

The mode of reproduction in sainfoin (Onobrychis viciaefolia Scop.)
by Ronald Lynn Haaland

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE in Agronomy
Montana State University
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Abstract:
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Self- and cross-fertility levels as estimated by percent seed set, were re-evaluated and found to be essentially equal. Plants selfed by bees exhibited higher fertilities than plants selfed by hand.

Inbreeding studies were initiated using seedling vigor, percent fertility and number of flowers per head as measurement criteria, In general, sainfoin was not adversely affected by inbreeding.

Four plant traits were observed and proposed for potential genetic markers: plant color, petiole color, bell shaped leaf and flowers with exposed styles.

Gene exchange was not observed in reciprocal non-emasculated crosses. All evidense leads to the hypothesis that sainfoin is highly self-fertile.

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Date March 1, 1970

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(ONOBRYCHIS VICIAEFOLIA SCOP.)

by

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A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree

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Agronomy

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ABSTRACT

An investigation of the mode of reproduction in sainfoin was made. Clones used in the study were from the variety Eski.

The floral morphology was found to exclude automatic self-pollination. Close examination of the style and stigma revealed appendages around the stigma that opened and closed.

Self- and cross-fertility levels as estimated by percent seed set, were re-evaluated and found to be essentially equal. Plants selfed by bees exhibited higher fertilities than plants selfed by hand.

Inbreeding studies were initiated using seedling vigor, percent fertility and number of flowers per head as measurement criteria. In general, sainfoin was not adversely affected by inbreeding.

Four plant traits were observed and proposed for potential genetic markers: plant color, petiole color, bell shaped leaf and flowers with exposed styles.

Gene exchange was not observed in reciprocal non-emasculated crosses. All evidense leads to the hypothesis that sainfoin is highly self-fertile.

INTRODUCTION

Sainfoin (Onobrychis viciaefolia Scop.) is gaining popularity in the United States and Canada as a forage crop. Breeding programs have been established to further develop sainfoins potential.

The mode of reproduction regulates plant breeding procedures. Previous literature indicates sainfoin is highly self-sterile and cross-fertile. Some observations indicated this was not the case.

The objective of this study was to thoroughly investigate the mode of reproduction in sainfoin. Studies on floral morphology, self- and cross-fertility, inbreeding, potential genetic markers and gene exchange in reciprocal crosses were conducted to accomplish this objective.

REVIEW OF LITERATURE

General

Sainfoin (Onobrychis viciaefolia Scop.) is a perennial forage legume gaining popularity in the United States because of its apparent weevil resistance and its non-bloating characteristics (10). In general, sainfoin is adapted to dryland conditions and requires a minimum of 13 inches of precipitation per year (10, 12, 24). Research in Nevada (22) has indicated that sainfoin is more winter hardy than alfalfa and the variety Eski has shown good drought and winterhardiness in Montana (15). Breeding programs have been established in the United States, Canada and Romania to further develop sainfoin's potential (8, 19, 27, 28).

Floral Morphology

The inflorescence of sainfoin is a dense spike-like indefinite raceme (head). Sainfoin has a typical legume flower with large standard and keel petals and two very small wing petals. The keel encloses ten stamens, nine united and one free, and a long style leading to an ovary containing a single ovule. The flowers open from the base of the raceme upwards to the terminal buds (26).

The sainfoin flower is pink with red viens and has a high nectar content which makes it attractive to bees (4, 14, 26). Darwin, in 1878, noted that when a sainfoin field was cut, honey bees were driven to desperation attempting to obtain nectar from red clover in an adjoining field (13).

When a bee visits a sainfoin flower, the keel is depressed and the stigma and anthers come in contact with the insect body. When the insect leaves, the keel returns to its normal position allowing other insects to trip the same flower.(26).

Fertility and Inbreeding

In 1906, Knuth reported that the style becomes more erect as anthesis progresses, ultimately projecting from the keel cleft, thus excluding automatic self-pollination and ensuring cross-pollination (23, 26).

Fleishmann, in 1932, showed that Persian sainfoin (Onobrychis viciaefolia var. persica Sir.) set an average of 25.5 seeds per raceme under open-pollination but when the racemes were bagged the average number of seeds per raceme was 1.5 (18, 26). Thomson hand tripped sainfoin and found fertilities of 5.11, 0.98 and 51.55 percent for tripped, untripped and open-pollinated racemes respectively. He concluded that the degree of self-fertility in sainfoin was very low (26). Self-fertility studies in Hungary show tripped fertility of 5.79 and untripped fertility of 3.74 percent (5).

Carleton reported a two year average of self-fertility in sainfoin of 6.56 and 1.91 percent for tripped and untripped respectively. Mean cross-fertility values for sainfoin and alfalfa were 26.73 and 22.55 percent respectively.

Carleton concluded from these results that alfalfa and sainfoin are similar in their average levels of self-fertility and response to

tripping. Cross-fertility levels of the two crops were also similar in this study (6, 7, 8).

Hanna, in 1968, reported a mean self-fertility level in sainfoin of 4.8 percent with tripping. The mean fertility level of open-pollinated plants was reported as 35.6 percent (19).

Thomson grew selfed seed of sainfoin and found establishment to be poor and vigor reduced. There were no chlorophyll deficient plants in his material (26). Bosca and Hejja found inbreeding depressions of 26.5 and 30.2 percent for stem height and tillering respectively. They also found chlorophyll deficiency in some inbred plants (5).

Carleton found the mean inbreeding depression of seedling vigor for sainfoin and alfalfa was 29.5 and 28.1 percent respectively with one generation of selfing. He noted, however, that clones in both crops showed a range from 0 to more than 50 percent inbreeding depression (7).

Researchers working with sainfoin have concluded that sainfoin is highly self-sterile and exhibits a severe inbreeding depression upon selfing. However, evidence is accumulating which indicates that low self-fertility may not be the rule in sainfoin.

Carleton observed that seed set on plants selfed in cages by bees was much higher than would be expected from previous selfing studies (9). On isolated plants, Hanna found seed set of 1,000 to 2,500 seeds per plant. Again, this is greater than expected when earlier self-fertility estimates are taken into consideration (19).

Thomson grew two different types of sainfoin in adjacent blocks and found no gene exchange between the two types. He speculated that the lack of hybridization occurred because bees worked a small area and did not fly to other plants (26). Hanna and Carleton independently noticed lack of gene exchange in two-clone unemasculated crosses which indicates cross-fertilization did not occur (9, 20, 29).

Dubbs grew progeny rows from individual plants that were open-pollinated and was amazed at the lack of variability within individual rows (16). This lack of heterozygosity is another indication that crossing is not the general rule in sainfoin.

Hanson and co-workers found an unanticipated high degree of selfing in several two-clone crosses of alfalfa. The presence of reciprocal differences was also noted in these crosses. They concluded that additional research must be conducted to determine the extent of natural crossing in alfalfa (21).

Plant breeders have also concluded that further research must be conducted to determine the mode of reproduction in sainfoin (9, 19, 29).

Genetic Markers

Cultivated sainfoin is a polyploid having a base chromosome number of seven and a 2N number of 28. Either disomic or tetrasomic inheritance is possible but the mechanisms of inheritance have not been determined to date (7, 11). Genetic markers are essential for exact determinations of inheritance mechanisms and the extent of crossing and selfing in a crop (21). Genetic markers are slowly being accumulated in sainfoin.

The seedling leaf of sainfoin typically has one (uni), two (bi) or three (tri) leaflets. Hanna and Carleton have employed this characteristic as a genetic marker, using the different proportions of uni, bi, and tri as the measurement criterion. The type of inheritance has not been determined for this trait (7, 8, 9, 20).

White flower color has also been proposed as a genetic marker (7). The natural occurence of white flowers in sainfoin is very rare (7, 24), however, some white flowering clones have produced all white flowering progeny upon selfing (9). A genetic system for this marker has not been established but it is probably a recessive trait (9). White flower color in alfalfa is expressed by the homozygous recessive genotype (cccc) with one or more dominant alleles giving purple pigmentation of the flowers (2, 3).

Sainfoin normally has olive-green seeds, however, some plants have viable red colored seeds. The inheritance of this trait has not been characterized (7).

Carleton and Hanna have concluded that more nondeliterious genetic markers are needed to determine the mode of reproduction and inheritance in sainfoin (9, 20).

CHARACTERIZATION OF FLORAL MORPHOLOGY

Materials and Methods

An examination of floral morphology is the first step in determining the mode of reproduction of a plant. A study was initiated in February of 1969 to determine if the flower structure of sainfoin governed its mode of reproduction.

Flowers were collected at random from the variety Eski. Spacial relationship of the anthers and stigma within the flower were noted. Each flower was dissected and examined with a dissecting scope and photographed.

The sexual organs were examined more thoroughly because of their functional envolvement in reproduction. The entire style was removed from the pistle with a dissecting needle, stained with IKI, and observed with a compound microscope at 100X magnification. Photographs of the stigma were made through the microscope.

Results and Discussion

The sainfoin flower has a large standard and keel petal and two very small wing petals. The keel contains ten stamens, nine united and one free, and a long style leading to an ovary containing a single ovule (Plate 1). These results are in accordance with Thomson's findings (26).

In most flowers the style was found to protrude out of the keel cleft as anthesis progressed, however, in some flowers this was not the case. In all flowers, the style protrudes past the anthers. The

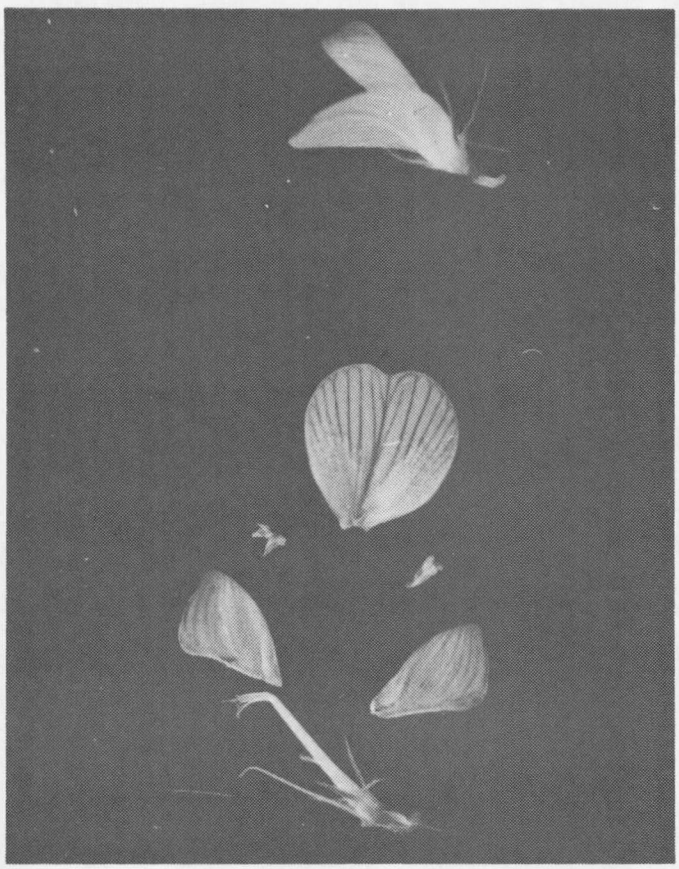

Plate 1. Intact flower above.
Disected flower below.

anthers shed thousands of pollen grains inside the keel but pollen was not observed on the stigma. This is probably due to the anther-stigma spacial relationship and indicates that automatic self-pollination is not possible in sainfoin. This does not, however, exclude the possibility of self-pollination by insects.

Microscopic observations of the female reproductive organs revealed appendages surrounding the stigma (Plate 2). The actual stigmatic surface absorbs more IKI than the appendages and is seen as the dark area inside the ring of appendages. As shown in Plates 3 and 4, the appendages may be closed over the stigmatic surface or open, exposing the stigma.

Stigmas from the flowers of different ages were examined to determine if the appendages open at a particular stage of floral development. Since sainfoin flowers open progressively from the bottom to the top of the raceme, all age groups could be obtained from a single raceme. Flowers that had reached the pink bud stage were considered very young; flowers just beginning to open, young; flowers at anthesis, mature; flowers partially closed after anthesis, old; and flowers completely wilted after anthesis, very old. The experiment was conducted on flowers from the greenhouse and flowers from the field.

A few flowers in the mature group had open appendages but the appendages were consistantly closed in all other age groups. Results from the greenhouse and the field were essentially the same. When the temperature in the greenhouse or the field became excessively warm,



Plate 2. Style and stigma of sainfoin showing stigmatic appendages.

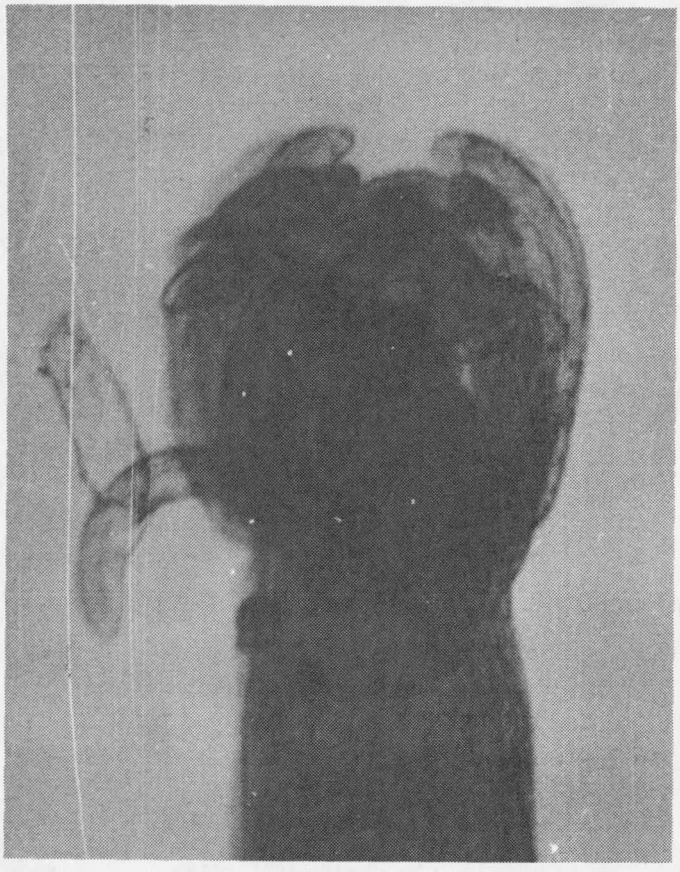

Plate 3. Style and stigma of sainfoin showing stigmatic appendages closed.

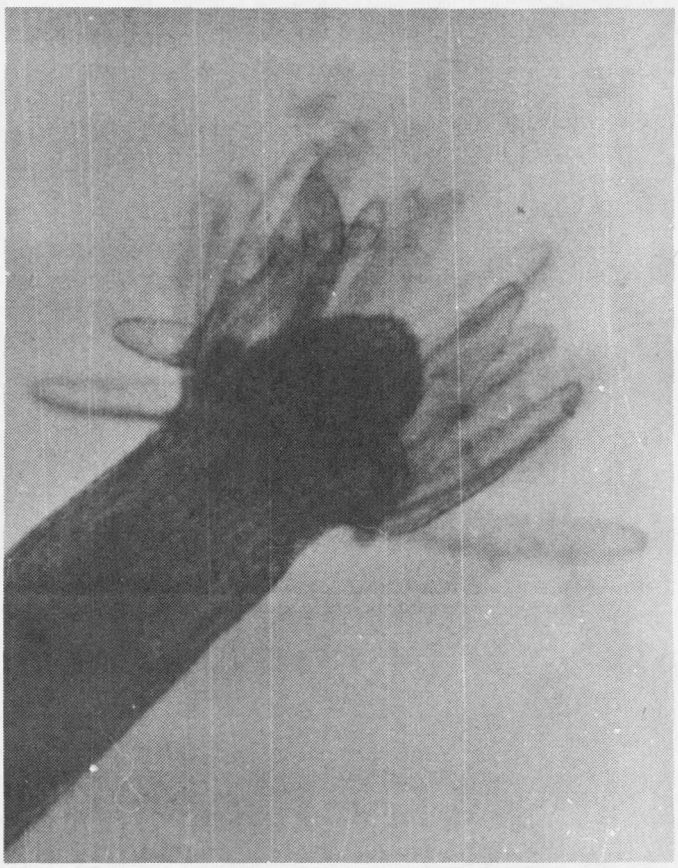

Plate 4. Style and stigma of sainfoin showing stigmatic appendages open.

the appendages were closed on all flowers regardless of the stage of maturity.

Because temperature was apparently involved in the movement of the appendages, further investigations were made to determine the stimulus for this movement.

Excised stigmas with attached styles were placed in a drop of water on a slide and observed under a microscope. When a stigma was found with open appendages, the slide was placed over a flame for two seconds. If the slide was observed soon enough after removal from the flame, movement from open to closed position could be seen. Movement from completely open to completely closed took about 15 to 20 seconds. The closing of the appendages was in short pulsating movements rather than a continuous smooth movement. In some cases, the appendages could be reversed from closed to open by placing cool water on the slide. Evidence points to temperature as a possible stimulus for appendage movement.

The actual mechanism for movement may be a temperature regulated osmotic pressure system. To determine if osmotic pressure was involved in appendage movement, mannitol solutions with osmotic pressures of 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 atmospheres were placed on open and closed appendages. The lowest pressure osmotic solution was applied first then through the intermediate solutions to the highest with observations being made between each treatment. Each solution was removed with blotting paper before the next one was applied.

The changing osmotic pressure had no apparent effect on appendage movement. In other words, the open appendages stayed open and the closed appendages stayed closed regardless of the osmotic pressure. The effect of mannitol on the appendage cells is not known and in fact may be detrimental. Thus, before any conclusive statements can be made other techniques should be utilized to determine the effect of varying osmotic pressure.

That temperature is the stimulus for appendage movement is not completely established. One must remember that these experiments were done on excised material which decreases the significance of inferences made about the intact organism. If temperature is the stimulus in the intact plant, it undoubtedly is less harsh than the flame used in this test and it probably brings about a slower movement of appendages. Further research utilizing more refined techniques and intact material is needed to completely determine the nature of appendage movement.

The functional significance of the stigmatic appendages has not been determined, however, since they are closed when the temperature is high it is logical to assume that protection of the stigmatic surface is their main function. This would lead one to assume that sainfoin has a stigma more vulnerable to environmental injury than other plants because other plants do not have protective appendages. In some plants the stigma is covered by a protective "membrane" that must be broken before pollination and fertilization can occur. In sainfoin the closed appendages could serve as the protective device

and then open up when the flower became more receptive for pollination thus eliminating the need for a stigmatic membrane. It is remotely possible that these appendages are nothing more than vistigal structures.

More reserach is needed to fully characterize the functional significance of these appendages and their possible relationship to mode of reproduction.

A RE-EVALUATION OF SELF AND CROSS-FERTILITY IN SAINFOIN

Materials and Methods

Because the observations made by Hanna and Carleton (20, 9) suggested something other than cross-pollination could be functioning in the reproduction of sainfoin, a re-evaluation of self and cross-fertility was made during the summer of 1969.

Eight clones from Eski were utilized in this study. Treatments consisted of the series of selfs and crosses shown in Table 1. Due to lack of facilities the treatments had to be made under different environmental conditions. Two selfs were made under plastic bubbles designed by Erb (17), four under 5' x 10' wire screen cages, and one in an isolated backyard plot. Eight crosses were made under the bubbles, two under 5' x 10' cages, seven in isolated back yard plots and one in a field plot. All crosses were unemasculated. A partial hive of bees was placed in each bubble and cage when the plants began to flower. This assured an adequate supply of pollinators to these treatments, however, the treatments made in back yard plots and in the field relied on indiginous pollinators so it was impossible to insure an adequate supply of pollinators.

Ten heads, randomly gathered within each treatment, were used to estimate the percent fertility. Percent fertility was calculated by the number of seeds per number of flowers.

A Duncan's multiple range test was used to detect differences between treatments. The environmental effects were not removed in this analysis.

Table I. Percent self- and cross-fertility in eight clones of sainfoin.[1/]

Treatment[2/]	% Fertility	Pollinating Environment
U_7 selfed	37.60 a	Screen Cage
$U_7 x T_9$	26.68 bcde	Screen Cage
$U_7 x U_{13}$	17.99 fghi	Backyard Plot
W_4 selfed	31.05 abc	Plastic Bubble
$W_4 x W_1$	32.62 ab	Plastic Bubble
$W_4 x W_3$ & W_1	32.76 ab	Backyard Plot
U_{13} selfed	27.05 abcd	Screen Cage
U_{13} OP	23.25 bcdef	Open Field
$U_{13} x U_7$	17.63 fghi	Backyard Plot
$W_3 x W_4$ & W_3	22.88 cedf	Backyard Plot
$W_3 x T_9$	21.75 defg	Plastic Bubble
$W_3 x T_{10}$	18.05 efghi	Plastic Bubble
W_1 selfed	7.84 jk	Plastic Bubble
$W_1 x W_4$	11.64 hijk	Plastic Bubble
$W_1 x W_4$ & W_3	9.68 ijk	Backyard Plot
$W_1 x T_{10}$	6.77 jk	Plastic Bubble
T_9 selfed	20.99 defg	Screen Cage
$T_9 x U_7$	6.76 jk	Screen Cage
$T_9 x T_{10}$	5.20 jk	Backyard Plot
$T_9 x W_3$	4.84 k	Plastic Bubble
T_{10} selfed	13.68 ghij	Screen Cage
$T_{10} x W_1$	13.66 ghij	Plastic Bubble
$T_{10} x W_3$	19.63 defgh	Plastic Bubble
$T_{10} x T_9$	4.43 k	Backyard Plot
H_1 selfed	26.83 bcd	Backyard Plot

1/ Each value is the mean fertility of ten heads. Means followed by the same letter are not significantly different at P = .05.

2/ W_3 was not selfed in this study. H_1 was not crossed in this study.

Results and Discussion

Self-fertility in isolation will give an indication of a plants mode of reproduction. If a plant has very low self-fertility in isolation, one can presume that the plant is not highly self-fertile and that cross-fertilization is probably the mode of reproduction. On the other hand, if a plant is highly self-fertile in isolation one can not assume that self-fertilization is the mode of reproduction because some crops that are highly self-fertile are mainly cross-pollinated (1).

In this study, the mean fertilities of all selfs and crosses is 23.01 and 16.08 percent respectively. These results are not significantly different and indicate sainfoin is highly self-fertile rather than highly self-sterile as reported in the literature.

Further support for this is found in the selfs and crosses of individual clones (Table I). In each clone the crosses were not significantly better than the self. In clones U_7 and T_9 the selfs were significantly better than the crosses. The self of W_1 was slightly less than the crosses of W_1 but the difference was not significant.

High and low fertility values were found under each environment. The apparent reason for this environmental variability was an insufficient number of pollinators in some treatments. This was particularly evident in the crosses, $T_9 x T_{10}$ and $T_{10} x T_9$, where bee activity was observed to be poor and the resulting fertilities were very low.

The conclusions from this study are obvious. Sainfoin is not highly self-sterile as previously reported, rather it appears to be highly self-fertile with some clones being more fertile than others. This study does not determine the mode of reproduction of sainfoin but it does rule out the contention that self-fertility is extremely low.

COMPARISON OF POLLINATION TECHNIQUES IN TWO CLONES OF SAINFOIN

Materials and Methods

A determination of the effectiveness of pollination techniques is needed to establish the accuracy of fertility estimates in plants. The low self-fertility estimates of sainfoin reported in previous literature may be due to the hand tripping technique used.

A study was initiated in the summer of 1969 to compare pollination techniques in sainfoin. Two clones, W_4 and U_{13}, were subjected to open pollination, selfing and crossing with bees, and selfing by hand.

The selfs and crosses with bees of W_4 were made in plastic bubbles and those of U_{13} in 5' x 10' wire screen cages. A partial hive of bees was placed in each enclosure when the plants began to bloom. The open-pollinated treatments were made close to other sainfoin plants and relied on indigenous pollinators.

The hand selfing treatment was applied to S_0 and S_1 plants in both clones by brushing an automobile key over the anthers and stigma. The treatment was applied daily for three weeks. The plants were kept isolate in 2' x 2' wire screen cages before and after the treatments were made.

Percent fertility was calculated by the number of seeds per number of flowers. An analysis of variance and a Duncan's multiple range test were used to determine significance of the treatments.

Results and Discussion

Duncan's multiple range test shows that open-pollination and bee pollination result in significantly higher fertilities than hand pollination (Table II).

The fact that hand selfing resulted in significantly lower fertilities than selfing with bees indicates that, previous self-fertility estimates in sainfoin were too low. These low estimates have been misleading and have lead to the biased conclusion that sainfoin is highly self-sterile and undoubtedly cross-pollinated.

Table II. Comparison of pollination techniques in two clones of sainfoin.

Treatments	Mean Fertility % W_4	Mean Fertility % U_{13}	Avg.[1]	Environment[2]
S_0 Open Pollinated	57.90	39.40	48.65 a	Field
S_1 Open Pollinated	51.50	40.40	45.95 a	Field
Selfed with bees	31.05	27.05	29.05 b	Enclosure
Crossed with bees	32.62	17.63	25.12 b	Enclosure
S_0 Selfed by hand	8.26	5.18	6.72 c	Enclosure
S_1 Selfed by hand	2.46	2.27	2.36 c	Enclosure

C. V. = 25.54%

1/ Means followed by the same letter are not significantly different at P = .05

2/ W_4 was selfed and crossed with bees in plastic bubbles.
U_{13} was selfed and crossed with bees in 5' x 10' screen cages.

INBREEDING DEPRESSION IN SAINFOIN AS ESTIMATED BY SEEDLING VIGOR

Materials and Methods

Whether or not a plant can withstand inbreeding will give an indication of its mode of reproduction. If a plant can be inbred without any adverse effects, it is probably naturally self-pollinated (1).

Two separate seedling vigor studies were made in 1968 to determine the effects of inbreeding on sainfoin.

In the first study, progeny from open-pollination (OP) were compared to progeny from the first generation of selfing (S_1). A split plot design was used, with nine clones as main plots and type of progeny as subplots. Ten seeds per subplot with five replications of each subplot were grown in 4" x 4" plastic pots in the greenhouse. The plants were watered with nutrient solution at appropriate intervals. After the seedlings had grown for two weeks, the OP and S_1 from each clone and each replication were thinned as a pair to an equal number of seedlings. The plants were harvested at six weeks of age by cutting them off just above the cotyledons. Dry weight in grams was used as the measurement criterion. A split-plot analysis of variance was used to analyze the data.

In the second study, progeny from selfs and crosses of four clones were compared. A randomized complete block design with ten replications was utilized. All other management practices were as in the first study. Measurements from each reciprocal cross were averaged. An analysis of variance was used to detect significant differences.

Results and Discussion

The conclusion to be drawn from these studies is obvious (Tables III and IV). With seedling vigor as a measurement criterion, sainfoin expresses no apparent inbreeding depression.

The C.V. of over 20% in both studies is probably related to the highly significant replication effect in both cases (Appendices XII and XIII). The replication effect may have been due to the method of thinning.

Table III. Seedling vigor in sainfoin as expressed by dry weight of six week old plants: S_1 vs OP progeny.

Clone	Mean Dry Weight in grams[1/]	
	OP	S_1
S-3	.110	.079
S-32	.100	.088
S-28	.100	.092
S-5	.088	.103
S-21	.103	.103
S-20	.102	.107
S-23	.093	.117
S-22	.120	.094
S-41	.104	.118
	.920	.901
	$\bar{X}$ = .102	$\bar{X}$ = .100

C.V. = 22%

1/ The OP and S_1 progeny were not significantly different at P = .05.

Table IV. Seedling vigor in sainfoin as expressed by dry weight of six week old plants: selfs vs crosses.

Parent Clone[2]	U-13	U-7	T-9	T-10
	Mean dry weight in grams[1]			
U-13	.104			
U- 7	.108	.104		
T- 9	.113	.097	.102	
T-10	.108	.114	.109	.094
	C.V. = 18%			

1/ Means were not significantly different at P = .05.

2/ Results from reciprocal crosses were averaged.

INBREEDING DEPRESSION IN SAINFOIN AS ESTIMATED BY A COMPARISON OF S_0 AND S_1 PERCENT FERTILITY

Materials and Methods

Fertility studies were used to further characterize the response of sainfoin to inbreeding. An estimate of inbreeding depression was made by comparing the percent fertility of S_0 and S_1 plants under open-pollination.

Ten clones from the variety Eski were used in this study. Five cuttings of each S_0 and ten seedlings of S_1 were established in 1968. Open-pollination fertility data was collected in 1969.

Measurements were made on ten heads randomly gathered from each treatment. Percent fertility was calculated by the number of seeds per number of flowers (26).

Data from the entire experiment were collected on June 24, 1969, and July 20, 1969. The results from the two dates were analyzed separately with an analysis of variance. Orthogonal pairing was used to determine significance of differences between S_0 and S_1 means (Appendix tables XIV and XV).

The same experiment was conducted on ten clones of alfalfa. Data were collected on July 20, 1969.

Results and Discussion

The mean inbreeding depressions of 1.9 and 0.4 percent fertility for S_0's vs S_1's in June and July respectively were non-significant (Table V). In June, the S_1 of S-16 was more fertile than the S_0 of

Table V. Inbreeding depression in sainfoin as estimated by a comparison of S_0 and S_1 fertility.

	Mean Percent Fertility					
	June			July		
Clone	S_0	S_1	$\bar{d}$ 1/	S_0	S_1	$\bar{d}$
S-16	16.8	35.4	-18.6**	15.0	43.2	-28.2**
S-44	27.5	34.7	- 7.2	26.7	52.6	-25.9**
S-40	24.9	14.0	10.9	44.2	32.9	11.3
S- 7	30.0	28.5	1.5	40.8	26.9	13.9*
S- 2	35.4	26.2	9.2	39.2	33.0	6.5
S-13	39.4	40.4	- 1.0	46.8	43.4	3.4
S-24	36.2	31.2	5.0	56.2	59.5	- 3.3
S-35	50.9	45.8	5.1	47.7	50.0	- 2.3
W- 4	57.9	51.5	6.4	81.1	63.6	17.5**
S-41	68.7	61.3	7.4	70.7	58.4	12.3*
Mean	38.8	36.9	1.9	46.8	46.4	0.4
	C.V. = 40%			C.V. = 28%		

1/ Mean differences ($d = S_0$ minus S_1) followed by * are significant at $p = .05$ and followed by ** are significantly different at $p = .01$.

S-16. In July, the S_1's of S-16 and S-44 were more fertile than the corresponding S_0's. In S-7, W-4 and S-41 the S_0's were more fertile than the corresponding S_1's.

Based on the mean of S_0's and S_1's sainfoin does not express an inbreeding depression in fertility however, clonal differences do exist. These differences may be due to inadequate sampling of the maternal genotype or actual differences in maternal genotypes. Further research is needed to characterize these differences.

The results from the alfalfa inbreeding study (Table VI) show a highly significant inbreeding depression in percent fertility for the mean of the ten clones.

Table VI. Inbreeding depression in alfalfa as estimated by a comparison of S_0 and S_1 fertility [1/].

Clone	Mean Percent Fertility S_0	S_1	$\bar{d}$
A-20	12.4	0	12.4
A-33	9.6	0	9.6
A-13	23.9	1.6	22.3
A- 6	10.9	0	10.9
A-51	20.6	3.0	17.6
A-23	15.9	0.8	15.1
A- 2	9.9	0.5	9.4
A-48	6.1	3.9	2.2
A-52	21.1	0.5	20.6
A-12	11.8	0.6	11.2
Mean	14.2	1.1	13.1**

C. V. = 27%

1/ A paired t-test of the mean of S_0's and mean of S_1's was significant at p = .01.

INBREEDING DEPRESSION IN SAINFOIN AS ESTIMATED BY A COMPARISON OF S_0 AND S_1 NUMBER OF FLOWERS PER HEAD

Materials and Methods

Flower number was used as another criterion to determine the effects of inbreeding on sainfoin. The same materials and methods of the previous experiment were used in this study with the exception that the number of flowers per head was used as a measurement of inbreeding. An analogous study in alfalfa was conducted at the same time. The analyses for these studies are shown in Appendix Tables XVI, XVII, and XVIII.

Results and Discussion

Inbreeding did not effect the number of flowers per head in sainfoin when averaged across all clones (Table VII). However, in June the S_0 of U-13 had more flowers than the S_1. In July, the S1 of S-16 had more flowers than the S_0 and the S_0 of S-44 had more flowers than the S_1.

Again sainfoin is apparently not effected by inbreeding but clonal differences do exist. These differences have not been characterized.

Alfalfa did not have a significant mean inbreeding depression in flower number per head (Table VIII). However, five out of ten clones did show an inbreeding depression which would indicate, inbreeding does lower the number of flowers per head of some clones.

There was no obvious correlation between percent fertility and number of flowers per head in either crop.

Table VII. Inbreeding depression in sainfoin as estimated by a comparison of S_0 and S_1 number of flowers per head.

	Mean Number of Flowers Per Head					
	June			July		
Clone	S_0	S_1	$\bar{d}$ [1/]	S_0	S_1	$\bar{d}$
S-16	36.1	46.4	-10.3	35.6	59.8	-24.2**
S-44	45.6	38.6	7.0	52.5	34.1	18.4**
S-40	55.9	56.1	0.2	57.7	52.3	5.4
S- 7	41.6	33.6	8.0	46.0	39.1	6.9
S- 2	54.5	48.3	6.2	58.9	48.1	10.8
U-13	51.0	39.2	11.8*	56.6	47.1	9.5
S-24	65.5	58.7	6.8	69.3	70.2	- 0.9
S-35	40.7	42.0	1.3	44.0	44.3	- 0.3
W- 4	45.3	42.7	2.6	55.3	44.4	10.9
S-41	53.8	48.8	5.0	53.3	52.0	1.3
Mean	49.0	45.4	3.6	52.9	49.1	1.8
	C.V. = 28%			C.V. = 24%		

1/ Mean differences ($\bar{d}$) followed by * are significant at p = .05 and followed by ** are significant at p = .01.

Table VIII. Inbreeding depression in alfalfa as estimated by a comparison of S_0 and S_1 number of flowers per head.

Clone	Mean No. flowers/head S_0	S_1	$\bar{d}$ [1/]
A-20	14.9	11.3	3.6*
A-33	15.0	6.4	8.6**
A-13	14.1	9.8	4.3*
A- 6	17.5	16.3	1.2
A-51	17.1	14.2	2.9
A-23	11.6	10.1	1.5
A- 2	18.7	14.1	4.6**
A-48	12.8	14.6	-1.8
A-52	16.7	16.7	0.0
A-12	15.1	9.3	5.8**
Mean	15.4	12.3	3.1

C.V. = 27%

1/ Mean differences ($\bar{d}$) followed by * are significant at P = .05 and followed by ** are significant at p = .01.

POTENTIAL GENETIC MARKERS IN SAINFOIN

Materials and Methods

Genetic markers are essential for estimating the amount of selfing and crossing in a plant, which is important in determining the mode of reproduction of the plant.

Seedlings and mature plants, of the sainfoin clones used in the preceeding studies, were screened for potential genetic markers. Descriptions were made of morphological abnormalities that were easily recognized and occurred more than once in a clone. Seed was saved from plants with abnormalities for further research.

Results and Discussion

Flower Morphology

Of 47 S_1 plants from clone T-9, five had abnormal flower morphology (Plates 5 and 6). The flowers matured but never opened up. The style protruded out of the closed flower by at least 3mm and sometimes more than 5mm. This mutant appears to be analogous to the exposed style mutant in alfalfa (3).

The five plants with the abnormal flower were easy to recognize, however, they set very few seeds when selfed or crossed. Therefore, this trait may be hard to utilize as a genetic marker.

An inheritance mechanism has not been worked out for this trait.

Leaf Morphology

Also in T-9 four S_1's out of 400 had abnormal seedling leaf morphology. The first leaf to emerge past the cotyledons was shaped like



Plate 5. Normal sainfoin flower.

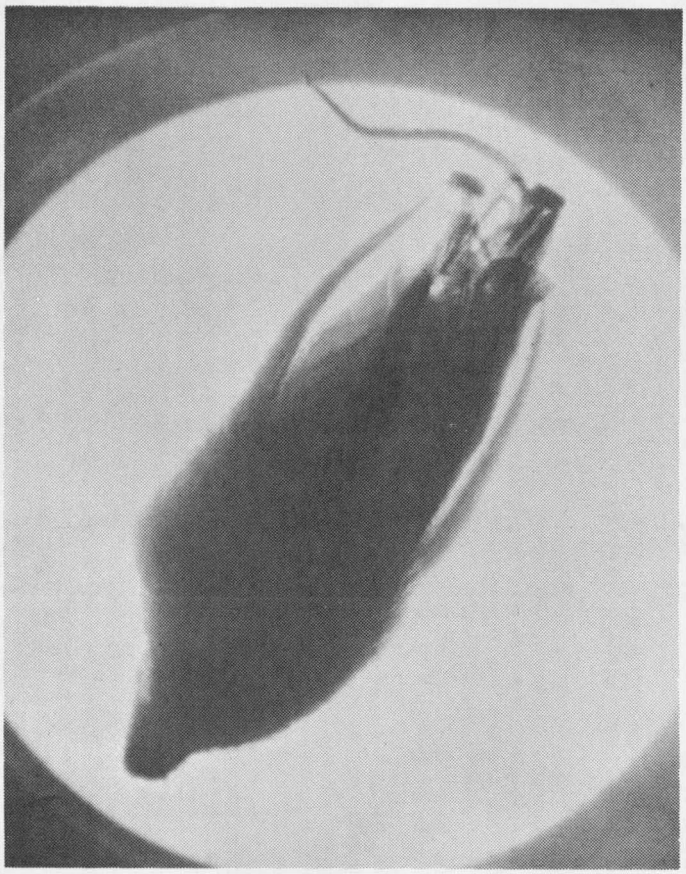

Plate 6. Abnormal sainfoin flower (exposed style mutant).

a trumpet bell. This trait may prove to be a desirable genetic marker because it is an easily recognizable seedling characteristic. Further research is needed to determine its mode of inheritance.

Plant Color

The Munsell plant color notation of U-13 and U-7 is light green (5GY 5/8) and of T-9 and W-4 is bluish green (2.5G 5/2). These colors are easy to distinguish by the time the plants are two weeks old. The colors hold true for the clones under different environments. This trait will be a good marker if care is taken not to include clones with intermediate colors that would be difficult to distinguish.

First Petiole Color

The first petiole of sainfoin seedlings amy be either red or green. In U-7, all plants have green petioles whereas in U-13, the ratio of red and green petioles is 7:5. Other clones have different proportions of red and green petioles. A genetic system has not been established for this trait.

ESTIMATES OF THE AMOUNT OF CROSSING IN SAINFOIN

Materials and Methods

Reciprocal unemasculated crosses (U_7 x U_{13} and U_7 x T_9) were made to estimate the extent of crossing in sainfoin. The traits used to determine if gene exchange had occurred were seedling leaf number (unifoliate vs trifoliate + bifoliate) red vs green petiole and blue vs light green color. The bi's and tri's were combined because it produced ratios that were easy to manipulate; there was no genetic basis for the combination.

All traits were assumed to be heritable but it was not known if one or the other was recessive. Seed was harvested from the female plants of the reciprocal cross, planted and seedlings examined for gene exchange.

Results and Discussion

When the trifoliates and bifoliates are bulked, both U-13 and U-7 selfed and the reciprocal cross show a 3:1 ratio of tri's to uni's. obviously, reciprocal differences can not be detected when both clones of the cross exhibit the same ratio. Clones with all uni's and all tri's will have to be crossed to detect gene exchange for this trait.

U_{13} has both red and green petioles in the ratio of 7:5 whereas U_7 has only green petioles. In the crosses used above, plants from U_{13} x U_7 had a ratio of 7:5, red to green, and plants from U_7 x U_{13} had predomenantly green petioles (Table IX). This indicates little or no gene exchange.

Plant color of U_7 selfed is light green (5GY 5/8) and plant color of T_9 selfed is bluish green (2.5G 4/2). In a two clone unemasculated cross made with bees, U_7 x T_9 gave all light green seedlings. The reciprocal, T_9 x U_7, gave all bluish green seedlings (Table IX). This again indicates there was no gene exchange, assuming plant color is a heritable trait.

These initial studies show sainfoin appears to be selfed with very little crossing taking place.

Table IX. A comparison of progeny of selfs and reciprocal crosses in sainfoin, for seedling marker genes.

Treatment[1/]	Traits: Seedling Leaf Number		Petiole Color		Plant Color	
	Unifoliate	Bifoliate & Trifoliate	Red	Green	Bluish Green	Light Green
U_{13} selfed	170	508	407	271	0	678
$U_{13} \times U_7$	206	582	476	312	0	788
U_7 selfed	107	389	0	496	0	496
$U_7 \times U_{13}$	212	494	18	688	0	706
T_9 selfed	155	379	210	324	534	0
$T_9 \times U_7$	13	25	2	36	38	0
$U_7 \times T_9$	36	84	1	119	0	120

1/ All selfs and crosses were made under wire screen cages with bees.

GENERAL DISCUSSION

Knuth, in 1906 (22) studied the mode of reproduction in sainfoin by examining its floral morphology. The stigma and style were found to protrude out of the keel cleft. This lead to the assumption that automatic self-pollination was not possible and that cross-fertilization was the mode of reproduction. Darwin's observation that bees are frequent and active visitors to sainfoin further substantiates this hypothesis.

The assumption that automatic self-pollination does not occur in sainfoin is a valid assumption not only because of the spacial relationship of the stigma and anthers but also because plants that are isolated from a pollen vector, do not set seed. However, the assumption that cross-fertilization is the mode of reproduction is not valid. If a plant has a mechanism of eliminating automatic self-pollination it does not necessarily imply it has a mechanism to eliminate self-pollination entirely. The fact that bees visit a plant frequently does not mean the plant has to be cross-pollinated. In the course of this research, several hours were spent observing honey bee movement in sainfoin.

A bee tends to work all open flowers on one plant before moving to another plant and in so doing probably distributes more self pollen on the plant than cross pollen. This would lead to a plant that was self-pollinated by bees.

Previous research (4, 8, 9, 18, 25) has shown self-fertility of sainfoin to be extremely low and fertility under open-pollination to be

high. This leads to the conclusion that sainfoin is cross-fertilized. The falicy of this conclusion lies in the fact that these self-fertility estimates were made by hand tripping the plants. The comparison of pollination techniques clearly indicates that sainfoin plants selfed by bees are much more fertile than sainfoin plants selfed by hand. The bees apparently do something that hand tripping does not. Bees may open the stigmatic appendages, allowing pollen to be deposited on the stigmatic surface whereas hand selfing may only carry pollen to the appendages and never get it to the stigmatic surface. The substitution of man made techniques for natural techniques has lead researchers to make biased and inadequate inferences about biological systems.

In general, a high degree of inbreeding depression has been reported in sainfoin. It has been suggested that clonal differences exist. In this research, sainfoin, on the average, did not express an inbreeding depression for seedling vigor, percent fertility and number of flowers per head. However, clonal differences did exist, with some clones showing a significant improvement of selfed plants over the mother plants. Depending on the clones chosen for study, sainfoin, in general, would or would not show a significant inbreeding depression.

Sainfoin has been grown as a forage crop for several hundred years. It is unfortunate that some reliable genetic markers have not been found. Researchers have avoided working with markers and crosses containing markers because they have not been able to interpret their results. Thomson noted lack of gene exchange between two types of sainfoin but

did not follow it up with further research (25). Both Hanna and Carleton noticed lack of gene exchange in two clone crosses but could not interpret the data based on the assumption that sainfoin is cross-pollinated. By simply assuming sainfoin is self-pollinated the lack of gene exchange in two-clone crosses can be explained. This paper further substantiates the self-pollination assumption by the lack of gene exchange in crosses between plants with red and green petioles and between bluish-green and light green plants.

Enough evidence has been accumulated to show that sainfoin is not highly self-sterile as previously reported but rather it is highly self-fertile. Further research must be done to determine the amount of natural crossing in sainfoin.

Sainfoin breeding programs will have to be re-evaluated and self-fertilization taken into consideration.

SUMMARY AND CONCLUSIONS

The mode of reproduction regulates plant breeding procedures. Sainfoin reportedly is highly self-sterile and is cross-fertilized. Present evidence indicates this may not be the case. Investigations were made to further characterize the mode of reproduction in sainfoin. The conclusions were as follows:

1. The sainfoin flower excludes automatic self-pollination.
2. The stigma is surrounded by appendages that open and close.
3. In general, self-and cross-fertility as estimated by percent seed set, were not significantly different.
4. Selfing by bees is much more effective than selfing by hand.
5. In general, sainfoin does not express an inbreeding depression for seedling vigor, percent fertility and number of flowers per head. Clonal differences do exist.
6. Some new potential genetic markers are plant color, abnormal seedling leaf, abnormal flower and petiole color.
7. Reciprocal unemasculated crosses had little if any gene exchange.

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APPENDIX

Table X. Analysis of variance of self- and cross-fertility in sain foin.

Variation	DF	SS	MS	F 2/
Total	249	38,112.99		
Treatment 1/	24	21,973.77	915.57	12.76**
Error	225	16,139.22	71.73	

C.V. = 46%

1/ Treatments consisted of crosses and selfs.

2/ ** = significance at p = .01.

Table XI. Analysis of variance of comparison of pollination techniques in two clones of sainfoin 1/

Variation	DF	SS	MS	F 3/
Total	11	4,059.54		
Clones	1	132.23	132.23	2.93
Treatments 2/	5	3,701.70	740.32	16.41**
Error	5	225.61	45.12	

C.V. = 26%

1/ W_4 and U_{13} were used in this study.

2/ Treatments in both clones were as follows: S_0 and S_1 open pollinated, selfs and crosses in isolation with bees, and S_0 and S_1 selfed by hand tripping.

3/ ** = significance at p = .01

Table XII. Analysis of variance of seedling vigor in sainfoin: S_1 vs OP progeny.

Variation	DF	SS	MS	F[1/]
Total	89	.06931		
Reps	4	.01924	.00481	6.68**
Clones (A)	8	.00305	.000381	.53
Error A	32	.02295	.00072	
Type Prog. (B)	1	.00009	.00009	.19
Type Proj. x Clone (AB)	8	.00708	.00088	1.87
Error B	36	.01690	.00047	

1/ ** = significance at p = .01.

Table XIII. Analysis of variance of seedling vigor in sainfoin: selfs vs crosses.

Variation	DF	SS	MS	F [1/]
Total	119	.0569		
Entry	11	.0050	.00045	1.32
Reps	9	.0183	.00203	5.97**
Error	99	.0336	.00034	

1/ ** = significance at p = .01

Table XIV. Analysis of variance of inbreeding depression in sainfoin as estimated by a comparison of S_0 and S_1 fertility in June, 1969. 1/

Variation	DF	SS	MS	F 2/
Clones	9	35,971.31	3,996.81	17.62**
Treatments within Clones				
S-16 (S_0 vs S_1)	1	1,729.80	1,729.80	7.62**
S-44 (S_0 vs S_1)	1	259.20	259.20	1.14
S-40 (S_0 vs S_1)	1	594.05	594.05	2.62
S- 7 (S_0 vs S_1)	1	11.25	11.25	.05
S- 2 (S_0 vs S_1)	1	423.20	423.20	1.86
U-13 (S_0 vs S_1)	1	50.00	50.00	.22
S-24 (S_0 vs S_1)	1	125.00	125.00	.55
S-35 (S_0 vs S_1)	1	130.05	130.05	.57
S- 4 (S_0 vs S_1)	1	204.80	204.80	.90
S-41 (S_0 vs S_1)	1	273.80	273.80	1.21
Error	171	38,567.90	226.87	
Total	200	78,295.36		

C.V. = 40%

1/ Individual degrees of freedom were partitioned by orthoganal pairing

2/ * = significance at p = .05, and ** = significance at p = .01.

Table XV. Analysis of variance of inbreeding depression in sainfoin as estimated by a comparison of S_0 and S_1 fertility in July, 1969. 1/

Variation	DF	SS	MS	F 2/
Clones	9	36,164.51	4,018.28	24.02**
Treatments within Clones				
S-16 (S_0 vs S_1)	1	3,976.20	3,976.20	20.78**
S-44 (S_0 vs S_1)	1	3,354.20	3,354.20	20.04**
S-40 (S_0 vs S_1)	1	638.45	638.45	3.81
S- 7 (S_0 vs S_1)	1	966.05	966.05	5.77*
S- 2 (S_0 vs S_1)	1	211.25	211.25	1.26
U-13 (S_0 vs S_1)	1	57.80	57.80	.34
S-24 (S_0 vs S_1)	1	54.45	54.45	.32
S-35 (S_0 vs S_1)	1	26.45	26.45	.16
W- 4 (S_0 vs S_1)	1	1,531.25	1,531.25	9.15**
S-41 (S_0 vs S_1)	1	756.45	756.45	4.52*
Error	170	28,442.89	167.31	
Total	199	76,167.91		

C.V. = 28%

1/ Individual degrees of freedom were partitioned by orthogonal pairing.

2/ * = significance at p = .05, and ** = significance at p = .01.

Table XVI. Analysis of variance of inbreeding depression in sainfoin as estimated by a comparison of S_0 and S_1 number of flowers per head in June, 1969. 1/

Variation	DF	SS	MS	F 2/
Clones	9	10,537.92	1,117.01	6.52*
Treatment within Clones				
S-16 (S_0 vs S_1)	1	530.45	530.45	3.09
S-44 (S_0 vs S_1)	1	245.00	245.00	1.43
S-40 (S_0 vs S_1)	1	.20	.20	.001
S- 7 (S_0 vs S_1)	1	320.00	320.00	1.87
S- 2 (S_0 vs S_1)	1	192.20	192.20	1.12
U-13 (S_0 vs S_1)	1	696.20	696.20	4.06*
S-24 (S_0 vs S_1)	1	231.20	231.20	1.35
S-35 (S_0 vs S_1)	1	8.45	8.45	.05
W- 4 (S_0 vs S_1)	1	33.80	33.80	.20
S-41 (S_0 vs S_1)	1	125.00	125.00	.73
Error	170	29,117.00	171.28	
Total	199	42,037.42		

C.V. = 28%

1/ Individual degrees of freedom were partitioned by orthoganal pairing.

2/ * = significance at p = .05.

Table XVII. Analysis of variance of inbreeding depression in sainfoin as estimated by a comparison of S_0 and S_1 number of flowers per head in July, 1969. 1/

Variation	DF	SS	MS	F 2/
Clones	9	11,341.52	1,260.17	8.30**
Treatment within Clones				
S-16 (S_0 vs S_1)	1	2,940.30	2,940.30	19.37**
S-44 (S_0 vs S_1)	1	1,692.80	1,692.80	11.15**
S-40 (S_0 vs S_1)	1	145.80	145.80	.96
S- 7 (S_0 vs S_1)	1	238.05	238.05	1.57
S- 2 (S_0 vs S_1)	1	583.20	583.20	3.84
U-13 (S_0 vs S_1)	1	451.25	451.25	2.97
S-24 (S_0 vs S_1)	1	4.05	4.05	.03
S-35 (S_0 vs S_1)	1	.45	.45	.00
W- 4 (S_0 vs S_1)	1	594.05	594.05	3.26
S-41 (S_0 vs S_1)	1	8.45	8.45	.06
Error	170	25,803.00	151.78	
Total	199	43,871.82		

C.V. = 24%

1/ Individual degrees of freedom were partitioned by orthogonal pairing.

2/ ** = significance at p = .01

Table XVIII. Analysis of variance of inbreeding depression in alfalfa as estimated by a comparison of S_0 and S_1 number of flowers per head. 2/

Variation	DF	SS	MS	F 2/
Clones	9	1,059.91	117.77	8.01**
Treatment within Clones				
A-20 (S_0 vs S_1)	1	64.80	64.80	4.41*
A-33 (S_0 vs S_1)	1	369.80	369.80	25.16**
A-13 (S_0 vs S_1)	1	92.45	92.45	6.29*
A- 6 (S_0 vs S_1)	1	7.20	7.20	.49NS
A-51 (S_0 vs S_1)	1	42.05	42.05	2.86NS
A-23 (S_0 vs S_1)	1	11.25	11.25	.76NS
A- 2 (S_0 vs S_1)	1	105.80	105.80	7.20**
A-48 (S_0 vs S_1)	1	16.20	16.20	1.10NS
A-52 (S_0 vs S_1)	1	0.0	0.0	0NS
A-12 (S_0 vs S_1)	1	168.20	168.20	11.44**
Error	170	2,498.50	14.70	
Total	199	4,436.16		

C.V. = 27%

1/ Individual degrees of freedom partitioned by orthogonal pairing.

2/ * = significance at p = .05 and ** = significance at p = .01.

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