas chromatograph equipped with a hydrogen flame ionization detector. Ethylene (C₂H₄) and C₂H₂ were separated in the 2 m X 8 mm OD stainless steel column packed with 80/100 mesh F-1 activated alumina (Supelco¹) and maintained isothermally at 130°C. Gas pressures entering the chromatograph were 24, 40, and 250 cm³ min⁻¹ for H₂, He, and compressed air, respectively. Retention times for C₂H₄ and C₂H₂ were 1.0 and 3.5 min, respectively, for this system. Ethylene peak heights were standardized with a dilution curve for C₂H₄ calibrating gas (1000 ppm C₂H₄ in He, Applied Sciences¹). Total nitrogenase activity (TNA) was expressed as μmol C₂H₄ plant⁻¹ h⁻¹, and also as μmol C₂H₄ plant⁻¹ for this study.

After the last gas samples were obtained, the plants were removed from the containers, and root and shoot dry weights (48 h X 50°C) were obtained. Initial acetylene reduction estimates of five plants were obviously erroneous due to leakage of incubation

flasks or syringes, and these data were discarded. Linear regression analysis was applied to the TNA at each sampling interval across plants to estimate linearity of C_2H_4 evolution with time. Simple correlation coefficients were computed across the 35 plants for all sampling intervals and root and shoot dry weights to determine the relationships among samples at different times and plant dry matter accumulation.

Results and Discussion

Acetylene reduction of Remont sainfoin under the conditions described proceeded linearly up to 12 h, then the rate declined presumably due to diurnal effects or inhibition by C_2H_2 (Fig. III-1). The means for the low 10%, high 10%, and all seedlings were 0.21, 1.24, and $0.73 \mu\text{mol } C_2H_4 \text{ plant}^{-1}$, respectively, at 1 h. The corresponding values at 12 h were 1.72, 5.59, and $3.49 \mu\text{mol } C_2H_4 \text{ plant}^{-1}$. Any incubation period during the linear phase would enable the selection of high or low $N_2(C_2H_2)$ -fixing sainfoin genotypes. The variability present among seedlings for TNA from 1 to 4 h indicated that these short intervals would be adequate for a selection program.

Across plants, all incubation periods were significantly correlated among each other except for several involving the 4 h sample (Table III-1). This could have been due to experimental errors because the samples could not be immediately analyzed. Mean TNA at 4 h was consistent with the linear increase with time, however, variability among the seedlings declined at 4 h. Acetylene reduction rates at 3 h were highly and significantly correlated with most incubation periods and dry matter accumulation. Seedlings comprising the high and low 10% of the population selected at 3 h would have been selected at most other sampling periods (Fig. III-2), thus 3 h was utilized in all subsequent determinations of TNA.

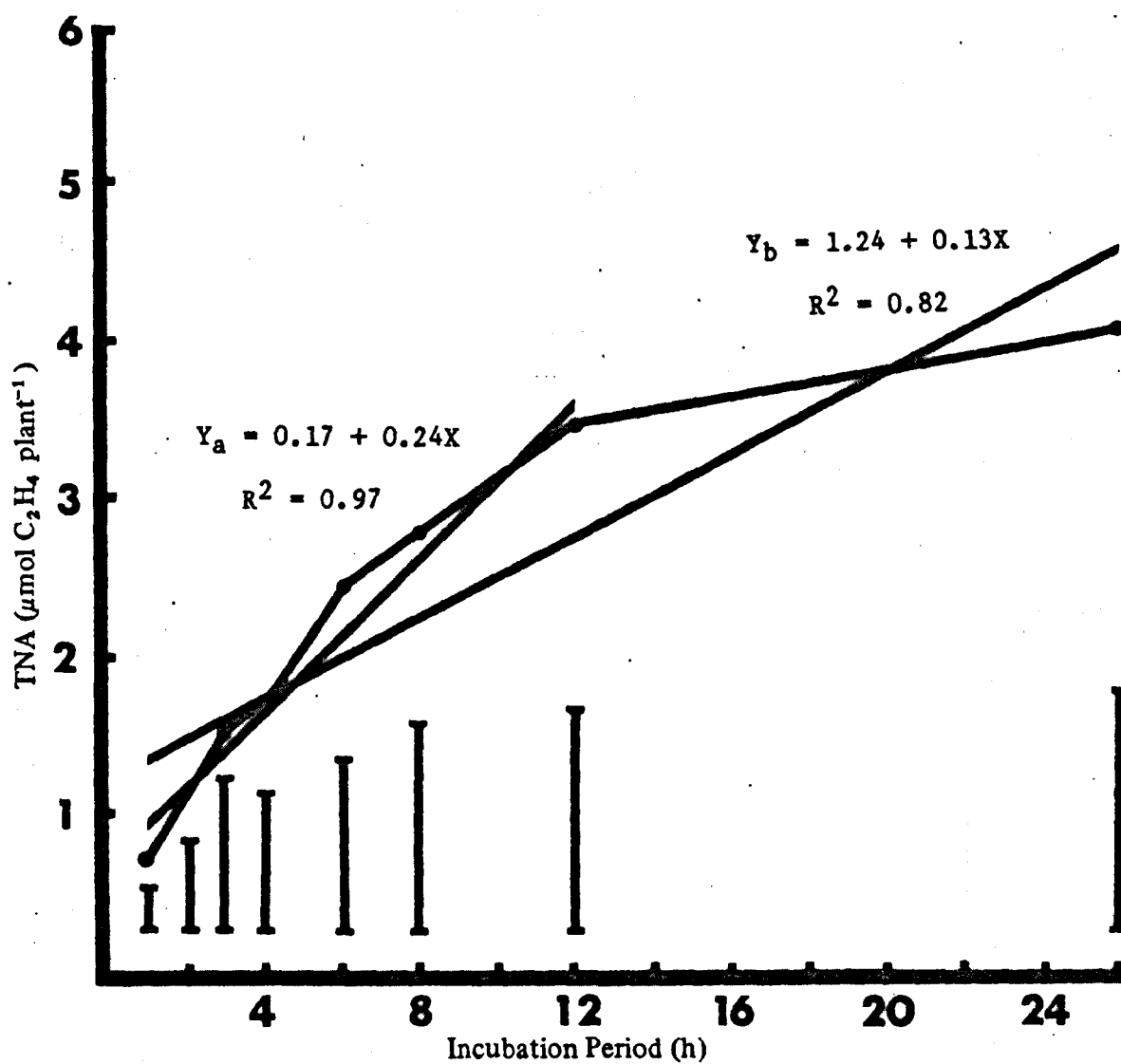


Fig. III-1. Time Course of Total Nitrogenase Activity (TNA) by 35 Sainfoin Seedlings and Regression Lines Excluding (a) or Including (b) the 26 h Sample. Vertical bars denote 1 standard deviation.

Table III-1. Correlations Among Total Nitrogenase Activity (TNA) at Eight Incubation Periods and Dry Matter Accumulation of 35 Remont Sainfoin Seedlings.

TNA ($\mu\text{mol C}_2\text{H}_4 \text{ plant}^{-1}$) Incubation period (h)	TNA ($\mu\text{mol C}_2\text{H}_4 \text{ plant}^{-1}$) Incubation period (h)							Dry Weight (mg)	
	2	3	4	6	8	12	26	Shoot	Root
1	0.81**	0.64**	0.27	0.52**	0.62**	0.63**	0.50**	0.61**	0.35*
2		0.73**	0.34*	0.66**	0.75**	0.74**	0.64**	0.73**	0.50**
3			0.34*	0.87**	0.95**	0.91**	0.73**	0.86**	0.35*
4				0.24	0.35*	0.45**	0.38*	0.57**	0.26
6					0.87**	0.78**	0.62**	0.78**	0.44**
8						0.88**	0.82**	0.84**	0.49**
12							0.88**	0.82**	0.46**
26								0.67**	0.45**
Shoot Dry Weight (mg)									0.53**

* and ** denote significance at the 5% and 1% levels, respectively.

Chapter IV

EFFECTS OF SEED SIZE ON GROWTH AND N₂ FIXATION OF REMONT SAINFOIN

Seed size is closely correlated to seedling vigor in sainfoin [17,42,43] and other forage legumes [1,7,8,9,10,17,45,60]. It was hypothesized that initial seed size (ISS) might affect growth and N₂ fixation estimates of seedlings grown under zero-N conditions. The objectives of this experiment were to: (1) establish the sequence of nodulation, growth, TNA, and net N accumulation in Remont sainfoin seedlings; (2) determine the effects of ISS on these parameters, and (3) examine how ISS effects might alter a breeding program to improve N₂ fixation of sainfoin.

Materials and Methods

Approximately 2.3 kg of dehulled Remont seed were passed through a series of sieves (2.58, 2.38, 2.18, and 1.98 × 12.7 mm). This resulted in five distinct seed size classes herein referred to as classes 1 to 5, from smallest to largest. Seed size distribution within Remont was determined as the number of seed per class, and seed weights were obtained from six replicate 25-seed samples per class. The seed were planted into 40 × 200 mm conetainers and inoculated at planting with 1 mL of a suspension (4.9×10^8 cells mL⁻¹) of the composite *Rhizobium* inoculum. All growth and assay conditions were similar to those previously described in Chapter III.

This experiment was conducted as a randomized complete block design with two blocks with five seedlings per treatment combination. The 30 treatments consisted of all combinations of the five seed size classes and six harvest dates (14, 28, 42, 56, 70 and 84 d)

providing a total of 300 seedlings. Percentage emergence was evaluated daily up to 10 d. At 14 d intervals, following planting, five random seedlings from each treatment per block were gently removed from the vermiculite and placed into a 250 mL Erlenmeyer flask for incubation in 10% C_2H_2 . Gas samples were transferred and stored in 7 mL Vacutainers (Becton-Dickinson¹) and analyzed in a Carle Instrument¹, Model 9702 gas chromatograph for this and all remaining experiments. A 3.2 mm OD $\times$ 2 m stainless steel column packed with 80/100 mesh F-1 activated alumina and maintained at 145°C provided good resolution of C_2H_2 and C_2H_4 . Flow rates for H_2 , He, and compressed air were 25, 40, and 300 cm³ min, respectively. Retention times for this system were 0.8 and 5.0 min for C_2H_4 and C_2H_2 , respectively.

After the TNA assay at each sampling data nodule counts, plant heights, and dry weights (48 h $\times$ 50°C) were obtained. By 42 d, TNA levels were conducive to evaluating all five single plants in each treatment. Thereafter individual seedlings were evaluated for TNA and dry matter accumulation. After the final harvest, roots and shoots were ground separately and analyzed by a micro-Kjeldahl procedure for N concentration. In addition, percentage N was determined from samples of the original seed size classes. Total N content was estimated by the product of percentage N and dry weight of shoots or roots.

The data were converted to an individual plant basis, and analyses of variance were computed for the means of all indices at each harvest date (excluding nodule number and TNA at 14 d), and combined across harvest dates. Orthogonal contrast coefficients for equally spaced treatments (seed size classes) were utilized to determine linear effects of ISS on each index. Linear regressions and correlations were utilized to determine relationships among the growth and N_2 fixation indices.

Results and Discussion

Considerable variability exists for seed size in Remont sainfoin (Table IV-1). Individual seeds weighing 3.7 and 35.2 mg were observed from ISS classes 1 and 5, respectively, closely corresponding to the range in seed weight for several *Onobrychis* spp. reported by Fransen [42]. Seed N content was closely related to seed size and weight.

Table IV-1. Seed Size and N Content of Five Remont Seed Classes.

Seed Size Class	Sieve Size (mm)	Median Size (mm)	% of Seed Lot	Dry Weight (mg seed ⁻¹)	N Content (mg seed ⁻¹)
1	< 1.98	1.9	27.6	9.7a	0.633a
2	1.98-2.18	2.1	18.8	12.6b	0.911b
3	2.18-2.38	2.3	34.0	15.3c	0.850b
4	2.38-2.58	2.5	15.6	18.7d	1.164c
5	> 2.58	2.7	4.0	21.6e	1.303c

Means followed by the same letter are not significantly different at the 5% level by Duncan's Multiple Range Test.

Seedlings from large seed emerged faster, had more nodules, and had higher N₂ fixation rates at most harvests (Fig. IV-1). Appreciable N increases had occurred by 28 d, and by 84 d seedlings from all ISS classes had accumulated over 700% the initial available N present in the seed (Fig. IV-2). Total plant N was highly indicative of N content in roots or shoots. Seed size effects were not significant at most harvest dates for TNA and several other traits, however, the combined analyses of variance across harvests (Table IV-2) detected significant differences among ISS classes for all indices except percentage N. Seedling age effects were significant for all indices, and the interaction of seedling age X seed size was only significant for nodule number per plant. These data indicate that differences

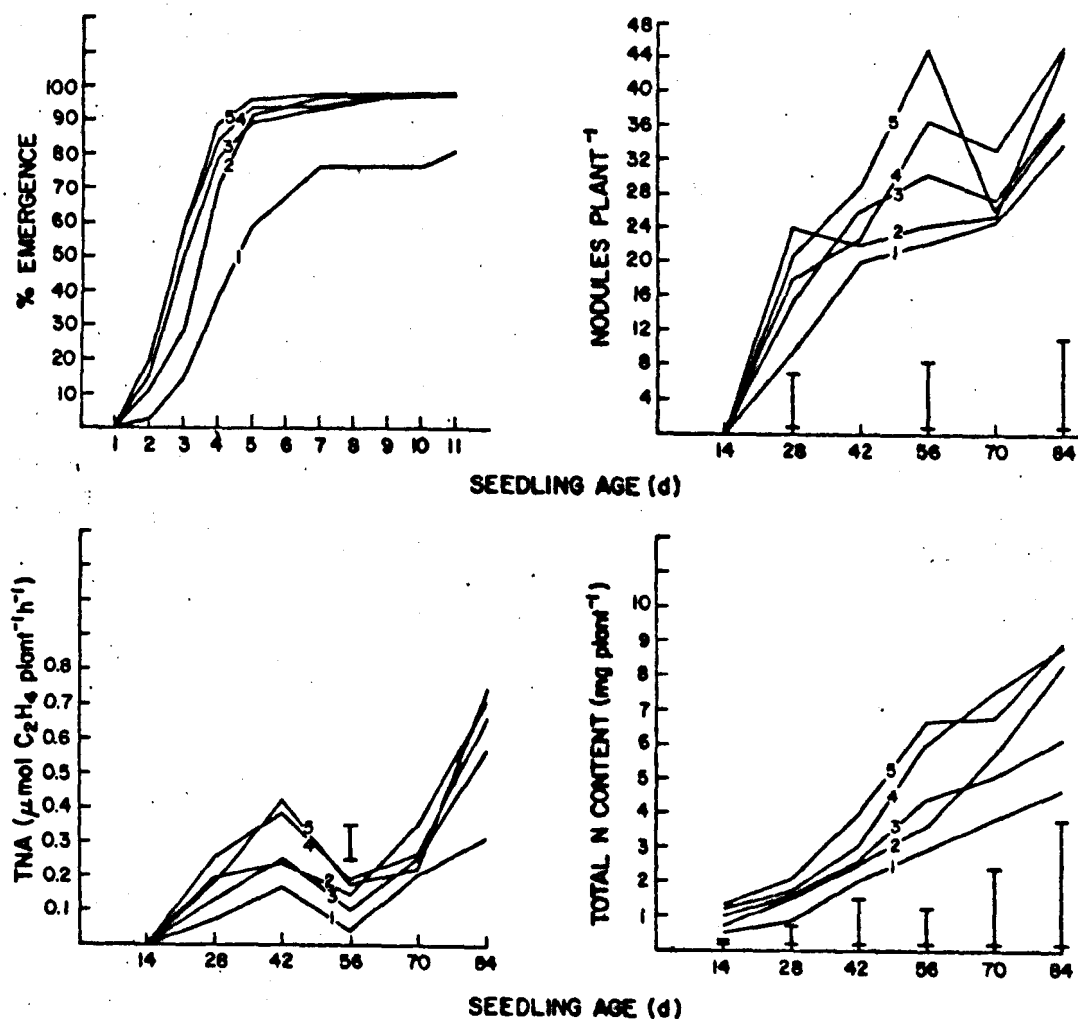


Fig. IV-1. Indices of Growth and N_2 Fixation of Remont Sainfoin Seedlings Arising From Five Seed Sizes and Evaluated at 14 d Intervals. Vertical bars denote protected least significant differences at the 5% level.

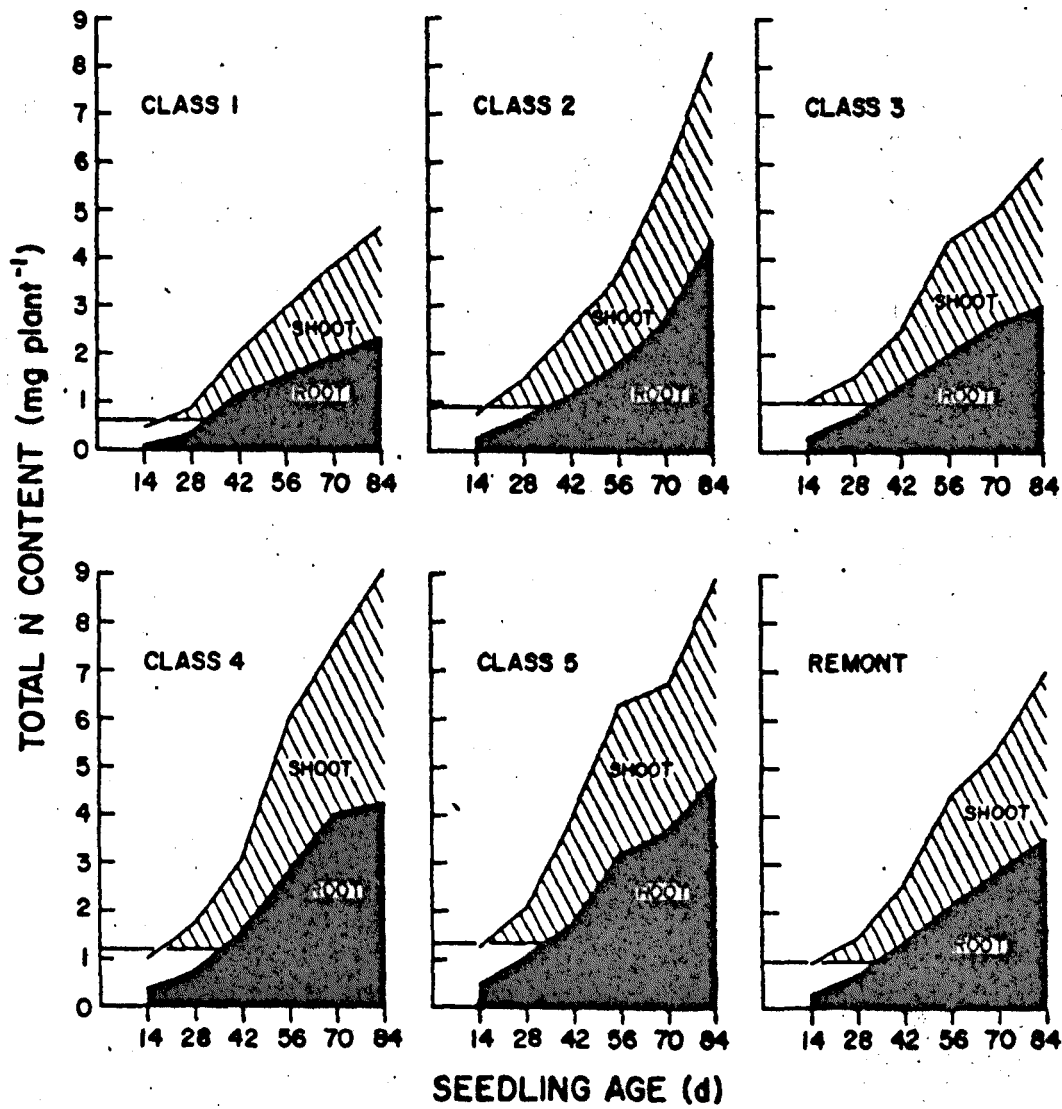


Fig. IV-2. Total N Content of Remont Sainfoin Seedlings Arising From Five Seed Size Classes and Evaluated at Six Harvest Dates. Horizontal Lines Denote Initial Seed N Content.

Table IV-2. Mean Squares for Six Traits Associated With Growth and N₂ Fixation by Remont Sainfoin Seedlings Arising From Five Seed Size Classes Evaluated at 14 d Intervals.

Source of Variation	d.f.	Nodules plant ⁻¹	TNA (μmol C ₂ H ₄ plant ⁻¹ h ⁻¹)	d.f.	Plant Height (mm)	Whole Plant		
						Dry Weight (mg plant ⁻¹)	N Concentration (%)	N Content (mg plant ⁻¹)
Block	1	46.08	0.59 [†]	1	82.13	640.27	59.60*	3.37*
Seed Size (S)	4	150.12**	6.67**	4	760.74**	8,711.17**	13.87	12.09**
Linear	1	485.76**	15.88**	1	2968.09**	31,415.09**	38.87	41.97**
Residual	3	38.23	3.58	3	6.24	1,143.20	5.53	2.13
Seedling Age (A)	4	713.05**	34.36**	5	125.19**	62,761.66**	424.28**	63.02**
S × A	16	50.38*	0.94	20	25.15	695.52	14.02	1.22
Error	24	20.68	1.21	29	22.72	403.02	9.98	0.64
CV%		16.1	38.2		8.2	16.9	9.4	20.8
R ² Index: Seed Size (mm)		0.81 ^{††}	0.60		0.98	0.90	0.70	0.87

[†] Mean squares for TNA and percentage N × 100.

^{††} Coefficients of determination calculated from orthogonal linear contrast coefficients for each index on initial seed size.

* and ** denote significance at the 5 and 1% levels, respectively.

due to ISS were manifested early, and maintained relatively consistent trends throughout the experiment.

Linear effects of ISS estimated from polynomial contrast coefficients for equally spaced treatments (Table IV-2) were significant for all traits except percentage N. Coefficients of determination (R^2) for seedling performance relative to ISS ranged from 0.60 for TNA to 0.98 for plant height from the combined analyses. Linear effects of ISS on growth are well illustrated by the regression of plant dry matter accumulation (Fig. IV-3) and total N content (Fig. IV-4) against ISS. Growth differences attributable to ISS (b values) generally increased with age, and became increasingly difficult to detect (reduced R^2 values) due to more variability at later harvest dates. These data indicate that growth and subsequently N_2 fixation under zero-N conditions are strongly and positively influenced by ISS.

Initially the reductions in TNA at 56 d and nodule number at 70 d (Fig. IV-1) were thought to be due to sampling or procedural errors. No changes in temperature, photoperiod, or light intensity of the growth chamber were observed which suitably accounted for these data. This phenomenon occurred in all seed size classes but did not appear to correspond to similar fluctuations in total dry weight or N content. However when these indices were considered on a growth rate basis (net change: mg d^{-1}), similar fluctuations were obtained (Fig. IV-5).

The seedlings underwent a slight reduction in the rate of nodule formation from 42-56 d, accompanied by reduced TNA at 56 d, and followed by diminished rates of dry matter accumulation and possibly nodule sloughing by 70 d. The pattern for rate of dry matter accumulation is similar to that obtained by Black [10] who conducted growth analyses of subterranean clover seedlings under zero-N conditions. Smith [97] found that nodule num-

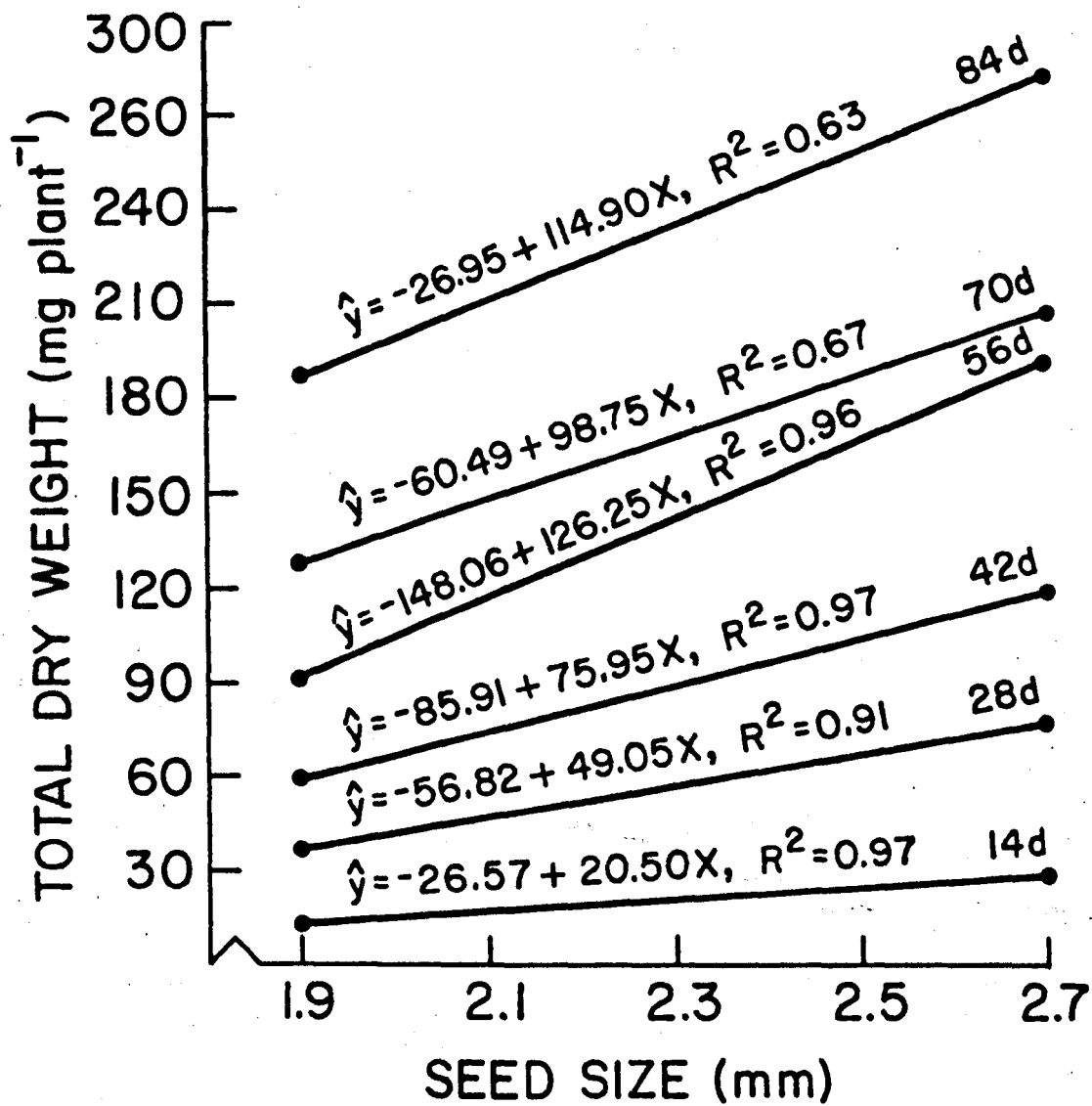


Fig. IV-3. Regression of Plant Dry Matter Accumulation at Six Harvest Dates Against Initial Seed Size.

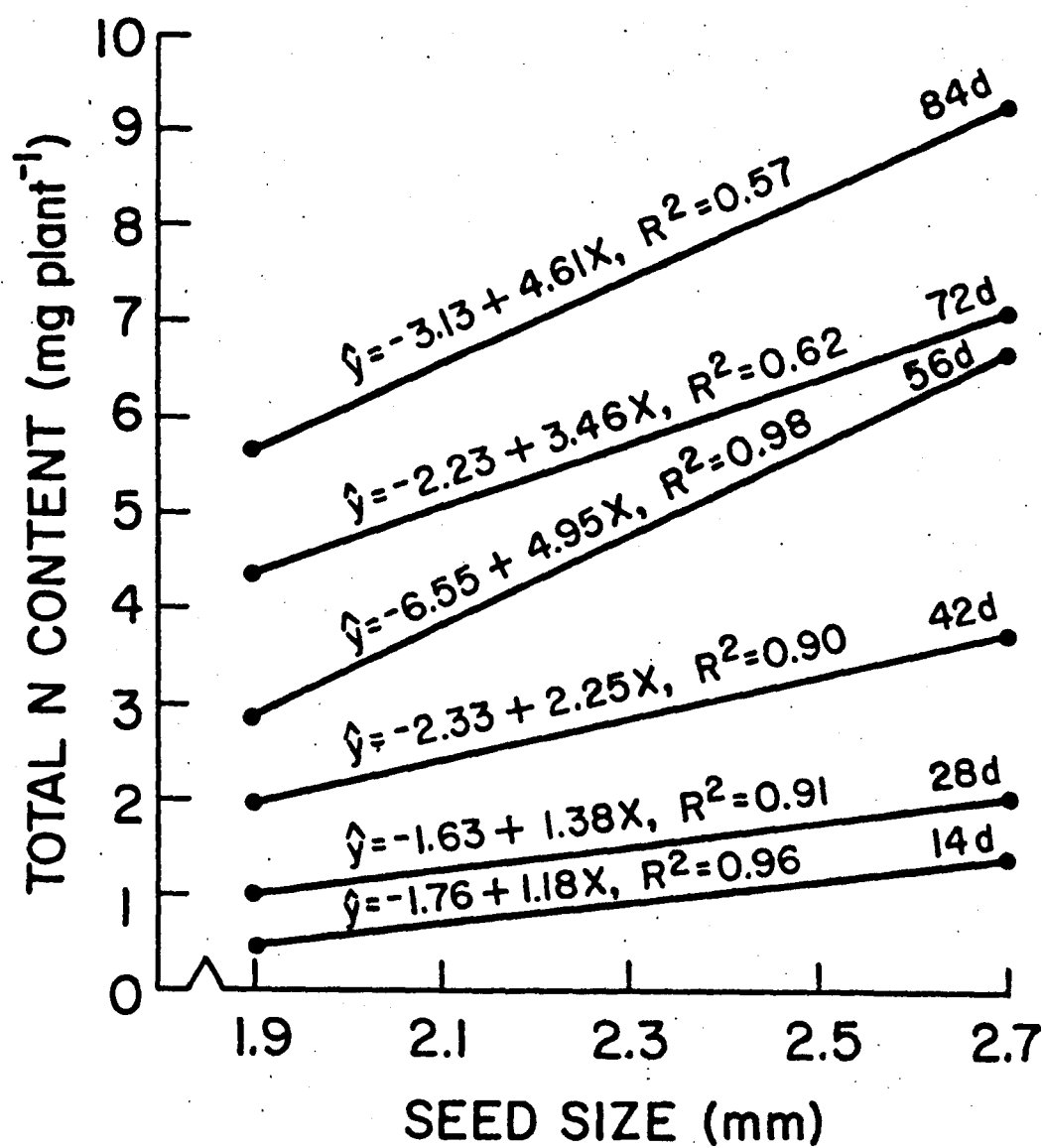


Fig. IV-4. Regression of Total N Accumulation at Six Harvest Dates Against Initial Seed Size.

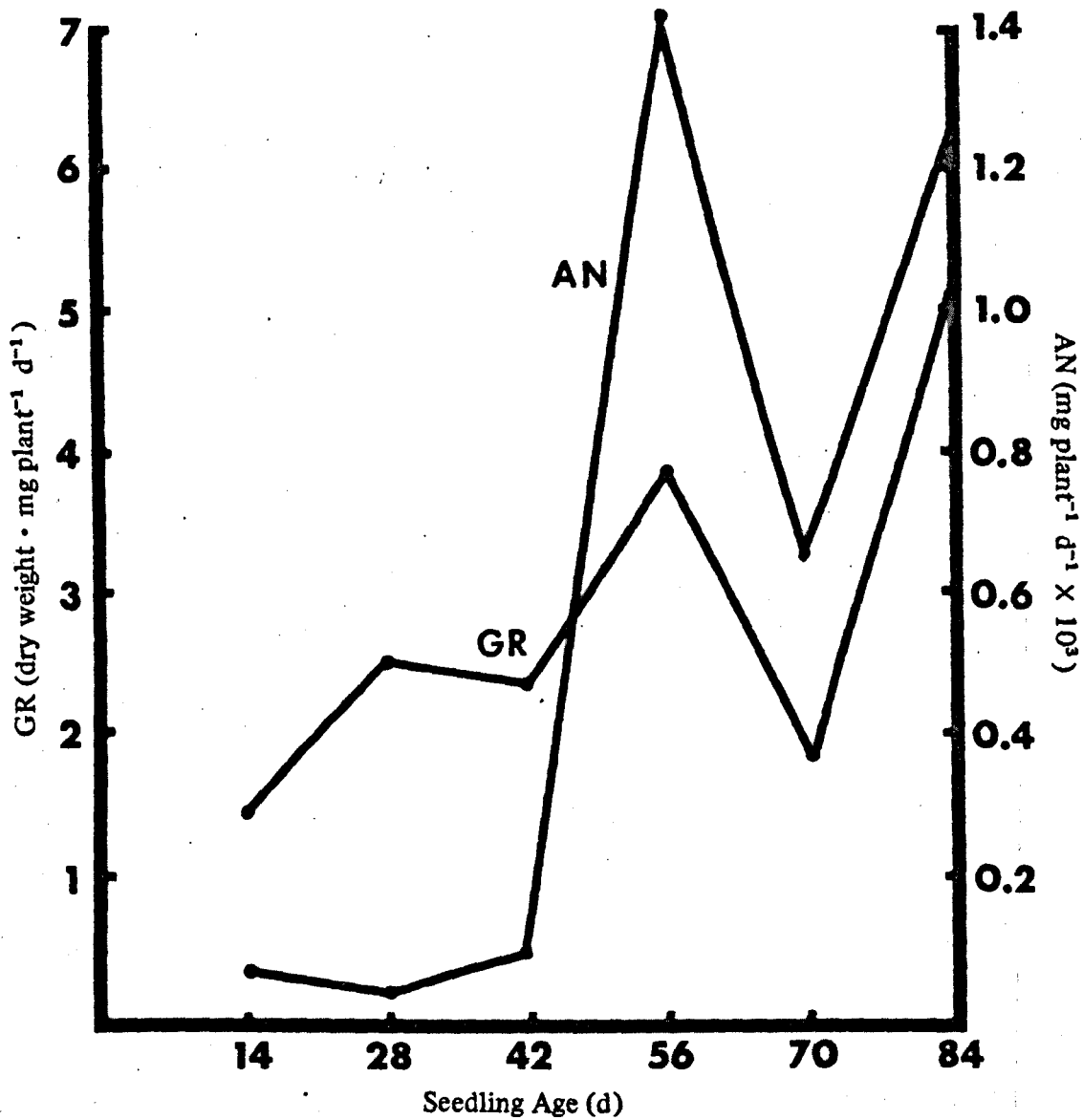


Fig. IV-5. Rates of Growth (GR) and N Accumulation (AN) of Remont Sainfoin Seedlings Evaluated at 14 d Intervals. Points represent means of 60 plants across seed sizes.

ber of alfalfa germplasms of varied dormancy levels declined at 49 d, followed by reductions in TNA at 56 and 63 d. Similarly, Cralle and Heichel [32] presented data for alfalfa and birdsfoot trefoil indicating fluctuations of TNA across time, however none of these workers have proposed explanations for this phenomenon. These oscillations may be indicative of competitive interactions within the legume seedling for substrates and energy to form new growth or fix N_2 [61]. Nodule sloughing and declines in TNA have been reported following removal of forage of white clover [96] and alfalfa [32]. Cralle and Heichel [32] found that recovery of TNA in alfalfa and birdsfoot trefoil grown in sand culture after partial shoot removal was correlated to the level of defoliation. They found that TNA following defoliation was more dependent upon leaf area than reserve carbohydrates. It appears that the interactions between growth and N_2 fixation of legume seedlings under zero-N conditions are established early and either of these factors could become limiting to the other during development. Further physiological examinations should be conducted to elucidate the interdependence of growth and N_2 fixation of the legume seedling under zero-N conditions. Regardless of the causes of the fluctuations in TNA and growth rate, seedlings from all seed classes responded similarly, as reflected by nonsignificant interactions of seedling age $\times$ seed size for most indices in the combined analyses of variance.

Relative growth rates (RGR) were computed for dry matter accumulation ($mg\ mg^{-1}\ d^{-1}$) to determine if seedlings from large seed were superior due to faster growth. No significant differences for RGR were detected among seed size classes (Table IV-3). In accord with the terminology of Blackman [11] these data indicated that differences in growth were attributable to the level of "initial capital" (seed size) rather than "rate of interest" (RGR). Black [10] reported that RGR of three subterranean clover cultivars

known to yield differently in field trials were independent of seed size or cultivar. He concluded that field performance was very closely correlated to the relative proportion of large seed within a cultivar. It appears that a similar situation could exist among sainfoin cultivars with varying distributions for seed size.

Table IV-3. Relative Growth Rates (plant dry matter accumulation) for Sainfoin Seedlings Arising From Five Seed Size Classes and Evaluated at 14 d Intervals.

Seed Size Class	Growth Period (d)					Mean
	14-28	28-42	42-46	56-70	70-84	
	-----RGR (total dry weight mg mg ⁻¹ d ⁻¹ × 10 ³)-----					
1	103*	47	56	20	39	53 a
2	174	37	35	23	42	62 a
3	127	35	35	16	19	46 a
4	101	43	46	23	26	48 a
5	123	43	48	0	39	51 a
Mean	126a	41 b	44 b	16 c	33 bc	52

*Tables values represent mean RGR across two replications of five random seedlings at each harvest.

Means followed by the same letter are not significantly different at the 5% level by Duncan's Multiple Range Test.

Of the indices examined which were significantly correlated to ISS, the least related and most variable trait was TNA. Initial seed size accounted for 40, 92, 65, 2, and 56% of the variability in mean TNA at 28, 42, 56, 70, and 84 d, respectively. Plant dry weight and TNA were significantly correlated for individual seedlings at 56, 70, and 84 d (Table IV-4). These values are lower than those previously reported for sainfoin [62] and alfalfa [37]. Intraclass correlations between TNA and dry weight were generally higher for larger ISS classes. These data demonstrate that considerable variability is present for TNA and dry weight even within classes of uniformly sized seed. It appears that efficient N₂-fixing

genotypes could be selected from within a seed class with only minimal confounding due to ISS. Cooper and Qualls [31] demonstrated that differences among birdsfoot trefoil seedlings of the same seed size were probably due to more efficient metabolism and partitioning of photosynthate. These factors would be related to efficient N_2 fixation of sainfoin seedlings.

Table IV-4. Regression Coefficients and Correlations (r) of Plant Dry Matter Accumulation and Total Nitrogenase Activity of Individual Sainfoin Seedlings Evaluated at 56, 70, and 84 d.

Seed Size Class	d.f.	Seedling Age					
		56 d		70 d		84 d	
		b_{yx}	r	b_{yx}	r	b_{yx}	r
1	8	0.107	0.13	0.012	0.46	0.015	0.47
2	8	1.223	0.48	0.30	0.75*	0.027	0.53
3	8	1.126	0.65*	0.013	0.37	0.060	0.88**
4	8	1.931	0.64*	0.025	0.77**	0.022	0.84**
5	8	1.56	0.65*	0.016	0.71*	0.014	0.47
Across all classes	48	1.116	0.63**	0.011	0.44**	0.028	0.70*

* and ** denote significance of r at the 5% and 1% levels, respectively.

In the breeding program at MSU to improve N_2 fixation of sainfoin, individual seedlings from uniformly sized seed are evaluated for TNA potential at 56 d, and selections comprising the high and low 10% are obtained. If selection had occurred in the present experiment ignoring seed size, all high and low selections at 56 d would have arisen from classes 4-5 and 1-3, respectively. Similar results would have occurred at 70 and 84 d. Selection for traits more closely to ISS such as total N or dry matter accumulation would have resulted in more extreme biasing due to ISS effects.

Variability in seed size of sainfoin is due to clonal differences, environmental conditions, and number and position of pods during development. Large seed has been correlated to heterosis in alfalfa [85]. Pederson and Barnes [85] found that alfalfa seed resulting from self-pollination were about 10% smaller than hybrid seed from the same plant. Carleton and Cooper [17] found that after removal of genetic effects by covariance analysis, the relationship between ISS and seedling vigor in sainfoin was significant within but not among clones. This indicated that the environmental conditions during seed development not only affected seed size, but also had a strong influence on subsequent seedling performance.

Regardless of the causes of variability in seed size within a single seed lot, data from this study indicate that ISS has a significant influence on juvenile growth and N_2 fixation of Remont sainfoin. It is not known if these effects are only limited to zero-N growth conditions. It appears that growth and TNA assays for breeding purposes should be conducted on seedlings arising from uniformly sized seed. The seed class utilized should probably represent the modal or median class to minimize possible genetic shift. After removal of the ISS effect, phenotypic variation in TNA or dry matter accumulation should better reflect genetic variability for N_2 fixation.

Chapter V

VARIABILITY AMONG SAINFOIN POPULATIONS FOR JUVENILE GROWTH AND $N_2(C_2H_4)$ FIXATION

Considerable variability is displayed among sainfoin cultivars and populations for seasonal production patterns under different management systems. The objectives of this study were to: (1) evaluate high yielding sainfoin populations for seedling growth and N_2 fixation under laboratory and field conditions, and (2) determine if any populations would serve as superior germplasm sources for a breeding program to improve N_2 fixation.

Materials and Methods

Seed of Eski, 'SK48', a Russian composite ("USSR"), Creston Eski composite ("Creston"), and two lots of Remont were sized as previously described. These cultivars and populations have performed well at several Montana locations and represent a range of seed size distribution and origin. A second Remont seed lot (Remont 2) which was harvested near Bozeman, Montana, and had been in cold storage since 1975 was included for comparative purposes. Eski and SK48 are one-cut cultivars released by the Montana Agricultural Experiment Station [41] and Poland, respectively. Creston is a composite of open-pollinated seed from Eski plants which had undergone 13 years of natural selection at Creston, Montana. The USSR lot was obtained by mixing seed in equal proportions from approximately 200 plant selections from a Russian introduction (PI number unknown).

Seed weights were obtained from six replicate 25-seed samples. Because the seed differed in age, size, origin and genetic constitution, standard germination tests [20] were

performed to detect potential differences in germinability and seedling vigor. Four replicates of 25 seeds per treatment combination (population $\times$ ISS class) were placed in 130 $\times$ 135 $\times$ 35 mm plastic boxes on blotter paper premoistened with 25 mL of sterile distilled water containing 0.25 mg of benomyl¹ to retard saprophytic fungi. The boxes were placed in an incubator set at 23°C, and germination percentages were determined daily until 11 d. Maguire's [73] speed of germination index was computed for each treatment.

Seeds with radicles extruded > 2 mm were removed daily, and transferred to blotters in a second set of boxes in the incubator. After 7 d growth in the second series of boxes, the radicles, cotyledons, and testa were separated, dried, and weighed. Stored food reserves of sainfoin cotyledons are exhausted by 9 d at 20°C [30], therefore radicle dry matter accumulation should reflect efficiency of utilizing initial substrates. Analyses of variance were computed for speed of germination values and radicle dry weights as indicators of seed condition and seedling vigor, respectively.

Seed of all 30 treatment combinations were planted into 25 $\times$ 160 mm containers, and inoculated with 1 mL of a suspension containing 3.2×10^8 cells mL⁻¹ of the composite *Rhizobium* inoculum. All growth and assay conditions were similar to those previously described. This experiment was conducted as a randomized complete block with two growth chambers designated as blocks. Five seedlings per treatment combination were grown in each block. Factorial treatments consisting of all 150 combinations of five ISS classes, six populations, and five harvest dates (14, 28, 42, 56, and 70 d) provided a total sample size of 1500 seedlings. At 14 d intervals, each treatment was evaluated for TNA and shoot dry weight (SDW). In the combined analyses of variance across harvest dates, populations were considered as random, and ISS classes and seedling age

were considered as fixed effects. Linear effects of ISS on SDW and TNA were estimated by orthogonal contrast coefficients for equally spaced treatments (ISS). Correlations were computed to determine relationships among ISS, SDW, and TNA.

In addition to the laboratory evaluations, these five populations (excluding Remont 2) were examined for forage yield and total harvested N under field conditions near Bozeman, Montana. These entries were seeded into 1.5 × 6.1 m plots (Bozeman silt loam agric pachic cryoboroll, fine silty, mixed) at a seeding rate of 44.8 kg of unsized PLS per ha. This experiment was conducted as a randomized complete block with four blocks. Prior to the only harvest during the year of seeding (1981) the plots received about 305 mm of combined precipitation from rainfall and sprinkler irrigations applied as needed.

Forage was harvested 84 d after seeding with a small plot flail harvester, and fresh weights were determined in the field. Random forage samples were obtained from each plot, dried (48 h × 50°C), and weighed. Dried samples were ground, and percentage N was determined by a micro-Kjeldahl procedure. Total harvested N was estimated by the product of percentage N and dry matter yield per plot. The data were converted to kg ha⁻¹ for these indices, and analyses of variance were computed.

Results and Discussion

The sainfoin populations varied considerably for seed size distribution and mean weight. Seed size distributions of Eski and Creston were skewed towards large seed, SK48 was skewed towards small seed, and the other populations were more randomly distributed (Fig. V-1). Seed of USSR were significantly heavier than seed of the other populations (Table V-1). No significant difference was detected between the two Remont lots for seed weight, and both Remont lots germinated significantly faster than all other populations.

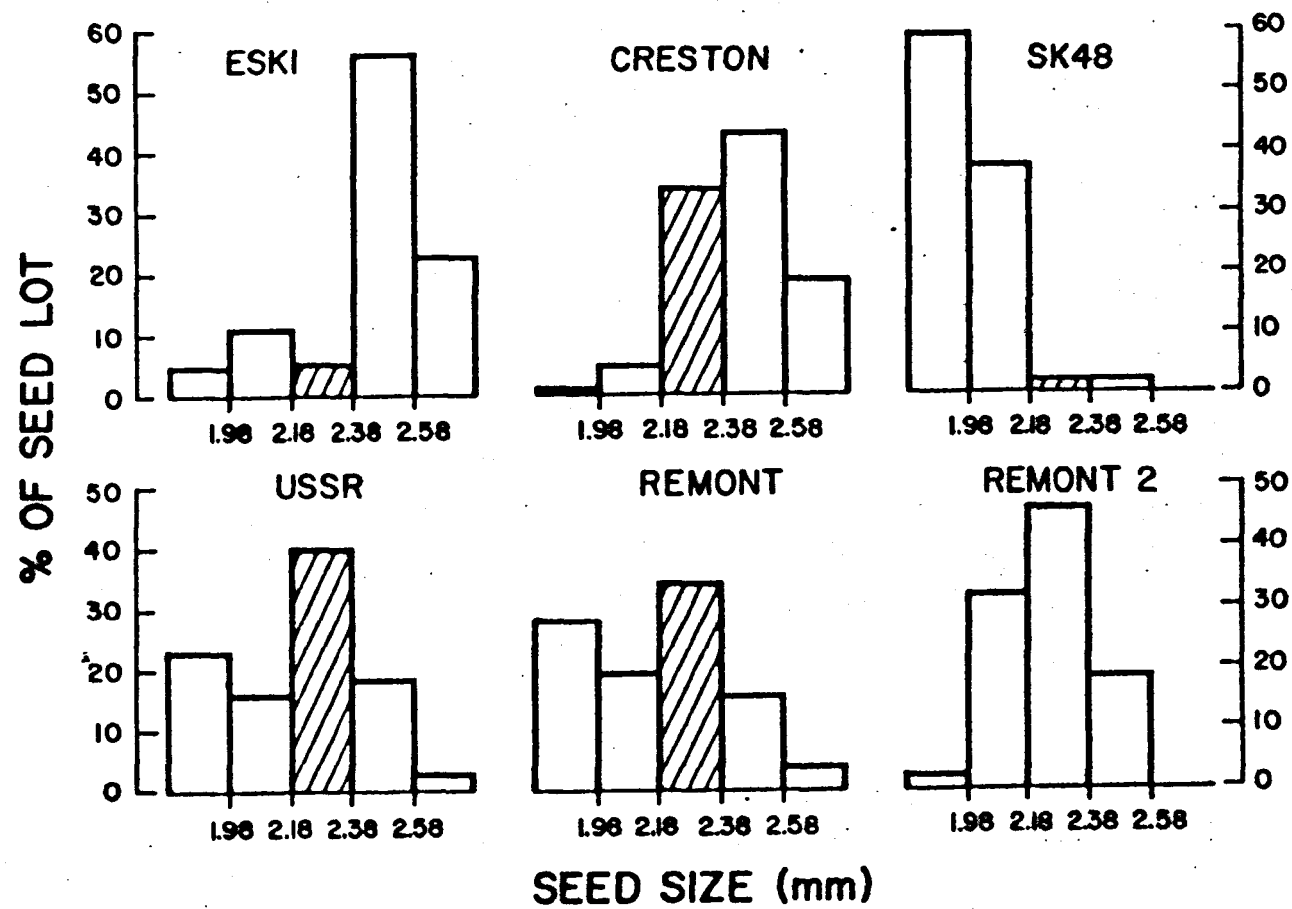


Fig. V-1. Seed Size Distributions of Six Sainfoin Populations. (Cross-hatched cells indicate seed size class used in subsequent experiments.)

Table V-1. Seed Weight, Speed of Germination, and Radicle Dry Matter Accumulation for Six Sainfoin Populations.

Popu- lation	Seed Weight (mg seed ⁻¹)						Speed of Germination						Radicle Dry Weight (mg)					
	Seed Size Class						Seed Size Class						Seed Size Class					
	1	2	3	4	5	Mean	1	2	3	4	5	Mean	1	2	3	4	5	Mean
USSR	12.7	15.1	17.0	20.1	24.4	27.8c	43.6	36.3	41.9	38.9	39.0	39.9b	2.8	3.2	3.2	3.9	6.6	3.9a
Eski	11.2	13.8	16.2	19.0	21.7	16.4b	25.4	35.8	40.4	37.5	42.8	36.4b	1.9	2.9	3.0	4.3	6.0	3.6a
SK48	10.0	13.2	15.9	19.0	21.9	16.0ab	23.7	19.9	23.3	20.0	23.7	22.1a	3.1	4.8	5.2	5.4	5.4	4.8a
Creston	10.0	12.8	15.5	18.2	21.4	15.6a	29.4	39.7	47.0	41.1	46.9	40.8b	1.9	2.7	3.2	3.4	6.1	3.4a
Remont	9.7	12.6	15.3	18.7	21.6	15.6a	31.1	44.9	51.1	48.3	53.1	45.8c	1.9	2.5	4.8	4.8	7.4	4.3a
Remont 2	9.1	11.9	15.2	18.3	21.2	15.1a	30.1	45.3	57.0	50.8	53.2	47.3c	1.8	2.3	3.5	5.7	5.7	4.9a
Mean	10.4a	13.3b	15.8c	18.9d	22.0e	16.1	30.5a	37.1b	43.7c	39.5bc	42.8bc	38.7	2.2a	3.2b	3.8bc	4.6c	6.0d	3.9

Means followed by the same letter are not significantly different at the 5% level by Duncan's Multiple Range Test.

Speed of germination may reflect the length of storage since these seed lots were obtained. Across populations, large seed germinated more rapidly than small seed. Dry weights of all seedling organs 7 d after germination were highly correlated to ISS (Fig. V-2) but were independent of population effects. Differences among populations due to genetic or environmental effects appeared to be manifested in speed of germination, however after germination all populations had similar vigor, dependent on ISS.

Seed size effects were significant for SDW but not TNA at all sampling dates, and across harvest dates (Table V-2). Linear effects of ISS on SDW in the combined analysis were highly significant ($R^2 = 0.91$, $P < 0.01$) when averaged across populations. Declines in rate of growth and TNA between 42 and 56 d were similar to those for Remont in the previous study, however less pronounced (Fig. V-3).

USSR had significantly higher SDW than Remont 2, Creston, and SK48 across all seed sizes and harvest dates (Table V-3). All populations were superior to SK48 for TNA. Adjusted values (weighted averages) of the populations were computed by weighting SDW and TNA values for each class with the frequency of ISS within each populations to predict the performance of these populations if a random seed lots had been utilized. Adjusted values were very similar to the means.

The relationships among ISS, SDW, and TNA were examined by linear regression analysis. Due to the treatment design statistically significant linear effects due to ISS were difficult to detect. Across populations and ISS, the correlations among ISS, TNA, and SDW declined steadily up to 70 d (Table V-4). Although SDW was highly correlated with ISS at 70 d, the relationships of ISS or SDW with TNA were nonsignificant by 56 d. These

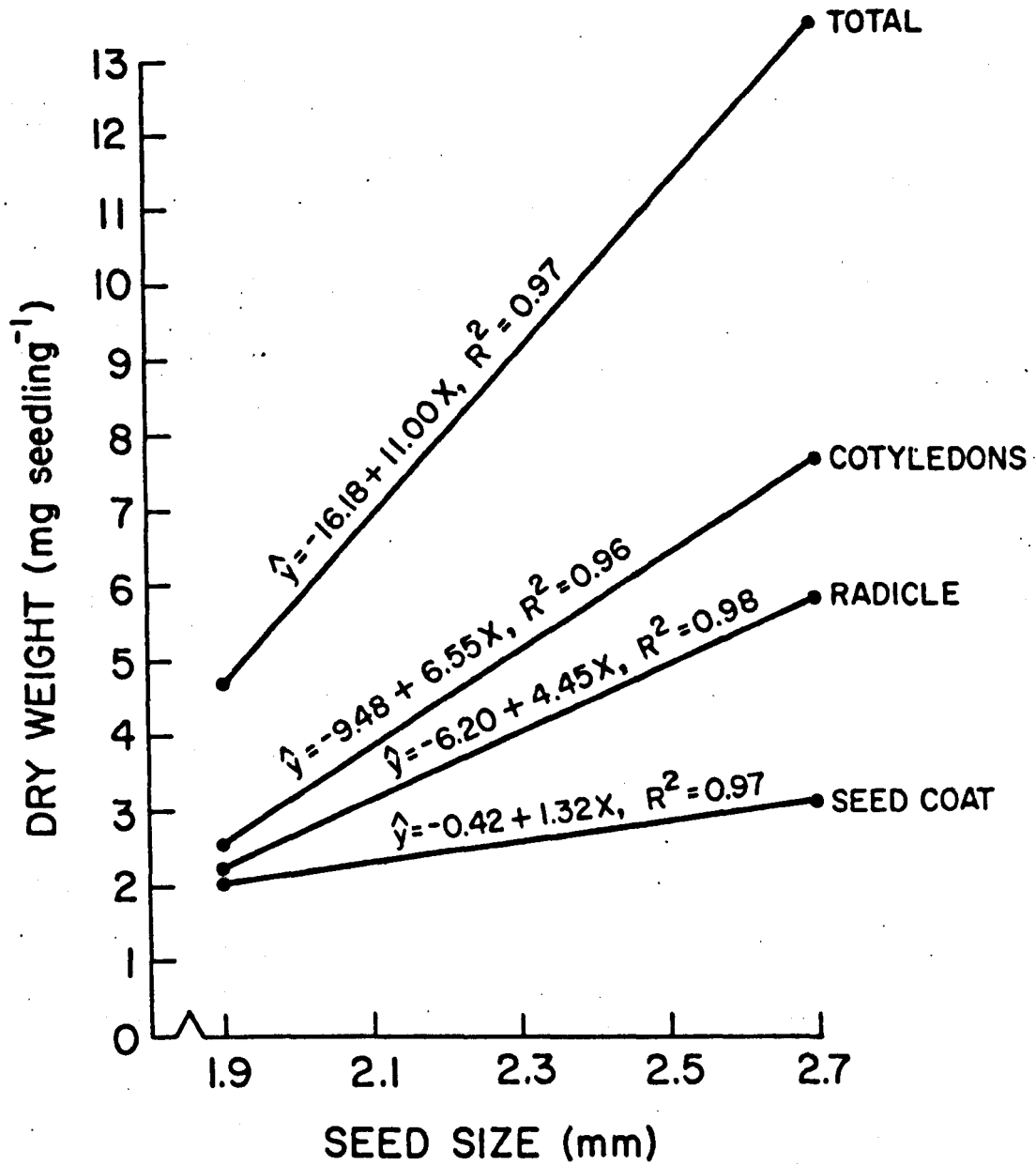


Fig. V-2. Dry Matter Accumulation in Sainfoin Seedling Organs 7 d After Germination as Affected by Initial Seed Size. Total dry weight represents cotyledons plus radicles.

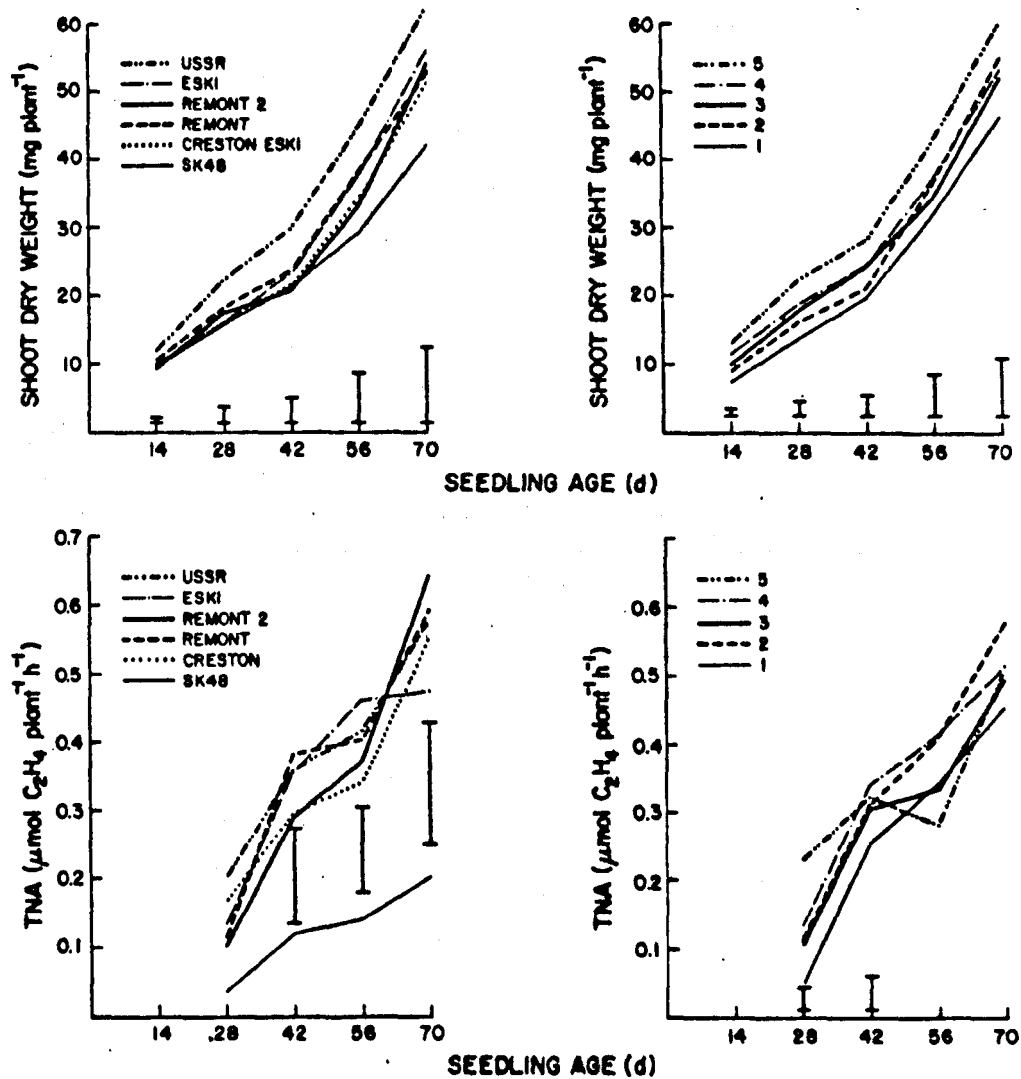


Fig. V-3. Shoot Dry Weight and Total Nitrogenase Activity (TNA) of Five Seed Size Classes of Six Sainfoin Populations Evaluated at 14 d Intervals. Vertical bars denote protected least significant differences at the 5% level.

Table V-2. Combined Analyses of Variance for Shoot Dry Weight (SDW) and Total Nitrogenase Activity (TNA) of Sainfoin Seedlings From Six Populations Evaluated at 14 d Intervals.

Source of Variation	SDW (mg plant ⁻¹)		TNA (μmol plant ⁻¹ h ⁻¹)	
	d.f.	MS	d.f.	MS
Block	1	13.09 [†]	1	0.83 ^{††}
Seed Size (S)	4	688.25*	4	0.57
Linear	1	2496.99**	1	0.76
Residual	3	85.37	3	0.51
Seedling Age (A)	4	17,379.38**	3	1.48**
Population (P)	5	598.02*	5	3.77**
S × A	16	38.74	12	0.26
S × P	20	56.16	20	0.35
A × P	20	74.66	15	0.45
S × A × P	80	24.01	60	0.23
Error	149	240.87	119	0.36

[†] SDW for seedlings at 14, 28, 42, 56, 70 d.

^{††} TNA mean squares × 10 for seedlings at 28, 42, 56, 70 d.

* and ** denote significance at the 5% and 1% levels, respectively.

data indicate that selection for efficient N₂(C₂H₂) fixation should occur no earlier than 56 d to minimize potential differences due to ISS.

SDW of all populations except USSR and SK48 were highly correlated to ISS (Table V-3). Interestingly these populations represented both extremes in seed size; USSR had the heaviest seed and SK48 was distributed towards small, light seed. The TNA of SK48 seedlings was correlated with ISS whereas the same relationship in USSR was negative. Correlations among ISS, SDW, and TNA of Remont and Creston seedlings were similar to those of SK 48 suggesting that ISS effects may be most important in sainfoin populations with a high proportion of light or small seed. This would extend the observation by Fransen and Cooper [43] that seed size is more important in small seeded species. The

Table V-3. Means and Adjusted Values for Shoot Dry Weight (SDW) and Total Nitrogenase Activity (TNA); and Correlation Coefficients (r) Among Initial Seed Size (ISS), SDW, and TNA for Six Sainfoin Populations.

Population	SDW (mg plant ⁻¹)		TNA ($\mu\text{mol C}_2\text{H}_4$ plant ⁻¹ h ⁻¹)		r		
	Mean	Adjusted Value	Mean	Adjusted Value	SDW: ISS	TNA: ISS	TNA: SDW
USSR	34.1a [†]	33.a [‡]	0.391a	0.385a	0.27 ^{††}	-0.67	0.50
Remont	28.7ab	27.6a	0.375a	0.372a	0.91*	0.71	0.71
Eski	28.5ab	30.7a	0.358a	0.379a	0.97**	0.45	0.40
Remont 2	27.0b	25.8a	0.353a	0.345a	0.91*	0.65	-0.12
Creston	26.5b	28.2a	0.339a	0.388a	0.91*	0.65	0.81*
SK48	23.7b	19.1b	0.124b	0.091b	0.71	0.93*	0.80*

[†] Means for SDW and TNA from combined analyses, $r_{sa} = 50$ and 40, respectively (see text for details).

[‡] Adjusted values = weighted averages from initial seed class frequencies.

^{††} Correlation coefficients computed for data at 28, 42, 56, and 70 d, * and ** denote significance at the 5 and 1% levels, respectively.

Means followed by the same letter are not significantly different at the 5% level by Duncan's Multiple Range Test.

Table V-4. Shoot Dry Weight (SDW), Total Nitrogenase Activity (TNA), and Correlation Coefficients (r) Among Initial Seed Size (ISS), SDW, and TNA for Sainfoin Seedlings Assayed at 14 d Intervals.

Seedling Age (d)	Mean		r		
	SDW (mg plant ⁻¹)	TNA (μmol C ₂ H ₄ plant ⁻¹ h ⁻¹)	SDW: ISS	TNA: ISS	TNA: SDW
14	10.1a [†]	—	0.99**	—	—
28	17.6b	0.13a	0.98**	0.92**	0.97**
42	23.2b	0.30b	0.97**	0.81*	0.70
56	36.1c	0.36b	0.91**	-0.35	0.43
70	53.3d	0.51c	0.82*	0.09	0.51

[†] Means from combined analyses, *rsp* = 60 for SDW and TNA.

* and ** denote significance of *r* at the 5 and 1% levels, respectively.

divergent behavior of the two Remont seed lots for these correlations is likely due to differences in environmental or physiological conditions during seed production. Although no significant differences were detected among the Remont seed lots for any index, the relationship between growth and N₂ fixation appears to vary among seed lots.

These data indicate that ISS may have differential effects on estimates of N₂ fixation among diverse populations. Ideally seed from all populations produced in the same environment should be utilized in examinations of juvenile growth and TNA. However since these were unavailable, the differential relationships among ISS and seedling performance of these sainfoin populations can not be attributed to either genetic or environmental factors, except possibly for Remont vs. Remont 2. The object of sizing seed by sieving is to minimize ISS effects and expose genotypic variability in N₂ fixation. From the previous experiment it appears that adequate variability was present for TNA within uniform ISS classes.

Under field conditions, all populations had significantly higher forage yields and total N accumulations than SK48 (Table V-5). These data are very similar to those obtained under laboratory conditions and in previous field evaluations [35] in which unsized seed had been utilized. Creston had a higher level of N accumulation than other populations, whereas its TNA had been low under zero-N conditions. Although these differences are not significant, these data suggest that Creston may be more dependent on soil N than the other populations.

Table V-5. Forage Yield and Total Harvested N of Five Sainfoin Populations in the Year of Seeding Near Bozeman, Montana.

Population	Forage Dry Matter	Total Harvested N
	----- kg ha ⁻¹ -----	
USSR	2958.6a	167.2a
Remont	2955.7a	159.6a
Eski	2902.8a	156.7a
Creston	2887.3a	207.9a
SK48	900.5b	71.1b

Means followed by the same letter are not significantly different at the 5% level by Duncan's Multiple Range Test.

SK48 has consistently been observed to be rosetted during the year of seeding [35] possibly due to photoperiod or thermal sensitivity. During subsequent harvest years SK48 has equal or superior forage production to any of the other populations. Krall [71] found that individual Remont plants maintained similar rankings for growth and N₂ fixation three years after planting. However, in the present study it appears that laboratory or field evaluations of SK48 during juvenile growth are not indicative of yield or N₂-fixing potential of SK48. These data indicate that caution should be observed in relating juvenile growth of sainfoin to performance of established plants. Although SK48 appeared to be

an inferior source population for improved N_2 fixation, this high yielding cultivar could be exploited for improved N_2 fixation. Further studies may be required to develop laboratory assays which better reflect growth and N_2 fixation potential of mature plants.

Chapter VI

EFFECTS OF INDIGENOUS AND COMMERCIAL *Rhizobium* INOCULANTS ON DIVERSE SAINFOIN POPULATIONS

The interaction of different *Rhizobium* strains with host genotypes for efficiency of N_2 fixation has been demonstrated in several forage legumes [14,16,23,50,88]. In order to operate a selection program for N_2 fixation within diverse germplasms, it is necessary to utilize an effective *Rhizobium* strain or composite [37]. The ultimate goal of the research program at MSU will be to present efficient population $\times$ inoculant combinations to field conditions. The objectives of this experiment were to: (1) examine variability for effectiveness of N_2 fixation among locally indigenous *Rhizobium* strains, (2) compare the effectiveness of indigenous and commercially available *Rhizobium* inoculants, (3) determine if the five diverse sainfoin populations are affected differentially by these inoculants, and (4) insure that the Nitragin Co.¹ composite utilized in other experiments was effective with all populations.

Materials and Methods

Single random plants were dug from each of the field plots described in the previous experiment (Chapter V). Since this trial was not inoculated, it was anticipated that the five diverse sainfoin populations might be inoculated by an array of different rhizobia. Four nodules were removed from each of the 20 plants and rinsed in water. The nodules were surface sterilized in 19% EtOH and 1% NaOCl for 3 min each, followed by three rinsing cycles in sterile water. Each nodule was aseptically transferred to a culture tube containing 10 mL of sterile distilled water. The nodules were crushed with a sterile stirring rod, and

the culture was allowed to incubate at room temperature for 4 h. A sterile loop was used to streak each culture on a petri plate containing YEM (0.1% yeast extract, 10% mannitol). The plates were streaked to facilitate the isolation of single colonies. After 3 d growth on plates, a single colony from each plate was transferred to slant tubes containing 10 mL of YEM. One week later the cultures were visually examined for color and texture, and the number of cultures was reduced to four per sainfoin population. The cultures selected were chosen to represent a range in visual characteristics in order to maximize the chances of obtaining variants for N_2 fixation.

Twenty-one 15 cm pots were sterilized in 1% NaOCl and filled with vermiculite which had been autoclaved (24 h $\times$ 121°C). Four seed of each population were surface-sterilized and planted into each pot for an infectivity assay. The 20 selected indigenous strains which had been increased in YEM broth for 6 d were equilibrated, and 75 mL (1.3×10^8 cells mL^{-1}) were applied to each pot. Seventy-five mL of uninoculated broth was applied to the extra pot for an uninoculated control. The pots were placed equidistantly, about 15 cm apart, in a growth chamber which had previously been disinfected with 1% NaOCl. The seedlings were irrigated daily with zero-N Hoagland's nutrient solution or distilled water which had been heated to $> 90^\circ\text{C}$ for 30 min and stored in sterile containers. The growing conditions were as described previously in Chapter III.

At 42 d plants were gently removed from the vermiculite and the nodules were counted (Table VI-1). Two strains originating from each population (except Creston) were selected which differentially nodulated the seedlings (few or many nodules). These eight isolates were presumed to be *Rhizobium* strains, and were utilized in the effectiveness

assay. In addition, one isolate from USSR which appeared to inhibit nodulation was selected. These nine isolates were maintained on YEM.

Table VI-1. Characteristics and Infectivity of Cultures Isolated From Nodules of Field-Grown Sainfoin.

Culture Number	Isolated From	Color	Texture	Infectivity		
				Number of:		Nodules Plant ⁻¹
				Plants	Nodules	
1	Creston	Y*	M [†]	19	0	0
2 ^{††}	Creston	B	G	19	167	8.8
3	Creston	W	M	16	0	0
4	Creston	DB	G	20	0	0
5	Eski	B	M	20	124	6.2
6	Eski	DB	M	9	10	1.1
7	Eski	B	M	19	26	1.4
8	Eski	B	M	19	10	0.5
9	Remont	DB	M	16	8	0.5
10	Remont	DB	M	20	73	3.7
11	Remont	W	G	19	40	2.1
12	Remont	DB	G	16	38	2.4
13	SK48	W	M	19	72	3.8
14	SK48	B	G	20	104	5.2
15	SK48	W	M	19	253	13.3
16	USSR	W	M	18	0	0
17	USSR	B	M	18	0	0
18	USSR	Y	MM	20	1	0.1
19	USSR	DB	GG	16	0	0
20	(Uninoculated control)	—	—	18	137	7.6

* Y=yellow, B=beige, W=white, DB=dark beige after 4 d on YEM.

† M=Milky, G=gelatinous.

†† Underlined cultures were selected and used for the effectiveness assay.

Several Nitragin Co.¹ *Rhizobium* inoculants which had been evaluated on Remont by Hill [62] were chosen to compare with the indigenous strains. The effective inoculants included 116A27, a *Petalostemum* sp. 124Z1, 116A8 + 116A27, and the composite (116A8, 116A12, 116A14, 116A15, and 116A17) which was extensively utilized through-

out all experiments. Ineffective inoculants chosen were strains 116A17 and 116A28. Cultures of these six inoculants plus the nine indigenous strains were increased in YEM broth, and equilibrated at 1.4×10^8 cells mL⁻¹.

Seed (Class 3) of each population were surface sterilized and planted into 40 × 200 mm conetainers filled with autoclaved vermiculite (24 h × 121°C). The vermiculite was inoculated at planting with 2 mL of inoculum (2.8×10^8 cells). Uninoculated controls were moistened with 2 mL of sterile YEM broth. The 80 factorial treatments were replicated three times as a completely random design. Inoculation treatments in conetainers were separated by at least 80 mm to reduce contamination. Sampling units were four seedlings (two seedlings in each of two conetainers), providing a total sample size of 960 plants.

The plants for this effectiveness assay were grown in the greenhouse, at 18 to 25°C. Supplemental lighting was utilized to increase the daylength to 16 h. The plants were irrigated weekly with Hoagland's zero-N nutrient solution and daily with tap water.

The plants were excavated at 56 d, and the four seedlings per treatment combination were placed into 250 mL flasks for the TNA assay. After the C₂H₂ incubation period nodules were counted, and the roots and shoots were bagged separately, dried (48 h × 50°C), and weighed. All data were converted to a per plant basis and analyses of variance were computed. The TNA values were blocked according to the three days required to assay all plants in a block. Population means for each index were regressed against Remont across *Rhizobium* inoculants to estimate inoculant specificities in relation to Remont.

Results and Discussion

Six single colony isolates from sainfoin nodules did not form nodules by 42 d (Table VI-1). The nodule crush technique is a common method of *Rhizobium* extraction [101], however the efficiency of recovery of *Rhizobium* by other workers is not known.

It is possible that several of the indigenous isolates were slow colonizers and had not formed nodules by 42 d. Although aseptic techniques were maintained throughout the infectivity assay, one of the most abundantly nodulated treatments was the uninoculated control. Hill [62] and Delaney [33] have observed similar difficulties in maintaining completely sterile conditions for sainfoin. In their studies and the present experiment, small white nodules were found profusely on uninoculated seedlings. It is possible that the contaminant rhizobia were present beneath the seed coat or entered through the ventilation system. Although the source of the contaminant is not known, the small white nodules are apparently ineffective [33] and are distinguishable from nodules formed by other *Rhizobium* strains.

Nine indigenous strains were chosen based on their divergent infectivity on each population. Only one infective strain was obtained from Creston and USSR. No correlation existed among the visual observations of culture morphology or the infectivity of isolates.

Highly significant differences due to *Rhizobium* inoculants were detected for all indices of N_2 fixation (Table VI-2). The difference between the nine indigenous isolates and four effective commercial inoculants as determined by orthogonal contrasts were significant only for TNA. Inoculants performed similarly on all populations as indicated by nonsignificant interactions for most traits. Across populations, only three inoculants

Table VI-2. Mean Squares for Traits Associated with N₂ Fixation of Five Sainfoin Populations Inoculated with 15 Indigenous *Rhizobium* Strains or Commercial Inoculants.

Source of Variation	d.f.	TNA ($\mu\text{mol C}_2\text{H}_2 \text{ plant}^{-1} \text{ h}^{-1}$)	d.f.	Nodules Plant ⁻¹	Dry Weight (mg plant ⁻¹)	
					Shoot	Root
Block	2 [†]	0.064	—	—	—	—
Population (P)	4	0.287**	4	65.558	1031.818**	1326.371**
Inoculant (I)	15 [‡]	0.418**	15	567.600**	2561.588**	3688.735**
Indigenous vs. Effective Commercial	1	4.809** ^{††}	1	17.320	128.650	152.122
Residual	14	0.104**	14	606.91**	2735.369**	3949.350**
P x I	60	0.036	60	22.710	136.921*	256.450
Error	158	0.035	160	33.287	92.031	197.545
CV%		65.0		46.9	21.1	24.3

[†] Three days were required to conduct the C₂H₂ incubation for the TNA assay.

[‡] Uninoculated controls were included in the analyses.

^{††} Orthogonal comparisons for all nine indigenous isolates vs. four commercial strains or composites previously shown to be effective with Remont [62].

(116A27, 116A8 + 116A27, and 124Z1) were significantly better than the uninoculated controls for TNA (Table VI-3).

The indigenous strains except USSR-18 resulted in significantly lower TNA than the nodules on uninoculated seedlings. From these data it initially appeared that contamination of uninoculated containers had occurred. However due to the random arrangement of the treatments, the containers with ineffective inoculants should have also become contaminated with the moderately effective contaminant inoculum. It is possible that inoculation with a high concentration of ineffective rhizobia precluded infection by the contaminant, therefore TNA was low. Although the presence of contaminant *Rhizobium* confounded this study, it was apparent that significant effects due to ineffectiveness or effectiveness of inoculants were obtained.

No direct relationship was observed between the population from which indigenous isolates were obtained and effectiveness. Isolates from Eski were least effective with Eski, whereas strains obtained from Remont or USSR were more effective with Remont or USSR, respectively than with other populations. The most effective indigenous strain with Remont was isolated from Creston, and this isolate was not significantly different from the best commercial inoculant with Remont. The indigenous isolates generally resulted in less TNA across all populations than 116A17 and 116A28, which had previously been shown by Hill [62] to be ineffective with Remont. These data indicate that locally indigenous rhizobia are inferior to even the least effective, commercially-available inoculants. With Remont, the commercial inoculants performed similarly to previous evaluations [62]. The 116A8 + 116A27 composite was the best inoculant for Creston and USSR. *Petalostemum* sp. 124Z1 was highly effective with four populations, but ineffective with SK48.

Table VI-3. Effects of 15 Indigenous or Commercial *Rhizobium* Inoculants on TNA of Five Sainfoin Populations.

Inoculant	Population					Mean
	Remont	Creston	USSR	Eski	SK48	
----- TNA ($\mu\text{mol C}_2\text{H}_4 \text{ plant}^{-1} \text{ h}^{-1}$) -----						
Indigenous strain isolated from:						
Creston (2)	0.539	0.089	0.101	0.087	0.029	0.169
Eski (5)	0.001	0.071	0.071	0.001	0.001	0.029
Eski (8)	0.387	0.141	0.360	0.071	0.068	0.205
Remont (9)	0.202	0.109	0.179	0.126	0.066	0.136
Remont (10)	0.260	0.097	0.066	0.087	0.057	0.113
SK48 (13)	0.218	0.170	0.151	0.205	0.071	0.163
SK48 (15)	0.168	0.270	0.153	0.282	0.080	0.191
USSR (16)	0.185	0.131	0.258	0.066	0.138	0.156
USSR (18)	0.303	0.359	0.423	0.560	0.134	0.356
Uninoculated						
Control	0.435	0.328	0.555	0.348	0.158	0.365
Nitragin Co. ¹ Strains						
116A17	0.437	0.510	0.395	0.350	0.224	0.383
116A28	0.246	0.331	0.280	0.382	0.158	0.279
116A27	0.550	0.644	0.566	0.667	0.378	0.561
116A8 + 116A27	0.323	0.760	0.733	0.535	0.372	0.546
<i>Petalostemum</i> sp.						
124Z1	0.593	0.574	0.718	0.623	0.140	0.530
Composite*	0.452	0.392	0.514	0.409	0.380	0.429
Mean	0.331	0.311	0.345	0.300	0.153	0.288

*The composite inoculum consisted of equal proportions of strains 116A8, 116A12, 116A14, 116A15, and 116A17.

LSD (5%) = 0.134 and 0.075 for comparing inoculant and population means, respectively.

Control seedlings had significantly more nodules than two commercial inoculants and all indigenous isolates (Table VI-4). Effectiveness of indigenous strains was generally related to number of nodules, however this was not true for the commercial inoculants. The least effective indigenous strain for TNA (Eski-5) formed the fewest nodules, whereas the least effective commercial strain (116A28) formed the highest number of nodules.

All commercial inoculants resulted in significantly higher root and shoot weights than the uninoculated control. This might be expected because shoot dry weight under zero-N conditions is a common selection criterion for commercial *Rhizobium* inoculants [101]. The most effective inoculant for these traits was the Nitragin Co.¹ composite, which increased shoot and root dry weights by 81 and 113%, respectively. The most efficient indigenous strain for TNA (Table VI-3) increased dry weights over uninoculated controls by 27%. Although most indigenous strains resulted in less TNA than uninoculated controls, shoot and root dry weights (Table VI-4) were not significantly different. These data indicate that growth and TNA are correlated across a wide range of N₂ fixation efficiencies, however within less heterogenous materials such as indigenous or commercial rhizobia this relationship diminishes.

The populations performed similarly to previous experiments across all inoculants. SK48 had significantly lower TNA than all other populations (Table VI-2). USSR had significantly higher shoot and root dry weights than all populations (Table VI-5). It appeared that this population was effectively nodulated by more inoculants and the contaminant than the other populations.

TNA of Creston and Eski were significantly correlated with TNA of Remont across all inoculants (Table VI-6c). Nodule number and dry weights of all populations were signifi-

Table VI-4. Effects of Indigenous or Commercial *Rhizobium* Inoculants on Mean Nodulation and Growth Across Five Diverse Sainfoin Populations.

Inoculum	Nodules Plant ⁻¹	Dry Weight (mg plant ⁻¹)	
		Shoot	Root
Indigenous strain isolated from:			
Creston (2)	5.2	34.4	45.2
Eski (5)	5.2	31.5	42.6
Eski (8)	7.9	36.8	51.0
Remont (9)	7.9	36.3	44.0
Remont (10)	5.2	35.5	47.8
SK48 (13)	8.8	39.7	51.5
SK48 (15)	7.9	32.4	47.5
USSR (16)	9.7	32.9	47.8
USSR (18)	14.1	45.8	52.8
Uninoculated			
Control	20.1	36.1	44.3
Nitragin Co. ¹ Strains			
116A17	16.7	62.3	74.2
116A28	20.6	52.2	72.2
116A27	20.2	64.7	76.9
116A8 + 116A27	15.2	63.7	76.0
<i>Petalostemum</i> sp. 124Z1	15.8	56.6	58.7
Composite*	18.0	65.2	94.4
Mean	12.3	45.5	57.9
LSD (5%)	4.1	6.9	10.1

*The composite inoculum consisted of equal proportions of strains 116A8, 116A12, 116A14, 116A15, 116A17. (This inoculum was utilized in all other experiments.)

Table VI-5. Nodulation and Growth of Five Sainfoin Populations Inoculated with 15 Indigenous or Commercial *Rhizobium* Inoculants.

Population	Nodules Plant ⁻¹	Dry Weight	
		Shoot	Root
USSR	13.7	52.8	70.1
Remont	10.6	44.6	56.3
Creston	13.1	45.3	56.3
Eski	12.4	44.8	53.3
SK48	11.9	39.9	53.6
Mean	12.3	45.5	57.9
LSD (5%)	NS	3.8	5.6

cantly correlated to Remont across all inoculants. However within indigenous or commercial *Rhizobium* inoculants, only root weights of SK48 were correlated to those of Remont. Although not significant, the relationships among the populations with Remont tend to be the strongest for commercial inoculants. These data indicate that the responses of all sainfoin populations exposed to an array of *Rhizobium* inoculants are very similar. The efficiency of N₂ fixation is most similar among the locally adapted populations—Remont, Eski, Creston. Interestingly this relationship does not exist between Eski and Remont when inoculated with rhizobia indigenous to the same farm where these cultivars were developed. It appears that there is little variability for effectiveness among indigenous *Rhizobium* isolates, possibly because N₂ fixation is not favored under field conditions, due to available soil N. Nonetheless, commercial *Rhizobium* inoculants are available which are highly effective with diverse sainfoin populations. Any effective commercial inoculant, with the possible exception of 124Z1, could be utilized for evaluation among or within diverse sainfoin populations for N₂ fixation. The abilities of these inoculants to colonize local soils

Table VI-6. Correlated Responses of Creston, USSR, Eski and SK48 Relative to Remont Across Indigenous (a), Commercial (b), and All (c) *Rhizobium* Strains as Determined by Regression Coefficients (b_{yx}) and Correlations (r).

Population	N	TNA ($\mu\text{mol C}_2\text{H}_4$ $\text{plant}^{-1} \text{h}^{-1}$)		Nodules Plant^{-1}		Dry Weight (mg plant^{-1})			
		b_{yx}	r	b_{yx}	r	Shoot		Root	
						b_{yx}	r	b_{yx}	r
a)									
Creston	9	0.022	0.04	0.169	0.05	0.105	0.10	-0.041	-0.06
Eski	9	0.129	0.11	0.096	0.05	0.158	0.12	0.226	0.36
USSR	9	0.213	0.25	0.278	0.12	-0.122	-0.10	-0.190	-0.22
SK48	9	0.019	0.07	0.696	0.34	-0.24	-0.29	0.185	0.37
b)									
Creston	6	0.333	0.27	0.202	0.29	0.399	0.47	0.542	0.52
Eski	6	0.634	0.63	-0.062	-0.11	0.156	0.24	0.234	0.41
USSR	6	0.582	0.45	0.177	0.36	0.496	0.47	0.716	0.54
SK48	6	0.051	0.05	0.454	0.38	-0.132	-0.12	0.721	0.85*
c)									
Creston	16 [†]	0.707	0.53*	0.627	0.59*	0.890	0.79**	0.653	0.74**
Eski	16	0.751	0.56*	0.766	0.79**	1.075	0.75**	0.703	0.70**
USSR	16	0.514	0.42	0.820	0.81**	1.104	0.71**	0.906	0.71**
SK48	16	0.353	0.47	0.625	0.73**	0.624	0.63**	0.673	0.87**

[†]Uninoculated controls were included in regression analyses.

* and ** denote significance at the 5% and 1% levels, respectively.

and compete for nodule sites should be assessed in order to maximize N_2 fixation by sainfoin.

Chapter VII

DIVERGENT SELECTION FOR $N_2(C_2H_2)$ RATES

Data from the previous experiments indicated that all sainfoin populations examined might serve as germplasm sources for increased N_2 fixation. Since these populations are known to perform well under Montana conditions, selection pressure for TNA might reduce their dependence on soil N. The objective of this study was to obtain divergent selections for TNA from within the five sainfoin populations and establish breeding populations of one-cut and multi-cut types for inheritance studies.

Materials and Methods

In this study 620 seedlings from Class 3 seed of the five sainfoin populations were grown under the conditions previously described. The *Rhizobium* inoculum density was 5.4×10^8 cells mL^{-1} . Approximately 35 seedlings of each population were assayed at 56 d in each of four blocks which were staggered at planting by 14 d. For the TNA assay each seedling was placed intact into a 1000 mL Erlenmeyer flask. Ten percent (100 cm^3) of the air was withdrawn and replaced with purified C_2H_2 . After the TNA assay, the seedlings were gently removed from the vermiculite and visually scored for degree of root fibrosity and nodulation. On a 1 to 5 scale plants with no or few nodules were assigned a score of 1 whereas abundantly nodulated seedlings were classified as 5. Similarly, seedlings with poorly developed tap roots were assigned a score of 1, and highly branched roots were classified as a 5. Whole plant fresh weights were obtained, and the seedlings were replanted into the vermiculite to await selection. All data were analyzed on a per-plant basis. Square root transformations were computed for TNA to reduce the correlation of means and

variance. Bartlett's test for homogeneity of variances was conducted for TNA to insure that analyses of variance were appropriate.

Bidirectional selections comprising the high and low 10% of each population were obtained. Approximately 13 seedlings were selected divergently for TNA from Eski, Creston, SK48, and USSR to compose two Syn 0 populations of single cut sainfoin types for high and low TNA. Forty-eight high and 51 low Remont selections from this study and previous evaluations composed a second set of Syn 0 populations. The seedlings were transplanted into four spatially isolated intercross blocks near Portales, New Mexico, in June, 1980. It was anticipated that the extended growing season would be conducive to high seed production during the year of establishment. However due to extremely high temperatures ($> 41^{\circ}\text{C}$) immediately after transplanting and during the remainder of the summer, over 90% of the plants in all populations died. Because the surviving plants would have provided insufficient numbers for broad germplasm pools, the selection program within single-cut sainfoin types was terminated. Further selection has resumed within Remont, and approximately 60 selections for high or low TNA should be available for intercrossing in August, 1982.

Results and Discussion

The five sainfoin populations were not significantly different for root or nodule scores (Table VII-1). The nodule score data agree with those in the previous study in which nodule numbers did not differ significantly among the populations inoculated with the Nitragin Co.¹ composite inoculum. The population variances for TNA were heterogeneous ($P < 0.01$), however after square root transformation, the analysis of variance was appro-

Table VII-1. Means and Variances of Five Sainfoin Populations for Indices Related to N₂ Fixation.

	N	Nodulation Score	Root Score	Fresh Weight (mg plant ⁻¹)	TNA (μmol C ₂ H ₄ plant ⁻¹ h ⁻¹)		
					Range	Mean	s ²
Remont	126	3.8*	3.7	446.9a	0-11.84	3.46a	3.79 [†]
Eski	125	4.0	3.5	387.3b	0-11.47	2.67b	2.68
USSR	123	3.7	3.6	454.1a	0- 6.34	2.59bc	2.29
SK48	123	3.6	3.4	405.7b	0- 7.33	2.23cd	3.26
Creston	123	4.0	3.6	410.9b	0- 7.11	2.17d	1.58
Mean	620	3.8	3.5	421.4	—	2.63	—
CV%	—	29.9	24.5	24.4	—	60.4 ^{††}	—

* Degree of nodulation and root fibrosity estimated on a scale of 1 to 5 with merit increasing as score increases.

[†] Within population variances.

^{††} Coefficient of variability for TNA after square root transformation was 37.7%.

Means followed by the same letter are not significantly different at the 5% level by Duncan's Multiple Range Test.

appropriate ($P < 0.10$). Remont seedlings had significantly higher TNA than all other populations. Interestingly USSR had similar plant fresh weights to Remont, yet was inferior to Remont for TNA. These data contrast with those of Hoffman and Melton [64] who reported no significant differences among 10 diverse alfalfa germplasm for TNA. In this study Creston had significantly lower TNA and total plant weight than Remont, Eski, and USSR. The variance for TNA indicated that substantial variability is present within all populations for improvement by selection.

Distributions for TNA were skewed towards inefficient N_2 fixation in all populations (Fig. VII-1) as previously described by Hill [62] for Remont. In contrast, whole plant fresh weight was normally distributed in all populations (Fig. VII-2). These data suggest that natural or artificial selection has reduced N_2 fixation of genetically diverse sainfoin populations without disturbing their yield potentials. A similar situation exists in alfalfa as several workers [64,92,100] have reported that many high-yielding alfalfa cultivars are inefficient in N_2 fixation. This indicates that selection pressure for high yield may be responsible for an increased dependence upon soil N, possibly accompanied by reduced N_2 fixation. In this study Creston had significantly lower TNA than Eski, its parent cultivar, however yields of these populations were equivalent under field and growthroom conditions. It appears that during the 13-year period of natural selection resulting in the Creston composite its N_2 -fixing potential has declined by about 20%. This does not appear to be associated with changes in specificity for *Rhizobium* strains as indicated in the previous experiment. The reduction in N_2 fixation could be due to inbreeding or genetic drift since the original Eski stand was reduced to about one plant 6 m^{-2} , however corresponding reductions in vigor or forage yield were not observed.

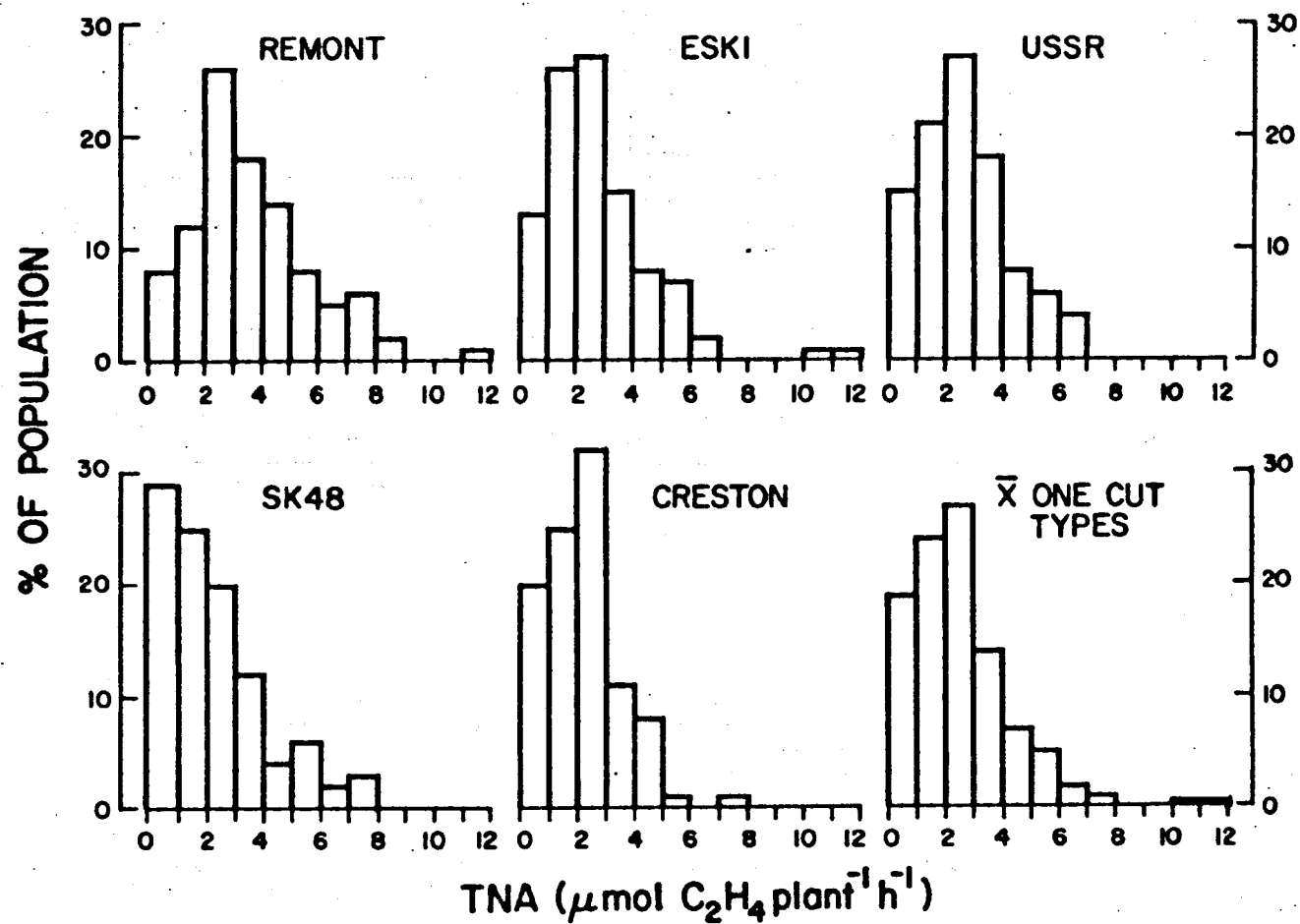


Fig. VII-1. Distributions for Total Nitrogenase Activity (TNA) Within Five Sainfoin Populations.

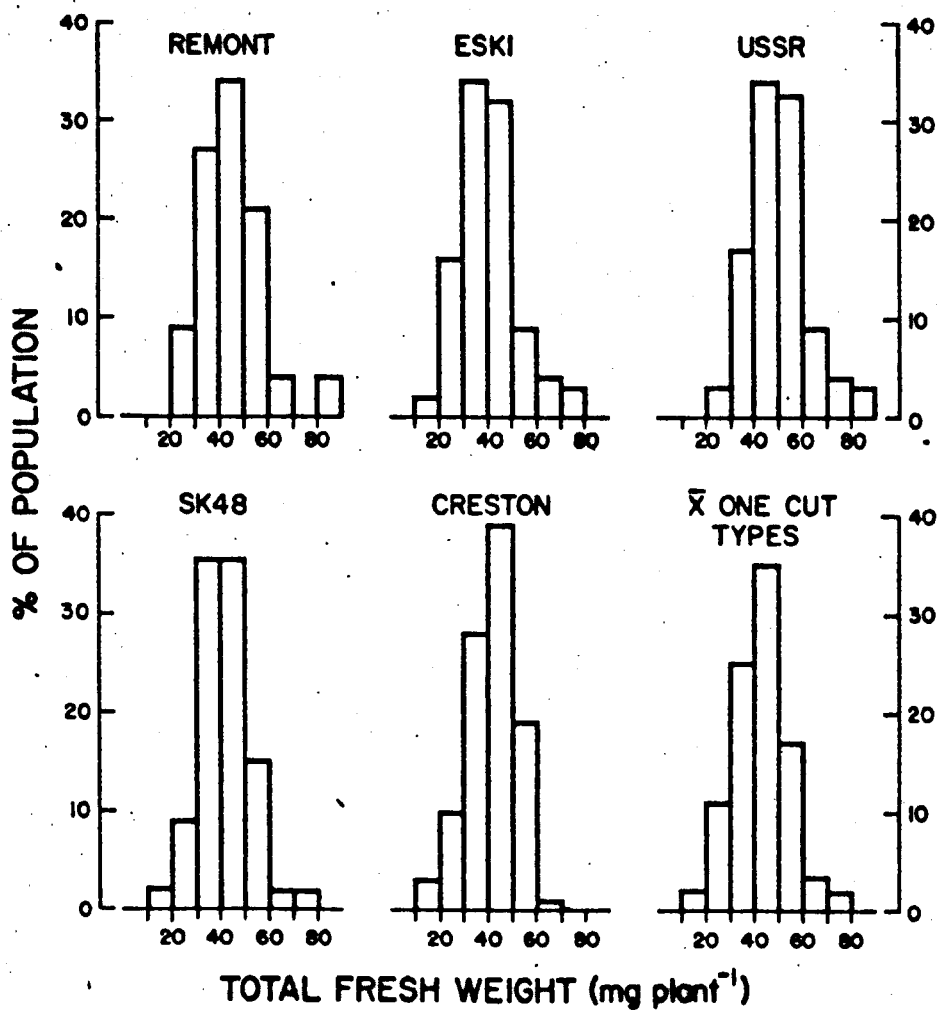


Fig. VII-2. Distributions for Total Fresh Weight Within Five Sainfoin Populations.

Further examinations should be conducted within Creston and Eski to determine if Creston has become more dependent on soil N, and if other related physiological traits were altered.

The data from this study and previous experiments indicated that Remont had higher N_2 fixation than other high yielding sainfoin cultivars. Although it appears that adequate variability is present to improve TNA of all populations, the most effective progress should be made with Remont. Since this cultivar is highly adapted to Montana conditions, improvement of N_2 fixation in Remont should advance without much breeding effort to maintain other agronomic characteristics such as uniform regrowth and yield potential. For these reasons it was decided to restrict subsequent breeding efforts for high TNA to selection within Remont.

Chapter VIII

INHERITANCE OF TOTAL NITROGENASE ACTIVITY IN REMONT SAINFOIN

Substantial response from selection for high TNA has been reported in alfalfa [37,63, 92,100]. Although progenies from sainfoin populations selected divergently for TNA were unavailable, an attempt was made to estimate the heritability of TNA from open-pollinated Remont families.

Materials and Methods

Eighty-three individual Remont seedlings were evaluated for TNA as previously described at 56 d. Selections comprising the low and high 10% based on TNA were removed and transplanted into the intercross blocks described in Chapter VII. The remaining 65 plants were transplanted into an isolated space-planted nursery on 0.6 m centers in June, 1980. Open pollinated seed was harvested in 1980 and 1981, and maintained as to the maternal plant. Adequate seed sized 2.18-2.38 mm was available for evaluation of 52 half-sib (HS) families. The seed were planted into 25 × 160 mm conetainers. The seedlings were grown in a greenhouse under conditions described in Chapter VI.

This experiment was conducted as a completely random design with four replications of each of the 52 HS families plus Remont as a check. Sampling units consisted of five seedlings per HS family, resulting in a total sample size of 1060 plants. After the TNA assay shoots were detached, dried (48 h × 50°C), and weighed. Analyses of variance were computed on data which had been converted to an individual plant basis. Correlations among TNA and SDW were determined, and heritability of TNA was estimated by parent-

offspring regression. Because the generations were evaluated in different environments, standard unit heritability (r) was substituted for the regression coefficient (b). In addition, narrow sense heritability estimates for SDW and TNA were also estimated from the variance components of the HS families.

Results and Discussion

Significant differences were detected among the HS families for SDW and TNA (Table VIII-1). Seven and six families were significantly better than Remont for TNA and SDW, respectively (not shown). One family had TNA significantly less than Remont.

Table VIII-1. Analyses of Variance for Total Nitrogenase Activity (TNA) and Shoot Dry Weight (SDW) of 52 Half-Sib Families of Remont Sainfoin.

	d.f.	MS	
		TNA	SDW
Among Families	52 [†]	0.029**	239.786**
Within Families	159	0.017	112.097
CV%	—	45.6	20.4

[†] Remont seedlings were included in the analysis of variance.
Mean squares are significant at the 1% level.

The distribution for TNA among the original 83 seedlings was skewed towards inefficiency (Fig. VIII-1a), as previously shown for Remont and other sainfoin populations. Removal of the high and low 10% of the plants by selection for TNA and low seed production by several clones resulted in a reduced mean and a more normal distribution in the base population. Distributions for TNA (Fig. VIII-1) and SDW (Fig. VIII-2) in the progenies

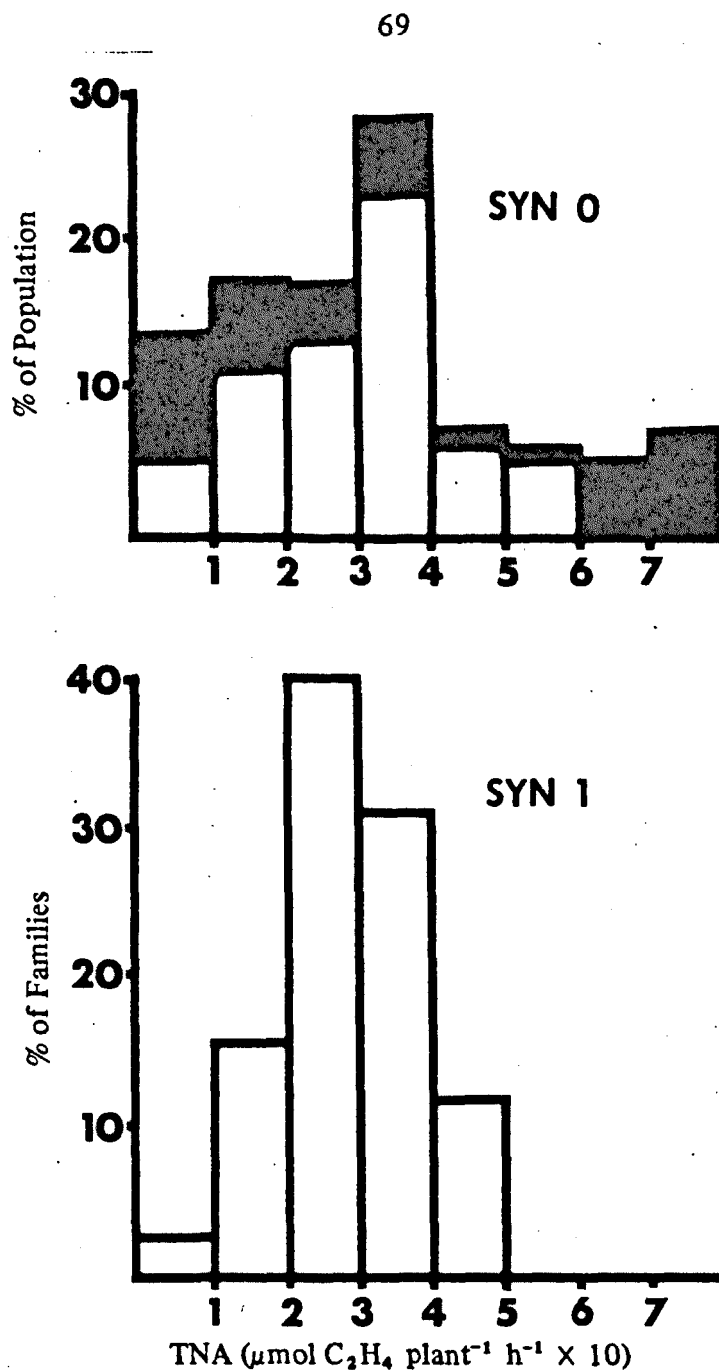


Fig. VIII-1. Distributions of Total Nitrogenase Activity (TNA) Among 83 Random Remont Seedlings (Syn 0) and 52 Subsequent Half-Sib Families (Syn 1). Shaded areas in Syn 0 designate plants removed as high or low TNA selections, or produced insufficient seed for evaluation.

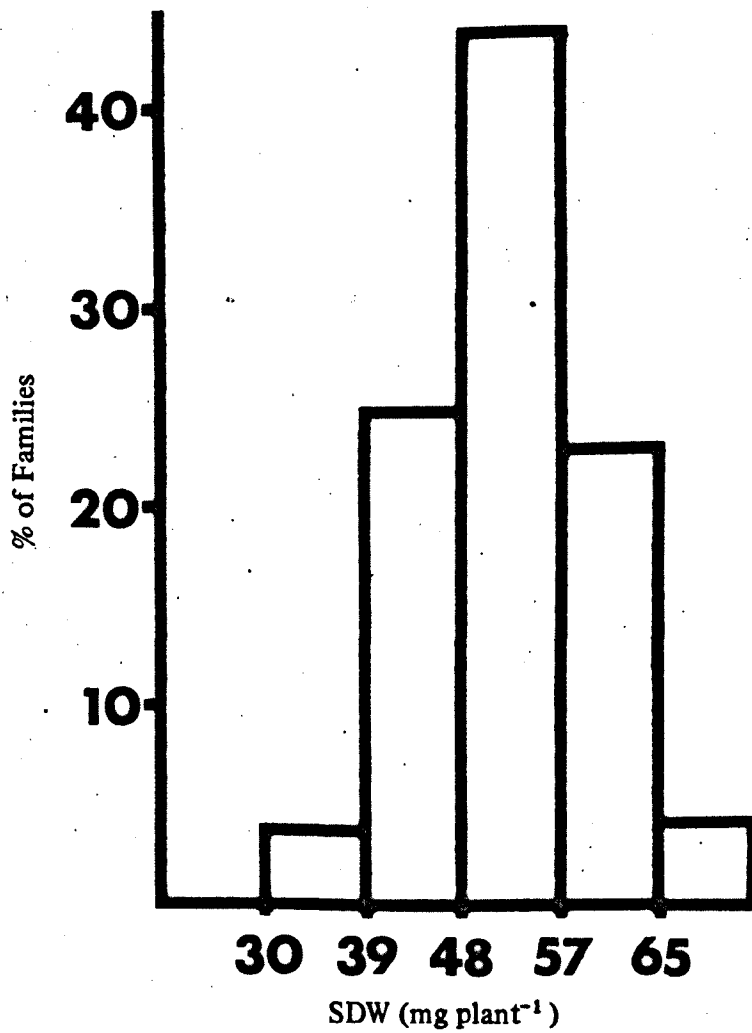


Fig. VIII-2. Distributions for Shoot Dry Weight (SDW) Among 52 Half-Sib Families of Remont Sainfoin.

were normally distributed. It appears that elimination of the few superior $N_2(C_2H_2)$ -fixing genotypes had a marked influence on the TNA distribution of the Syn 1 generation.

The correlation between SDW and TNA in the HS families was highly significant (Table VIII-2) and similar to previous experiments. The TNA and SDW of the progenies were not significantly correlated to TNA of the parents. The heritability estimate based on parent-offspring regression was 14%. It is likely that the removal of individuals representing extremes in TNA in the Syn 0 coupled with the high level of unexplained error was responsible for this low value. The correlation between SDW and TNA in the Syn 1 similar to previous experiments indicates that TNA values of the HS progenies were probably not erroneous.

Table VIII-2. Correlations (r) Among Shoot Dry Weight (SDW) and Total Nitrogenase Activity (TNA) of 52 Remont Seedlings (Syn 0) and Their Subsequent Half-Sib Families (Syn 1).

	TNA (Syn 1)	TNA (Syn 0)
	-----r-----	
SDW (Syn 1)	0.53**	-0.20
TNA (Syn 1)	—	0.14

** denotes significance at the 1% level.

Narrow sense heritability estimates for TNA and SDW based on variance components were 62 and 88%, respectively. These estimates are similar to those reported for alfalfa [37,100], but higher than the estimate for TNA, based on parent-offspring regression. Significant differences among individual plants or HS families were difficult to detect in

absence of the seedlings representing extremes in TNA. Although TNA exhibited the most error variation of all traits examined in these studies, it appears that selection for TNA would be effective. It is likely that the heritability of TNA is actually higher than was estimated by parent-offspring regression, therefore substantial progress should be made through selection.

Due to the low frequency of elite $N_2(C_2H_2)$ -fixing genotypes in a population and the time and labor requirement for the TNA assay, it appears that the selection programs proposed by Duhigg et al. [37] and Viands et al. [100] may be appropriate. These workers suggested that large populations of alfalfa seedlings grown under zero-N conditions could be initially screened for SDW or plant height. Elite genotypes would then be evaluated for TNA. Based on the significant and repeatable correlations between SDW and TNA, and the apparently high levels of error associated with measuring TNA, it is likely that this approach may be useful in improving N_2 fixation in sainfoin.

Chapter IX

SUMMARY AND CONCLUSIONS

The inability of sainfoin to fix adequate levels of N_2 may limit its forage production and stand longevity. The objectives of this research were to evaluate several sources of variability for N_2 fixation by juvenile sainfoin, and to determine if improvement through conventional plant breeding techniques is feasible.

Total nitrogenase activity (TNA) was closely related to dry matter and N accumulation, therefore this assay should be an effective selection tool for improving N_2 fixation of sainfoin. Acetylene reduction proceeds linearly up to 12 h, and incubation periods of 3 h provide reliable estimates of TNA to facilitate large numbers of seedlings in a breeding program.

Seedlings from large seed emerge faster, have more nodules, and higher accumulations of dry matter and N at most evaluations up to 84 d. The superiority of seedlings from large seed is not related to differences in relative growth rates. Seedlings selected for high or low TNA at 56, 70, or 84 d arose consistently from large and small seed size classes, respectively. Therefore seed size should be considered when evaluating sainfoin during juvenile growth.

Locally indigenous *Rhizobium* strains isolated from five diverse sainfoin populations were less effective for TNA than the least effective commercially-available inoculants. The *Rhizobium* inoculants behaved similarly on all sainfoin populations, therefore any effective inoculant would be suitable for a plant selection program.

Considerable variability exists among and within diverse sainfoin populations for growth and $N_2(C_2H_2)$ fixation. In general, Remont was superior and SK48 was inferior

to the other populations in growthroom and field evaluations. Adequate variability for TNA was present within all populations for potential improvement of N_2 fixation. The most effective progress will likely be made by selection within Remont, a high yielding Montana cultivar.

Narrow sense heritability estimates for TNA from 52 Remont HS families were 14 and 62, respectively, from parent-offspring regression and variance components. Although these values are low, it is apparent that progress could be made by selection. Since progress might be slow due to low heritability estimates, preliminary selection based on growth would be useful for reducing the number of individuals to be evaluated by TNA.

Future research should be conducted to determine if: (1) laboratory estimates of N_2 fixation adequately predict mature plant performance, (2) effective *Rhizobium* strains can be established in fields containing ineffective indigenous rhizobia, (3) sainfoin populations selected for high TNA have reduced dependence upon soil N reserves.

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