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THE EFFECT OF INTERSPECIFIC HYBRIDIZATION
ON CERTAIN GENETIC RATIOS
IN SWEET CLOVER

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INTRODUCTION

During recent years sweet clover has become more and more recognized as an important agricultural crop. Consequently, considerable effort has been placed in the improvement of its agronomic characters. The breeding of improved varieties made more intensive studies of the crop necessary. Genetic studies have been conducted on many characters. In a number of studies, inter- and intraspecific fertility has been determined and techniques of selection have been developed.

In many of the genetic studies simple mendelian segregation occurred. At the Dominion Forage Crops Laboratory at Saskatoon, where particular emphasis is placed on the development of coumarin-deficient strains of sweet clover, a study of the mode of inheritance of coumarin content was undertaken in 1954. Many of the F_2 progenies segregated 3:1 for high vs. low coumarin content. The same segregation occurred with respect to free vs. bound coumarin. In some F_2 progenies however, unexpected ratios of approximately 8 high-coumarin to 1 coumarin-deficient plants occurred. Some type of complementary inheritance was suggested as a possible explanation for these ratios.

The present study was undertaken to determine the cause of the unexplained segregation in several F_2 populations. A new method of testing sweet clover seeds for coumarin was evaluated with regard to its applicability in breeding work.

LITERATURE REVIEW

Previous Genetic Studies

Inheritance of coumarin content.

Stevenson and White (30) crossed low- and high-coumarin selections. The progenies were tested by the method described by Stevenson and Clayton (28). The F_1 was intermediate in coumarin content and the F_2 fell into two fairly distinct classes. They concluded that the low-coumarin character was inherited as a simple recessive.

Rinke (20) crossed a low-coumarin plant (.186% coumarin) by a high-coumarin plant (.511%). A modification of Stevenson and Clayton's method of testing was employed. The F_1 contained an average of .159% coumarin and consequently the low-coumarin character was assumed to be dominant. In the F_2 , two classes were distinguished. Selfed progenies of the low-coumarin parent ranged in their coumarin content from .06 - .29%, thus F_2 plants, falling within this range were considered to display the low-coumarin character. Selfed progenies of the high-coumarin parent ranged in their coumarin content from .30 - .60%, and consequently F_2 plants, falling within this range, were considered to display the high-coumarin character. On the basis of this division, 22.7% of the F_2 population was high in coumarin and 77.3% was low in coumarin. He therefore concluded that one factor pair was involved in coumarin inheritance; the low-coumarin character being dominant.

Horner and White (12) crossed low-coumarin plants with several high-coumarin strains. The F_1 progenies were high in coumarin content and thus the factor or factors for high-coumarin appeared to be

dominant. The F_2 results gave a good fit to a 3:1 ratio. The low-coumarin F_2 plants bred true in the F_3 . Nine high-coumarin F_2 plants bred true for high coumarin in F_3 and 22 segregated 3:1. These F_3 data fit closely the expected 1:2 ratio of true-breeding to segregating lines. These results clearly indicate that low coumarin is inherited as a simple recessive.

Smith (24, 25) crossed Melilotus dentata x Melilotus alba. The hybrids were all chlorophyll-deficient. They were grown to maturity when grafted onto M. officinalis. These hybrids were self sterile but seed was set on backcrossing to M. alba. The backcross populations indicated that two genes were involved in coumarin inheritance; a recessive gene reduces the coumarin content from 2.5 - .5%, and a second gene, displaying no dominance, reduces the coumarin content to .1%.

Webster (33), using embryo culture, crossed high-coumarin M. officinalis x low-coumarin M. alba. In the F_2 population 382 plants contained coumarin and 80 were coumarin-deficient. These results do not fit a one factor ratio but the author suggested a single recessive gene for low coumarin.

Goplen (9) determined the mode of inheritance of coumarin content from diallel crosses involving the variety Arctic, (containing "free coumarin"), the variety Pioneer (containing "bound" coumarin) and some coumarin-deficient selections. The latter were derived from the M. dentata x M. alba cross, made by Smith (24), backcrossed to M. alba several times. Two of these were of the Alpha type.

The coumarin content in the F_1 was approximately one-half the

average of the selfed progeny of the high-coumarin parent. The F_2 population of the Arctic x Pioneer cross segregated 91 free coumarin : 28 bound coumarin. On the basis of a 3:1 ratio, a P-value of .95 - .50 was obtained. It was concluded that free coumarin is dominant over bound coumarin.

The cross, coumarin-deficient (C-50-CP-4-3-9-1) x Pioneer, segregated 3:1 in the F_2 for bound coumarin vs. coumarin-deficient ($P=.50-.20$). Both parents in this cross were homozygous for the gene for bound coumarin.

In a similar cross (C-50-CP-7-1-1-1-1) x Pioneer the F_1 contained free coumarin and the F_2 provided a good fit to a 9:3:4 ratio ($P=.50-.20$). The coumarin-deficient parent of this cross carried the gene for free coumarin because the Pioneer plant used was the same as in the previous cross.

The F_1 population of the coumarin-deficient (C-50-CP-4-3-9-1) x Arctic cross contained free coumarin and the F_2 segregated 9:3:4, ($P=.50-.20$). In the similar cross with the same Arctic plant and C-50-CP-7-1-1-1-1 as coumarin-deficient parent, the F_1 contained free coumarin. In the F_2 , 419 free coumarin containing plants and 50 deficient plants were observed. The author suggested complementary three-factor segregation and an excellent fit to a 57:7 ratio was obtained ($P=.95-.50$). Similar results were obtained in the cross of coumarin-deficient S-50-240-3-1 x Arctic.

Several coumarin-deficient plants of the three parental lines were intercrossed among themselves but none of their progeny produced coumarin.

Goplen (9) concluded that a dominant major gene is responsible for coumarin production. A second gene governs the type of coumarin. It only attains expression in a coumarin-containing plant. Furthermore, complementary action between two independent genes was hypothesized.

Inheritance of the dwarf-branching or Alpha type of growth.

In a field of Arctic sweet clover, Kirk (13) observed some "aberrant" types in 1924. They exhibited "constricted branching, tufted foliage, numerous primary stalks and well developed much branched crowns". Progenies from these plants were grown and six out of ten families segregated for the normal "sweet clover" type and "aberrant" type.

Elders (6) observed a dwarfed, bushy plant in a plot at the Brandon Experimental Station. The dwarf character was found to be inherited as a simple recessive. Natural crossing between the two types of sweet clover was observed to the extent of 10 - 20%.

In a nursery containing plants of both types, Kirk (16) observed an average of 57% natural crossing. In a study of 43 F_2 families a good fit to a 3:1 ratio was obtained for each individual family, as well as for the whole population. The typical sweet clover type of growth is completely dominant. Different types of dwarf-branching plants were observed and the author concluded that possibly two or more recessive factors for dwarfness occur. A large proportion of the Alpha types could be recognized in the seedling stage but accurate counts could be obtained only in the fall of the first year and in the

second year of growth.

Clarke (1) intercrossed various strains of the Alpha type and also crossed them with Arctic. In all but two F_2 families of the crosses with Arctic, no significant deviations from 3:1 ratios were observed. On intercrossing Alpha types, the F_1 and F_2 progenies were also dwarfed, except in three cases in which normal F_1 plants appeared, and the F_2 progenies segregated. In these exceptional progenies two different genes for Alpha type occurred. Consequently a 9:7 segregation was expected in the F_2 . Since no double recessives could be observed, a 9:6 ratio was expected, and a close fit to it was obtained. No convincing evidence for the hybrid origin of the Alpha type from crosses of sweet clover x alfalfa was discovered and it is assumed that they arose by simple gene mutation.

Stevenson (27) observed the growth type in progenies of Alpha type x Arctic. Only normal types occurred in the F_1 , and the F_2 -results closely fitted the 3:1 ratio ($P = .70 - .50$). Thus the common type of growth is dominant over the Alpha type.

Goplen (9) found that, under greenhouse conditions, neither the selfs of the Alpha parent nor any F_2 segregates exhibited the bushy type of growth. Therefore, it was assumed that the expression of the gene or genes involved depends on the environment.

Inheritance of seed pigmentation, seed permeability, and seed color.

Kirk and Stevenson (18) crossed plants of an inbred line which produced purple-spotted seed with common white plants. The latter

produced clear golden-yellow seed. The seed of the F_1 showed purple markings. Seed pigmentation appeared to be a dominant character.

Stevenson (27) crossed an Alpha-type plant, which bred true for the mottled condition with an Arctic plant which was true breeding for clear seed. In the F_2 , 114 plants produced mottled seed and 34 plants produced clear seed. Assuming a 3:1 segregation, a P-value of .50 - .70 was calculated. In the F_3 , 26 lines bred true for mottled seed, 63 segregated and 19 bred true for clear seed. For the bulk of the segregating lines, a P-value of .90 was calculated for a 3:1 ratio. It was concluded that the mottled condition is dominant, and it is dependent on a single factor difference.

Stevenson (28) collected mature seed from 10,982 single plants and found that only .66% of it was permeable to moisture. In the second inbred generation, seeds were produced which showed brownish, sunken areas on their seed coat, and were permeable. These sunken spots coincide with the area of moisture absorption.

White and Stevenson (35) selected two strains for high permeability (98.2%). These selections show a large proportion of seed with the sunken brownish spots. Spotted seeds were about 96% permeable. A highly permeable line was crossed with plants of the Alpha variety. The F_1 produced only non-spotted seed. The F_2 population segregated and revealed that high permeability is recessive. The number of factors controlling permeability could not be determined from the data; and it was concluded that the character was inherited in a complex manner.

Downey, Greenshields and White (3) classified F_2 progenies of permeable x non-permeable seed crosses. All crosses gave good fits to 3:1 ratios with P-values of .95 - .50. The heterogeneity χ^2 indicated that all families were homogeneous, and no linkage was observed between the factors for spotting and for free coumarin. Simple recessive inheritance of seed coat spotting was concluded. Modifying genes may be involved. The environment influenced greatly the penetrance and expressivity of the character. The highest degree of spotting was observed in open pollinated seed; it was less in seed set in the greenhouse and was the lowest in selfed seed set under selfing bags. The authors concluded that seed coat spotting is controlled by one major gene; the degree of expression being the result of a balance between environment and one or more modifying genes.

Fowlds (6) observed three distinctly different seed colors in sweet clover. The golden yellow seed color results from a yellow seed coat and yellow embryo. In green seed, both seed coat and embryo were green; and in pale yellow seed, the seed coat was white and the embryo yellow. The seed produced by a F_1 hybrid, green female x yellow male, was pale green. In this case the seed coat was green and the embryo yellow. F_2 results indicated that green was recessive to yellow, but the results were not a good fit to a 3:1 ratio.

Inheritance of various other characters.

Smith (23) intercrossed annual and biennial forms of M. alba. In the F_2 generation, 1,567 annual and 479 biennial types occurred. These results fit a 3:1 ratio and a dominant gene was assumed to cause

the plant to mature in one season. The occurrence of a dominant mutation was therefore demonstrated.

In a number of F_2 progenies of Alpha x Arctic sweet clover, Clarke (1) observed approximately 25% pale green seedlings. The statistical analysis revealed that they represent the homozygous recessive class of one factor pair segregating for normal green and pale green seedling color. In several progenies a deficiency in the pale green class was noted. It was due to a relatively high mortality rate in this class. The progeny of one cross closely fitted a 9:7 ratio, therefore the presence of two different factors for pale green was concluded.

Kirk (17) reported on the flower color inheritance in the progenies of a natural hybrid of M. alba x M. officinalis. It was found later however, that it was a hybrid between M. alba x M. suaveolens. Four different colors were observed in the F_2 and the segregation indicated that at least two and possibly three factors are involved. In all progenies, where yellow-flowered plants were expected, a deficiency of such plants was noted.

Gettys and Johnson (8) selected two self-sterile and cross-fertile plants of M. officinalis and crossed them to a self-fertile inbred line of annual M. officinalis. The F_1 plants were backcrossed to both parents. About half of the F_1 plants were cross-fertile with one parent and half were cross-fertile with both parents. An oppositional-factor type of sterility was assumed. The F_1 of self-sterile x self-fertile parents was self fertile, thus the self-fertility allele is dominant.

Interspecific Hybridization

Kirk (15) intercrossed M. alba and M. officinalis. Approximately 60% seed was set but only shrunken seed, incapable of germination, developed. This indicated that "we are dealing with a rather wide cross or a high degree of incompatibility".

Similar results were obtained by Stevenson and Kirk (29). In intergeneric crosses with *Medicago* abortive seeds were produced when sweet clover was used as female parent.

Webster (33) cultured excised hybrid embryos of M. officinalis x M. alba crosses on artificial nutrient media. Two F₁ plants grew to maturity. They produced about 75% stainable pollen. A high degree of homology between the genomes of the two species was observed, but some abnormality was evident. The F₂ populations did not segregate normally, neither for coumarin content, nor for seed color. In most cases a deficiency was observed in the recessive class. Since 25% of the pollen was aborted, normal F₂ ratios could not be expected and they did not occur.

Greenshields (11) studied the embryology of interspecific crosses in *Melilotus*. In reciprocal crosses between M. alba and M. dentata a considerable degree of incompatibility was evident. Genetic unbalance, as well as competition between embryo and endosperm may be responsible for abortion of the seeds at early stages, however some seeds reach maturity. In M. officinalis x M. alba crosses the embryos developed for 14 days but endosperm is sparse or absent. The embryo failure may be due to nutritional deficiencies caused by genetic

unbalance in the endosperm.

Stephens (31) studied interspecific hybrids in cotton. Structural differences between the genomes of interfertile species were evident. These differences may be large enough that they may be observed cytologically or they may be allelic differences. In many cases these differences involve segments of chromosomes which are not large enough to be detected by cytology. When partly homologous chromosomes pair, crossing-over is reduced in region of structural differentiation. In progenies of interspecific hybrids clear cut genetic ratios are not obtained. Monofactorial segregations yield blurred F_2 ratios, and skewed backcross ratios are often obtained, when transferring genes from a distantly related species to another. In all cases there is a marked deficiency in the class containing the gene from the donor parent. In successive backcrosses the normal 1:1 ratios are more closely approached. The deficiency in the class containing the introduced gene suggests that it is carried on a partially homologous chromosome and that the gametes containing it are at a selective disadvantage. Crossing-over is assumed to take place, to some extent at least, between partially homologous chromosomes; thus under the influence of selection "foreign" chromatin is progressively removed and the ratios tend to become normal. Another result of structural differentiation was observed in that, on backcrossing hybrids, blocks of linked genes were introduced rather than individual genes. Certain cases of complementary allelism have been shown not to be due to allelism but to the interaction of non-homologous but closely adjacent loci.

Methods of Coumarin Analysis.

Ufer (32) developed a rapid quantitative method of coumarin testing based upon fluorescence. If coumarin is heated in potassium or sodium hydroxide and irradiated with fluorescent light, bright yellow-green fluorescence results. High- and low-coumarin samples could be distinguished readily.

Slatensek and Washburn (22) converted coumarin into the soluble fluorescent salt with sodium hydroxide. The samples were classified as "high" or "low". Using a fluorometer, quantitative readings were obtained.

White, Savage and Johnston (34) modified the above method by reducing to one-fifth the amount of leaf material and reagent used. The necessity of taking an aliquot is also eliminated.

Coplen, Greenshields and White (10) demonstrated that only actively growing leaves from the tip of the branches give reliable tests. A considerable amount of coumarin is lost in drying. The authors collected two or three fresh leaves from growing plants. They were placed in a 3/4 x 6" test tube containing approximately 10 ml. of 2.5 N sodium hydroxide. The tubes were then heated at 97°C for two hours and read before an ultra-violet lamp. The samples were scored visually on a scale from one to five, one being coumarin-deficient and five high-coumarin. For quantitative analyses, they heated 100 mg. of fresh leaf material in 15 ml. of 2.5 N sodium hydroxide for 2 1/4 hours at 97°C. The samples were then cooled off to room temperature and 1 ml. of the solution pipetted into a volumetric flask containing 99 ml. of distilled water. After tipping, the solution was

put into a cuvette, irradiated before an ultra-violet lamp for ten minutes and then read in a Coleman photofluorometer.

A colorimetric method of testing is outline by Clayton and Iarmour (2). Coumarin is extracted from leaf material with 95% ethyl alcohol. A deep crimson color is then produced by coupling coumarin to diazotized p-nitroaniline in alkaline solution. The intensity of the developed crimson color is proportional to coumarin concentration and can be used for colorimetric measurements.

A short colorimetric procedure for coumarin determination is outlined by Smith (26).

McAllister and Davis (19) outlined a method by which a small portion of the seed was tested. The remaining portion was germinated.

Dworak (4) selected low coumarin plants on the basis of the odor of dried leaf samples.

Many workers have demonstrated that the coumarin content in sweet clover varies with the stage of development and between different parts of the plants (7, 10, 21, 28, 32, 35).

MATERIALS AND METHODS

Plant Material

F₁ seed of similar types of crosses as studied by Goplen (9) was planted in greenhouse flats and then transplanted to a field nursery. The original crosses involved selections of the variety Arctic, containing free coumarin, the variety Pioneer, containing bound coumarin, and three coumarin-deficient selections, which were derived from M. dentata x M. alba crosses made by Smith (24).

From the F₂ population C-50-CP-7-1-1-1-1 x Arctic (9), which on the basis of a 57:7 segregation, was assumed to display complementary inheritance, 52 coumarin-deficient plants were selected. Ten of these were diallel crossed and also crossed with all the other 42 plants. If the hypothesized complementary gene action was operating, some of the crossed progeny were expected to show high coumarin content. The ten plants, used in the diallel crosses were also crossed onto some coumarin-deficient F₂ plants of the populations that had segregated 3:1 for coumarin to coumarin-deficient plants.

The abnormal segregation in certain F₂ populations could have been caused by preferential pollen functioning known as certation effects. To detect certation effects F₁ plants which segregated 3:1 in the F₂, and F₁ plants which segregated approximately 8:1, and their coumarin-deficient parents were grown in the greenhouse and reciprocally intercrossed in the following manner:

Throughout this thesis the plants used in determining the causes of unexplained segregation in certain F_2 populations are designated as follows.

TABLE 1. Designation of the parental plants used in the study of abnormal segregation.

Designation	Description of plant	Strain	F_2 segregation in crosses	
			with Arctic	with Pioneer
N	Normal plant	C-50-CP-4-3-9-1	3:1	3:1
D	Abnormal plant	C-50-CP-7-1-1-1-1	approx. 8:1	3:1
D ₁	Abnormal plant	C-50-CP-7-1-1-1-2	approx. 8:1	--
D ₂	Abnormal plant	S-50-240-3-1	approx. 8:1	--
A	Arctic		--	--
P	Pioneer		--	--

Consequently F_1 plants segregating 3:1 in the F_2 are referred to as normal F_1 's, those segregating approximately 8:1 are referred to as abnormal F_1 's.

In these crosses an attempt was made to estimate the proportion of selfs occurring. When crossing, four racemes were emasculated at a time.

One of these was chosen at random and left unpollinated. The percentage of selfed seed was subsequently determined. In the cross (D x P) x (D x A) the female parent was heterozygous for free coumarin, the male parent homozygous. The progenies containing bound coumarin, being the homozygous recessive class, represent one-quarter of the selfs which actually occurred. Thus a reliable estimate of the percentage of selfs was obtained and the ratios were corrected accordingly. From the normal and abnormal F_1 , pollen was examined and the proportion of unstainable pollen grains was determined. Root tips and buds were collected for cytological examination.

The inheritance of the low-coumarin and permeable-seed characters was determined in F_2 progenies derived from hybrids of permeable seeded M. alba selections x coumarin-deficient F_3 progenies of the M. officinalis x M. alba hybrids, cultured by Webster (33). Since penetrance and expressivity of the permeable-seed character are low under greenhouse conditions, this population was grown in a field nursery.

In order to evaluate a new testing procedure 15 seeds each of the variety Arctic, the variety Pioneer and a coumarin-deficient selection were tested, by the method described by McAllister and Davis (19). These seeds were then planted in greenhouse flats and the plants re-tested after eight weeks. For a further study reciprocal crosses were made between coumarin-deficient M. alba strains and plants of the variety Arctic, and the hybrid seed was then tested by the same method.

Methods of Crossing and Selfing

All the crossing and selfing was done in a greenhouse from which bees were excluded. On the female parents racemes, with a few unopened florets at the tip, were selected for emasculation. The unopened florets and the oldest ones at the bottom of the raceme were removed. The petals were then pulled off with tweezers and the anthers were removed with vacuum through a fine glass nozzle (1 mm. diameter). Each raceme was approached from the bottom and progressively emasculated to the tip, thus avoiding injury to the pistils. This procedure was facilitated by the use of a binocular (3.5 x magnification), which was worn by the operator. In all coumarin-deficient x high-coumarin crosses the deficient plant was used as the female parent. Possible selfs appeared in the F_1 as coumarin-deficient individuals and they were eliminated.

Selfing was performed by rolling the racemes lightly between the thumb and forefinger. The operators hands were washed and dried after each plant, thus avoiding contamination.

Methods of Sampling

For the rapid fluorometric test, two or three fresh leaves from the tip of the plant were collected and immediately placed in a test tube containing approximately 10 ml. of 2.5 N sodium hydroxide. The tubes were corked and kept in the dark until the time of analysis (10).

For the quantitative analysis, 100 mg. of fresh leaf material was collected from near the top of the plant. It was immediately placed

in a test tube containing 15 ml. of 2.5 N sodium hydroxide and corked. If the samples were not analysed immediately, they were stored in the dark.

The samples for the colorimetric test consisted of one newly opened leaf from near the top of the plant. This leaf was placed in a test tube containing two ml. of 95% ethyl alcohol. The tubes were corked and allowed to stand for 24 hours until analysis.

In the testing procedure described by McAllister and Davis (19) small portions of the seeds are tested for coumarin, using the rapid fluorometric method. Seeds were scarified and placed on wet paper towels for three to five hours. When they had swollen, the tips of the cotyledons were cut off with a razor blade and placed in a test tube containing two ml. of 2.5 N sodium hydroxide plus three mg. of dried alfalfa leaf. About 80 seed samples were tested by the colorimetric procedure. In these cases the cut off portions of the seeds were placed in test tubes containing one ml. of 95% ethyl alcohol.

Methods of Coumarin Analysis

Most of the material studied was tested by the rapid fluorometric method described by White, Savage and Johnston (34) and modified by Goplen, Greenshields and White (10). The test tubes containing approximately 10 ml. of 2.5 N sodium hydroxide and the fresh leaf samples were heated in a waterbath at 97°C for two hours. They were then cooled off to room temperature and read before an ultra-violet lamp. The samples were visually scored from one to five (1, 1+, 2, 2+...5).

Readings of 2+ and higher indicated the presence of coumarin, samples reading two and lower were considered to be void or to contain only a trace of coumarin.

The colorimetric method described by Clayton and Larmour (2) and modified by Smith (26) was used to distinguish between free and bound coumarin. The procedure is outlined as follows:

"Add 2 c.c. of 95% ethyl alcohol to a test tube, add 1 c.c. of 4% sodium carbonate (23.3 gm. of sodium carbonate per 500 c.c. of distilled water). Tip. Cork loosely. Heat in waterbath at 70°C for ten minutes. Cool in refrigerator for one-half hour. Now add 1 c.c. of diazonium solution. Tip. Allow to stand for 20 minutes before reading."

Samples classified as positive developed a deep red color. In the negative samples no color change appeared.

For quantitative tests the method described by Slatensek and Washburn (22) and modified by Slatensek (21) and Goplen et al. (10) was used. By this method, the total coumarin content is measured. One hundred mg. of fresh leaf material was placed in a test tube containing 15 ml. of 2.5 N sodium hydroxide; corked loosely and heated at 97°C for 2 $\frac{1}{4}$ hours. The samples were then cooled to room temperature with tap water. One ml. of the solution was pipetted into a volumetric flask containing 99 ml. of distilled water and thoroughly mixed. The solution was put into a photofluorometer cuvette, irradiated before a ultra-violet lamp for ten minutes and then read in a photofluorometer equipped with B-1 and PC-1 filters. The instrument was first calibrated by the use of standard solutions of known concentration.

Using the testing procedure described by McAllister and Davis (19)

small portions of seeds were tested by the rapid fluorometric and by the colorimetric methods. The amount of solvent used was considerably reduced for both tests in view of the fact that only very small amounts of tissue were analysed. For the colorimetric test, the coumarin was extracted with one ml. of 95% ethyl alcohol. The amounts of reagents used for subsequent testing (sodium carbonate and diazonium solution) were also reduced to one-half. For the fluorometric procedure two ml. of 2.5 N sodium hydroxide were used.

The remaining portion of each seed was germinated on blotting paper in petri dishes and grown for several weeks. These plants were then retested by the rapid fluorometric method.

Classification of Permeable and Non-permeable Seed

Mature, open-pollinated seed was collected from F₂ plants in the field nursery. Where possible, samples of approximately 200 seeds were harvested. They were threshed on a corrugated rubber mat and then examined with a binocular using 15 x magnification. The brownish, sunken spots in the seed coat of permeable seeded plants could be observed readily.

Methods of Cytological Examination

The proportions of unstainable pollen were determined by collecting pollen from newly opened florets and placing it on clean slides. A drop of acetocarmin stain was added and the coverglass slightly pressed. After placing the slides under the microscope, 10 to 15 different

fields showing pollen were chosen at random and the number of stained and unstained pollen grains was counted. The percentage of stainable pollen was calculated.

Meiotic metaphase chromosomes were observed in pollen mother cells. Buds were collected and immediately placed in 3:1 alcohol-acetic acid fixative. They were stored in this solution in the refrigerator for about one week. The anthers were then dissected from the florets under a binocular using 60 x magnification. During dissection the buds were prevented from drying out by adding glycerin to the fixative. The anthers were placed on clean slides and a drop of acetocarmin was put on the material. After applying the cover slip the slide was heated on a hot-plate for about one minute and then squashed.

From the plants to be studied, cuttings were rooted in running water in a tin trough. The root tips were collected in bottles containing 3:1 alcohol-acetic acid and stored in the refrigerator for about one week. The meristematic parts of the roots were then placed on a slide and acetocarmin stain was added. The tissue was well squashed with a soft iron needle, covered and heated. Cells showing late prophase stages were located under low power and chromosome counts were made, using the oil immersion objective.

EXPERIMENTAL RESULTS

Complementary Gene Action

The main objective of this study was either to prove the complementary gene action hypothesis, proposed by Goplen (9), or to find an alternative explanation for the unusual ratios of approximately 8:1, which occurred in some F_2 populations of coumarin-deficient x Arctic crosses. In the two crosses D x A and D_2 x A marked deficiencies occurred in the low-coumarin class. They were explained on the basis of a 57:7 ratio, which was ascribed to the segregation of two additional, complementary factors, C and D. The coumarin-deficient parents were assumed to possess one of these factor pairs in the homozygous dominant condition. The Arctic parents were thought to contain the second complementary gene pair in homozygous dominant condition. The coumarin-deficient F_2 segregates would be of the three following genotypes, with their relative frequency as indicated:

3 cucuBBC-dd
3 cucuBBccD-
1 cucuBBccdd

From these F_2 populations 52 coumarin-deficient segregates were selected. Ten of these were diallel crossed and crossed with all the other 42 plants. Some of the progeny were expected to possess the two complementary factors in the dominant condition, C-D-, and thus exhibit high coumarin content. The ten plants were also crossed with some coumarin-deficient F_2 segregates of the N x A cross. A total of 1,601 plants,

406 of the diallel group and 1,195 of the others, were tested by the fluorometric method. The observed readings ranged from 1 to 2+. From each class, 1, 1+, 2 and 2+ ten plants were sampled and quantitatively tested with the photofluorometer. All the samples gave zero readings. It was concluded therefore that all plants in this population were void of coumarin and thus complementary genes were not involved. Consequently, a different explanation for the off ratios was sought.

Environmental Influences

Goplen (9) studied his populations in the greenhouse. The conditions there differ considerably from natural field conditions. In order to detect possible environmental influences on the F_2 ratios, F_2 populations from the same types of crosses were grown in the field. They were tested in the fall of the first season and again in the second season, prior to flowering. At this time they were also tested by the colorimetric method to distinguish between free and bound coumarin. In the spring of the second season it was noted that a large amount of winter killing had occurred. Several populations indicated strongly, that the coumarin-deficient segregates were affected more severely by winter killing. Consequently, the segregation for high vs. low coumarin, obtained in the fall, was considered to be reliable. The segregation for free vs. bound coumarin was obtained only on the surviving high-coumarin plants. The results from these populations are summarized in table 2.

TABLE 2. Segregation of coumarin-deficient x Arctic and coumarin-deficient x Pioneer hybrids in the F₂

Cross	F ₂ segregation							
	high coum.	low coum.	ratio	P-value for 3:1 ratio	free coum.	bound coum.	ratio	P-value for 3:1 ratio
N x A	240	69	3.5:1	.50-.20	129	48	2.7:1	.95-.50
N x P	99	20	4.9:1	.05-.01	--	76	--	--
D x A	560	100	5.6:1	<.01	283	--	--	--
D x P	75	20	3.7:1	.50-.20	30	16	1.9:1	.20-.10
D ₂ x A	390	64	6.1:1	<.01	360	--	--	--

The ratios obtained in these populations show the same trend as the ones observed by Goplen (9). It appears that in the progenies of all crosses small deficiencies occur in the coumarin-deficient class. This however may be due to the fact that in each cross a number of plants appeared, which gave doubtful readings and these were included in the high-coumarin class. With the colorimetric test a distinction between bound-coumarin and coumarin-deficient plants is not possible, but the excess of bound-coumarin segregates in the two segregating populations indicate that a number of these doubtful plants were wrongly classified. The crosses N x A, N x P and D x P segregated 3:1 for high vs. low and the crosses D x A and D₂ x A show marked deficiencies in the low-coumarin classes.

The segregation for free vs. bound coumarin in the F₂ of the crosses N x A and D x P fits a 3:1 ratio. The plants containing

free coumarin appear to be affected by winter killing to about the same extent as plants containing bound coumarin. On the basis of this assumption it was possible to calculate the dihybrid ratios. Three quarters of the plants containing coumarin which were winter killed were added to the free-coumarin class, one quarter was added to the bound-coumarin class. The dihybrid segregations obtained in this manner are presented in Table 3.

TABLE 3. Two factor segregation in the F_2 of the crosses N x A and D x P

Cross	F_2 segregation			P-value for 9:3:4 ratio
	free coumarin	bound coumarin	no coumarin	
N x A	176	64	69	.50-.20
D x P	52	23	20	.50-.20

These results give good fits to the theoretical expectation of a 9:3:4 ratio.

These results therefore furnish further proof for the assumption of one factor pair, segregating for high vs. low coumarin (Cu, cu), the low-coumarin gene being recessive. A second factor pair segregates for free vs. bound coumarin. The gene for bound coumarin (b) is recessive. This factor pair attains expression only in the presence of Cu.

The F_2 of the cross D x A also segregated for normal growth type and Alpha type. It has been established by several workers (1, 16, 27),

that the normal type is conditioned by a dominant factor and the Alpha type by its recessive allele. In this population however 273 normal types and 40 Alpha types occurred, a ratio of approximately 7:1. This population therefore exhibits deficiencies in the genotypic classes displaying the genes from the D parent for both the low-coumarin and the Alpha character.

Certation Effects

Neither genic nor environmental causes for the off ratios could be detected. Therefore the possibility that the pollen of the genotype Cu of the hybrid (D x A) is more effective in fertilization than pollen of the genotype cu was investigated. The F₁ plants (N x A), (C x P) and (D x A) and the coumarin-deficient parents N, D and D₁ were reciprocally intercrossed. Crosses between F₁ plants, with plants (N x A) and (D x P) as male parent, were expected to segregate 3:1. Crosses with plant (D x A) as male parent were expected to show a deficiency in the low-coumarin class. In the backcrosses with the coumarin-deficient plants as male parents 1:1 ratios were expected in all cases. The opposite backcross with plants (N x A) and (D x P) should segregate 1:1 whereas with plant (D x A) a deficiency in the low-coumarin class was expected.

The selfs in these populations could not be detected on the basis of their coumarin content. Since they tend to dilute the expected ratios, it was attempted to estimate the proportion of selfs. While crossing, four racemes were emasculated at a time and one was chosen at random to be left unpollinated. From 569 florets emasculated in this way 24.4% set seeds. The ratios in the progenies of

the above crosses were therefore corrected with 24.4% selfs. For these corrections selfs of F_1 (D x A) were assumed to segregate 8:1, and selfs of F_1 (N x A) and F_1 (D x P) were assumed to segregate 3:1. In the cross F_1 (D x P) x F_1 (D x A) the female parent was of the genotype CucuBb, the male parent was of the constitution CucuBB. Progenies containing bound coumarin, Cu-bb, represented the homozygous recessive class of the selfs and the total proportion of selfs in this cross was determined at 26.2%. The two estimates correspond closely and it is assumed that the corrected ratios represent the true segregation. The results of these crosses are presented in Table 4.

TABLE 4. Segregation in reciprocal crosses and backcrosses of normal and abnormal F_1 's, and their coumarin-deficient parents.

Cross	female parent	male parent	normal segregation on one factor basis	Segregation			
				high	low	observed ratio	P-value
1	$F_1(DxA)$	$F_1(NxA)$	3:1	164	40	4.1:1	.10-.05
2	"	$F_1(DxP)$	3:1	206	79	2.6:1	.50-.20
3	$F_1(NxA)$	$F_1(DxA)$	3:1	163	31	5.1:1	< .01
4	$F_1(DxP)$	"	3:1	217	55	3.9:1	.10-.05
5	$F_1(NxA)$	N	1:1	69	49	1.4:1	.10-.05
6	"	D	1:1	58	27	2.1:1	< .01
7	$F_1(DxA)$	N	1:1	31	19	1.6:1	.10-.05
8	"	D	1:1	39	24	1.6:1	.10-.05
9	"	D_1	1:1	38	33	1.1:1	.95-.50
10	D_1	$F_1(NxA)$	1:1	16	18	.9:1	.95-.50
11	"	$F_1(DxA)$	1:1	7	0	--	--

In cross No. 1 a segregation of 3:1 was expected. The results obtained fit that ratio. The P-value is very low due to an excess of high-coumarin segregates. Since the selfed progeny of the male parent of this cross closely fits a 3:1 ratio, these results indicate that the female gametes of the genotype *cu* are probably at a slight selective disadvantage.

Normal segregation occurs in cross No. 2. In cross No. 3 the expected deficiency in the low coumarin class is shown. It is not as

marked however as reported by Goplen (9). This may be due to the fact that an F_1 which gives a normal segregation in the F_2 was used as female parent. For a deficiency as large as indicated by a ratio of approximately 8:1 to occur, it may be necessary to use F_1 (DxA) as male and female parent. A similar off ratio was expected in cross No. 4. Although the segregation fits a 3:1 ratio, the P-value is very low, .10 - .05, and some irregularity is probably present.

In the crosses 2 and 4 the same type of low-coumarin plant is involved on the female and the male side. Since the ratios in these crosses fit the normal 3:1 segregation more closely than the ratios in the crosses 1 and 3, these results may indicate that some degree of incompatibility exists between the plants D and N. On the basis of this assumption the segregation in the selfed populations of these plants should be normal, however this is not the case. The incompatibility appears to exist between plant D and A, and not at all or to a smaller degree between plants D and P, and between N and A. The functioning of the male and female gametes is apparently affected by this incompatibility. In the pollen the irregularities may be somewhat more pronounced as indicated by comparing cross 1 and 3. The deficiency of plants without coumarin is greater when D is on the male side of the cross. In certain plant combinations, for example in the crosses 2 and 4, no selection against the gametes carrying cu can be shown, and reasonably good fits to a 3:1 ratio of coumarin vs. no coumarin are obtained.

The segregation in backcross No. 6 deviates significantly from a 1:1 ratio whereas in backcross No. 5 no significant deviation is

observed. As previously observed, the gametes carrying *cu* from plant N are not completely compatible with the *cu* carrying gametes from plant D. The low P-value in backcross 5 may also reflect that the recessive gametes are at a selective disadvantage, regardless of what plant they were contributed by. The same is true for backcrosses No. 7 and No. 8.

Backcross No. 9 gives an excellent fit to a normal 1:1 ratio. It seems therefore that whatever the cause for irregular segregation, it is only expressed in the progenies of certain plants and plant combinations. Backcross No. 10 shows that F_1 (N x A) produces equal amounts of *Cu* and *cu* gametes and they all function in combination with the *cu* gametes of plant D_1 . Too few plants were obtained in backcross No. 11 to arrive at definite conclusions. The results nevertheless suggest that when F_1 (D x A) was used as the male parent the *Cu* carrying pollen was more successful than *cu* pollen in effecting fertilization.

The examination of pollen of (D x A) and (N x A) F_1 plants revealed that by both types 14-16% unstainable pollen grains were produced. Roottips and pollen mother cells were studied cytologically and no irregularities in chromosome pairing and chromosome number could be observed.

Effects of Interspecific Hybridization

Coumarin-deficient F_3 segregates of the *M. officinalis* x *M. alba* cross, cultured by Webster (33), were crossed with a high coumarin,

permeable seeded strain of M. alba as male parent. In these crosses the plants derived from the interspecific cross served as donor of the cu gene, the M. alba strain contributed the gene for seed permeability. Eleven families with a total of 619 plants were tested for coumarin and for seed permeability. The results of this study are presented in Tables 5 and 6.

TABLE 5. Segregation for individual characters in the F₂ of low-coumarin non-permeable seed x high-coumarin permeable seed crosses.

Family	high coumarin	low coumarin	non-permeable seed	permeable seed
1	32	7	36	3
2	14	3	10	7
3	28	3	26	5
4	22	2	15	9
5	104	19	100	28
6	33	2	24	11
7	98	15	92	21
8	36	3	25	14
9	77	8	56	29
10	41	6	36	11
11	<u>51</u>	<u>10</u>	<u>51</u>	<u>10</u>
Total	547	78	471	148

TABLE 6. Dihybrid segregation in the F₂ of low-coumarin non-permeable seed x high-coumarin permeable seed crosses.

Family	high coumarin		low coumarin	
	non-permeable seed	permeable seed	non-permeable seed	permeable seed
1	29	3	7	--
2	7	7	3	--
3	23	5	3	--
4	13	9	2	--
5	81	28	19	--
6	22	11	2	--
7	77	21	15	--
8	22	14	3	--
9	48	29	8	--
10	30	11	6	--
11	<u>41</u>	<u>10</u>	<u>10</u>	<u>--</u>
Total	393	148	78	--

The results in Table 6 show that the two recessive genes do not occur together in the same plant. Therefore the theoretical ratios for the segregation of high-vs. low-coumarin and non-permeable vs. permeable seed are modified to 4:1. The pooled data of the segregation for coumarin content in Table 5 do not fit the 4:1 ratio ($P =$ less than .01). A marked deficiency in the low-coumarin class is shown. The heterogeneity χ^2 for the eleven families gives a P-value greater than .99. It is therefore in order to pool the results since all the families are homogeneous for the deficiency in the low-coumarin class.

The segregation for non-permeable vs. permeable seed of the pooled results gives a very poor fit to a 4:1 ratio ($P = .05-.02$).

In this case the deficiency is in the non-permeable class. The heterogeneity χ^2 shows, however, that the families are heterogeneous and the results may not be pooled. The families 2, 4, 8 and 9 deviate significantly from a 4:1 ratio with large deficiencies in the non-permeable classes. The ratios in the families 3, 5, 7, 10 and 11 give good fits to the theoretical ratio with P-values from .95-.30. In the families 1 and 6 poor fits to the 4:1 ratio are obtained with P-values of .10-.05. In family 1 the permeable class is deficient whereas in family 6 too few non-permeable segregates were obtained.

The dihybrid segregation deviates significantly from the modified theoretical ratio of 9:3:3. Since the heterogeneity χ^2 gives a P-value of .05-.02, the pooled segregation is of little significance. The segregation in the individual families either deviate significantly from the theoretical ratio or show very low P-values.

The results in these populations show that the genotypic classes displaying the genes contributed by the offspring of the interspecific cross very often show deficiencies. It appears therefore that irregular segregation is due to the effect of interspecific hybridization.

Testing Sweet Clover Seeds for Coumarin

To evaluate the testing procedure described by McAllister and Davis (19) 15 seeds each of the varieties Arctic and Pioneer and of a coumarin-deficient selection were tested. All the samples of the high-coumarin varieties gave a green fluorescence by the rapid fluo-

rometric method (10) indicating coumarin, while on the basis of the same test the samples of the coumarin-deficient selection contained no coumarin. These seeds were planted in greenhouse flats and the plants were tested again after eight weeks. Similar results were obtained; the Arctic and Pioneer plants showed a high coumarin content, the plants of the coumarin-deficient selection were void of coumarin.

Subsequently 204 seeds were obtained from reciprocal crosses between coumarin-deficient strains and the variety Arctic. One hundred and twenty-five of these were tested by the fluorometric procedure. In half of the samples the cut-off portion of the seed coat plus the tip of the cotyledons was included, in the other half only the tips of the cotyledons were tested. The seeds were germinated and the plants grown from them were tested after eight weeks.

Most of the seeds produced on the Arctic plants gave strong readings for high-coumarin content. A few doubtful readings were also obtained. The majority of the plants grown from these seeds exhibited high-coumarin content. A small portion of doubtful readings of 2+ were also obtained. The seeds produced on the coumarin-deficient plants gave low readings in most cases. About 10% of the samples were classified as doubtful and 5% showed high coumarin content. The plants grown from these seeds were high in coumarin content with the exception of a few coumarin-deficient selfs.

The colorimetric test gave similar results. Positive readings were obtained from all the seeds produced on Arctic plants. Seeds

from the reciprocal crosses gave negative tests. About 10% doubtful readings were obtained in the latter group.

The exculsion of the seed coat from the sample did not influence the obtained readings.

All the seeds, except the selfs of the crosses with coumarin-deficient plants as female parent, contained the gene Cu for coumarin production. These results, however, indicate that the coumarin content of the seed is determined by the female parent and not by the genotype of the embryo.

DISCUSSION

In the progenies of crosses between the same types of plants as studied by Goplen (9) similar ratios were observed. The crosses $N \times A$, $N \times P$ and $D \times P$ segregated 3:1 in the F_2 for high vs. low coumarin whereas the F_2 of the crosses $D \times A$ and $D_2 \times A$ marked deficiencies occurred in the coumarin-deficient classes. The segregation for free vs. bound coumarin in the progenies of the $N \times A$ and $D \times P$ crosses gave a close fit to a 3:1 ratio. The dihybrid segregation closely fitted the theoretical expectation of a 9:3:4 ratio. The assumption of one factor pair segregating for high vs. low coumarin (Cu, cu), the coumarin-deficient gene being recessive, and a second factor pair segregating for free vs. bound coumarin (B, b), the bound-coumarin gene being recessive, is further substantiated. The genes B and b attain expression only in a plant containing the gene Cu .

The F_2 progeny of the cross $D \times A$ segregated for normal growth type vs. Alpha type. In contrast to the results of other workers (1, 16, 27), a ratio of approximately 7 normal types: 1 Alpha type was observed. The deficiency of Alpha types in the F_2 progeny of the $D \times A$ cross is assumed to be due to the same cause as the deficiency of low-coumarin segregates in the same population.

From the F_2 of a $D \times A$ cross studied by Goplen (9) 52 coumarin-deficient segregates were selected. Since all intercrosses among these plants produced only coumarin-deficient progenies it is concluded that complementary genes are not involved. It is assumed therefore

that irregular segregation in certain populations is due to non-genic causes. The same off ratios were observed in D x A progenies grown in the field and in the greenhouse, and the unusual ratios do not appear to be attributable to environmental influences. The study of the crosses designed to detect certation effects indicates that the deficiency in the low-coumarin classes in the F_2 of D x A and D_2 x A crosses can not be attributed to preferential pollen functioning alone. Some type of incompatibility exists between plants D and A. It either does not exist between plants D and P and plants N and A or it exists to a smaller degree only. The results show that the male and the female gametes are affected by this incompatibility, its effects appear to be more pronounced in the pollen. The F_1 plants (N x A) and (D x A) produce equal proportions of stainable pollen, although in the plant combinations D x A and D_2 x A there is a deficiency of effective cu-carrying pollen, or both pollen and eggs. It is possible that pollen and eggs carrying the gene cu effect fertilization as readily as gametes carrying Cu but that some embryos of the genotype cucu abort before maturity.

It is evident that in the progenies of crosses with plants D and N selection occurs against cucu types. In the selfed progenies of most crosses with plant D this selection is strong enough to cause significant deviations from normal one factor ratios. In the progenies of crosses with plant N the selection against cucu types is present to a lesser degree and thus low P-values are often calculated.

The segregation in the progenies of the crosses between permeable-

seeded strains of M. alba and coumarin-deficient F_3 segregates of a M. officinalis x M. alba cross show significant deviations from normal ratios. No low-coumarin, permeable-seeded plants were obtained. The two recessive genes together either are lethal or male and female gametes of this genotype in combination are unsuccessful in fertilization. The segregation for high vs. low coumarin content deviates significantly from a modified theoretical 4:1 ratio. The class containing the low-coumarin gene, contributed by the offspring of an interspecific cross, is deficient. All the families studied are homogeneous for this deficiency.

For the non-permeable vs. permeable seed segregation heterogeneous results were obtained for the 11 families. The observed results fit the modified ratio of 4 non-permeable to 1 permeable seeded plants in seven families. These families were homogeneous and the pooled results give a very close fit to the 4:1 ratio. The remaining families showed marked deficiencies in the non-permeable class. In this case again the class containing the gene from the offspring of the interspecific cross shows the deficiency. With respect to seed permeability it appears that normal segregation occurs in some families but not in others.

Stephens (31) reported that in the progenies of interspecific crosses in cotton skewed backcross ratios and blurred monofactorial ratios are obtained. These are ascribed to structural differences between the genomes involved in the interspecific crosses. He further noted that these structural differences are too small to be

detected cytologically. In successive backcrosses the ratios become more normal. Since all the low coumarin strains of sweet clover are derived from an interspecific cross between M. dentata and M. alba made by Smith (24), similar structural differences between the genomes of these two species are assumed to be responsible for the irregular segregation in certain populations. In successive generations the plants become more stabilized with respect to these structural differences. Depending on the degree of stabilization in the coumarin-deficient plants normal or abnormal segregation occurs in crosses with M. alba. For the permeable seed character normal segregation occurs in some crosses while others show deficiencies in the non-permeable class. In the progeny of the interspecific cross M. officinalis x M. alba cultured by Webster (33) only the low-coumarin character was selected. It is probable that some F₃ segregates carry the gene pair for this seed character on a pure M. alba chromosome, while others carry them on cross-over chromosomes. In crosses with M. alba the plants of the first group segregate normally whereas in crosses of M. alba with the latter group irregularities occur.

Thus it is concluded that the irregular segregation for coumarin content, seed permeability and Alpha type in certain sweet clover populations is not due to genic causes but it is associated with the effect of interspecific hybridization. In intraspecific crosses normal mendelian ratios occur whereas in interspecific crosses and backcrosses abnormal ratios are obtained. These are due to structural

differences between the chromosomes of the species involved in the interspecific cross. It is probably that the gametes carrying structurally differentiated chromosomes are less effective in fertilization and in certain cases some of the zygotes resulting from the fusion of two such gametes abort. In the coumarin-deficient strains of sweet clover the chromosomes displaying structural differences are apparently stabilized to various degrees, and therefore in crosses with pure species normal ratios occur in some cases, while in others deficiencies are present.

The test of sweet clover seed for coumarin content revealed that seeds produced on plants containing coumarin also contain coumarin, regardless of the genotype of the embryo of the seed. Similarly seeds produced on coumarin-deficient plants are in most cases also coumarin-deficient. Thus with regard to coumarin content the genotype of the embryo is generally not expressed in the seed and this testing procedure can not be relied upon as a rapid method of determining the presence or absence of coumarin in breeding material of sweet clover.

SUMMARY

1. Two genes are involved in the inheritance of coumarin content in sweet clover. The main gene Cu is responsible for the production of coumarin. The gene B conditions free coumarin in a coumarin-containing plant.
2. The Alpha type of growth is conditioned by one recessive gene. There is no linkage present between the coumarin genes and the gene for Alpha type.
3. Seed permeability is inherited as a simple recessive. This character is independent from the other characters studied.
4. The genes for seed permeability and low coumarin together either are lethal or male gametes containing these two genes are not effective in fertilization in combination with female gametes of the same genotype.
5. With respect to the four characters, coumarin content, type of coumarin, growth type and seed permeability simple mendelian segregation occurs in the progenies of some crosses while in others irregular ratios are obtained.
6. The irregular segregation in certain F_2 populations is not due to the segregation of complementary genes. The environment does not significantly affect the F_2 ratios.
7. The irregular segregation is due to non-genic causes. It is ascribed to the effect of interspecific hybridization. In the progenies of crosses with interspecific hybrids the classes displaying the genes contributed by the interspecific hybrid show marked deficiencies.

These deficiencies are ascribed to structural differences between the chromosomes of the species involved in the interspecific crosses. The structural differences between corresponding chromosomes apparently are too small to cause meiotic irregularities and to be detected cytologically. The gametes carrying structurally differentiated chromosomes are assumed to be at a selective disadvantage. Male and female gametes are affected. In some plant combinations the embryos containing such chromosomes are aborted. In successive generations the chromosomes become stabilized and normal ratios are then observed.

8. The testing method, by which portions of the seed are analysed for coumarin content, can not be applied to breeding work. The coumarin content in the seed corresponds with that of the female parent in most cases but not with the genotype of the embryo.

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