Variability for traits associated with N□b2□s□s 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 Philosphy in Crop and Soil Science
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Abstract:
Poor symbiotic nitrogen (N2) 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 Na fixation by juvenile sainfoin and to determine if improvement could be made through selection.

Growth and N a fixation patterns of‘Remont’ seedlings evaluated at 14 d intervals to 84 d were closely related to initial seed size (ISS). The N a(C jH a) 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 N2 (C2H2) 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 N2 (C2H2) 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

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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 $N_2(C_2H_2)$ 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.

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 plane 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 (N_2) 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 N_2 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 N_2 fixation. Improvement of N_2 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 N_2 Fixation

Hardy and Holsten [58] characterized measurements of N_2 fixation into three categories: (1) growth, morphology, and leghemoglobin content; (2) various N analyses including ^{15}N 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 N_2 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 N_2 fixation.

Nodule number, mass, and weight have been used as indicators of N_2 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 N_2 fixation, therefore these criteria should only be used as qualitative indicators of N_2 fixation [5,14].

Hardy et al. [56] reported that pink coloration of nodules, attributable to presence of leghemoglobin, was associated with active N_2 fixation; whereas white, green, or brown nodules were ineffective. Visual estimates of nodule color have been used as qualitative evidence for N_2 fixation by several workers [23,37,63,64]. LaRue and Child [72] reported a fluorescent measurement of the porphyrin component of leghemoglobin which is 10^3 times more sensitive than other estimates of nodule color. Although leghemoglobin concentration is related to active N_2 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 if 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 F_2 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 N_2 fixation. Several genes located on different chromosomes were involved with symbiosis.

Nutman [78,82] described five simply inherited genes involved with N_2 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 X high crosses had acetlyene reduction rates more than 200% higher than those of the low X low crosses. Progeny from the low X 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 N_2 Fixation in Sainfoin

Sainfoin appears to differ from the other forage legumes which are being exploited for their high N_2-fixing capacities. Low N_2 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 N_2 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 N_2-fixing sainfoin genotypes when inoculated with selected *Rhizobium* inoculants, and (3) presentation of an efficient *Rhizobium* inoculant × sainfoin population "package" to field conditions. Sainfoin rhizobia exhibit broad variability for their effects on N_2 fixation. Hill [62] evaluated 16 single *Rhizobium* strains, seven of which increased C_2H_2 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 N_2 fixation of sainfoin by host selection. Cecil [25] found considerable variability in C_2H_2 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 N_2 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 N_2 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 multicut hay systems or pasture [36]. The seed lot utilized was commercially obtained, certified seed which had been extensively utilized for *Rhizobtum* 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 *Rhizobtum* 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 conetainer 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 cm^3 of air were removed with a syringe and replaced with purified acetylene (C_2H_2). The plants were allowed to incubate in the C_2H_2, and sampled after 1, 2, 3, 4, 6, 8, 12, and 26 h. Gas samples were withdrawn into 1-cm^3 disposable syringes. The needles on the sampling syringes were inserted into rubber stoppers to prevent leakage prior to gas analysis.

Gas samples (1 cm^3) were injected into a Beckman[1] Model GC 2-A gas chromatograph equipped with a hydrogen flame ionization detector. Ethylene (C_2H_4) and C_2H_2 were separated in the 2 m × 8 mm OD stainless steel column packed with 80/100 mesh F-1 activated alumina (Supelco[1]) and maintained isothermally at 130°C. Gas pressures entering the chromatograph were 24, 40, and 250 cm^3 min^{-1} for H_2, He, and compressed air, respectively. Retention times for C_2H_4 and C_2H_2 were 1.0 and 3.5 min, respectively, for this system. Ethylene peak heights were standardized with a dilution curve for C_2H_4 calibrating gas (1000 ppm C_2H_4 in He, Applied Sciences[1]). Total nitrogenase activity (TNA) was expressed as μmol C_2H_4 $plant^{-1}$ h^{-1}, and also as μmol C_2H_4 $plant^{-1}$ for this study.

After the last gas samples were obtained, the plants were removed from the conetainers, and root and shoot dry weights (48 h × 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 μmol C_2H_4 plant^{-1}, respectively, at 1 h. The corresponding values at 12 h were 1.72, 5.59, and 3.49 μmol C_2H_4 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.

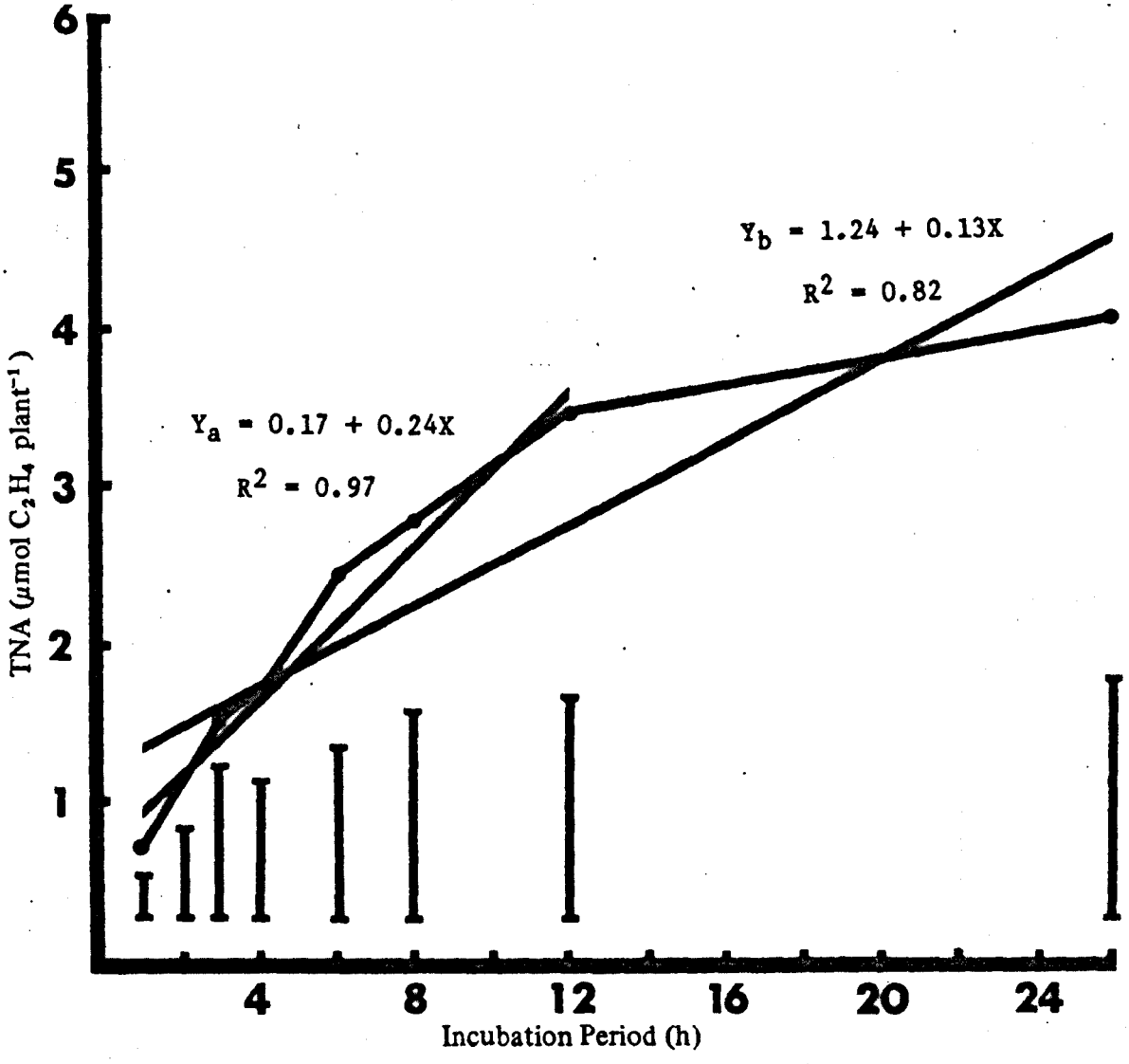

Fig. III-1. Time Course of Total Nitrogenase Activity (TNA) by 35 Sainfoin Seedlings and Regression Lines Excluding (a) or Including (b) the 26 h Sample. Vertical bars denote 1 standard deviation.

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

TNA (μmol C_2H_4 $plant^{-1}$) Incubation period (h)	TNA (μmol C_2H_4 $plant^{-1}$) 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 N_2 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 N_2 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 N_2 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-Dickinson[1]) and analyzed in a Carle Instrument[1], Model 9702 gas chromatograph for this and all remaining experiments. A 3.2 mm OD X 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 cm^3 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 X 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 N_2 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

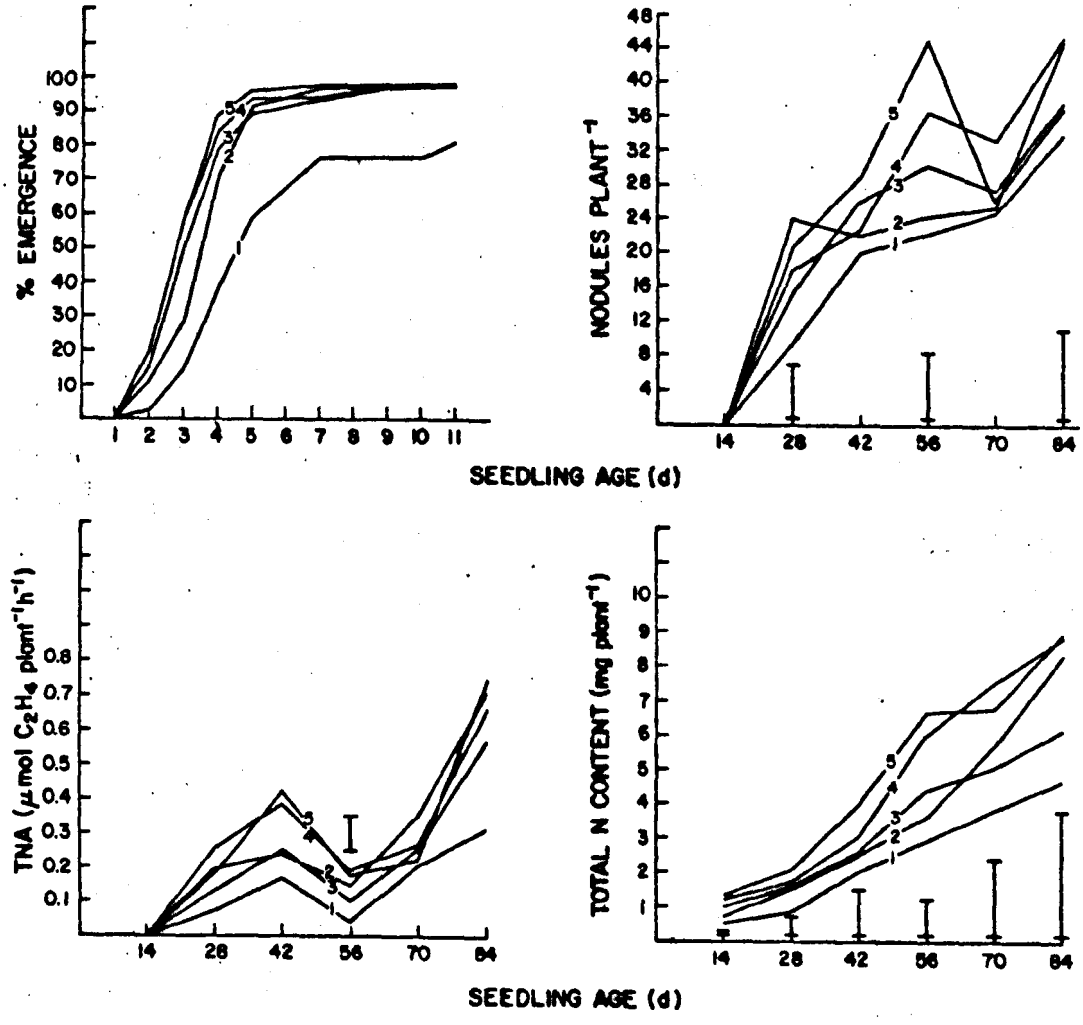

Fig. IV-1. Indices of Growth and N_2 Fixation of Remont Sainfoin Seedlings Arising From Five Seed Sizes and Evaluated at 14 d Intervals. Vertical bars denote protected least significant differences at the 5% level.

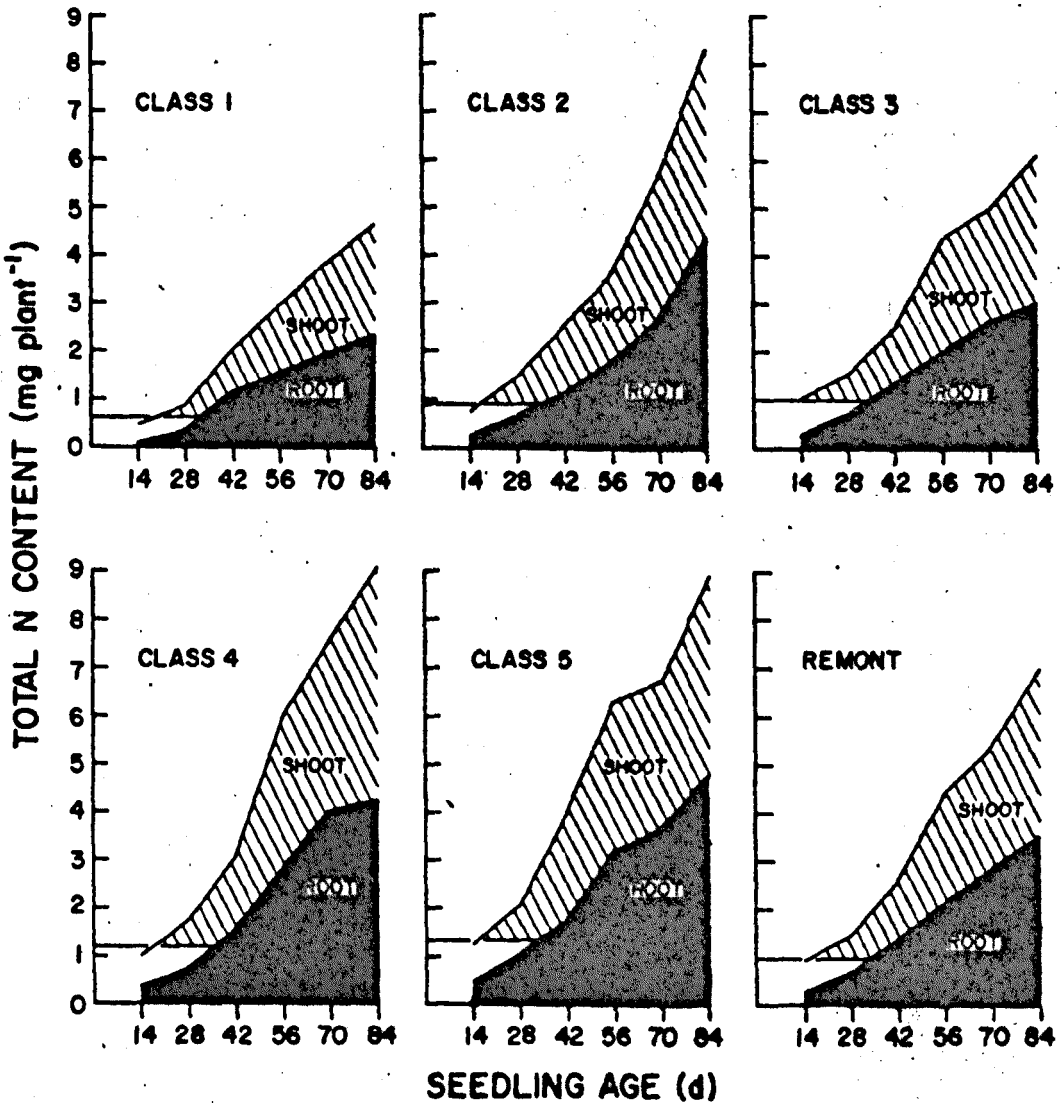

Fig. IV-2. Total N Content of Remont Sainfoin Seedlings Arising From Five Seed Size Classes and Evaluated at Six Harvest Dates. Horizontal Lines Denote Initial Seed N Content.

Table IV-2. Mean Squares for Six Traits Associated With Growth and N_2 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_2H_4 plant^{-1} h^{-1})	d.f.	Plant Height (mm)	Whole Plant: Dry Weight (mg plant^{-1})	Whole Plant: N Concentration (%)	Whole Plant: 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 X 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 X 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-