



Reproduction and genetics of sainfoin (*Onobrychis viciaefolia* Scop.) as they relate to its breeding
by William Jay Knipe

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of
DOCTOR OF PHILOSOPHY in Crop and Soil Science

Montana State University

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Abstract:

Sainfoin pollen collected in the greenhouse had lower in-vitro germination than alfalfa, alsike clover, birdsfoot trefoil, and cicer milkvetch. The addition of yeast extract to the media increased germination of greenhouse collected alfalfa pollen but had no effect on sainfoin. This addition increased germination of field produced sainfoin pollen. Sainfoin pollen collected in the field was affected by time of day collected and genotype but not by age of flower. In-vitro germination was not correlated to percent seed set, indicating pollen collected at any time of day could successfully be used for making artificial crosses. Stigma receptivity was affected by time of day with pollinations after 5:00 P.M. being most successful. Sainfoin flowers were emasculated by 5 or 10 seconds immersion of the staminal column in 47.5 percent alcohol and removing pollen by rubbing the stamens between the thumb and forefinger. The latter method was faster to apply and gave higher seed set following artificial pollinations.

Three white-flowered plants were different genetically since only one segregated upon selfing. The segregation observed fit a 3:1 ratio which did not differentiate between disomic and tetrasomic inheritance.

A plant heterozygous for the exposed stigma mutant was selfed and the progeny fit a 15 normal to 1 exposed stigma ratio. The trait may be controlled by two independent duplicate genetic factors. This ratio suggests that sainfoin is an allopolyploid.

Estimates of percent crossing varied under different planting arrangements. When a plant was surrounded by pollen parents in an open-pollinated nursery, over 90 percent crossing occurred, but in single-cross arrangements under bubble isolation 8 to 28 percent crossing took place.

A significant inbreeding depression was observed for plant weight under field conditions. In the greenhouse, inbreeding depressions were estimated for rooting ability of cuttings and plant height.

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March, 1972

ACKNOWLEDGMENT

The author wishes to extend sincere appreciation to Dr. Albert Carleton, his major professor, for his guidance during the course of the study and preparation of this manuscript. He also extends gratitude to other members of his committee, Professor Robert Eslick, Dr. Eugene Sharp, Dr. Clee Cooper, and Dr. James Sims for reviewing the manuscript.

The author also extends gratitude to his wife, Ardith, for her cooperation throughout his graduate work. He also thanks the rest of his family, Kay and Gertrude, for their understanding and support throughout his graduate career.

He acknowledges the financial assistance provided by the Montana Agricultural Experiment Station in the form of an assistantship. Without this assistance, graduate school would have been impossible for him.

WJK

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ABSTRACT

Sainfoin pollen collected in the greenhouse had lower in-vitro germination than alfalfa, alsike clover, birdsfoot trefoil, and cicer milkvetch. The addition of yeast extract to the media increased germination of greenhouse collected alfalfa pollen but had no effect on sainfoin. This addition increased germination of field produced sainfoin pollen. Sainfoin pollen collected in the field was affected by time of day collected and genotype but not by age of flower. In-vitro germination was not correlated to percent seed set, indicating pollen collected at any time of day could successfully be used for making artificial crosses. Stigma receptivity was affected by time of day with pollinations after 5:00 P.M. being most successful. Sainfoin flowers were emasculated by 5 or 10 seconds immersion of the staminal column in 47.5 percent alcohol and removing pollen by rubbing the stamens between the thumb and forefinger. The latter method was faster to apply and gave higher seed set following artificial pollinations.

Three white-flowered plants were different genetically since only one segregated upon selfing. The segregation observed fit a 3:1 ratio which did not differentiate between disomic and tetrasomic inheritance.

A plant heterozygous for the exposed stigma mutant was selfed and the progeny fit a 15 normal to 1 exposed stigma ratio. The trait may be controlled by two independent duplicate genetic factors. This ratio suggests that sainfoin is an allopolyploid.

Estimates of percent crossing varied under different planting arrangements. When a plant was surrounded by pollen parents in an open-pollinated nursery, over 90 percent crossing occurred, but in single-cross arrangements under bubble isolation 8 to 28 percent crossing took place.

A significant inbreeding depression was observed for plant weight under field conditions. In the greenhouse, inbreeding depressions were estimated for rooting ability of cuttings and plant height.

CHAPTER I

INTRODUCTION

Forage crops are utilized in the cattle and sheep industry as their basic source of feed. Much of the effort to improve forage crops has been on legumes, particularly alfalfa (Medicago sativa L.). This introduced species has become the most widely utilized forage legume in the United States. Alfalfa causes bloat and is subject to severe damage from the alfalfa weevil.

Sainfoin (Onobrychis viciaefolia Scop.) is a recently reintroduced forage legume which does not cause bloat and is not affected by the alfalfa weevil. Sainfoin has been successfully used as a pasture and hay legume in Europe for many years. It is becoming an acceptable substitute for alfalfa under some conditions in Montana and other Northern Rocky Mountain States.

Sainfoin is relatively shorter lived, more susceptible to root-rot diseases, and not as competitive with weeds as alfalfa. Forage yields of sainfoin need improvement, particularly under irrigation. Basic knowledge as to the mode of reproduction, genetics, and on techniques for controlled crossing are needed in sainfoin. To fulfill this need, a series of specific studies were undertaken.

1. Affect of prevailing environmental conditions, time of day collected, and genotypes on pollen viability were studied.

2. The relationship between in-vitro pollen germination and percent seed set was estimated.

3. Stigma receptivity as affected by time of day was investigated.

4. An effective technique to make controlled emasculated crosses was sought.

5. Genetic studies of the inheritance of flower color and the inheritance of exposed stigma in sainfoin were initiated. Determinations on whether sainfoin is genetically an auto- or allopolyploid were made.

6. Percent crossing under three different planting arrangements was estimated.

7. Inbreeding depressions resulting from self-pollination were estimated.

CHAPTER II

REVIEW OF LITERATURE

Description and History of Sainfoin

Sainfoin is a long lived, deep rooted perennial. Roots may extend 20 feet into the soil and be two inches in diameter with an extensive development of lateral roots. Leaves are pinnately compound with 13 to 15 leaflets per leaf. Flowers are usually dark pink in color and occur in an erect determinate raceme. Occasionally flowers will be white instead of pink (1, 53, 57).

Sainfoin seed is borne in a single seeded pod. The pods are indehiscent, bean shaped, bilaterally compressed, have a rough net-veined appearance, and are brown when mature. The seed is kidney shaped, yellowish green to dark brown in color, and is quite large for a forage legume. The seed is approximately nine times the size of alfalfa seed by weight (20, 57, 71).

Sainfoin has been cultivated in Europe and Russia for several centuries. In Europe, it was first cultivated in France and its culture was first described in 1629. It was grown in Germany during the 17th century and in Italy during the 18th century (57, 61).

Sainfoin has excellent forage quality, good drought, and cold tolerance, and does not cause bloat in ruminants when pastured. Europeans have reported a satisfactory yield and productive stands for as long as 20 years. Sainfoin led to the profitable cultivation of

dry calcareous land which had previously been nearly useless (1, 3, 4, 14, 41, 46, 48, 53, 57, 61).

To summarize the European and Russian literature, one would conclude that sainfoin has been successfully and profitably cultivated, as forage for livestock for several centuries. It produced an adequate amount of high quality forage under most conditions. When compared to alfalfa, its performance was variable. It produced less than, or more than, alfalfa, depending upon local conditions (1, 3, 10, 22, 46, 47, 48).

Sainfoin in North America

Sainfoin was first officially tested in the United States during the late 1800's and early 1900's. These early tests did not lead to wide acceptance of sainfoin in this country, probably because it was compared to alfalfa for hay production under conditions which were optimum for alfalfa. Also, inferior strains may have been used in the tests (20, 28, 34, 35).

In several Northwestern states and Canada, sainfoin is being evaluated and compared to alfalfa and other forage crops for hay and pasture production. For hay production, its relative performance was variable when compared to red clover, trefoil, and several grasses (18, 20, 26, 34, 55, 56, 58, 68). It performs best, relative to other forages, on dry, cobbly, calcareous soils (37, 57, 69). Under irrigation and on heavy moist soils, persistence was poor. Sainfoin

production declined markedly after two or three harvest years (18, 23). It is suspected that root and crown diseases may cause the decline (54). In Montana, Rhizobia strains have not been effective on sainfoin and sainfoin crops are often nitrogen deficient. This may make plants more susceptible to diseases and winter kill (13, 18, 23, 56, 59, 62).

Sainfoin lacks the ability to compete effectively with weeds. Good weed control is needed to enable sainfoin to become well established (64).

In feeding trials with beef cattle, sainfoin was equal to or superior to alfalfa hay when consumption, rate of gain, and digestibility were measured. Sainfoin hay is lower in crude protein than alfalfa hay but higher in nitrogen free extract (40, 69). Sainfoin is resistant to the alfalfa weevil and can be used as an alternate hay crop for alfalfa in heavily invested areas (18, 28).

Pasture trials with beef cattle and sheep indicate that sainfoin is an excellent non-bloating legume. Its palitability is unsurpassed by other forage crops, and it has adequate drought resistance and winter hardiness (23, 26, 34, 37, 49).

Sainfoin seed may be utilized as a protein source. Eski sainfoin has produced as much as 1,000 pounds of seed per acre. Seed yield can probably be increased through plant breeding. Hulless Eski sainfoin is about 35 percent protein, and the protein quality is good (25, 37).

Sainfoin seed production is good throughout Montana. The germination of sainfoin seed is good, but seed may begin to lose its viability after only one year in storage (17, 37, 39, 55, 57, 68, 71).

The greatest potential for sainfoin use is as a dryland pasture crop. It could be grown advantageously for hay in areas where alfalfa is not well suited (13, 56, 69).

There are several sainfoin breeding programs in the United States and Canada. Traits under consideration are persistence, quality, growth habit, disease resistance, seed yield, and forage yield. Genetic variation exists for all of these traits. Several varieties have been released in Montana and Canada. These varieties have all resulted from selection among and within adapted plant introductions. Basic knowledge regarding reproduction in sainfoin must be obtained before more sophisticated breeding can be done (16, 20, 21, 27, 34, 40, 69).

The culture of sainfoin is similar to that of other perennial legume forages, except in Montana it may benefit from nitrogen fertilization (16). It is seeded approximately one to two inches deep at 35 pounds per acre in pure stands on irrigated land and 26 pounds per acre on dryland. For hay, sainfoin should be harvested during the early bloom stage as quality decreases rapidly with increasing maturity (20, 39, 58, 59).

Pollen Physiology and Stigma Receptivity

Sainfoin pollen exhibits lower in-vitro germination and has shorter pollen tubes than alfalfa pollen. Boron was essential for in-vitro sainfoin pollen germination but even with boron germination percentages were 20 percent or less (31).

In-vitro pollen germination has been studied in other species (35, 50, 51, 70). Often these studies involved correlating in-vitro pollen germination with percent seed set or percent fertility of plants under field or greenhouse conditions. In general, the correlations between in-vitro pollen germination and percent seed set were low. Pollen with less than 5 percent germination often caused fertilization (50).

Artificial medias affect pollen germination and pollen tube elongation. Pollen of different species require different media for germination, some species of pollen require only water while others require chemical substances which are present in the stigmatic fluid (50).

Chemical substances which are known to enhance in-vitro pollen germination are sugar (usually sucrose), boron, calcium, and sometimes organic acids (8, 31, 38, 50, 51, 70). The function of these substances is not known. Evidence indicates that sucrose is more than an osmotic agent because for in-vitro germination of pollen from many species other sugars will not substitute for sucrose (8, 38, 50). When the

pollen tubes penetrate the style, an increase in carbohydrates occurs. Concentrations of 10 to 30 percent sucrose resulted in optimum germination of pollen collected from several species of Leguminosae (38, 50).

Pollen is often deficient in boron and in nature this deficiency is compensated for by high levels of boron in female flower parts. Possible functions of boron are to increase absorption and translocation of sugars, for synthesis of pectic materials and adenosine triphosphate activation (70). The effect of boron is specific since other elements will not substitute for it. Optimum germination of Leguminosae pollen has resulted from .010 to .015 percent boric acid in solutions of sucrose (50, 70).

During in-vitro pollen germination, a "population effect" has often been observed. This refers to the phenomenon of groups of pollen grains having higher germination than isolated pollen grains. Calcium alleviated the "population effect" in over 100 species. Some pollen requires the calcium ion for optimum germination, while other pollen requires it only for maximum tube elongation. Concentrations of .03 percent calcium carbonate in solutions of sucrose plus boron gave optimum pollen germination and tube elongation in many plant species (50, 51).

In-vitro germination of red clover pollen showed that amino acids, or yeast extract, enhanced pollen germination when added to a solution containing sucrose, boron, and calcium. The response was influenced by the environment in which the pollen producing plants

were grown (43, 44).

In-vitro pollen germination may be influenced by germinating media, age of pollen, nutrition of plants, and temperature before, during, and after anthesis. Pollen germination is dependent upon many factors, thus it is highly variable. Pollen germination may vary from flower to flower on the same plant (8, 38, 43, 44, 50, 51, 70).

Properties of the pistil influenced pollination and fertilization of Lotus spp. The stigmatic membrane must be ruptured before pollen germination will occur. The stigmatic fluid stimulates pollen germination. In some species, the membrane ruptured autonomously when ovules reached maturity; these species were highly self-fertile and self-pollination was common. Other species required insects (bees) to rupture the membrane before effective pollination would occur. This is a mechanism which enhances cross-pollination. When the bee visits a flower, the membrane is ruptured and pollen is deposited on the stigma. These species exhibited a marked inbreeding depression upon selfing (11, 60, 63).

Genetics

There are no reported genetic studies in sainfoin; however, some cytological work has been done. Most cultivated species of sainfoin are tetraploids with $2N = 28$ chromosomes and $X = 7$. One species, Onobrychis crista-galli L., has $2N = 16$ chromosomes with $X = 8$. There are also

several diploid species (21, 30). Evidence indicates O. viciaefolia Scop. is a tetraplois with $2N = 28$ chromosomes and $X = 7$. Sainfoin tetraploids are either autopolyploids or segmental allopolyploids. Upon the observation of chromosome pairing phenomenon at metaphase I of meiosis, primarily bivalents were formed with a few multivalents and univalents. The author concluded sainfoin was either an autopolyploid or an allopolyploid of the Primula kewensis type (30). An allopolyploid of the Primula kewensis type is derived from doubling the chromosomes of an F_1 hybrid where considerable chromosome pairing has occurred. An allopolyploid of the Primula kewensis type would be equivalent to a segmental allopolyploid. These reported cytologic findings give little information on what genetic segregation ratios to expect in sainfoin. The multivalent formation at metaphase I of meiosis suggests that there is some homology between genomes; however, studies are needed to establish whether sainfoin will exhibit disomic or tetrasomic inheritance for a specific trait (2, 30, 65).

White flower color and exposed style are rare mutants in sainfoin and have been proposed as genetic markers. Both traits were suspected to be inherited as simple recessives (19, 33). Type of first leaf (unifoliate, bifoliate, or trifoliate), and red seed coat have been suggested as genetic markers (16).

The inheritance of flower color has been studied in alfalfa and red clover. In both species, the inheritance is complex with several loci involved. Some loci (genes) seem to be inherited in an additive

manner and other genes influencing the same trait may show dominance and epistasis. The genes present in different combinations will result in different segregation ratios (5, 6, 12, 24, 66, 72). Alfalfa flower color may be purple, yellow, variegated, or white (5, 12, 24). In contrast to this, sainfoin flower color is either white or pink (16, 57).

The exposed style mutant has been reported and studied in alfalfa. Its inheritance was tetrasomic, but the homozygous recessive type was not found as often as expected. This deviation from expected was attributed to gametic selection against gametes carrying the recessive allele (6, 52).

Reliable genetic markers are needed to determine the mechanism of inheritance in sainfoin. Genetic markers would be useful in estimating gene exchange in unemasculated crosses using bees as pollinators (32, 33).

Mode of Reproduction and Inbreeding in Sainfoin

Early reports indicated sainfoin was almost completely cross-pollinated and highly self-sterile. Progeny resulting from self-pollination exhibited a marked inbreeding depression (7, 67). The conclusions on the mode of reproduction were reached by comparing percent seed set under conditions of open-pollination with bees, artificial self-pollination by hand, and no pollination (plants were isolated from insects). Under these conditions, it was found that 25 to 50 percent

of the open-pollinated flowers set seed, 5 percent of the self-pollinated flowers set seed, and 3 percent of the flowers receiving no pollination set seed (19, 29, 45, 67). If bees were used to self-pollinate sainfoin, the seed set was not significantly different from open-pollination (19, 33). A significant inbreeding depression for seedling vigor and fertility did not occur when S_0 and S_1 plants were compared. Gene exchange was not observed between single crosses made with bees without emasculation. These findings led the investigator to suggest the possibility that sainfoin is self-pollinated rather than cross-pollinated (19, 32, 33).

CHAPTER III

IN-VITRO POLLEN GERMINATION AND STIGMA RECEPTIVITY

Viability of Greenhouse Collected Pollen

Materials and Methods. In-vitro pollen germination studies were conducted using pollen collected in the greenhouse during 1970 and 1971. Germination media used was a solution of 20 percent sucrose, .03 percent calcium carbonate, and .01 percent boric acid. Agar was used in some studies instead of the liquid solution. The above described solution was selected after evaluating several solutions containing other levels of sucrose, calcium, and boron. Solutions tested contained 10, 20, 30, and 40 percent sucrose with two levels of calcium and two levels of boron. Calcium levels were .015 and .030 percent calcium carbonate. Boron levels were .005 and .010 percent boric acid.

Preliminary studies showed greenhouse collected sainfoin pollen to have poor in-vitro germination. In-vitro germination of sainfoin pollen was compared to that of alfalfa, cicer milkvetch, alsike clover, and birdsfoot trefoil pollen during April and May of 1970.

Pollen was obtained from freshly opened randomly selected flowers in the greenhouse and germinated in the liquid solution. The flowers were held above small plastic cups containing about three milliliters of the germinating media and tripped so pollen fell into the liquid. After four hours, at room temperature, germination percentages were determined by stirring the media and placing a drop of the fluid

on a slide, covering with a cover slip and observing under 10 X magnification. Percent germination was determined by counting 100 pollen grains in three different areas on each slide. Pollen was considered germinated if there was an observable germ tube.

In addition to studies using the germinating media previously described, greenhouse collected pollen of alfalfa and sainfoin was placed in the standard liquid media containing different levels of yeast in 1971. Clone A₂₀ was the sainfoin pollen source, while alfalfa pollen came from clone C₈₉. Levels of yeast used were 0, 250, 500, 1000, and 1500 ppm.

Results and Discussion. Sainfoin pollen had less than 3 percent germination; whereas, cicer milkvetch, birdsfoot trefoil, clover, and alfalfa pollens germinated in excess of 70 percent in the 1970 study (Table 1).

TABLE 1. IN-VITRO POLLEN GERMINATION FROM SEVERAL LEGUME SPECIES GROWN IN THE GREENHOUSE IN 1970

Species	Mean germination ^{1/} %
Sainfoin Clone 1	0 a
Sainfoin Clone 2	2.07 a
Birdsfoot trefoil	70.60 b
Alfalfa	78.10 c
Alsike Clover Clone 1	85.33 d
Alsike Clover Clone 2	89.19 d
Cicer milkvetch	90.03 d

^{1/}Means followed by the same letter are not different at p = .05
(Duncan's New Multiple Range Test)

When alfalfa and sainfoin pollen were put into solutions containing yeast in 1971, alfalfa pollen germination increased but sainfoin did not (Table 2).

TABLE 2. IN-VITRO POLLEN GERMINATION OF GREENHOUSE PRODUCED ALFALFA AND SAINFOIN POLLEN AT DIFFERENT LEVELS OF YEAST IN 1971

Species	Yeast added to media	Mean germination <u>1/</u>
	ppm	%
Sainfoin A ₂₀	0	.95 a
Sainfoin A ₂₀	1500	1.11 a
Sainfoin A ₂₀	0	2.86 a
Sainfoin A ₂₀	1000	3.33 a
Sainfoin A ₂₀	500	4.00 a
Sainfoin A ₂₀	250	8.10 a
Alfalfa C ₈₉	0	29.84 b
Alfalfa C ₈₉	1000	87.71 c
Alfalfa C ₈₉	250	93.81 d
Alfalfa C ₈₉	500	95.87 d

1/ Means followed by the same letter are not different at $p = .05$
(Duncan's New Multiple Range Test)

The reason for low germination of greenhouse produced sainfoin pollen is not known; however, it has been observed by Carleton (20) and myself that sainfoin plants do not grow well in the greenhouse.

Possibly the reduced vigor of sainfoin grown in pots in the greenhouse is associated with reduced pollen vigor and germination.

Clone A₂₀, which had poor in-vitro pollen germination in the greenhouse, set 42.1 percent seed upon selfing, and clone W₄ set 14.0 percent seed when pollinated with greenhouse collected pollen from A₂₀. In-vitro pollen germination was not closely associated with percent seed set. The artificial germinating media may not have contained a necessary factor or factors for a high pollen germination percentage. This factor, or factors, would be present in-vivo; thus, in-vitro germination would not be a good estimate of in-vivo germination. Also, when a stigma is pollinated, so much pollen is placed on it that pollen with low germination can affect fertilization. I suspect the latter factor to be of greatest importance as it was observed, in artificial pollinations, that the amount of pollen placed on the stigma was very important in obtaining satisfactory seed set.

Viability of Field Collected Pollen

Materials and Methods. The influence of time of day collected on viability was studied with pollen collected from randomly selected field grown sainfoin plants in July of 1970. Pollen was collected from three flowers at 8:00 A.M., 11:00 A.M., 2:00 P.M., and 5:00 P.M. Care was taken to select flowers that had opened that morning. Pollen from each flower at each collection time was germinated in media consisting

of the solution previously described plus agar.

The effect of flower age on pollen viability was determined in July of 1971. Ten freshly opened flowers and ten flowers one, two and three days old were selected at random from five plants. The flowers within each age group from the five plants were mixed and pollen was taken from this mixture. Pollen was placed in plastic cups containing the germinating media previously described. Percent germination was determined as previously described for the study involving greenhouse produced pollen.

In addition to the effect of age of flower on pollen viability, the effect of two levels of yeast (500 and 1,000 ppm) and one level of alanine plus glycine was tested. The amino acids were both present in the standard solution at 100 ppm each. These additional treatments were applied to pollen of freshly opened flowers in an effort to determine if adding yeast or amino acids to the standard germinating media would enhance the germination of field produced sainfoin pollen.

Results and Discussion. Pollen collected at 8:00 A.M. had 29.8 percent germination, and this was higher than the other three collection times (Table 3, page 18). Pollen collected later in the day had 23 to 24 percent germination, and this would probably affect fertilization after artificial pollination as effectively as would pollen having 29 percent in-vitro germination.

TABLE 3. PERCENT GERMINATION OF POLLEN COLLECTED AT DIFFERENT TIMES OF DAY IN THE FIELD DURING 1970

Times	Percent of germination ^{1/}
8:00 A.M.	29.83 b
11:00 A.M.	24.17 a
2:00 P.M.	23.13 a
5:00 P.M.	23.75 a

^{1/} Means followed by the same letter are not different at $p = .05$ (Duncan's New Multiple Range Test)

Pollen from individual sainfoin plants had 21.1 to 32.5 percent germination (Table 4). Although this represented a significant difference, any of the clones would be satisfactory as pollen parents for controlled pollinations.

TABLE 4. PERCENT GERMINATION OF FIELD COLLECTED POLLEN FROM EIGHT CLONES, ON JULY 18, 1970

Clones	Percent of germination ^{1/}
2	21.10 a
5	21.50 a
1	21.83 a
6	22.23 a

Table 4 (Continued)

Clones	Percent of germination ^{1/}
3	25.57 ab
8	27.50 ab
7	29.50 b
4	32.50 b

^{1/} Means followed by the same letter are not different at $p = .05$
(Duncan's New Multiple Range Test)

Flower age did not significantly affect pollen viability (Table 5, page 20). Pollen from flowers of any age can be used for pollinations prior to wilting of the flower. Sainfoin flowers will wilt within three or four days after opening even though they are not pollinated. The addition of yeast to the germinating media did enhance germination of the field collected pollen (Table 5).

The field collected pollen was germinated on the agar media in 1970 and had 20 to 30 percent germination. This was much higher germination than exhibited by greenhouse collected pollen germinated in liquid media in 1970. The use of agar instead of liquid media may have caused this difference. It was determined in 1971 that there was no difference between germinating medias for field and greenhouse collected pollen.

TABLE 5. PERCENT GERMINATION OF FIELD PRODUCED POLLEN TAKEN FROM FLOWERS OF DIFFERENT AGE, AND POLLEN TREATED WITH TWO LEVELS OF YEAST AND ONE LEVEL OF ALANINE PLUS GLYCINE

Age of pollen	Pollen treatment	$\bar{X}$ % germination ^{1/}
New pollen	None	5.08 ab
1 day old pollen	None	5.91 ab
2 day old pollen	None	4.13 a
3 day old pollen	None	3.01 a
	500 ppm yeast	14.14 c
	1000 ppm yeast	10.69 bc
	100 ppm glycine plus 100 ppm alanine	8.36 abc

^{1/} Means followed by the same letter are not different at $p = .05$ (Duncan's New Multiple Range Test)

Relationship Between In-vitro Pollen Germination and Percent Seed Set

Materials and Methods. During the summer of 1970, nine clones representing a range of self-fertility were selected. Self-fertility was determined after selfing flowers by hand while cross-fertility was determined after open-pollination of flowers. Pollen was taken from randomly selected flowers of these nine clones and germinated on agar media. The percent pollen germination of these clones was correlated to their mean percent self- and cross-fertility which had been determined in 1968.

In another study, five clones were selected in 1971. Pollen from these clones was germinated in the liquid culture as previously described. These mean percent germination values were then correlated to percent self- and cross-fertility of the clones determined in 1971. The self- and cross-fertility values were obtained following self- and cross-pollinations made by hand.

Results and Discussion. Correlation coefficients for self- and cross-fertility and in-vitro pollen germination in 1970 were .01 and .44, respectively. Neither of these correlations were significant at $p = .05$ (Table 6).

TABLE 6. MEANS FOR IN-VITRO POLLEN GERMINATION DETERMINED IN 1970, AND SELF- AND CROSS-FERTILITY DETERMINED IN 1968

Clones	Self-fertility ^{3/}	Cross-fertility ^{2/}	Pollen germination ^{1/}
	%	%	%
S ₃₉	26	52	17.17 a
S ₂₆	24	67	33.83 c
S ₄₁	20	67	16.83 a
S ₁₃	11	69	24.50 ab
S ₃₅	13	70	37.33 c
S ₂₄	12	69	30.17 a
S ₇	4	37	17.17 a
S ₄₄	3	35	26.33 b
S ₄₀	2	35	23.50 ab

^{1/} Means followed by the same letter are not different at $p = .05$ (Duncan's New Multiple Range Test)

^{2/} $r = .44$ for cross-fertility and in-vitro pollen germination. n.s.

^{3/} $r = .01$ for self-fertility and in-vitro pollen germination. n.s.

The correlation coefficients .85 and .65, for cross- and self-fertility, respectively, and in-vitro pollen germination in the 1971 study were non-significant (Table 7). In-vitro pollen germination is not very useful in predicting plant fertility.

TABLE 7. MEANS FOR IN-VITRO POLLEN GERMINATION AND SELF- AND CROSS-FERTILITY IN 1971

Clones	Self-fertility ^{3/}	Cross-fertility ^{2/}	Pollen germination ^{1/}
	%	%	%
W ₁	4.99 a	3.68 a	.67 a
U ₇	8.34 a	12.16 b	10.00 bc
U ₁₃	10.53 a	18.00 c	7.00 abc
W ₃	15.04 a	10.63 b	3.33 ab
W ₄	18.66 a	25.25 d	12.00 c

^{1/} Means followed by the same letter are not different at $p = .05$ (Duncan's New Multiple Range Test)

^{2/} $r = .85$ for cross-fertility and in-vitro percent pollen germination. n.s.

^{3/} $r = .65$ for self-fertility and in-vitro percent pollen germination. n.s.

Stigma Receptivity as Influenced by Time of Day

Materials and Methods. Five clones were selected which were known to represent a range from low to high in fertility. Each of these clones was represented by a row of four space planted cuttings. One

plant of each clone was uncovered from 8:00 A.M. to 11:00 A.M., one was uncovered from 11:00 A.M. to 2:00 P.M., one was uncovered from 2:00 P.M. to 5:00 P.M., and one was uncovered from 5:00 P.M. to 8:00 A.M. the following day. In this way, one plant of each clone was exposed to bee activity for a constant time interval each day at four different times of the day. At the beginning of the study, ten racemes on each plant were tagged. The plants were covered and uncovered at the specified times each day until all flowers of each raceme opened. Percent seed set was then calculated for each raceme, of each plant, by dividing the number of seeds on a raceme by the number of flowers.

Results and Discussion. A significant difference in percent seed set was found for clones (Table 8). The clones had been selected to represent a range in fertility so they were expected to be different. Each clonal mean was significantly different from other means.

TABLE 8. MEAN PERCENT SEED SET FOR FIVE CLONES SELECTED TO REPRESENT A RANGE IN FERTILITY

Clone	Seed set ^{1/}
	%
S ₁₉	44.9 e
S ₂₄	32.6 d
S ₅₄	24.2 c
S ₄₀	16.9 b
S ₂	8.2 a

^{1/} Means followed by the same letter are not significantly different at $p = .05$ (Duncan's New Multiple Range Test)

Percent seed set for the 5:00 P.M. exposure interval was significantly higher than that at 8:00 A.M., 11:00 A.M., or 2:00 P.M., and there was no significant difference between the 8:00, 11:00, or 2:00 P.M. exposure times (Table 9). Bee activity was observed to subside greatly after 8:00 P.M. so a longer exposure to bees did not cause the higher seed set at the 5:00 P.M. treatment.

TABLE 9. OPEN-POLLINATED SEED SET AT FOUR DAILY EXPOSURE INTERVALS

Time	Seed set ^{1/}
	%
8:00 A.M. - 11:00 A.M.	21.80 a
11:00 A.M. - 2:00 P.M.	23.90 a
2:00 P.M. - 5:00 P.M.	19.80 a
5:00 P.M. - 8:00 A.M.	36.10 b

^{1/}Means followed by the same letter are not significantly different at $p = .05$ (Duncan's New Multiple Range Test)

Flowers of a raceme do not all open in one day. Anthesis progresses upward on sainfoin racemes and flowers continue to open all day long (Table 10, page 24). If ovaries of open flowers became over mature and began to degenerate by the following morning, pollinations made late in the day would have involved a higher percentage of freshly opened flowers than pollination made earlier in the day. Bubar has

established that ovaries begin to disintegrate within a few hours after anthesis in Lotus spp. (11).

TABLE 10. NUMBER OF FLOWERS OPENING ON DIFFERENT RACEMES DURING A.M. AND P.M. FOR DIFFERENT SAINFOIN CLONES ON CLEAR DAYS AT BOZEMAN

Clone	Day	Number of flowers opening Two raceme total		
		A.M.	P.M.	Total
U ₁₃	6-30-71	3	7	10
U ₁₃	7-1-71 <u>1/</u>	5	13	18
U ₁₃	7-8-71	6	6	12
W ₁	6-30-71	8	14	22
U ₇	7-1-71	6	12	18
W ₃	7-8-71	7	11	18
W ₄	7-8-71	7	8	15

1/ Only one raceme, values doubled

Conclusions

1. Sainfoin pollen collected in the greenhouse does not germinate in-vitro as well as some other legumes in the same media.

2. Sainfoin pollen collected at any time of day, from any genotype, from flowers of any age, will probably function equally in artificial pollinations.

3. In-vitro germination percentage for sainfoin pollen is not always indicative of its ability to effect fertilization.

4. It may be advantageous to make artificial pollinations after 5:00 P.M. if possible.

CHAPTER IV

ARTIFICIAL POLLINATION IN SAINFOIN

Materials and Methods

Genetic studies require absolute control of pollination. A technique to emasculate and artificially pollinate sainfoin plants has not been described. Two emasculation techniques were used.

The standard petal was removed from the flower with forceps after anthesis. The keel was then pushed away from the staminal column so that stamens, stigma, and style were exposed. Flowers of some racemes were then dipped into a solution of 47.5 percent alcohol for five seconds and rinsed in water and some were immersed for 10 seconds followed by rinsing. Pollen from a selected source was applied to stigmas that had dried for 10 to 15 minutes. Pollen was collected on a fingernail by tripping flowers onto it; and stigmas were dipped into this pollen. A control, flowers emasculated but not pollinated, was included. Four clones were used for the 1970 study. Plants were caged to prevent insect cross pollination.

A second treatment (the "pinch method") consisted of removing pollen from open flowers by rubbing stamens between the thumb and forefinger. This is possible in sainfoin because the staminal column will protrude when pressure is applied to the base of the keel. To avoid self-pollination, the remainder of the raceme possessing unopened flowers was removed. Artificial pollinations were made as

previously described. One control group was emasculated as described but not pollinated and another control received no emasculaton or pollination. Four and five clones were used for the greenhouse and field studies, respectively.

Results and Discussion

The five-second alcohol treatment resulted in adequate emasculaton as flowers receiving this treatment had only 0.5 percent seed set without artificial pollination. The 10 second alcohol treatment resulted in 2.7 percent seed set when followed by artificial pollination. This was significantly different from the 11.4 percent seed set following the five second alcohol treatment (Table 11). The 10 seconds in the alcohol solution apparently damaged the stigmatic tissue more than did the five second immersion.

TABLE 11. MEAN PERCENT SEED SET FOLLOWING ALCOHOL EMASCULATION TREATMENTS AND ARTIFICIAL CROSS-POLLINATION IN THE FIELD DURING 1970

Treatment		Time immersed in alcohol seconds	Number of flowers treated	Percent flowers forming pods
Emasculaton	Pollination			
yes	yes	10	614	2.73
yes	yes	5	235	11.35 ^{1/}
no	no	5	218	0.46

^{1/} Percent seed set for the five second treatment was significantly higher than that for the 10 second treatment at $p = .05$

The five second alcohol treatment resulted in adequate emasculation and still allowed for enough seed set to conduct genetic studies; however, the alcohol treatment was slow to apply. A more rapid means to accomplish emasculation was needed.

The flowers in the greenhouse set 15.4 percent seed following artificial pollination of flowers emasculated by the pinch method. The pinch method of emasculation was 100 percent effective. Untreated flowers had 2.6 percent seed set (Table 12). The pinch method resulted in effective emasculation and still allowed for adequate seed set following controlled pollinations, and it was much faster to apply than the alcohol treatments.

TABLE 12. MEAN PERCENT SEED SET FOLLOWING "PINCH" EMASCULATION AND ARTIFICIAL POLLINATION IN THE GREENHOUSE DURING 1971.

Treatment		Number of flowers treated	Percent flowers forming pods
Emasculation	Pollination		
yes	yes	837	15.41
yes	no	175	0
no	no	353	2.55

The pinch method, when used in the field during 1971, resulted in effective emasculation. Flowers emasculated but not pollinated set 1.1 percent seed. Flowers receiving no treatment set 2.0 percent and

flowers receiving cross-pollination after emasculation set 14.8 percent seed (Table 13). The maximum amount of contamination that could have been expected to occur was about 7 percent. If a mass of foreign pollen is applied to a stigma, the amount of contamination resulting from selfing may be reduced.

TABLE 13. MEAN PERCENT SEED SET FOLLOWING PINCH EMASCULATION AND ARTIFICIAL CROSS-POLLINATION IN THE FIELD DURING 1971

Emasculation	Type of pollen applied	Number of flowers treated	Percent flowers forming pods
yes	cross	1984	14.77
yes	none	281	1.07
no	none	397	2.02

The percent seed set after emasculation could probably be increased if bees were used for pollination. Honey bees are better pollinators because they probably apply more pollen directly to the end of the stigma than I did. If a stigmatic membrane is present, bees may be more adept at rupturing it. Bubar has reported such a requirement in other legume species (11).

The honey bee lands on sainfoin flowers between the keel and standard petals. It lands with its head at the base of the keel and its abdomen will be at the tip of the keel; this forces the end of

the stigma directly into the pollen load on the underside of the bee. The bee will then move its legs in a kicking motion and move its abdomen from side to side for several seconds prior to leaving the flower.

Plants used in the artificial crosses were different in effectiveness as females but not as males (Table 14 below and Table 15, page 32). These same five clones differed significantly in their in-vitro pollen germination percentages (Table 7, page 22).

TABLE 14. PERCENT SEED SET BY FIVE SAINFOIN FEMALE PARENTS FOLLOWING PINCH EMASCULATION AND CROSS-POLLINATION IN 1971

Clone	Percent seed set ¹ / ₁	Number of pollen parents
W ₁	3.68 a	4
W ₃	10.63 b	4
U ₇	12.16 b	4
U ₁₃	18.00 c	3
W ₄	25.25 d	3

¹/ Means followed by the same letter were not different at $p = .05$ (Duncan's New Multiple Range Test)

TABLE 15. PERCENT SEED SET WHEN FIVE SAINFOIN CLONES WERE USED AS POLLEN PARENTS FOR ARTIFICIAL CROSSES

Clone	Percent seed set <u>1/</u>	Number of female parents
U ₇	12.60	3
U ₁₃	13.02	3
W ₄	13.43	4
W ₁	14.22	4
W ₃	15.57	4

1/ These means were not different from each other at $p = .05$

Conclusion

The best emasculation technique used in this investigation was the "pinch" method.

CHAPTER V

GENETICS

Materials and Methods

Three white-flowered clones designated W_1 , W_3 , and W_4 and two pink-flowered clones designated U_7 and U_{13} were selfed and the progenies were classified for flower color. Clones W_1 , W_4 , U_7 , and U_{13} were selfed in 1969 in isolation by honey bees. Clone W_3 was selfed by hand manipulation of flowers in 1967. Selfed seed of these clones was planted in the field during 1970, and plants were classified for flower color.

Clones W_3 and W_4 were reciprocally crossed to U_7 and U_{13} in 1970. Resulting progenies were grown in the greenhouse and classified for flower color in 1971. During 1971, the three white-flowered clones were crossed in all possible combinations and reciprocally crossed to U_7 and U_{13} .

Flowers of clone W_4 are white if contrasted to normal dark pink flowers, but they are not pure white and appear light pink when contrasted to the other white-flowered clones. Flowers of W_3 have a white keel and standard which has prominent pink veins. Flowers of W_1 have both a white keel and standard. Veins are faintly colored only on the basal one-half of the standard. Flowers of clones U_7 and U_{13} are of the normal dark pink color.

Clone T₉, a plant known to be heterozygous for the exposed stigma mutant, was selfed and resulting seed was planted in the field in 1970. This progeny was classified in 1971 for exposed stigma.

Results and Discussion

Clone W₄, upon selfing, segregated for flower color. Twenty-six plants of 136 had very white flowers which were similar to the flowers of clone W₁. The remaining 110 plants had light pink flowers like the mother clone. This ratio fit a 3:1 at $p = .100 - .250$. Light pink was dominant to white.

This 3:1 ratio does not allow for differentiation between disomic and tetrasomic inheritance. With tetrasomic inheritance and random chromosome assortment, the simplex condition (Aaaa) will give a 3:1 segregation ratio upon selfing which is the same ratio that is expected with disomic inheritance (2, 9).

Three plants of cross W₄ X U₇ and three plants which resulted from U₇ X W₄ bloomed. Reciprocal differences were not observed; therefore, the progenies were the result of crossing and not selfing. Flower color of all progeny was a little lighter than that of the dark pink parent. To determine whether dominance or incomplete dominance was being expressed, one would want to evaluate more F₁ progeny under field conditions.

One progeny from the cross W₄ X U₁₃ bloomed in 1971. This

plant had lighter pink flowers than U_{13} and were identical to $W_4 \times U_7$ progeny.

The 33 selfed progenies from W_3 did not segregate for flower color and had flowers like the mother clone. The 40 selfed progenies from W_1 did not segregate for flower color, and flowers were like those of W_1 . The number of plants classified was relatively small, but both W_3 and W_1 appeared to be homozygous for flower color. Progenies from crossing W_3 to U_{13} and U_7 in 1970 did not bloom in 1971.

When clones U_7 and U_{13} were selfed, no segregation occurred for flower color. Forty-five and 26 S_1 progenies of U_7 and U_{13} were examined respectively. This progeny number is small when determining inheritance in a tetraploid, but data suggests that the pink-flowered clones are homozygous for flower color.

Twenty out of 220 S_1 progeny from T_9 had the exposed stigma trait, and the remaining 200 were normal. These data fit a 15:1 ratio at $p = .10$ to $.05$. This ratio can be produced by two independently segregating duplicate genetic factors and is often observed in allopolyploids.

Conclusions

1. White flower color and exposed stigma are heritable and can be used as genetic markers in sainfoin.
2. White flower color and exposed stigma are recessive to pink

flowers and normal stigmas.

3. Sainfoin appears to be an allopolyploid.

CHAPTER VI

PERCENT CROSSING IN THE FIELD AND UNDER BUBBLE ISOLATION

Top-Cross and Line-Cross Progeny

Materials and Methods. In 1969, seed was harvested from four multicut sainfoin lines which had been top-crossed to Eski, a single-cut type. Each multicut line had been planted between two rows of Eski in the field at Bozeman in 1967. Rows were 20 feet long and on one foot centers. Pollination was by bees under field conditions.

Seed from the multicut lines was designated as top-cross progeny. This seed, remnant multicut line (line-cross progeny) and Eski seed were planted in the field in 1970. Single row entries were evaluated for the ability to bloom the year of establishment, for early spring growth, and regrowth after cutting in 1971.

The percentage bloom was determined as the percentage of plants which bloomed in each row. Early spring growth was determined from two samples on May 20, 1971. Twenty culms were then randomly taken from each sample and weighed individually. Weights were expressed as grams per culm. Regrowth following first cutting was measured from three samples on August 27, 1971. These samples were then dried and weighed. Weights were expressed as grams per sample.

Results and Discussion

Eski differed from multicut lines in percentage bloom the year of establishment, early spring growth and regrowth following first harvest.

When the top-cross and line-cross progeny were compared for the traits measured, considerable crossing between Eski and the multicut lines was evident (Figure 1, page 39; Fig. 2, page 40; and Fig. 3, page 41). Only the regrowth trait showed significant differences between top-cross and line-cross progeny. The other two traits had similar trends but line-cross and top-cross progeny did not differ significantly.

The mechanism of inheritance of the traits is not known; therefore, it is not possible to determine the exact percent crossing. Had the percent crossing been known, the mechanism of inheritance of the traits could have been determined. Since line-cross and top-cross progeny differed, some crossing occurred.

The differences between top-cross and line-cross progeny varied for individual lines. The top-cross progeny ranged from equal to the midparent to almost equal the high parent (Figure 1, page 39). The midparent is defined as the average of the two parents. Progenies were subject to sampling errors in that each row may be contained too few progeny to adequately sample the population. Also, the trait may have been affected by the environment. The average values of the top-cross and line-cross progeny was considered to be better evidence for

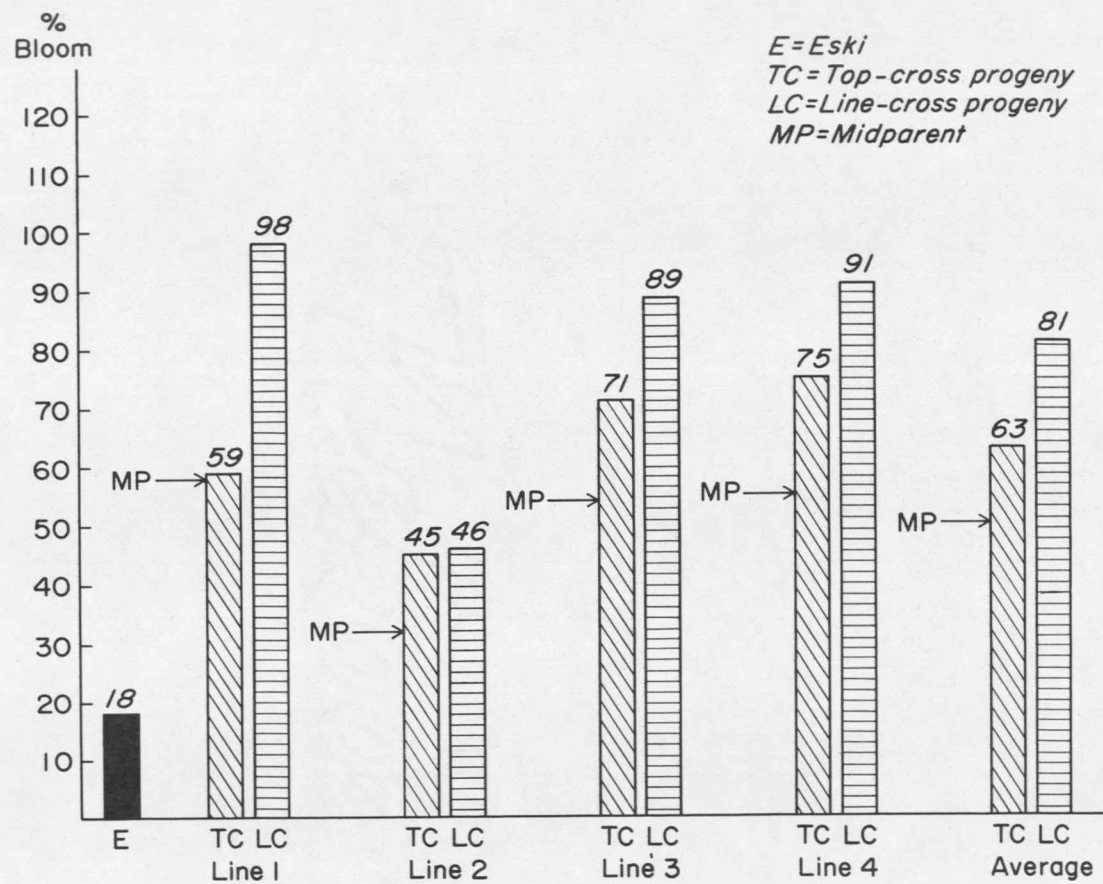


FIGURE 1. PERCENT BLOOM THE YEAR OF ESTABLISHMENT FOR ESKI, TOP-CROSS AND LINE-CROSS PROGENY, AND THEIR RESPECTIVE MIDPARENTS

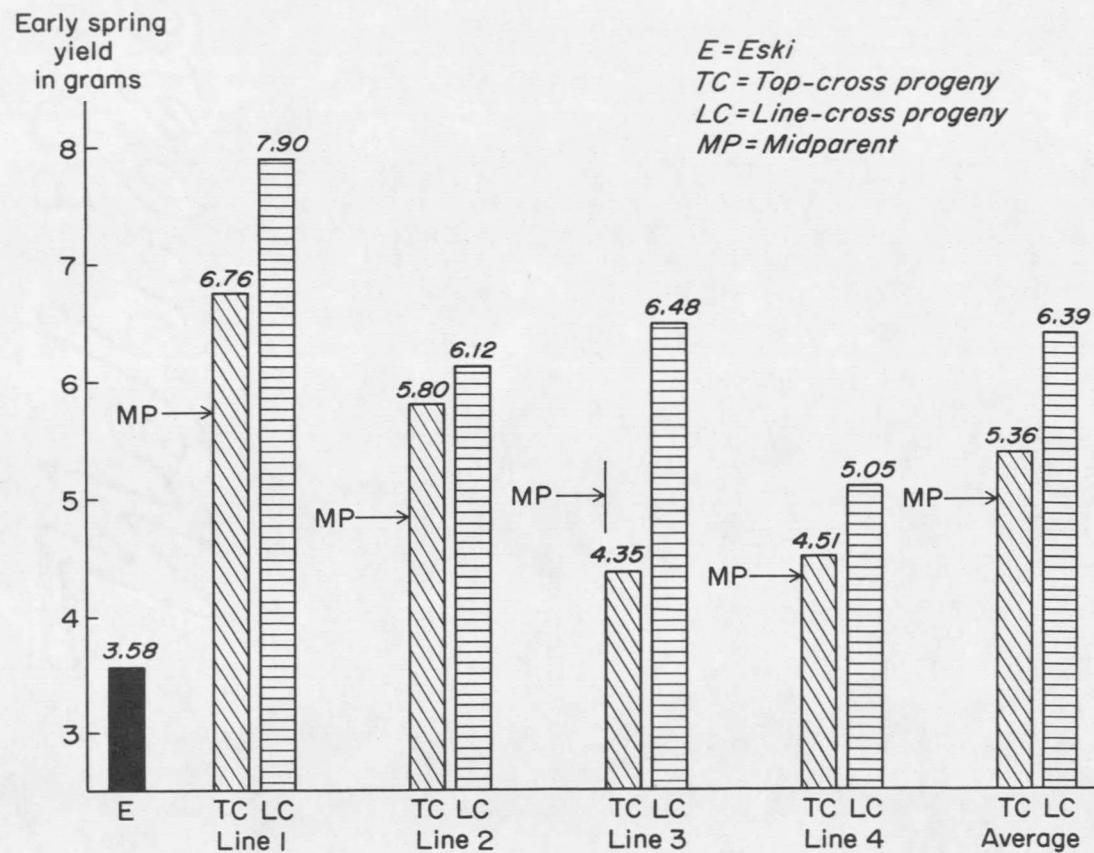


FIGURE 2. EARLY SPRING GROWTH IN GRAMS PER CULM FOR ESKI, TOP-CROSS AND LINE-CROSS PROGENY AND THEIR RESPECTIVE MIDPARENTS

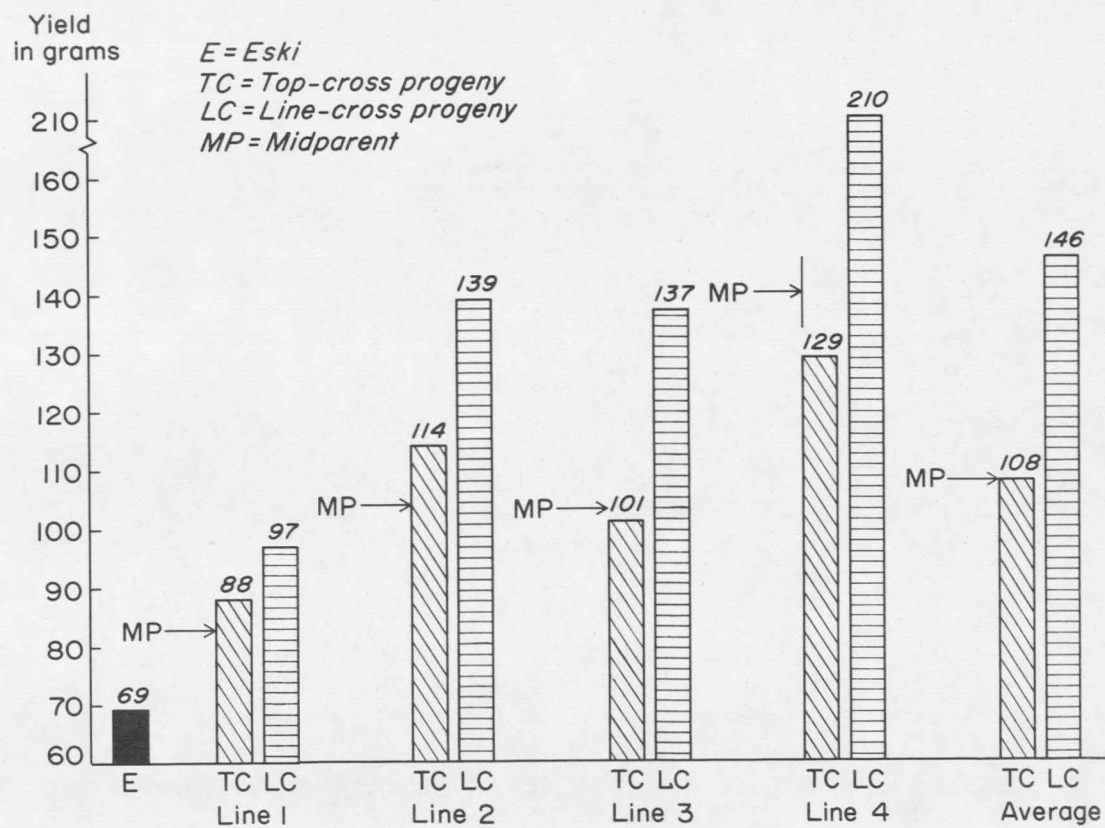


FIGURE 3. REGROWTH IN GRAMS PER SAMPLE OF ESKI, TOP-CROSS AND LINE-CROSS PROGENY AND THEIR RESPECTIVE MIDPARENTS

crossing than any of the lines individually.

Percent Crossing in an Open-pollinated Nursery

Materials and Methods. Seed was harvested from three white-flowered plants which were surrounded by rows of pink-flowered plants. Each white-flowered plant was about two feet, in all directions, from a row of pink-flowered plants.

Seed was harvested from the white-flowered plants in 1969 and seeded in rows in 1970. The progeny were classified for flower color in 1971.

Results and Discussion. Progeny from the three white-flowered plants had 91, 91, and 97 percent pink flowers (Table 16, page 43). These three plants had produced only white-flowered plants upon selfing. Under the planting arrangement described, 91 to 97 percent cross-pollination occurred. Although the maternal plants were about two feet from the pollen parents, they were quite large and lodged, so racemes of the white-flowered maternal plants were intermixed with racemes of the pink-flowered pollen plants.

Percent Crossing Under Bubbles

Materials and Methods. Four single crosses involving white- and pink-flowered clones were made. Each single-cross consisted of two

TABLE 16. PINK-FLOWERED PROGENY PRODUCED BY WHITE-FLOWERED MATERNAL CLONES WHEN SURROUNDED BY PINK-FLOWERED PLANTS UNDER FIELD CONDITIONS

Clone	Number of progeny observed	Number of white-flowered progeny	Number of pink-flowered progeny	Percent pink-flowered progeny
W ₃	37	1	36	97
W ₄	47	4	43	91
W ₆	56	5	51	91

cuttings of one white-flowered clone and two cuttings of a pink-flowered clone. The plants were spaced about three feet apart with both cuttings of each clone in the same row. Seed was harvested from the white-flowered clones and planted in rows in 1970. The progenies were classified for flower color in 1971. The two white-flowered clones were W₁ and W₃. The pink-flowered clones were T₁₀, T₉, and U₇.

Results and Discussion. The percentage pink-flowered progenies was 28, 27, and 12 percent when W₃ was placed in isolation with T₁₀, T₉, and U₇, respectively. Eight percent of the progeny from cross W₁ X T₁₀ had pink flowers (Table 17, page 44).

Clone T₁₀ bloomed later than did W₃, but both T₉ and U₇ bloomed at the same time as W₃ so differential blooming did not cause the large amount of selfing observed in all of these single-crosses.

TABLE 17. PINK-FLOWERED PROGENY PRODUCED BY WHITE-FLOWERED MATERNAL CLONES WHEN FUNCTIONING IN SINGLE CROSSES WITH PINK-FLOWERED CLONES UNDER BUBBLES

Cross	Number of progeny	Number of white flowered progeny	Number of pink flowered progeny	Percent pink flowered progeny
$W_3 \times T_{10}$	116	84	32	28
$W_3 \times T_9$	83	73	10	12
$W_3 \times U_7$	51	37	14	27
$W_1 \times T_{10}$	76	70	6	8

In the case of $W_1 \times T_{10}$, the differential blooming may have caused the large amount of selfing which occurred. Another possible cause for the large amount of selfing occurring in all the single crosses was bee movement. Honey bees will work all flowers in a vicinity before leaving. Under the space planted conditions in this study, plants did not lodge and all flowers in close proximity tended to be from a single plant. Thus, when the bee worked the first two or three flowers on a plant; it had mostly cross-pollen on its body but after that it carried predominantly self pollen to the remainder of the flowers. Without an effective incompatibility system, this would lead to a large amount of self-fertilization. This would explain the findings of Carleton and Haaland who failed to obtain gene exchange

in single crosses made as described (19, 32). This same problem of selfing among single-crosses of alfalfa has been described (15, 36, 42).

Conclusions

Proximity and number of pollen parents in relation to the female parent are the primary factors determining crossing percentage in sainfoin.

CHAPTER VII

INBREEDING

Materials and Methods

Five cuttings of 10 clones were space planted in rows in the field at Bozeman during 1968. In each row beyond the five cuttings, 10 S_1 progeny of the clone were space planted. All plants were three feet apart and there were three feet between rows.

During August, 1971, the surviving plants of each mother clone and the first five S_1 plants were cut and weighed individually.

One-hundred cuttings were taken from S_0 , S_1 , and S_2 plants of five clones in April of 1971. S_0 plants were vegetative cuttings taken from each clone. S_1 and S_2 plants were from self-pollination of S_0 and S_2 plants, respectively. Twenty cuttings were taken from each clone and mixed. One-hundred cuttings were randomly taken from this mixture. S_2 progeny were sampled in the same way. The cuttings were placed in a rooting bench and after three weeks scored for rooting ability. A cutting was considered rooted if it had a root one-fourth ($\frac{1}{4}$) inch long. Data were expressed as percentage rooted.

Rooted S_0 , S_1 , and S_2 plants were transplanted in rows into a greenhouse bench. Plants were fertilized weekly with a complete nutrient solution. After three weeks, each plant was cut off at ground level and plant height was measured to the nearest one-fourth inch. Measurements were made from the base of the plant to the

furthermost leaf tip on the main stem.

Results and Discussion

Inbreeding depression for plant weight exhibited by the field planted material ranged from 14 to 64 percent, with an average of 39 percent (Table 18). Inbreeding also reduced rooting ability of cuttings.

TABLE 18. MEAN FORAGE YIELD IN OUNCES PER PLANT OF S_0 AND S_1 PROGENY GROWN IN THE FIELD AT BOZEMAN

Clone	S_0	S_1	$\bar{d}$	Percent reduction $S_0 - S_1$
S_{14}	23.20	20.00	3.20	14
S_2	26.20	18.20	8.00	31
S_{52}	26.40	11.00	15.40	58
S_{13}	29.00	18.00	11.00	38
S_{40}	33.00	15.00	18.00	55
S_{54}	33.25	30.80	12.45	37
S_7	33.75	25.20	8.55	25
S_{39}	34.00	27.50	6.50	19
S_{44}	38.80	14.00	24.80	64
S_{41}	40.75	23.00	17.75	44
Mean	31.84	19.27	12.57 ^{1/}	39

^{1/} This mean difference was significant at $p = .05$

Seventy-nine percent of S_0 cuttings rooted compared to 33 and 22 percent for S_1 and S_2 cuttings, respectively (Table 19). S_2 plants used in the study were one year old and more vigorous than the older S_0 and S_1 plants. In spite of this, cuttings taken from the S_0 plants rooted much better than cuttings taken from S_2 plants. S_2 progeny of some clones performed better than did the respective S_1 progeny.

TABLE 19. MEAN PERCENT ROOTING OF CUTTINGS TAKEN FROM S_0 , S_1 AND S_2 PROGENY OF FIVE CLONES AT BOZEMAN IN 1971 ^{1/}

Clone	Percent of plants rooting		Percent reduction $S_0 - S_1$	S_2	Percent reduction $S_0 - S_2$
	S_0	S_1			
U ₁₃	95	25	79	36	62
S ₃₅	85	30	65	4	95
W ₁	74	61	18	18	76
T ₁₀	72	27	63	12	83
U ₇	69	22	68	41	41
Mean	79.0 a	33.0 b	58.6	22.2 b	71.4

^{1/} Means followed by the same letter are not different at $p = .05$ (Duncan's New Multiple Range Test)

Prognies exhibited significant inbreeding depression for plant height. The inbreeding depression for this trait ranged from 4 to 55

percent from S_0 to S_1 progeny with an average of 28 percent (Table 20). From S_0 to S_2 , the average reduction was 35 percent. S_2 progeny of U_7 exhibited a 16 percent increase in plant height over the respective S_0 progeny. The other three clones had 15 to 62 percent reduction from S_0 to S_2 progeny. S_2 progeny of clone S_{35} was not obtained due to poor rooting. This clone exhibited only 4 percent reduction from S_0 to S_1 . Of the five clones involved, these two had the weakest S_0 plants and they exhibited the least amount of inbreeding depression (Table 20).

TABLE 20. MEAN PLANT HEIGHT OF S_0 , S_1 , AND S_2 PROGENY OF FIVE CLONES GROWN IN THE GREENHOUSE AT BOZEMAN DURING 1971 ^{1/}

Clone	S_0 inches	S_1 inches	Percent reduction $S_0 - S_1$	S_2 inches	Percent change $S_0 - S_1$
U_{13}	12.19	5.46	55	4.64	-62
S_{35}	9.21	8.81	4	--	--
W_1	9.15	6.71	27	4.03	-56
T_{10}	8.16	6.39	22	6.97	-15
U_7	7.14	5.75	19	8.25	+16
Mean	9.17 a	6.63 b	28	5.97 b	-35

^{1/} Means followed by the same letter are not significantly different at $p = .05$

Conclusions

1. Sainfoin will have an inbreeding depression upon self-fertilization.
2. When producing synthetic varieties, a sufficient number of parental clones must be used to prevent excessive sib-mating among Syn. one, two, and three progeny.

CHAPTER VIII

SUMMARY AND CONCLUSIONS

To facilitate control of pollination in sainfoin, basic features of pollen and stigmas were studied. Different emasculation techniques were compared and a method to artificially cross sainfoin was developed. Genetic studies were initiated on flower color and an exposed style mutant. Percent crossing was estimated among sainfoin genotypes under three different planting arrangements. Inbreeding depressions were estimated. Results and conclusions from these studies were:

1. Alfalfa, cicer milkvetch, birdsfoot trefoil and alsike clover pollen collected in the greenhouse germinated in-vitro in excess of 70 percent, whereas sainfoin pollen had less than 3 percent in-vitro germination. Greenhouse collected alfalfa pollen responded significantly to the addition of yeast to the germinating media but greenhouse collected sainfoin pollen did not. Field produced sainfoin pollen had 5.08 to 32.50 percent germination. The germination of this pollen was influenced by genotype and time of day collected. Yeast extract enhanced the germination of the field collected pollen. The germination of this pollen was not significantly affected by age of flower. In-vitro pollen germination was not significantly correlated with percent seed set. Pollen from any genotype collected at any time of day would serve effectively in artificial pollinations.

Stigma receptivity was influenced by time of day, with pollinations after 5:00 P.M. being most effective. If possible, artificial pollination of sainfoin flowers should be made late in the afternoon or early evening.

2. Rubbing stamens between the thumb and forefinger resulted in as effective emasculation as did immersing exposed staminal columns in 47.5 percent ethyl alcohol for five or ten seconds. Alcohol treatments were slow to apply. Flowers emasculated with the five and ten second alcohol treatments set 11.4 and 2.7 percent seed, respectively, after pollination. Physically removing pollen from flowers by rubbing stamens between the thumb and forefinger was faster to apply than the alcohol treatments, and 14.8 percent of flowers emasculated in this way set seed following artificial pollination. There was no difference between clones in their effectiveness when serving as pollen parents; however, some clones served more effectively as females than others.

3. Upon selfing W_4 , a clone with light pink flowers, 136 progeny fit a 3:1 ratio for light pink- to white-flowered progeny. These data did not allow for differentiation between disomic and tetrasomic inheritance, but they did indicate this gene was inherited dominantly.

Two white-flowered clones did not segregate for flower color upon selfing. The 33 selfed progenies of W_3 were similar in flower color to the mother clone as were the 40 selfed-progenies of W_1 . The selfed-progenies of two pink-flowered clones did not segregate for

flower color. F_1 progeny from pink- and white-flowered crosses could be distinguished from either parent. The progeny number observed was small and they were in the greenhouse so it was not concluded whether this gene or genes were showing dominance or incomplete dominance.

When selfed-progenies of a clone heterozygous for the exposed style mutant were examined, 200 were normal and 20 exhibited the exposed style trait. This fits a 15:1 ratio at $p = .10$ to $.05$. This indicated that two independent duplicate genetic factors may control the inheritance of the trait. Sainfoin may genetically resemble an allopolyploid rather than an autopolyploid.

4. Percent crossing between clones planted in single-cross arrangements ranged from 8 to 28 percent. When three white-flowered plants were surrounded by pink-flowered plants in the field, 91, 91, and 97 percent crossing occurred. To achieve maximum cross-pollination in single crosses, the maternal plant should be surrounded by several pollen plants.

5. Inbreeding depression for plant weight of the field grown plants was from 14 to 64 percent with an average of 39 percent. Rooting ability decreased as inbreeding increased. Inbreeding depression for plant height in the greenhouse ranged from 4 percent to 55 percent from S_0 to S_1 with an average of 28 percent.

Sainfoin breeders should take care to have enough parental clones in synthetics to prevent excessive sib-mating among Syn one, two, and three progeny.

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