IDENTIFICATION AND INHERITANCE OF FLOWER PIGMENTS IN DIPLOID *MEDICAGO* SPECIES

Thesis for the Degree of Ph. D.

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Richard L. Cooper

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This is to certify that the

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presented by

Richard L. Cooper

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Fred C. Elliott
Major professor

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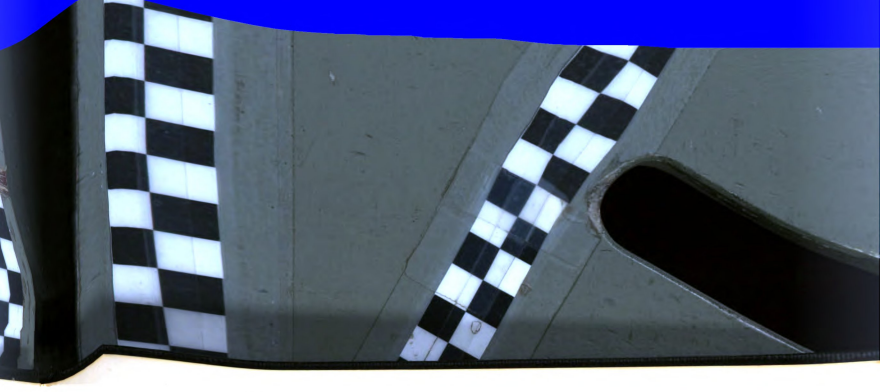

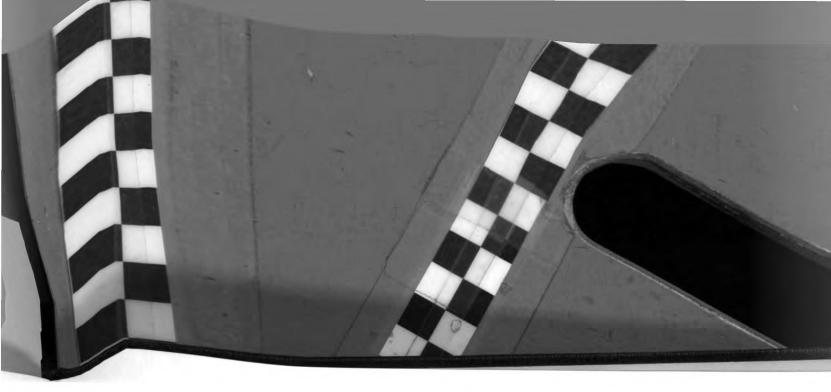

IDENTIFICATION AND INHERITANCE OF FLOWER PIGMENTS IN DIPLOID *MEDICAGO* SPECIES

By

Richard L. Cooper

AN ABSTRACT

Submitted to
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Department of Farm Crops

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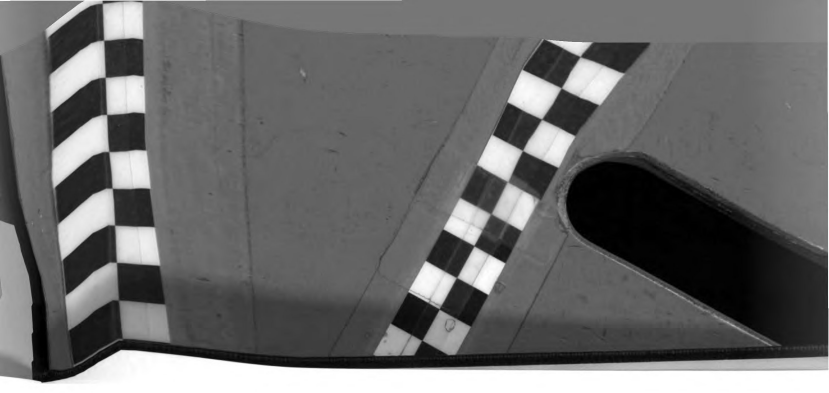


ABSTRACT

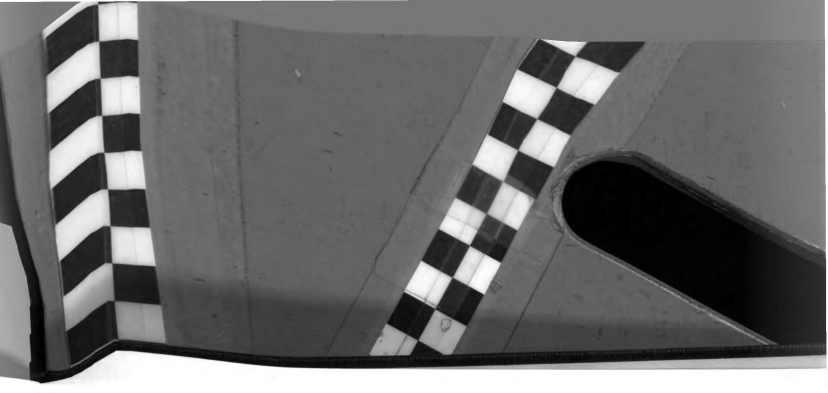
IDENTIFICATION AND INHERITANCE OF FLOWER PIGMENTS IN DIPLOID MEDICAGO SPECIES

by Richard L. Cooper

Flower color has been used as a convenient tool for the study of tetraploid inheritance in alfalfa. Such studies have been only partially successful because of the complexities of tetraploid inheritance and the phenotypic interaction of flower pigments. A technique whereby the complicating factors of tetraploid segregation and phenotypic interactions are removed would be useful in determining the inheritance pattern of flower color in alfalfa.

From the F_2 intercross progeny of a tri-species diploid hybrid (M. gaetula x M. falcata) x M. sativa, 42 flower color types were identified. These plants were used as source material in an intensive crossing and selfing program of the various color types. Flower pigments were separated chromatographically from 700 plants within 23 segregating families, identified, and their inheritance patterns determined.

Three anthocyanin pigments were found in every flower containing anthocyanin. The production of these pigments was under control of a single dominant gene.



Richard L. Cooper

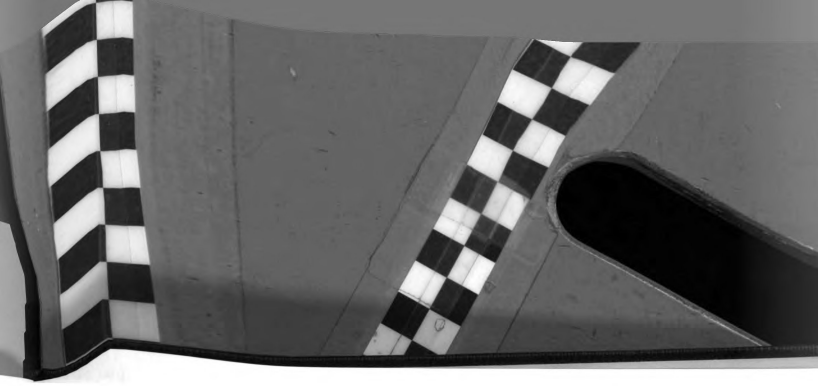
Nine flavonol pigments consisting of six quercetin glycosides and three kaempferol glycosides were also observed. Two quercetin glycosides and one kaempferol glycoside were present in every plant examined. Although a definite segregation pattern was not established, two hypotheses were proposed for the inheritance of quercetin pigments.

Two kaempferol glycosides exhibited independent segregation in a 3:1 ratio indicating each was controlled by a single dominant gene.

The carotenoid pigment in alfalfa flowers was identified as xanthophyll ester and exhibited a 1:4:6:4:1 segregation in some families, indicating control by quantitative factors at two loci.

Phenotypic correlation of pigments indicated that blue and purple colors were due to mixtures of three anthocyanin pigments. Evidence obtained indicated that kaempferol glycosides produce a phenotypic effect by copigmentation with anthocyanin pigments. Certain intensity levels of quercetin glycosides produced a yellowing effect, but yellow flower color intensity was most closely associated with intensity of xanthophyll pigments.

From the inheritance of flower pigments and their phenotypic correlation, an inheritance chart for flower color in diploid alfalfa is proposed.



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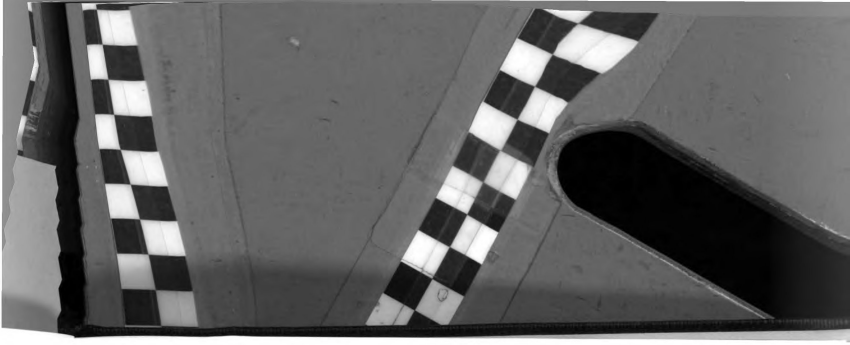

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INTRODUCTION

Flower color has been used as a convenient tool for the study of inheritance in tetraploid alfalfa. These studies have been only partially successful because of the phenotypic interactions of the blue and yellow pigments, and the complications resulting from tetraploid segregation.

The availability of natural diploid alfalfa provides a tool whereby the inheritance of flower color can be studied without the complication of tetraploid inheritance. Once an inheritance pattern is established at the diploid level, it may serve as a useful guide in the interpretation of tetraploid inheritance.

Separation of the component pigments and study of their inheritance individually removes the phenotypic interactions encountered in a study of flower color inheritance. From the inheritance of these pigments, an explanation of flower color inheritance in diploid alfalfa can be obtained.

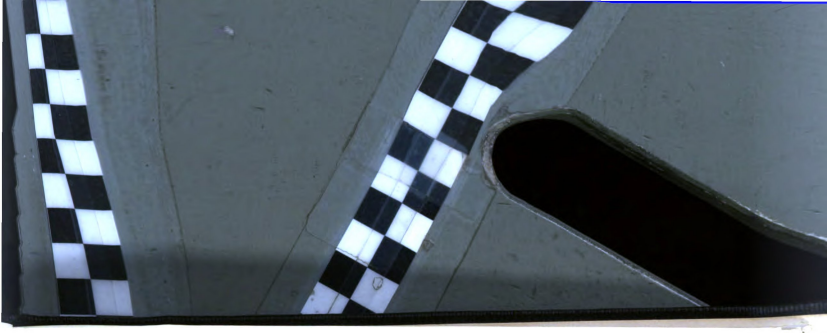
The purpose of this study was to (1) identify the flower pigments in alfalfa (2) study the inheritance of these pigments (3) use this information to explain flower color inheritance in diploid alfalfa.

REVIEW OF LITERATURE

Flower Color Inheritance in Alfalfa

Flower color has been used as a convenient tool for studying inheritance in tetraploid and diploid alfalfa. Earlier investigations were with tetraploid alfalfa. In these studies, flower colors were grouped into three main classes "purple", which included purple and variegated flowers, "yellow", which included all gradations of yellow, and "white". Using this system of classification, 3:1, 15:1 and 63:1 F_2 ratios of purple to non-purple were observed from crosses between tetraploid M. sativa (purple flowered) and M. falcata (yellow flowered) and also in crosses between purple and white flowered M. sativa plants. Most investigators explained these ratios on the assumption of disomic inheritance and segregation for one to three supplementary (duplicative) factors for anthocyanin production (Lepper and Odland 1939; Armstrong and Gibson 1941; Weihing 1948). Stanford (1951) obtained the same ratios but pointed out that they could be explained either on the basis of tetrasomic or disomic inheritance.

The occurrence of purple flowered progeny from white x white or white x yellow crosses has been cited as evidence of complementary gene action (Lepper and Odland 1939; Weihing 1948). Complementary gene action in diploid alfalfa was reported by Twamley (1955) who obtained 9:7 ratios in some F_2



families of a sativa-falcata diploid cross.

Yellow color inheritance has not been as extensively studied because of the epistatic effect of purple pigment and the quantitative nature of yellow color segregation. Grouping all gradations of yellow into a single class, Odland and Lepper 1939, proposed a single gene for yellow pigment production. Weihing 1948, in a study of F_2 segregating families observed 3:1, 15:1 and 63:1 segregation of yellow to white. From these observations he proposed at least 3 factors for yellow pigment production.

A more intensive study of yellow pigment inheritance was made by Twamley (1955) in both diploid and tetraploid plants. From the distribution into intensity classes of 85 yellow F_2 plants of a diploid sativa-falcata cross, he proposed yellow pigment production was controlled by four quantitative genes, or more probably by four factors, some of which are quantitative and some which are qualitative.

Flower Pigments in Alfalfa

Flower pigments in alfalfa can be divided into three main classes, anthocyanin (blue and red pigments), anthoxanthins (yellow sap soluble pigments) and carotenoids (yellow plastid pigments). These three classes of pigments were first reported by Twamley (1955) who identified the anthocyanin as primarily malvidin. Lesins (1956), however, using paper chromatography was able to separate four anthocyanin

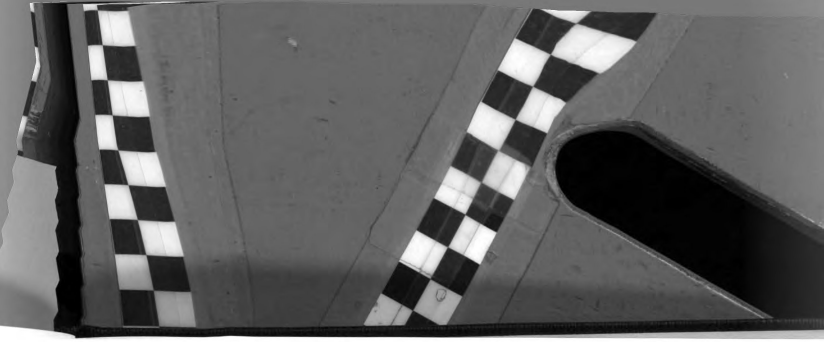
[illegible]

pigments from alfalfa, and identified their aglycones as delphinidin, petunidin and malvidin. Davies (1958) identified delphinidin, cyanidin and malvidin, as well as the anthoxanthins, quercetin, kaempferol, myricetin, and tricin from extracts of alfalfa flowers.

In a histological study of alfalfa flowers, Lesins (1956), observed that the coloring pigments were located in the epidermal layers of the petals. In variegated flowers, the coloring matter consisted of yellow carotenoid plastids, anthocyanins and anthoxanthins. Green color was explained as a result of a side by side purple-and-yellow cell mosaic occurring when not all cells had both purple and yellow pigments. When numerous cells contained both yellow and purple pigments, a color subtraction phenomenon was produced, giving dark color.

Genetics of Flower Pigments

Anthocyanins and anthoxanthins.-Much of the knowledge about inheritance of anthocyanin and anthoxanthin pigments stems from the research of Scott-Moncrieff 1936, Beale 1939, and Lawrence 1935. These investigators combined the information on chemical structure of flower pigments (Robinson 1933) with the accumulated knowledge of flower color inheritance, and established the first examples of the relationship between single genes and a simple biochemical difference in higher plants.



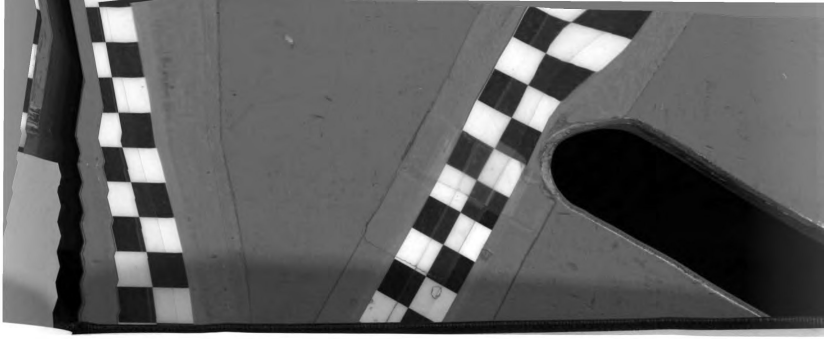
All flavonoid pigments arise from a common C_{15} precursor and are elaborated into the different classes by processes of hydroxylation, methylation, glycosidation and acylation (Harborne 1958). Specific examples of these types of action have been reviewed by Scott-Moncrieff (1936) and Lawrence et al (1940). More recent examples have been found by the use of paper chromatography to separate complex mixtures of pigments (Geissman et al 1954; Dodds and Long 1955; Nordstrom 1956; Alston and Hagen 1958; Harborne and Sherratt 1958).

Of particular pertinence to the study of flower pigment inheritance in alfalfa are the genetic studies of flower color in sweet pea, Lathyrus odoratus. Beale (1939) reported that some varieties of sweet peas contained a mixture of delphinidin, petunidin, and malvidin glycosides. He also observed the presence of an anthoxanthin, which in the presence of anthocyanin formed weak additive complexes, making the anthocyanin bluer than it would be in absence of anthoxanthin (copigmentation). This presence of anthocyanidin mixtures and copigmentation effects of anthoxanthins was confirmed by the recent work of Pecket (1960). Based on observations of a number of different Lathyrus species he concluded that the bluing of the wings within a species or variety and the bluish red to blue flower color of other species is due primarily to the copigmentation action of the anthoxanthins.

Carotenoids.-One of the first genetic studies on carotenoid production was reported by Mangelsdorf and Fraps (1931), in a study of yellow endosperm color inheritance in corn. They found that zeaxanthin, the principle pigment in yellow endosperm was controlled by a single quantiative factor Y. Because of the triploid (3N) condition in the endosperm, four dosage levels of Y, (yyy, yyY, yYY, and YYY) were possible with the four corresponding phenotypic classes (white, pale yellow, dilute yellow, and deep yellow endosperm color).

LeRosen et al (1941) in an inheritance study of lycopene, the red carotenoid pigment in tomato, found production of this pigment controlled by a single dominant gene.

In an intensitive review of the literature on flower color inheritance, Clark et al (1960) compiled inheritance charts for each of 75 different species. Of these 75 species, only 4 species had genetic factors identified as affecting carotenoid production. These were (1) Mimulus cardinalis with a single quantitative factor for carotenoid production, segregating 1:2:1 (Brozek 1932; Vickery and Olsen 1956) (2) Tropaeolum majus with two complementary genes for production of carotenoid (Moffett 1936; Sutton 1939) (3) Eschscholtzia californica, in which carotenoid production is controlled by a single dominant gene (Douwes 1943) (4) Primula sp. with a single dominant gene for carotenoid production (Frimmel 1932).



Chromatographic Identification of Pigments

Anthocyanins.-The earlier color and distribution tests developed by Robinson (1931) for the identification of anthocyanins, have largely been replaced by paper chromatography. Bate-Smith (1948) was the first to use paper chromatography for separation and identification of flavonoid pigments. Since that time, paper chromatography has been used by many investigators for separation of complex mixtures of flower pigments (Bate-Smith 1950, 1954, 1956; Bate-Smith and Westall 1950; Geissman et al 1954, 1955; Nordstrom et al 1953, 1956; Hayashi 1954, 1957; Harborne 1958; Alston and Hagen 1958; and many others. A very excellent review of the paper chromatographic procedures used in identification of anthocyanin pigments was published by Harborne (1958).

Extensive lists of R_f values for numerous anthocyanins and anthocyanidins, in various solvent systems, are available in the literature, serving as useful guides for tentative identification of flower pigments (Bate-Smith 1950; Geissman 1954, 1955; Harborne 1958). For more positive identification, these authors suggest that unknown pigments should be compared directly with an authentic sample on the same chromatogram (cochromatography), preferably in two or more solvent systems.

Anthoxanthins.-In general the chromatographic procedures used for the separation and identification of anthocyanins are equally applicable to anthoxanthins. Techniques

for extraction of anthoxanthin and their hydrolysis for obtaining the aglycones have been reported by several investigators (Geissman 1954, 1955; Nordstrom 1953, 1956; Bate-Smith 1954; Roberts 1956).

Several published lists of R_f values for the anthoxanthins are available for use in tentative identification of extracted anthoxanthin pigments (Bate-Smith 1950, Gage et al 1951; Geissman 1954, 1955; Roberts 1956).

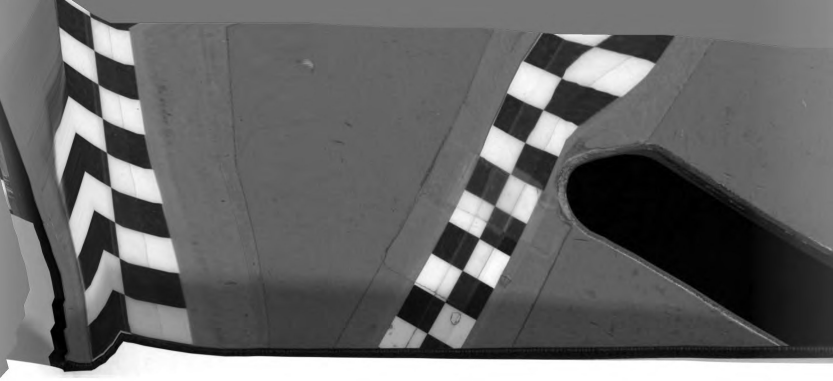
Carotenoids.-Separation of carotenoid pigments has been primarily by use of adsorption columns. Procedures have been worked out and summarized by Strain (1943) and Goodwin (1955). An excellent method for preparation of columns by a wet packing method was described by Williams (1948).

MATERIALS AND METHODS

In the F_2 intercross population of a trispecies hybrid between diploid *M. sativa* and the F_1 (*M. gaetula* X *M. falcata*), forty different color types were obtained, ranging from white to yellow, through green (variegated) to purple. Color types were identified by comparison with freshly picked flowers from standard color plants. Whenever a new color was found, it was given a new number and was made a standard plant for that phenotype. An effective color chart was made by placing freshly picked flowers from each of the standard plants into water-filled glass vials carried in a tray.

Two plants, representative of each flower color phenotype, were moved into the greenhouse for use in a crossing program. Special emphasis was placed on crosses of purple and variegated flowered plants to yellow and white flowered plants. Yellow by white crosses were also made as well as crosses within the major color classes. Several plants were selfed, with particular emphasis on the variegated flower types. Since there were no plants with the orange yellow flower color of *M. falcata*, three different sources of *M. falcata* were also included in this crossing program.

All flowers were emasculated using Lesins (1955) technique of dipping the tripped flowers into 57% ethanol and rinsing quickly in fresh water. When nearly dry, the adhering anthers and pollen were removed by air suction. As a



check, 100 flowers of a self-fertile clone were emasculated and not pollinated. Only 2 pods and one seed developed. Pollination was accomplished by transferring pollen from plant to plant with a tooth pick. Racemes were then tagged as to date and parentage.

After maturity, seed was harvested from the various crosses, germinated on moist filter paper in petri dishes, and transplanted to the greenhouse into 2"X2" peat pots. The pots were placed on top a 4" layer of sand. A strip of mesh wire was stretched about 5" over the top of the bench to support developing seedlings. In this manner, 1000 seedlings were grown to the blooming stage in one 3'X20' greenhouse bench, without transplanting.

Using the fresh flower color chart, the progeny were classified as to phenotype. Special emphasis was made to group phenotypes within progenies so it would not be necessary to analyze chromatographically all plants. A maximum of five plants within each phenotype were analyzed, but if segregation for pigments appeared in these five plants, additional plants were examined.

For chromatographic analysis, a minimum of 20 flowers per plant were needed, with a maximum of 30 flowers. For many plants it was necessary to make three or four collections to obtain the minimum number of flowers. To insure that the same plants were used in subsequent collections, each seedling was tagged as to cross and plant number.

When flowers were collected the petals were separated from the other flower parts by clipping off the standard, tripping the flower and pulling off the wings and keel in a unit. The petals were put in small coin envelopes labeled as to cross, plant number and color type, and placed back in the envelope box. By storing the box in a cool dry place the small petals dried readily and could be held for 2 or 3 months with no apparent breakdown of pigments.

Extraction Procedure.-Using the technique developed by Vickery and Olsen (1956), the plastid borne, carotenoid pigments were extracted by placing 3 mg of dried alfalfa petals in a mortar and grinding in petroleum ether. A few grains of sand were added for abrasive action. The ether extract was decanted off and centrifuged to remove any suspended material. As soon as the residue in the mortar had dried, 3 ml of .1N HCl was added for extraction of the sap soluble anthocyanin and anthoxanthin pigments. The tissue was ground further in this acid solution and then, while the residue was still in suspension, poured into the test tube containing the dried precipitate from the petroleum ether extract. The solution was stirred, centrifuged and the supernatant extract poured off. In some flowers, particularly the bright yellow types, the residue after acid extraction still contained some yellow plastid pigment. This was removed by adding 3 ml of isopropyl alcohol to the residue, stirring it up and letting it set 10 to 15 minutes. Centrifuging and

decanting off the yellow isopropyl alcohol extract left a colorless white residue. For convenience in centrifuging, extractions were done on two petal samples at a time.

Chromatographic Analysis.-The acid soluble pigments, anthocyanins and anthoxanthins, were separated and identified by use of paper chromatography. Inital comparisons between ascending and descending chromatography indicated that the ascending technique described by Vickery and Olsen (1956), with some modification, would more aptly meet the requirements needed. Whatman No. 1 filter paper was cut into 14 1/2 X 1 1/2 inch strips and the pigment to be tested spotted 3 cm from the bottom. The paper was attached to the proper size cork and then inserted into a large test tube (15" X 2") such that the bottom of the paper was 0.5 cm in the appropriate developing solvent. The cork sealed the tube, permitting the formation of a saturated atmosphere.

Since most of the acid extracts were rather dilute solutions, it was necessary to concentrate the pigment by repeated application of the extract to the paper. To speed up this procedure, a mimeoscope was utilized for light as well as heat to increase rate of evaporation. The standard procedure was to apply twenty drops from a glass rod which had been rounded on the end by heating. The same glass rod could be used from sample to sample by rinsing it in running water and wiping it with a clean cheese cloth. Although this is not as precise as the use of micropipettes, it proved both satisfactory and expedient.

The prepared strips were placed in twenty test tubes, supported in wooden racks and placed inside a chromatocab. The insulated chromatocab decreased temperature fluctuation on the tube walls. Under tnese conditions, a very uniform solvent front was obtained with readily repeatable separation of pigments.

Solvent systems.-Four solvent systems were used in this study and are listed with abbreviation, composition, and runing time in Table 1.

Table 1. Solvent systems used for the chromatography of flavonoid pigments.

Abbreviation	Composition	Ratio V/V	Running Time hrs.
BAW[1]	n-butanol-acetic acid-water	4:1:5*	20
HAc-HCl[2]	water-acetic acid-conc. HCl	82:15:3	8
15% HAc[3]	water-acetic acid	85:15	6
Forestal[4]	acetic acid HCl-water	30:3:10	15

[1]Bate-Smith 1950, Gage, et al 1951, Harborne 1958; [2]Harborne 1958; [3]Gage et al 1951; [4]Bate-Smith 1954, Harborne 1958.
*Top phase used

The BAW solution was the standard solvent used in chromatographic separation of the flower pigments in the raw plant extracts. The three other solvents were used for identification of the purified pigments. BAW was equilibrated three days in a separatory funnel before the lower phase was removed and the upper phase distributed in 30 cc aliquots into the test tubes. The BAW in the tubes was replaced with

fresh solution at least every two weeks, depending on the frequency of use. In descending chromatography the filter paper strips, spotted with the extract, were equilibrated 24 hours with the lower phase of the BAW solvent system before the top phase was added to the top tray (Bate-Smith 1950). The running time for the descending BAW was only 9 hours.

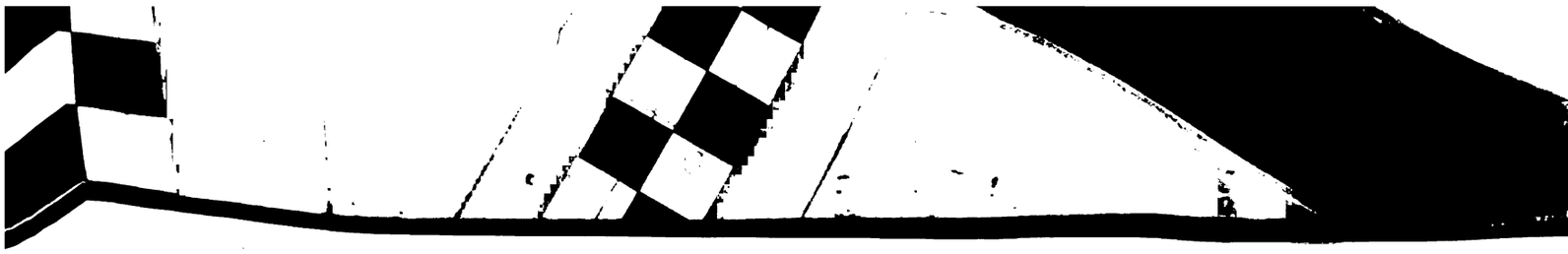
For purposes of identification, the solvent systems used were those for which published R_f values of flavonoid pigments were available. The R_f values for anthocyanins were determined in BAW and HAc-HCl and the R_f values for the anthoxanthins in BAW and 15% HAc. For the aglycones of the anthocyanins, (anthocyanidins), R_f values were determined in "Forestal" solvent and on 1% HCl acid washed paper in BAW. The acid washing was needed to prevent fading out of the anthocyanidins (Harborne, 1958). The R_f values for the anthoxanthin aglycones were determined in the "Forestal" solvent only. The "Forestal" solvent was particularly effective for aglycone analysis because any glycosides remaining as a result of incomplete hydrolysis have a very high R_f value in this solvent and hence are not confused with the lower R_f values of the aglycones (Bate-Smith 1950).

An important aid in locating the flavonol pigments on the chromatograms was a blank tube containing 30 cc of ammonium chloride in the bottom. When the chromatograms were inserted into the ammonia vapors, the flavonol spots were intensified to a bright yellow. Also, in some cases, ultra

violet light was used in combination with ammonia vapor to locate very dilute spots.

Purification of Pigments.-For purification of individual pigments, a series of 30 chromatograms were run of the concentrated extracts from plants containing the appropriate pigments. The pigment bands were then cut from the dried chromatograms and placed in 15 ml vials. To these 30 strips, approximately 3 cc of .1N HCl was added as an eluting solvent. Elution with the more commonly used 1% HCl or 1% methanolic HCl (Harborne 1958) did not prove satisfactory as the anthoxanthin glycosides were readily hydrolyzed in this solution at room temperature. Pigment eluted in .1N HCl remained primarily as the glycoside even after one month storage in the refrigerator.

Hydrolysis of glycosides.-In order to identify the aglycones, acid hydrolysis of the purified glycoside was used. Three ml of .1N HCl containing the eluted pigment was made 2N by adding concentrated HCl. This solution was then heated in a boiling water bath, in absence of light, for 10 minutes (Nordstrom 1956). Hydrolysis at 100°C for 20 minutes in the light, as recommended by Bate-Smith (1954) was too severe for the dilute solution of eluted anthocyanins, and the pigments were lost. The aglycone was separated from the hydrolyzate by partitioning into a few drops of isoamyl alcohol. The isoamyl fraction was then used in spotting the chromatograms (Bate-Smith 1954).



For the anthoxanthins, hydrolysis of the eluted pigment in 1N HCl for 10 minutes gave nearly complete hydrolysis (Roberts 1956). A more severe treatment of 2N HCl for 15 minutes destroyed the eluted pigment although it was satisfactory for hydrolysis of the raw extract. Here again the isoamyl alcohol was used to separate the aglycone from the hydrolyzate.

Identification of pigments.-For identification, the R_f value for each of the purified glycosides was determined in both an organic and an aqueous solvent. In addition, the R_f value for the aglycone of each pigment was determined in two solvent systems for the anthocyanidins and one solvent system for the anthoxanthin aglycones (Table 1). These R_f values were compared with previous reported R_f values and where available, were cochromatogrammed with authentic compounds. The compounds obtained and their sources are listed in table 2.

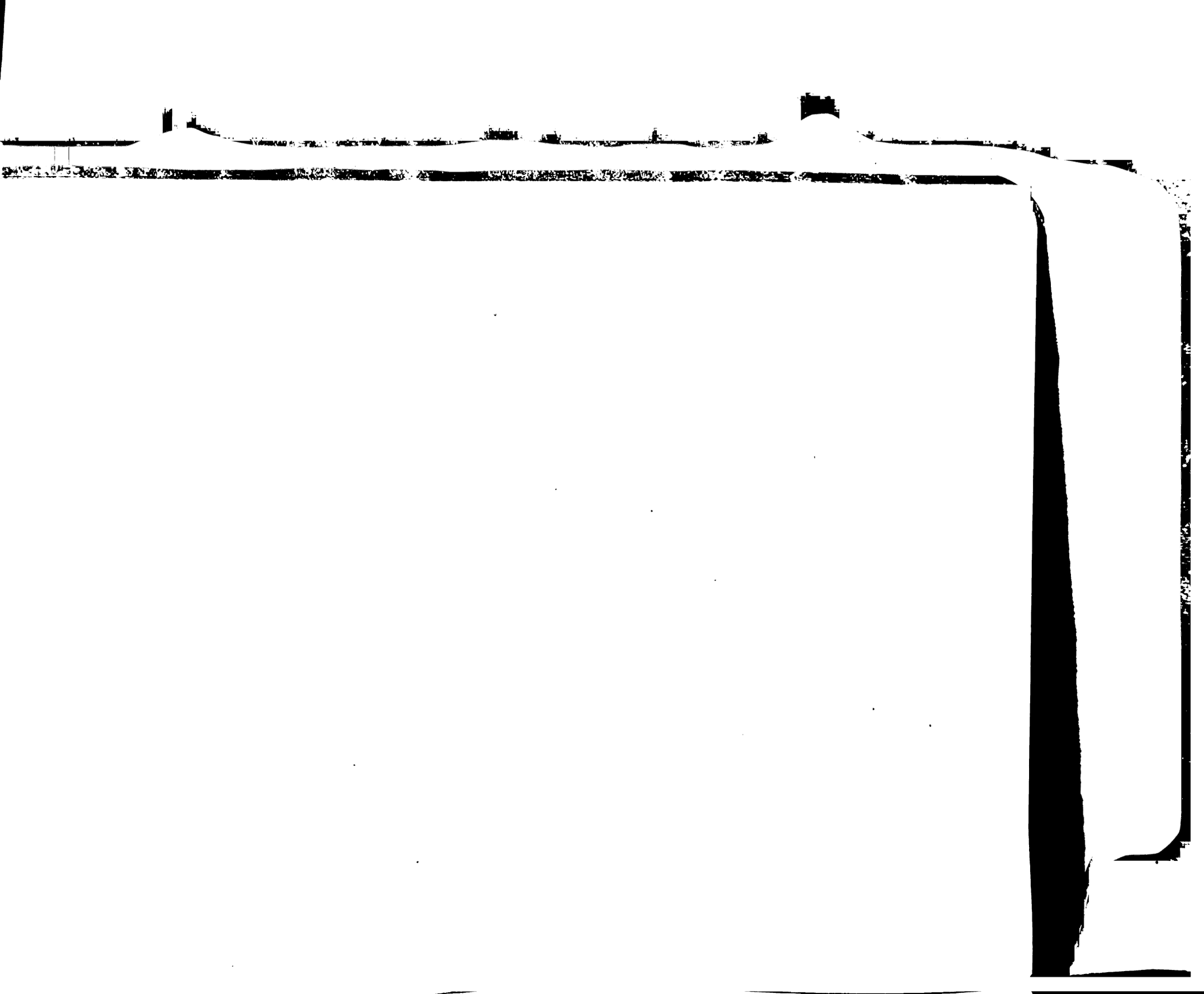


Table 2. Authentic compounds and their source

Pigment	Source
Delphinidin 3,5 diglucoside Delphinidin 3 monoglucoside	J. B. Harborne, John Innes Horticultural Institute Hertford, England
Malvidin 3,5 diglucoside Kaempferol	Mann Chemical Company 135 Liberty Street New York 6, N. Y.
Quercetin Rutin Crystalline Carotene	General Biochemicals Laboratory Park Chagrin Falls, Ohio
Delphinidin Malvidin	Hydrolysis of their respective standard glycosides
Petunidin Cyanidin	Hydrolyzed extract of maroon petunia and of red rose, respectively

Carotenoid Intensity.-It was noted that there were several levels of intensity for the ether soluble carotenoid pigments. These were rated visually, at the time of extraction as 0, if no visible yellow was obtained in the ether extract, Tr, if a trace of yellow pigment, and +, ++, or +++ with increasing intensity of yellow pigment. The residual plastid pigments, extracted with isopropyl alcohol were found to be directly proportional to the intensity of the ether extract.

Partition test for carotenoids.-A simple test for the separation of carotene and xanthophylls is to partition the ether soluble pigment between petroleum ether and 80% methanol. Carotene, being more soluble in petroleum ether, remains in that phase, whereas xanthophyll will be accumulated in the

methanol phase. However, if xanthophylls are esterified with fatty acids, which often may be the case in raw plant extract, this test can be misleading for the xanthophyll esters are retained in the petroleum ether phase, appearing to be carotene (Strain 1945). To prevent such an error in identification, the raw petroleum ether extract was saponified for 15 minutes in alcoholic KOH* to break possible ester linkages. To remove the hydrolyzed xanthophyll from the alcoholic KOH, the solution was diluted in half with water and then shaken with an equal volume of petroleum ether. The xanthophyll was partitioned into the ether phase where it was washed twice by shaking with an equal volume of 50% ethyl alcohol and once with water, to remove any residual KOH. This saponified pigment was then partitioned between petroleum ether and 80% methanol to determine if the pigment was primarily xanthophyll or carotene.

Adsorption chromatography.-Adsorption columns were prepared for separation of component pigments in the petroleum ether extract. Glass tubing, 1 cm in diameter was cut in 30 cm lengths and heated in a blow flame 2 inches from one end to form a constriction for support of a cotton plug.

*Dissolve 10 gms of KOH in 5 ml of water, cool, and then add 95 ml of absolute ethyl alcohol.

In all columns, the wet packing system described by Williams (1948) was used. For the separation of xanthophyll or xanthophyll esters from carotene, a column was prepared with the bottom 15 cm packed with a 2:1 mixture of Celite and MgO (activated)*, the next 4 cm consisting of Ca $(OH)_2$ and finally a two cm plug of Na_2SO_4. A highly concentrated solution of petroleum ether extract from 0.5 gm of M. falcata flowers was used and the pigment adsorption bands compared with that of B carotene from standard crystalline carotene. Xanthophylls and xanthophyll esters are adsorbed in the Ca $(OH)_2$ layer while the carotene pigments move through the Ca $(OH)_2$ into the MgO layer (Strain 1942).

For the resolution of xanthophyll components, a column of 1:1 Celite to activated MgO was prepared by wet packing under vacumn in dichloroethane (ethylene dichloride) Strain 1945). The saponified petroleum ether extract from 0.5 gm of M. falcata flowers was concentrated by evaporating the ether solution to near dryness and then dissolving in 3 ml of dichloroethane. This solution was then added to the top of the Celite-MgO column and developed in dichloroethane.

Analysis of Data.-The observed genetic ratios for pigment inheritance were tested against expected values, using the X^2 method for determining goodness of fit.

*Formerly Micron brand adsorptive magnesia, now call Sea Sorb 43, Fisher Scientific Company, Chicago 51, Illinois

EXPERIMENTAL RESULTS

Three anthocyanin and nine flavonol pigments were separated from the .1N HCl extracts of diploid alfalfa flowers and identified by use of paper chromatography. Carotenoid pigments were extracted with petroleum ether and were separated and identified on adsorption columns and by partition tests (Figures 2a and 2b).

Identification of anthocyanins

Anthocyanin glycosides.-Three distinct anthocyanin glycosides were separated out on the paper chromatograms (Figure 2a). The R_f values for these pigments were determined in BAW and HAc-HCl, and where available, the unknown pigment was cochromatogrammed (run side by side on the same chromatogram) with an authentic compound, Based on these R_f values and comparison with known standards or previously reported R_f values, these three pigments were identified as delphidin 3,5 diglucoside, petunidin 3,5 diglucoside, and malvidin 3,5 diglucoside (Table 3).

(a) Purple

(b) Variegated

(c) Yellow

(d) White

Figure 1. Flower color types in diploid alfalfa

(a)Anthocyanins(b)Anthoxanthins(c)B Carotene(d)Xanthophyll Esters

Figure 2. Paper chromatograms in BAW of (a) anthocyanins and (b) anthoxanthins and $Ca(OH)_2$-MgO adsorption columns of (c) B carotene and (d) xanthophyll esters.

Figure 5. Paper chromatograms in DAW of (a) anthocyanins and (b) anthoxanthins and $Ce(SO_4)_2$-H_2O adsorption columns of (c) carotene and (d) xanthophyll esters.

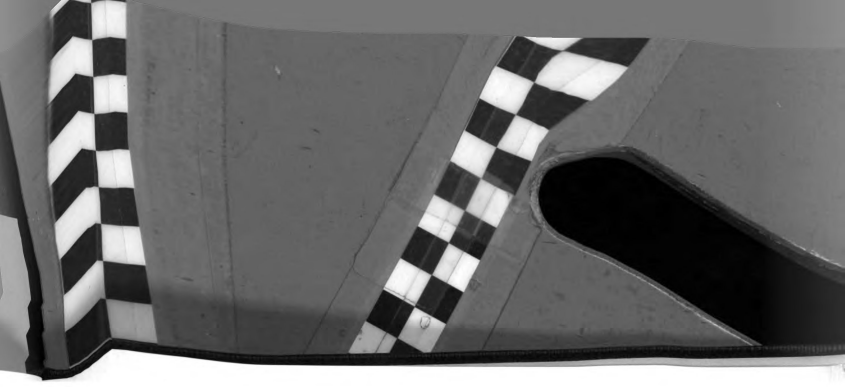


Table 3. R_f values of anthocyanins in BAW and HAc-HCl

BAW (4:1:5)				
Delphinidin 3,5 diglucoside	0.15	0.15	0.15	0.11
Petunidin 3,5 Diglucoside*	0.21	-	0.24	-
Malvidin 3,5 diglucoside	0.25	0.25	0.31	0.22
HAc-HCl (82:15:3 H_2O)				
Delphinidin 3,5 diglucoside	0.36	0.36	0.32	-
Petunidin 3,5 diglucoside	0.43	-	0.32	-
Malvidin 3,5 diglucoside	0.53	0.53	0.42	-

*Tentative identification
(a) Harborne 1958 (b) Bate-Smith 1950

The delphinidin and petunidin glycosides formed a purple band on the BAW chromatogram whereas the malvidin glycoside was mauve in color. These observations were in agreement with those reported by Harborne (1958) for these three pigments. Although an authentic source of petunidin 3,5 diglucoside was not available, the intermediate position of this pigment, between the delphinidin and malvidin glycosides, its color, and its R_f value in comparison to a previously reported R_f value in BAW (Harborne 1958), strongly supported this identification.

Anthocyanin aglycones.-After hydrolyzing the purified anthocyanin glycosides 10 minutes in 2N HCl, three different aglycones were obtained. These were identified as delphinidin, petunidin, and malvidin, by cochromatogramming with authentic compounds using Forestal solvent and BAW as the

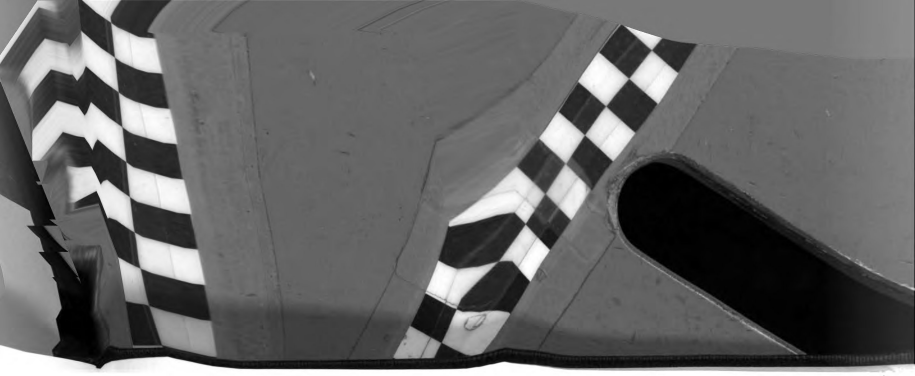
developing solvents (Table 4).

Table 4. R_f values of anthocyanin aglycones in Forestal solvent and BAW

	Alfalfa Extract	Authentic Compounds	Previous Values (a)	(b)	(c)
Forestal					
($10H_2O$:30HAc:3HCl)					
Delphinidin	0.32	0.32	0.32	0.30	0.30
Petunidin	0.46	0.46	0.46	0.48	0.45
Malvidin	0.68	0.68	0.60	0.63	0.60
Cyanidin	-	0.53	0.49	0.50	0.50
BAW (4:1:5)					
Delphinidin	0.36	0.36	0.42	-	-
Petunidin	0.48	0.48	0.52	-	-
Malvidin	0.58	0.58	0.58	-	-
Cyanidin	-	0.65	0.68	-	-

*Run on acid washed paper
(a) Harborne 1958 (b) Pecket 1958 (c) Bate-Smith 1954

The observed R_f values closely agree with those previously reported. Since Davies (1958) had reported that one of the anthocyanins in alfalfa was a cyanidin glycoside, the R_f values for cyanidin were also determined in the two solvent systems (Table 4). None of the three anthocyanins isolated from the alfalfa flowers gave R_f values in these two solvents corresponding to those of cyanidin.



Identification of anthoxanthins

Anthoxanthin glycosides.-Nine anthoxanthin glycosides were separated from alfalfa flower extract by paper chromatography in BAW and 15% HAc solvents. All but two of these glycosides had R_f values less than 0.5 (Table 5).

Table 5. R_f values of anthoxanthins in BAW and HAc

	BAW		15% HAc
	Ascending	Descending	Ascending
Authentic Compound			
Rutin (previous values)*	0.57	0.57	0.62
Rutin (observed)	0.46	0.56	0.63
Quercetin glycosides			
A	0.15	0.12	0.69
B'	0.18	0.15	0.67
B	0.27	0.26	0.54
C'	0.29	0.28	0.59
C	0.33	0.34	0.65
D	0.36	0.42	0.51
Kaempferol glycosides			
E	0.42	0.46	0.73
F	0.61	0.68	-
G	0.76	0.79	-

*Gage et al 1951

These values are considerably lower than any R_f values previously reported for anthoxanthin glycosides.

Since previously reported R_f values were obtained by descending chromatography this method was used as a check against the ascending method used in this study. The descending method failed to significantly alter the R_f values from those obtained by the ascending method (Table 5).

The pigments were tentatively identified as flavonol or flavone glycosides based on their forming a bright yellow appearance when exposed to ammonia vapors in visible light and a brown or brownish yellow appearance under ultraviolet light (Geissman 1955). Since there were no authentic flavone or flavonol compounds with such low R_f values for comparison, exact identity of the unknown anthoxanthin glycosides was not possible. However, each of the glycosides were characterized by their R_f values in the BAW and 15% HAc solvent systems (Table 5) and the aglycone of each glycoside determined (Table 6).

Anthoxanthin aglycones.-Each of the nine anthoxanthins were hydrolized ten minutes in 1N HCl and the aglycone partitioned from the hydrolyzate with isoamyl alcohol. The six glycosides with the lowest R_f values (A thru D) were found to be quercetin derivatives while the other three (E thru G) were derivatives of the aglycone kaempferol (Table 6). These aglycones were identified by cochromatogramming with the authentic samples of quercetin and kaempferol.

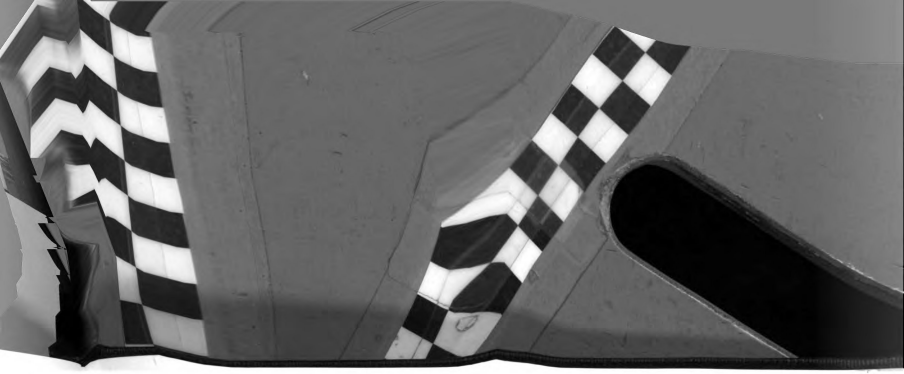


Table 6. R_f values of anthoxanthin aglycones in Forestal solvent

Anthoxanthin	Aglycone	Authentic Compound
		(a) Quercetin
A	0.51	0.52
B'	0.57	0.57
B	0.58	0.57
C'	0.60	0.60
C	0.59	0.58
D	0.56	0.56
		(b) Kaempferol
E	0.76	0.76
F	0.74	0.75
G	0.73	0.73

The quercetin glycosides were yellow to orange yellow and formed less diffuse spots than the pale yellow kaempferol glycosides (Figure 2b).

Identification of carotenoid pigments

Xanthophyll Ester.-When the petroleum ether extract was added to a $Ca(OH)_2$-MgO adsorption column and developed with petroleum ether, nearly all the yellow pigment was adsorbed in the $Ca(OH)_2$ layer in a bright yellow band (Figure 2d), indicating that the carotenoid pigment was primarily

xanthophyll (Strain 1945). A trace of orange yellow pigment moved through the $Ca(OH)_2$ layer into the MgO zone and was identified as B carotene by comparison with an authentic sample of crystalline carotene (85% B carotene).

The xanthophyll was eluted from the $Ca(OH)_2$ layer into petroleum ether and shaken with an equal volume of 80% methanol. The pigment remained in the ether phase, indicating that the xanthophylls were in an esterified form. By hydrolysis in alcoholic KOH, the ester linkages were broken and when the hydrolyzed pigment was partitioned between ether and 80% methanol, the pigment moved into the methanol phase, verifying its identification as xanthophyll.

Separation of the hydrolyzed pigment on a column of MgO, using dichloroethane as the developing solvent, resulted in the development of at least two and possibly three separate bands of xanthophyll. However, since no authentic samples of xanthophyll were available for comparison, positive identification of these pigments was not possible.

Segregation patterns of individual pigments

The general procedure followed in the study of segregation patterns was to formulate a genetic hypothesis for inheritance of a given pigment based on the large S_1 progenies of two variegated plants, g346 (56 plants) and 18s (43 plants). This hypothesis was then further tested with the additional smaller families.

Anthocyanins.-The three anthocyanin pigments were inherited as a unit with either all or none of the three pigments present. There was some evidence of differences in relative amounts of the three anthocyanin pigments, but no definite segregation pattern or phenotypic effect was observed. The ratio of plants having anthocyanin (P) to those without (p) fit a 3:1 ratio in the selfed progeny of two variegated plants and either a 1:1 or 1:0 in crosses of blue or variegated plants to yellow or white flowered plants. No anthocyanin containing progeny were obtained from crosses between yellow x yellow, yellow x white, or white x white plants (Table 7).

Table 7. Observed segregation for anthocyanin production

Cross	P	p	X^2	Probability
g346 ⊗	40	16		
18s ⊗	31	12		
Observed total	71	28		
Expected (3:1)	74.3	24.7	0.587	0.50 > P > 0.30
H474 X 6s	13	9		
38 X 18s	5	8		
34s X 22506B19	15	9		
18s X 7s	5	5		
H474 X 59-101-(2)	6	7		
38 X 10s	7	6		
Observed total	51	44		
Expected (1:1)	47.5	47.5	0.501	0.50 > P > 0.30
40 X 16s	22	0		
40 X 14s	15	0		
40 X 38s	11	0		
40 X 59-101-2	10	0		
30 X 7s	11	0		
C73 X 7s	17	0		
36 ⊗	15	0		
Observed total	101	0		
38s X 59-101-2	0	10		
38s X 8-2	0	12		
4s X 35	0	10		
35 X falcata-1	0	19		
35 ⊗	0	8		
Observed total	0	59		

Kaempferol glycoside F.-Of the nine anthoxanthin glycosides, only kaempferol glycosides F and G exhibited segregation in enough families to permit genetic anlaysis. The ratio of plants containing kaempferol glycoside F to those without, (f), showed a good fit to expected 3:1 and 1:1 ratios (Table 8). Other families were non-segregating either for presence or absence of this pigment.

Table 8. Observed segregation of kaempferol glycoside F

Cross	F	f	X^2	Probability
g356 ⊗	44	12		
H474 X 6s	17	4		
40 X 38s	9	2		
38s X 38s	9	3		
38s X 10s	10	3		
Observed total	89	24		
Expected (3:1)	84.7	28.3	0.87	0.50 > P > 0.30
40 X 16s	10	12		
38 X 18s	6	7		
30 X 7s	6	5		
C73 X 7s	9	8		
Observed total	31	32		
Expected (1:1)	31.5	31.5	0.016	0.90 > P > 0.80
18s ⊗	0	43		
18s X 7s	0	10		
Observed total	0	53		
40 X 14s	15	0		
40 X 59-101-2	10	0		
38s X 59-101-2	10	0		
H474 X 59-101-2	13	0		
34s X 22506B19	24	0		
35 X falcata-1	19	0		
4s X 35	10	0		
35 ⊗	8	0		
36 ⊗	15	0		
Observed total	124	0		

Kaempferol glycoside G.-Segregation for kaempferol glycoside G is similar to that observed for glycoside F. The ratio of present to absent fit very well with expected values of 3:1 and 1:1. Also there were non-segregating families in which all plants either contained the pigment or none contained the pigment.

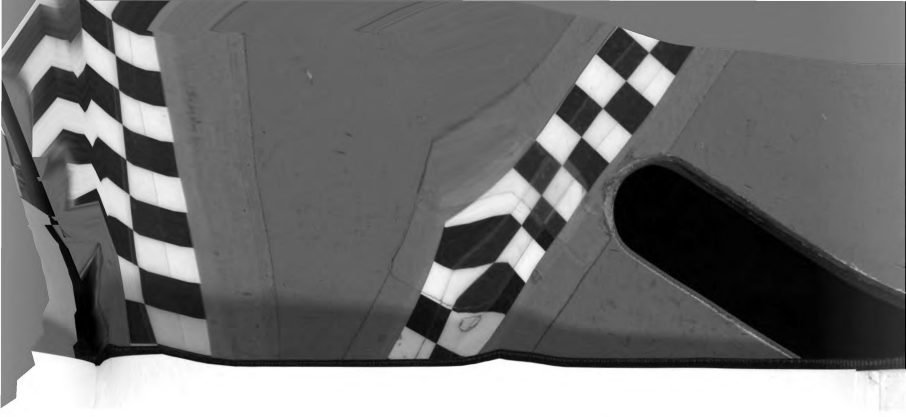


Table 9. Observed segregation of kaempferol glycoside G

Cross	G	g	x^2	Probability
g346 ⊗	42	14		
40 X 38s	9	2		
C73 X 7s	12	5		
38 X 10s	8	5		
Observed total	71	26		
Expected (3:1)	72.7	24.3	0.16	0.70 > P > 0.50
38s X 8-2	5	7		
H474 X 6s	11	10		
38 X 18s	4	9		
30 X 7s	4	7		
Observed total	24	33		
Expected (1:1)	28.5	28.5	1.42	0.30 > P > 0.20
18s ⊗	0	43		
34s X 22506B19	0	24		
Observed total	0	67		
40 X 16s	22	0		
40 X 14s	15	0		
40 X 59-101-2	10	0		
38s X 59-101-2	10	0		
H474 X 59-101-2	13	0		
35 X falcata-1	19	0		
4s X 35	10	0		
35 ⊗	8	0		
36 ⊗	15	0		
Observed total	122	0		

Xanthophyll.-The xanthophyll pigments were extracted with petroleum ether and given a quantitative rating of o, Tr, +, ++ and +++, depending on the intensity of yellow pigment in the extract. The frequency distribution within these classes approached a 1:4:6:4:1 (+++ : ++ : + : Tr: 0) for the 43 S_1 progeny of clone 18s, and a 1:2:1 (+:Tr:0) for the 56 S_1 progeny of clones g346 (Table 10).

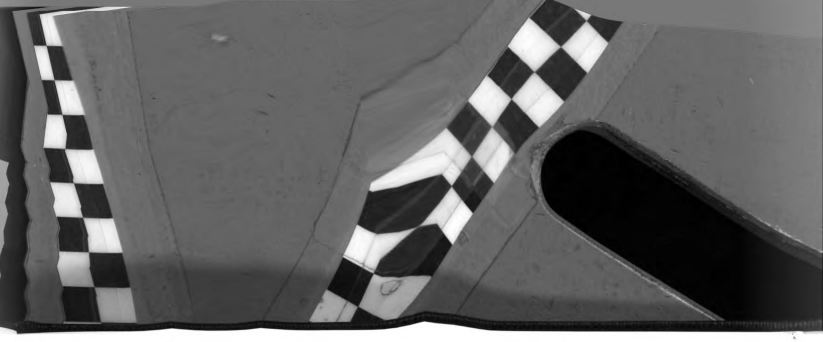


Table 10. Observed segregation of xanthophyll

Cross	YYYY	YYY	YY	Y	y	X^2	Probability
18s ⊗	0	10	20	12	2		
Expected (1:4:6:4:1)	2.6	10.8	16.2	10.8	2.6	4.35	$0.50 > P > 0.30$
g346 ⊗			13	26	17		
Expected (1:2:1)			14	28	14	0.85	$0.70 > P > 0.50$

Most families were too small for determining a fit to a five class distribution, but when 0 and Tr plants were grouped into one class and +, ++ and +++ plants into a second class, a good fit to expected ratios was obtained. The 18s progeny gave a very good fit to a 11:5 (≥+:Tr) ratio and the g346 progeny fit at 1:3 distribution. Other ratios observed for smaller families were 3:1, 1:1, and non-segregation for all + or greater or all Tr or 0 plants (Table 11).

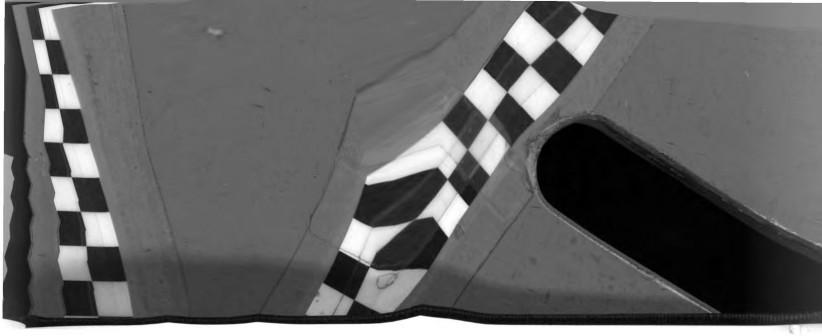
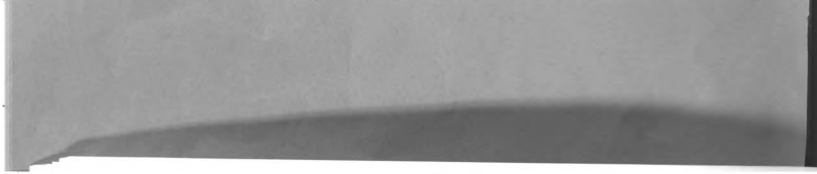


Table 11. Observed segregation of xanthophyll into combined classes

Cross	≥YY	≤Y	X^2	Probability
18s ⊗	30	13		
Expected (11:5)	29.6	13.4	0.017	0.95 > P > 0.90
g346 ⊗	13	43		
40 X 38s	3	9		
30 X 7s	3	8		
Observed total	19	60		
Expected (1:3)	19.8	59.2	0.043	0.90 > P > 0.80
38 X 18s	8	5		
38 X 10s	9	4		
Observed total	17	9		
Expected (3:1)	19.5	6.5	0.82*	0.50 > P > 0.30
38s X 8-2	6	6		
H474 X 6s	11	10		
18s X 7s	5	5		
Observed total	22	21		
Expected (1:1)	21.5	21.5	0.024	0.90 > P > 0.80
40 X 59-101-2	10	0		
38s X 59-101-2	10	0		
H474 X 59-101-2	13	0		
34s X 22506B19	24	0		
35 X falcata-1	19	0		
Observed total	76	0		
40 X 16s	0	22		
40 X 14s	0	15		
C73 X 7s	0	17		
4s X 35	0	8		
35 ⊗	0	10		
36 ⊗	0	15		
Observed total	0	87		

*Yates correction for sample size less than 40 and with 1 degree of freedom.

There was some evidence that the level of refinement of the extraction procedure permitted the occasional error of placing some Tr plants into the O classification resulting in an excess of O type plants. However, grouping the Tr



and 0 plants into one class overcame this difficulty, resulting in a good fit to expected ratios.

Joint segregation of pigments

In order to detect possible linkage relationships in the inheritance of the pigments studied, their joint segregation patterns were analyzed. Because of the multiple classes involved, only the large S_1 progenies of clone g346 and 18s were used for these analyses.

Anthocyanin and Kaempferol glycoside F.-The joint segregation for the presence or absence of anthocyanin (P) and kaempferol glycosice F gave a very good fit to the 9:3:3:1 ratio expected from independent inheritance of these two pigments (Table 12).

Table 12. Joint segregation of kaempferol glycoside F. and anthocyanin P.

Cross	F-P-	F-pp	ffP-	ffpp	Total
g346 ⊗	31	12	10	3	56
Expected(9:3:3:1)	31.5	10.5	10.5	3.5	
$X^2 = 0.195$		D.F. = 3		$0.98 > P > 0.95$	

Anthocyanin and kaempferol glycoside G.-The observed ratio fit very closly the 9:3:3:1 ratio predicted on the assumption of independent inheritance of anthocyanin production and kaempferol glycoside G (Table 13).

Table 13. Joint segregation of kaempferol glycoside G. and anthocyanin P.

Cross	G-P-	G-pp	ggP-	ggpp	Total
g346 ⊗	30	12	11	3	56
Expected	31.5	10.5	10.5	3.5	

$X^2 = 0.380$ D.F. = 3 $0.95 > P > 90$

Kaempferol glycosides F and G.-A good fit to the expected 9:3:3:1 dihybrid ratio was obtained for the joint segregation of the two kaempferol glycosides F and G (Table 14).

Table 14. Joint segregation of kaempferol glycosides F. and G.

Cross	F-F-	F-gg	ffG-	ffgg	Total
g346 ⊗	32	10	12	2	56
40 ⊗ 38s	6	2	2	1	11
Total observed	38	12	14	3	67
Expected (9:3:3:1)	37.8	12.5	12.5	4.2	

$X^2 = 0.551$ D.F. = 3 $0.95 > P > 0.90$

Anthocyanin, kaempferol glycosides F and G.-In the 56 S_1 progeny of clone g346, a very good fit was obtained to the predicted trihybrid ratio 27:9:9:9:3:3:3:1, based on independence of inheritance of anthocyanin production (P), kaempferol glycoside F, and kaempferol glycoside G (Table 15).

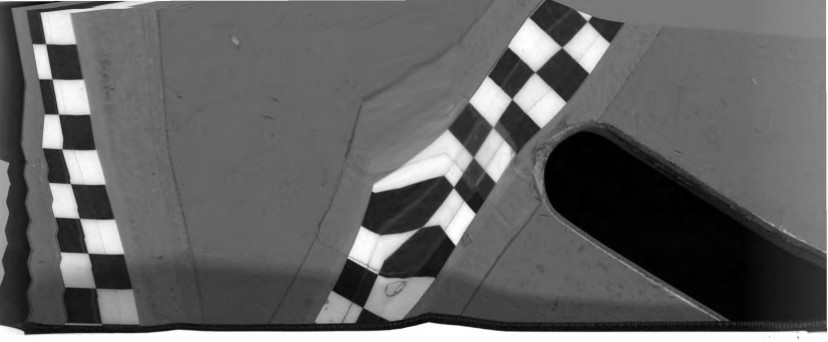


Table 15. Joint segregation of kaempferol glycosides F and G and anthocyanin P.

Cross	PFG	PFg	PfG	pFG	Pfg	pFg	pfG	pfg
g346 ⊗	22	9	8	9	2	3	3	0
Expected (27:9:9:3:3:3:1)	23.6	7.9	7.9	7.9	2.6	2.6	2.6	.875

X^2 = 1.84 D.F. = 7 0.98 > P > 0.95

Xanthophyll and anthocyanin.-Progenies were too small to give a valid approximation to the ten possible (1:4:6:4:1 X 3:1) or 6 possible (1:2:1 X 3:1) classes. Also, the occasional misclassification of plants containing trace amounts of xanthophyll (Tr) as (0) type plants caused marked deviations from predicted joint segregation. However, by grouping the xanthophyll classes into those with at least two plus factors (≥YY) for xanthophyll production and those with one or none (≤Y), a good fit was obtained to the 6.6: 3:2.2:1 ratio (ie 11:5 X 3:1) expected, assuming independent inheritance of xanthophyll and anthocyanin production (Table 16).

Table 16. Joint segregation of xanthophyll and anthocyanin

Cross	≥YYP	≤YP	≥YYp	≤Yp	X^2	Probability
18s ⊗	21	10	9	3		
Expected (6.6:3:2.2:1)	22.1	10	7.4	3.4	0.829	0.90 > P > 0.80
g346 ⊗	9	31	4	12		
Expected (3:9:1:3)	10.5	31.5	3.4	10.5	0.50	0.95 > P > 0.90

Xanthophyll and kaempferol glycoside F.-Using the combined classes for xanthophyll, the joint segregation of xanthophyll and kaempferol glycoside F approximated the 3:9:1:3 ratio expected assuming independent inheritance of these two pigments (Table 17).

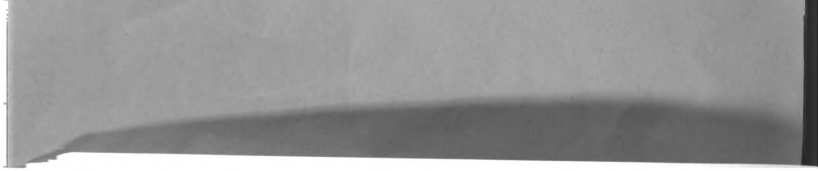
Table 17. Joint segregation of xanthophyll and kaempferol glycoside F

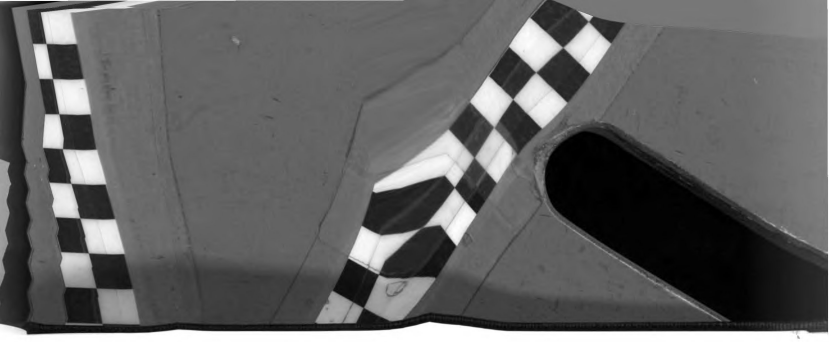
Cross	≥YYF	≤YF	≥YYf	≤Yf	X^2	Probability
g346 ⊗	13	30	0	13		
Expected (3:9:1:3)	10.5	31.5	3.5	10.5	4.75	0.20 > P > 0.10

Xanthophyll and kaempferol glycoside G.-The predicted 3:9:1:3 ratio, assuming independent inheritance was closely approximated by the joint segregation of xanthophyll and kaempferol glycoside G (Table 18).

Table 18. Joint segregation of xanthophyll and kaempferol glycoside G

Cross	≥YYG	≤YG	≥YYg	≤Yg	X^2	Probability
g346 ⊗	12	30	1	13		
Expected (3:9:1:3)	10.5	31.5	3.5	10.5	2.66	0.40 > P > 0.30

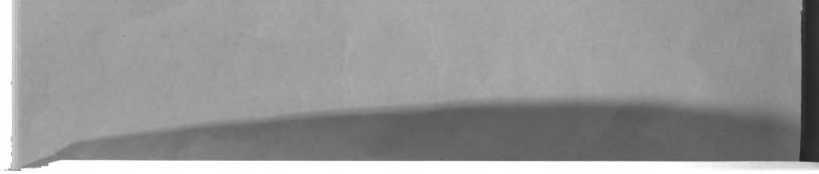




Additional evidence of segregation

Anthocyanin intensity.-In certain crosses, there was marked variability in anthocyanin intensity. However, because of a mixture with anthoxanthin pigments in the raw extract and the separation of the anthocyanin pigment into three distinct bands on the paper chromatograms, it was difficult to give a reliable visual rating for anthocyanin intensity per se. In some plants the relative amounts of the three pigments seemed to vary, but in general there appeared to be a rather constant equilibrium between the concentration of the three pigments. There was not enough data to formulate a definite inheritance pattern, but the differences in anthocyanin concentration could account in part for some of the phenotypic variation within the proposed genotypic classes.

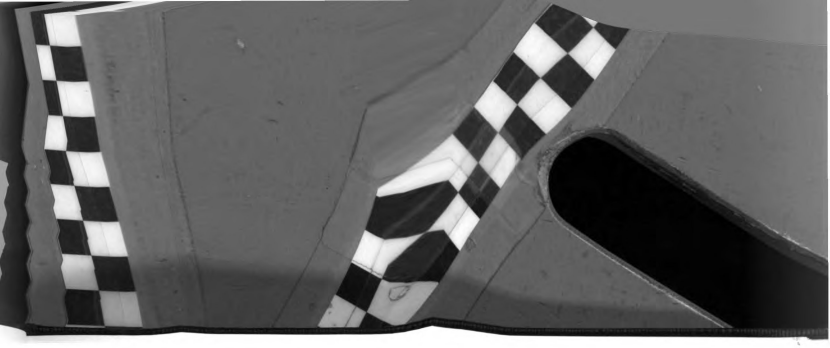
Bud color.-Among the yellow and white flowered plants, there appeared to be segregation for a trace of anthocyanin in the buds (pink buds). In nearly all crosses having yellow or white progeny, both pink budded and yellow or white budded types appeared and in about equal numbers. There was some evidence of an environmental effect on penetrance, however. In field classification of pink budded versus non-pink budded plants, it was observed the same plants which had pink buds on the first reading, failed to show the pink buds on a later reading or vice versa. Also, one of the white flowered plants which had white buds all summer in the field, developed pink buds when brought into the greenhouse.



Because of this possible penetrance effect and the fact that the maximum number of yellow flowered plants in any one family was only 16, no genetic hypothesis for the inheritance of this trait was proposed.

Intensity of quercetin glycosides.-In most families, all plants contained the quercetin glycoside A, but in a few families segregation for presence or absence of this pigment occurred. In one cross, 40 X 165, neither of the parents nor their 21 progeny contained this pigment. In other crosses there was evidence of quantitative differences in the amount of glycoside A among the progeny but no segregation for presence or absence of this pigment. Also, there seemed to be a correlation between the intensity of quercetin glycoside A and the amount or presence of quercetin glycosides B', B, and C'. In the progeny from crosses containing a M. falcata parent, the glycoside A was usually intense and the quercetin glycosides B', B and C' were also present. In most other plants, quercetin glycoside A was present in the complete absence of or only trace amounts of B', B, and C'. In no plants, however, were the glycosides B', B and C' found in the absence of A.

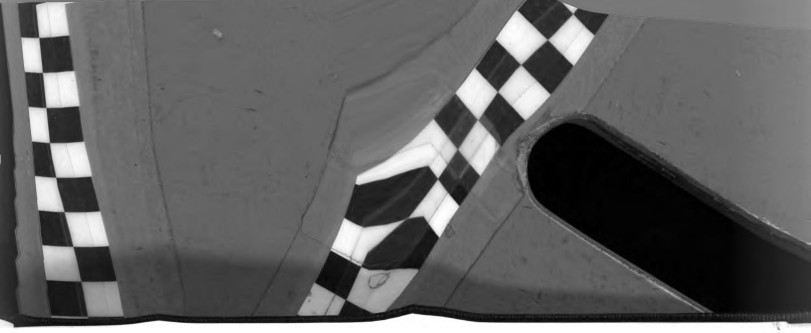
The quercetin glycosides C and D, along with kaempferol glycoside E, were found in every plant examined, including 18 white flowered plants. However, when the intensity of quercetin glycosides A, C, and D, in the white flowered S_1 progeny of clone 35, were compared with the intensity of these pigments in yellow flowered progeny from a cross between clone 35 and M. falcata, there was a marked higher intensity in the yellow flowered plants.



Phenotypic effects of pigments

Anthocyanins.-The anthocyanins impart the reddish-blue or purple color to alfalfa flowers. The apparent wide range in anthocyanin intensity results in a corresponding range in flower color. In flowers, however, the phenotypic effect of anthocyanins are also modified by anthoxanthin copigments and the background effect of the yellow xanthophyll pigment. No definite correlation between a certain phenotype and certain quantitative balance of the three anthocyanin pigments was observed. Flower color ranged from very light blue, with only a small amount of anthocyanin, to dark blue or purple in which the concentration of the anthocyanin was considerably higher. In some yellow and white flowered plants, a trace amount of anthocyanin was evident in the buds, giving a pink color. As the flowers opened the trace of anthocyanin disappeared.

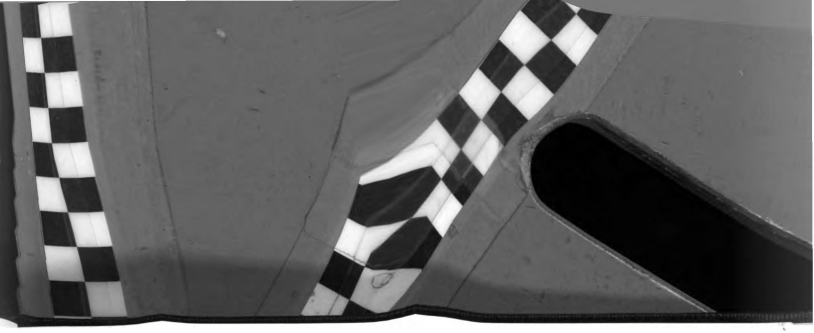
In plants with variegated flowers, the buds or freshly opened flowers contained considerably more anthocyanin than flowers several days old. The decreased anthocyanin intensity, resulting from aging, permitted the yellow xanthophyll pigment to show through. In this transition period from nearly purple to nearly yellow, the variegated flower types are observed, and at a certain ratio of blue to yellow, a green phenotype often occurs. This green gradually fades out to a smudgy yellow in many flowers.



Quercetin glycosides.-There was some evidence that higher concentrations of quercetin glycoside gave a phenotypic effect. In plants containing only a trace amount of xanthophyll pigment, there were two levels of yellow intensity. Quercetin glycoside A was absent in the lighter yellow flowers but present in the more intense yellow flowers. At higher concentrations of xanthophyll, no marked phenotypic effect of the quercetin glycosides was evident. Although the white flowered plants contained quercetin glycosides A, C, and D, there were no phenotypic effects. However, when these flowers were placed in ammonia vapors, they turned yellow indicating presence of flavonol pigments. No "true white" flowers were found in which the ammonia vapor test was negative. Yellow flowers and variegated flowers, however, contained considerably higher amounts of quercetin glycosides than did white flowers. M. falcata clones or progeny of a cross with M. falcata parentage were noticeably higher in the concentration of quercetin glycosides.

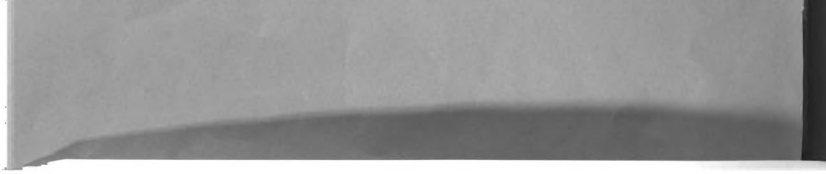
Additional evidence of the phenotypic effect of quercetin glycosides is the appearance of a trace of light yellow color in blue flowers which do not contain xanthophyll pigment. These flowers have a slight bluish green appearance, but do not fade out to the smudgy yellow of variegated flowers containing xanthophyll.

Kaempferol glycosides F and G.-Although the pale yellow color of kaempferol glycosides did not appear to impart a

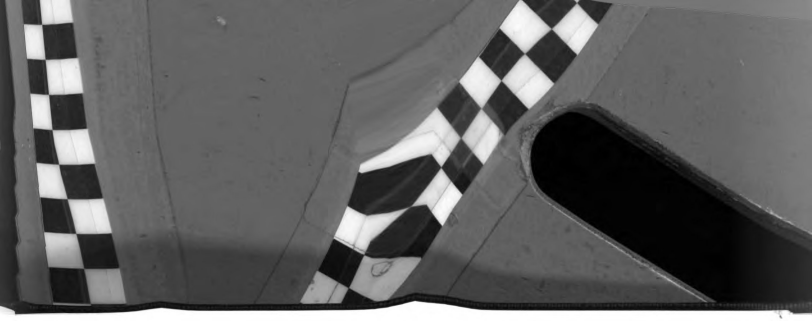


Kaempferol glycosides F and G.-Although the pale yellow color of kaempferol glycosides did not appear to impart a direct yellowing effect on the phenotype, there was some evidence for copigmentation with anthocyanin, particularly for glycoside G. The maroon or red wine phenotype nearly always was associated with the absence of glycoside G and the presence of at least one factor for xanthophyll. In the non-xanthophyll plants (yyyy), presence of both kaempferol glycosides F and G was associated with blue flowers whereas absence of F or G or both was associated with reddish blue types. These latter two classifications were not absolute since some reddish blue and blues occurred in the same genotypes. However, the relative frequency of the reddish blue types in comparison to blue was considerably higher in plants with f, g or fg genotypes.

Xanthophyll.-The concentration of xanthophyll in the flower extract appeared to be directly correlated with the intensity of yellow color in the flowers. The five levels of xanthophyll pigment intensity, +++, ++, +, Tr, and O had four corresponding phenotypic classes of orange yellow, bright yellow, yellow, light yellow, and white. However in plants containing a trace (Tr) level of xanthophyll, two different yellow intensities were observed, indicating the presence of other yellow pigments with phenotypic effect. In plants containing + or greater amounts of xanthophyll, only one phenotype per level of xanthophyll was observed.



In plants containing both anthocyanins and xanthophyll, the xanthophyll has an important background effect on the phenotype of the flowers. Plants high in both anthocyanin and xanthophyll pigments appeared very dark purple to almost black. In other types the yellow background effect produced a maroon or reddish wine color. As the anthocyanin fades on aging, the yellow xanthophyll becomes more evident and the phenotype change from purple to a variegated type. Each level of xanthophyll produces a somewhat different phenotypic effect in combination with anthocyanin, but because of the continuous variation in the degree that the anthocyanin had faded, specific genotypic-phenotypic relationships were difficult to establish. High intensity of xanthophyll pigment produces an orange-yellow background effect in variegated flowers, while at lower xanthophyll intensities, the background color is yellow to pale yellow.



DISCUSSION

Because of the difficulty in collecting flowers, each sample of flowers was used for the extraction of both carotenoids and flavonoid pigments. In most flowers, all the carotenoid pigment was removed by the petroleum ether extract, but in plants containing considerable amounts of carotenoids, a yellow residue was observed following the ether and acid extractions. This yellow pigment could readily be removed by isopropyl alcohol, leaving a white residue.

The amount of residual pigment extracted by the isopropyl alcohol was directly proportional to the intensity of yellow pigment obtained in the ether extract. Since, in this study, the relative amounts of pigment from plant to plant were more important than the absolute amount it was not deemed necesary to combine the carotenoid extracts.

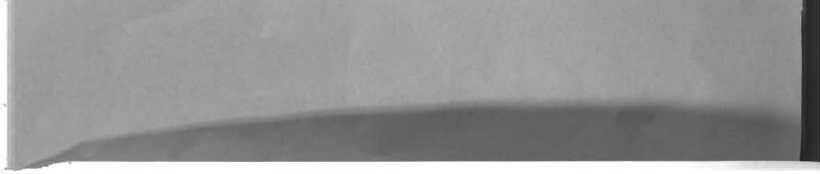
Ascending chromatography was used in preference to descending chromatography for this study. In preliminary tests, ascending chromatography in the 15" test tube gave better separations of pigments and more consistent results than descending chromatography in a large glass chromatographic jar. An additional advantage of using the test tubes was that the solvent could be used for several runs whereas with the descending method, it was necessary to remove the solvent after each run and clean the equipment. Also the

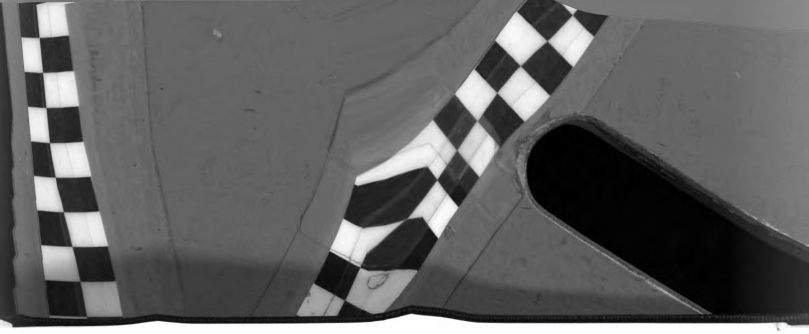
small air volume of the test tubes permits rapid saturation of the atmosphere by the volatile solvent, eliminating an equilibrating period often needed when larger containers are used. The simplicity of both set-up and use, with good separations and consistent results made this procedure particularly well suited for the chromatographic analysis of large numbers of plants from segregating populations.

Because of the dilute concentration of pigments obtained, it was necessary to concentrate the pigments by repeated spotting of the chromatogram. The light from the mimeoscope used was beneficial in locating spots for repeated application and speeding up evaporation of the solvent. The glass rod used in spotting the chromatograms gave fairly consistent size drops at each application and was satisfactory for the purpose of this study. However, for a strictly quantitative study micropipettes should be used for spotting chromatograms.

Identification

Anthocyanins.-Three anthocyanin pigments were found in all purple flowered plants examined and were identified as the 3,5 diglucosides of delphinidin, petunidin and malvidin. These findings are in agreement with Lesins (1956) who found delphinidin, petunidin and malvidin glycosides in both tetraploid and diploid alfalfas. Davies (1958), using Forestal solvent, reported similar results with the exception of finding cyanidin instead of petunidin. Because of





this discrepancy in the literature, both petunidin and cyanidin authentic samples were used in this study. R_f values of these two pigments were very close in Forestal solvent but their marked separation in BAW solvent positively identified the anthocyanidin from alfalfa as petunidin. The small difference between the R_f value of cyanidin and of petunidin in Forestal solvent (the only solvent used by Davies) suggests the possibility of a missidentification.

The occurrence of anthocyanin mixtures derived from delphinidin and its methylated derivatives, petunidin and malvidin is not uncommon and has been reported in several species (Lawrence et al 1939). Beale (1939) first reported this combination of pigments in Lathyrus odoratus (sweet pea) and more recently Pecket (1960) in a survey of Lathyrus species, found several species containing mixtures of delphinidin, petunidin and malvidin glycosides.

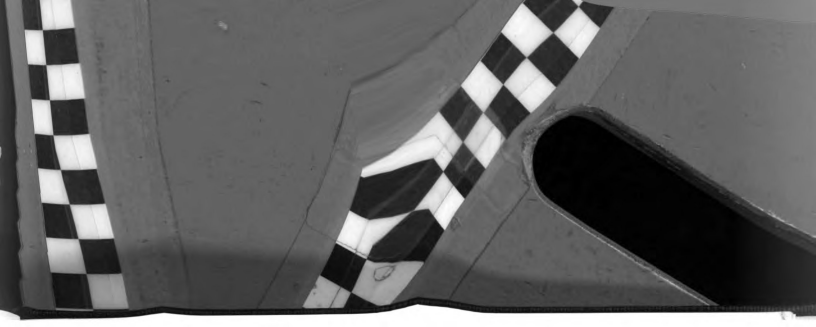
Lesins (1956) reported the separation of four anthocyanins and a trace of the aglycone delphinidin from raw extract of alfalfa flowers. The anthocyanins were identified as two delphinidin glycosides, a petunidin glycoside and a malvidin glycoside. These results differ from those reported here by the presence of the aglycone delphinidin and a second glycoside of delphinidin. This could be due to actual differences in plant material or differences in technique used.

Quercetin.-Six quercetin glycosides were identified based on their characteristic yellowing in ammonia vapor,

brownish yellow color under ultra violet light, and positive identification of their aglycone as quercetin. These pigments ranged in R_f values from 0.12 for quercetin glycoside A to 0.28 for quercetin glycoside D. All of these R_f values are considerably below any previous reported values for quercetin glycoside (Bate-Smith 1950, Gage and Wender 1951).

A satisfactory explanation for these observations is difficult. When the chromatographic procedure used (BAW, ascending) was checked against and authentic sample of rutin (quercetin 3-rhamnodiglucoside), the observed R_f values were only slightly below previously reported R_f values. Also this same procedure was used for anthocyanin separations and gave good approximations to R_f values reported for these pigments. Altering the technique by using descending chromatography in a large chromatographic jar and equilibrating the paper 24 hours with the lower phase of the BAW solvent (as used by Bate-Smith 1950) failed to significantly alter the R_f values. Extraction procedure, paper and solvent used were similar to those used by previous investigators. Therefore, the possibility that the low R_f values obtained were due to differences in technique does not seem likely.

The basic C_{15} molecule, common to all flavonoid pigments, is elaborated into different classes by the processes of hydroxylation, methylation, glycosidation and acylation (Lawrence and Price 1940, Harborne 1958). In a study of



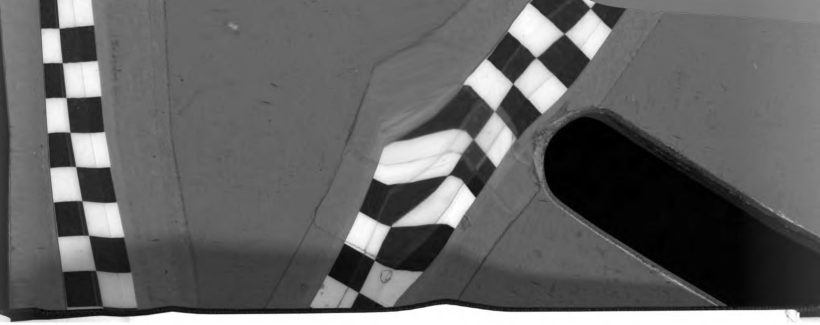
the relationship between the structure of flavonoid molecules and their R_f values in BAW, Bate-Smith and Westall (1950) observed that in general, as the number of hydroxyl groups and/or number of glucose units increase, R_f is decreased, whereas methylation and acylation increase the R_f values of the basic pigments.

Since the hydroxylation and methylation patterns are determined at the aglycone level and acylation increases R_f values, the only structural modification that can explain the low R_f values obtained is different glycosidation patterns. Anthoxanthins in general have considerably more glycosidic variability than anthocyanins. At least five glycosidic derivatives of quercetin have been reported in the literature. In the BAW solvent, these pigments range in R_f values from 0.57 for rutin (quercetin-3-rhamnoglucoside) to 0.82 for quercetrin (quercetin-3-rhamnoside) (Gage and Wender 1951). The only yellow to orange flavonoid pigment reported with an R_f value within the 0.12 to 0.28 values obtained in this study was a glycoside of aueresidin, an orange aurone pigment found in Antirrhinum majus (Geissman 1954). Chromatogramming of an extract from yellow flowers of snapdragon verified this report, but the bright orange color of this pigment in ammonia vapor readily distinguished it from the bright yellow color of the quercetin glycosides obtained from alfalfa.

An alternative explanation for such low R_f values is the possibility that the quercetin glycosides occur in a complex molecule in alfalfa. The analytical procedures needed to determine such a complex, or the chemical structure of the glycosides beyond the identification of their aglycone, was beyond the scope of this study and must await further investigation.

Kaempferol.-Three kaempferol glycosides were identified by their characteristic color reactions in ammonia vapor and under ultra-violet light, and by positive identification of their aglycone as kaempferol. The kaempferol glycosides formed pale yellow spots on the chromatograms, usually visible only when intensified in ammonia vapor. This was in contrast to the brighter yellow of the quercetin glycosides.

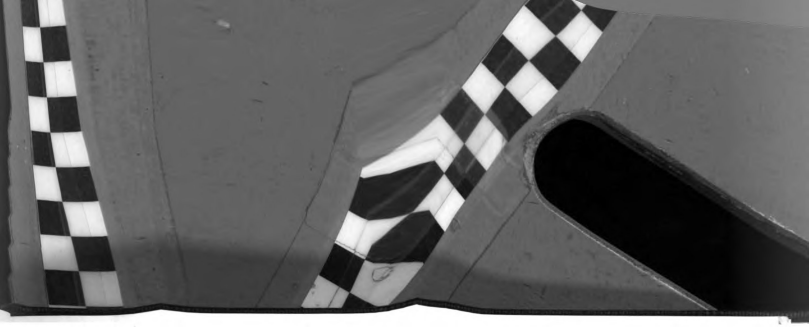
The R_f values of the three kaempferol glycosides in BAW solvent were 0.35, 0.54 and 0.71. Two previously reported R_f values in BAW were 0.51 for robinin (kaempferol-3-dirhamnogalactose) and 0.75 for kaempferitrine (3-dirhamnoside). The appoximation of these two R_f values, by the kaempferol glycosides F and G respectively, suggests a tentative identification of these pigments. However, since authentic samples were not available for comparison, these tentative identifications could not be verified. At least three other kaempferol glycosides have been reported in the literature, 3-glucoside (astragalin), 3-rhamnoglucoside, and 3-rhamodiglucoside, but their R_f values in BAW had not been determined (Roberts 1956). The possibility exists, however that one of these corresponds to the third unidentified kaempferol glycoside.



Of interest is that robinin (kaempferol-3dirhamnogalactose, sometimes called kaempferol-3-robinoside, was first identified from the flowers of Robinia pseudoacacia (Honey locust, a legume tree) and that kaempferitrin (kaempferol-3-dirhamnoside) as well as other kaempferol rhamnosides were obtained from Acacia linifolia, and Acacia decurrens, two members of a large genus of flowering trees and shrubs belonging to the legume family (Sannie 1952). If the tentative identification of these pigments is correct, this suggests a possible taxonomic significance of these kaempferol glycosides.

Carotenoids.-The carotenoid pigments in alfalfa flowers were identified as primarily xanthophyll esters with a trace of B carotene. This identification was based on partition tests between petroleum ether and 80% methanol, before and after saponification, and their differential adsorption in a $Ca(OH)_2$-MgO column. Xanthophyll esters behave as carotene in the partition test, remaining in the ether phase, and could be mistaken for carotene if this were the only test used. This is a possible explanation of the previous report (Twamley 1955) that the carotenoid pigment isolated from alfalfa was primarily carotene. The xanthophyll pigment was separated into two and possibly three components on a MgO column, using dichloroethane as the developing solvent. Lack of authentic samples of xanthophylls did not permit identification of these components. However, the traces of B carotene found suggest that these xanthophyll esters may be hydroxylated derivatives of B carotene. One of

the components, with the least adsorbancy on the MgO column was pale orange in color, approximating the color of zeaxanthin (3,3'-dihydroxy B carotene), in Strain's (1945) color plate. Zeaxanthin and two other B carotene derivatives, cryptoxanthin (3-hydroxy B carotene) and eschscholtzanthin (3,3'-dihydroxydehydro B carotene) have been isolated from flowers (Goodwin 1955). Numerous other carotenoid pigments have been isolated from flowers, however, so that the above possibilities are not conclusive.

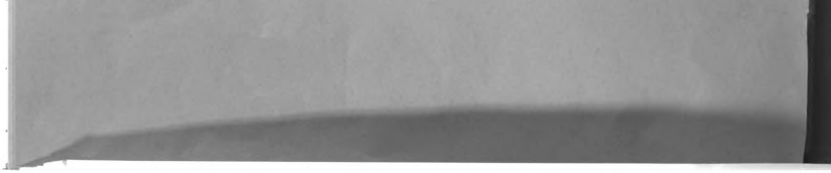


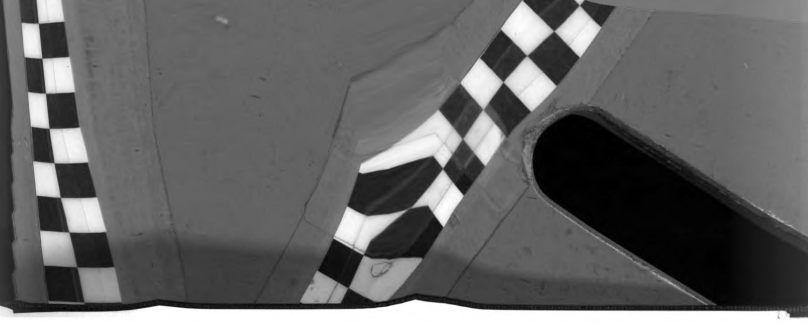
Pigment inheritance

Anthocyanins.-Production of anthocyanin was controlled by a single dominant gene. The absence of complementary gene action as reported by Twamley (1956) and others is probably due to homozygosity of one of the complementary factors for anthocyanin production.

The three anthocyanin pigments identified were inherited as a unit, all three being present in every plant containing anthocyanin. These observations were similar to those reported by Lesins (1956) with the exception that in two tetraploid crosses, segregation for the malvidin glycoside occurred. However, it was noted that malvidin was only present in the plants which also contained delphinidin and petunidin derivatives.

Since the only anthocyanidins obtained from alfalfa flowers were trihydroxylated in the 2 phenol ring of the benzopyrilium nucleus, alfalfa must be homozygous for a gene or genes determining the trihydroxylating pattern. In addition, since every plant with anthocyanin, contained delphinidin plus its two methylated derivatives, petunidin and malvidin, alfalfa must also be homozygous for a methylating gene or genes. The occurrence of all three anthocyanidins in a mixture has been attributed to incomplete methylation by Lawrence et al (1939). According to Lawrence, this methylating process may be either the straight forward methylation of delphinidin, or of a





precursor which in the absence of complete methylation gives rise to delphinidin. The incomplete methylation theory seems applicable to the situation in diploid alfalfa and in general to tetraploid alfalfa, with the possible exception of a gene segregating for malvidin production in tetraploid alfalfa (Lesins 1956).

Genes determining the glycosidation pattern for anthocyanins appeared to be in a homozygous condition, and there was no evidence of acylating genes.

Since anthoxanthins were found in every plant examined, the gene affecting the production of anthocyanin must exert its influence in the biosynthetic pathway after formation of the C_{15} precursor, which is common to both anthocyanin and anthoxanthins. Whether this also applies to both complementary factors reported in diploid alfalfa by Twamley (1955), and reported in tetraploid alfalfa by various investigators, depends on the finding of a "true white" plant, indicating a block preventing formation of C_{15} nucleus and hence of all flavonid pigments. "True white" flowers are defined as white flowers which fail to turn yellow in ammonia vapor (Bate-Smith 1955). If even a trace of anthoxanthin is present, flowers will turn a pale yellow. Some supposedly "true white" tetraploid alfalfa flowers, obtained from Clements (1961), turned pale yellow in the ammonia vapor test and on chromatogramming showed at least two of the quercetin glycosides (A and D). Based on these observations it appears doubtful, that "true whites" in

the sense used by Bate-Smith (1955) exist in alfalfa.

Quercetin.-Two of the six quercetin glycosides were present in every plant examined. The four other glycosides exhibited segregation in some crosses, but data was insufficient to formulate a definite inheritance pattern.

It was observed that quercetin glycoside A (fig. 2b) was present in nearly all plants. However, a few plants were obtained in which this glycoside was absent. When such plants were crossed, none of their progeny contained this pigment. This suggested that the absence of quercetin glycoside A was controlled by a gene or genes in the homozygous recessive condition.

Additional observations indicated that the intensity levels of glycoside A could be divided into at least four classes (0, Tr, + , ++), and that as the intensity of quercetin glycoside A increased, there was also an increase in intensity of the other quercetin glycosides. Quercetin glycosides B', B, and C' were very dilute pigments and were found only in plants containing fairly intense levels (≥+) of quercetin. The simultaneous increases in intensity of all six quercetin glycosides suggests an intensifier gene or genes for the aglycone, quercetin.

From these observations, two hypothesis for inheritance of quercetin glycosides have been proposed, (1) the inheritance of quercetin glycosides is controlled by quantitative factors at two loci, affecting the intensity of the aglycone, quercetin and segregating 1:4:6:4:1. An alternative hypothesis (2) is that production of quercetin glycoside A is controlled by a

single dominant gene and that quercetin intensity is controlled by a single quantitative factor segregating 1:2:1.

The presence of quercetin glycosides A, C, and D in white flowers, indicated that not all levels of intensity produce a phenotypic effect. To test the hypotheses for quercetin inheritance and to determine genotypic-phenotypic relationship, crosses should be made between non-xanthophyll (yyyy) yellow flowered plants and the distribution of yellow intensity types observed in the segregating generations. In this manner, the epistatic effect encountered in this study resulting from the presence of yellow xanthophyll pigments, can be eliminated.

Kaempferol.-One of the kaempferol glycosides, E, was present in every plant examined while the other two, F and G exhibited independent segregation in a 3:1 ratio. Thus, it was concluded that production of each of the kaempferol glycosides F and G was controlled by a single dominant and independent gene.

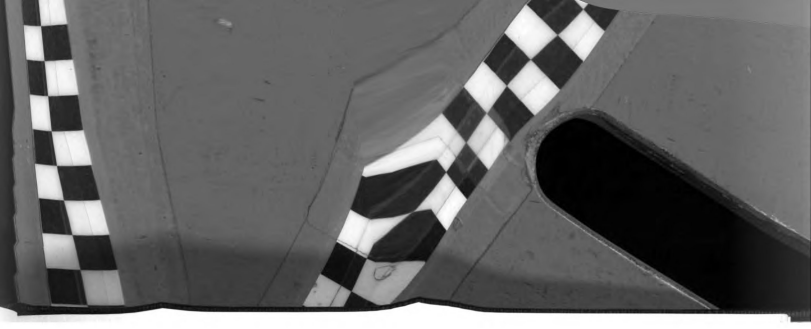
Since the three kaempferol glycosides differ only in their glycosidic pattern, it suggests that genes F and G must produce their effect by determining the kind and/or position of glycoside units on the kaempferol molecule. Glycosidation genes have been reported for anthocyanins (Lawrence 1940), but to the author's knowledge, no specific examples of gene action controlling the glycosidic nature of

kaempferol have previously been reported. Determining the exact action of the genes involved here, depends on positive identification of the kaempferol glycosides, which was not possible in this study.

Carotenoids.-The carotenoid pigments, identified primarily as esters of xanthophyll, segregated into a 1:4:6:4:1 ratio for intensity in the 43 S_1 progeny of a variegated flowered clone 18s. Based on this evidence plus supplementary evidence from twenty other crosses, it appeared that xanthophyll inheritance in flowers of alfalfa is controlled by quantitative factors at two loci, ($Y_1y_1Y_2y_2$).

Of particular interest is the close agreement between the observed segregation for xanthophyll and quercetin pigments in diploid alfalfa, and the hypothesized segregation for these pigments proposed by Twamley (1955), who based his hypotheses on the segregation of yellow color intensity in 85 yellow F_2 plants from a purple x yellow diploid cross.

That a similar inheritance pattern occurs for yellow pigments in tetraploid alfalfa is indicated by the 13 intensity classes Twamley was able to discern in 257 F_2 plants of a yellow x white tetraploid cross. Earlier reports of a single factor Y for yellow pigment production in tetraploid alfalfa could be an artifact from the bulking of all purple and variegated plants and of all gradations of yellow into single classes. The 38:10:1 (purple:yellow:white) ratio



obtained from the F_2 of a purple times yellow tetraploid was explained as approximating a 12:3:1 ratio (Lepper and Odland 1939). However, the observed ratio of 48 non white: 1 white, more nearly approaches the 63:1 disomic ratio expected based on two quantitative genes segregating for yellow and a 3:1 segregation for anthocyanin production. Under this hypothesis, all of the blue plants with exception of 3/64 would contain at least one factor for xanthophyll production.

Weihing's (1948) observations of F_2 family segregation from a white times yellow tetraploid cross is also in accord with a two factor hypothesis for xanthophyll production.

Joint segregation.-The joint segregation data indicated that genes segregating for the production of anthocyanin, kaempferol glycosides F and G, and xanthophyll were independently inherited. Based on the segregation patterns of these genes in 21 families, genotypes were proposed for the 23 parent clones. These genotypes together with observed phenotypes are listed in table 19. In some crosses, there was some indication that intensity of quercetin glycosides was associated with intensity of xanthophyll, but this could be due to the homozygous condition in M. falcata of the plus factors for the production of each of these pigments.

Other factors.-Although a quantitative study of anthocyanin intensity was not made, marked differences were apparent.

It was noticed however, that some of these apparent differences were actually due to the presence of anthoxanthins

Table 19. Phenotypes and genotypes proposed for parental clones

Parental	Phenotype			Genotype
	General	Hort. Color Chart		
15p*	reddish blue	Imperial purple	33	$PPffFFy_1y_1y_2y_2$
3p*	variegated	Pod green	61	$PfF\_G\_Y_1y_1Y_2y_2$
g346	variegated	Pod green	61	$PpFfGfY_1y_1y_2y_2$
18s	variegated	Pod green	62/2	$PpffggY_1y_1Y_2Y_2$
H474	maroon	Violet purple	733/1	$PpFfggY_1y_1y_2y_2$
30	maroon	Imperial purple	33/1	$PPFfggY_1y_1y_2y_2$
40	light blue	Mineral violet	635/2	$PPFfGgy_1y_1y_2y_2$
10s	blue-yellow	Orchid purple	31/3	$PpFfGgY_1y_1Y_2y_2$
C73	blue	Spectrum violet	735	$PPFfGgy_1y_1y_2y_2$
36	blue green	Lavender violet	637	$PPFFGGy_1y_1y_2y_2$
34s	violet	Spectrum violet	735	$PpFFggy_1y_1y_2y_2$
38s	yellow	Sulphur yellow	1/2	$ppFfGgY_1y_1Y_2y_2$
38	yellow	Sulphur yellow	1/1	$ppFfGfY_1Y_1y_2y_2$
6s	yellow	Sulphur yellow	1/2	$ppFfGgY_1y_1Y_2y_2$
7s	light yellow	Dresden yellow	64/2	$ppffGgY_1y_1y_2y_2$
14s	light yellow	Dresden yellow	64/2	$ppFFGGY_1y_1y_2y_2$
16s**	pale yellow	Primrose yellow	601/3	$ppFfggY_1y_1y_2y_2$
8-2**	pale yellow	Primrose yellow	601/3	$ppFfggY_1y_1y_2y_2$
59-101-2	orange yellow	Lemon yellow	4	$ppFFGGY_1Y_1Y_2Y_2$
falcata-1	orange yellow	Lemon yellow	4	$ppffggY_1Y_1Y_2Y_2$
22506B19	orange yellow	Orange yellow		$ppFfggY_1Y_1Y_2Y_2$
35	white	White		$ppFFGGy_1y_1y_2y_2$
4s	white	White		$ppffGGy_1y_1y_2y_2$

*Original Parents: 15p= M. sativa; 3p= (M. gaetula x M. falcata)
**Differ from light yellow types by absence of quercetin glycoside A

causing blending or copigmenting effects, or to the background effects of the xanthophyll pigments.

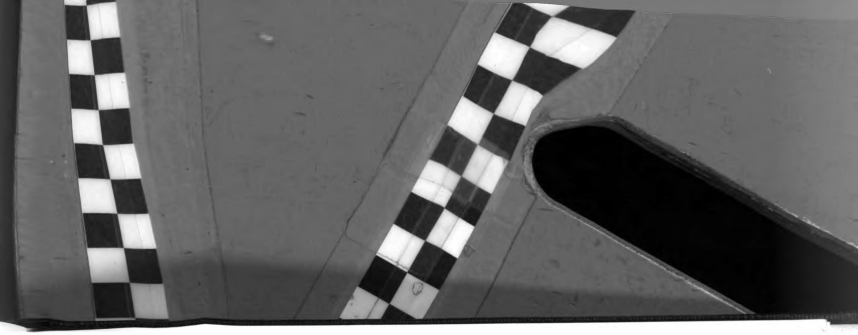
The inverse relationship of anthocyanin production versus anthoxanthin production, as proposed by Robinson (1931) was not especially evident. In fact, very definite contradiction of this hypothesis was obtained by finding many very pale blue flowers with very small amounts of anthoxanthin.

Because of the blending and copigmentation effects of anthoxanthins, it would be desirable to separate the anthoxanthins from the anthocyanins by exhaustive extraction of the acid extract with ethyl acetate (Bate-Smith 1950), before quantitative measuring of anthocyanin intensity. This was not done by Twamley (1955) and, thus, could account for some of the variability observed.

All 14 families which contained yellow and white progeny segregated for a trace of anthocyanin in the buds and in general approximated a 1:1 ratio. One exception was a 3:1 segregation for presence of pink buds in the 16 non-purple progeny of g346 x. However, because of the small numbers of non-purple plants in each family, and possible penetrance effect, influenced by environment, a definite genetic hypothesis was not possible.

Phenotypic correlation

Anthocyanins.-The presence of the gene for anthocyanin production is expressed in the light blue to purple color of alfalfa flowers. Anthocyanin intensity ranged from very dilute, almost white flowers to deep purple. It was evident,

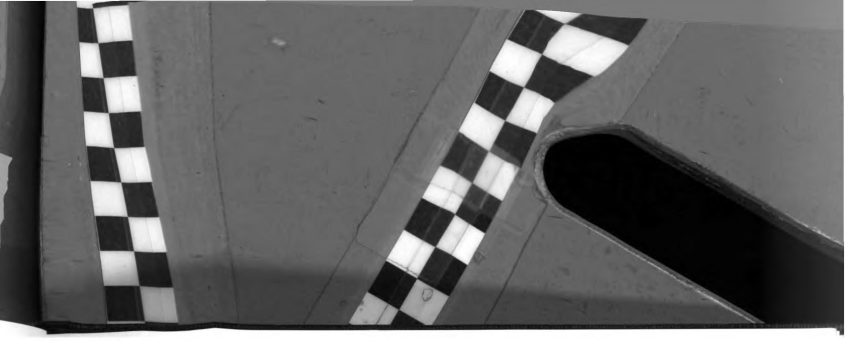


however, that much of the phenotypic variability in purple color was due to the blending or copigment effects of anthoxanthins and background effects of xanthophyll.

Pink buds on yellow or white flowers have been observed in many genera and usually are attributed to the accumulation of anthocyanin diluter genes (Paris et al 1960). According to Lawrence (1940) such genes produce their effect by altering the rate of pigment synthesis. This serves as a possible explanation of the penetrance effect of pink buds in alfalfa and points out the need to adjust the environment to permit expression of these effects in an inheritance study.

Kaempferol.-The dilute yellow color of kaempferol glycosides, and the presence of all three in white flowers indicate these pigments impart little or no yellow color to alfalfa flowers. There is evidence, however, that these pigments produce an important phenotypic effect by copigmentation with anthocyanins.

Of 30 plants with a maroon phenotype, observed in the segregating progenies, none were found which contained both kaempferol glycosides F and G. Seven contained only glycoside F, three only glycoside G and twenty contained neither glycoside F or G. In addition, all maroon phenotypes contained at least one plus factor for xanthophyll. In plants having no xanthophyll, there was a significantly greater number of reddish blue flowered plants in the Fg, fG, and fg

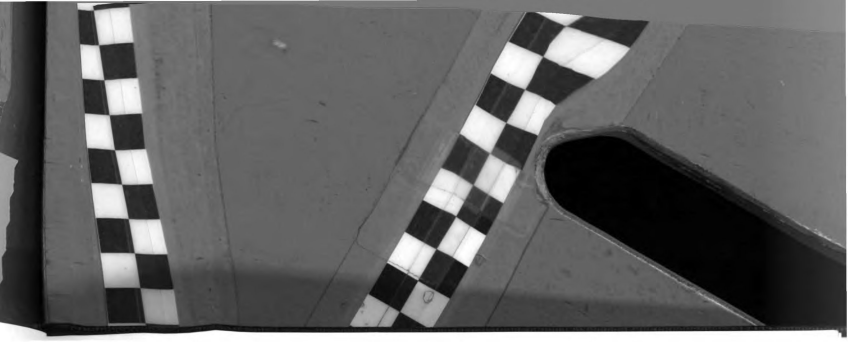


classes than was found in the FG genotypes. There was some overlapping of phenotypes between these classes, however.

Thus, based on these observations, it is proposed that glycosides F and G function in a copigment action, complexing with anthocyanins to give a bluing effect. This would explain the predominance of reddish blue types in the fg genotypes and the low frequency of these types in FG genotypes. Likewise, the maroon phenotype is a result of the combination of reddening effect by absence of kaempferol glycoside and the background effect of xanthophyll.

Quercetin-The exact phenotypic effects of the quercetin glycosides are difficult to determine because of the epistatic effect of the yellow xanthophylls. However, the appearance of traces of yellow in some phenotypes absent for a plus factor for xanthophyll indicated a phenotypic effect. Additional evidence was observed in the segregation of yellow color intensity within a single genotypic class for xanthophyll. The presence of quercetin glycosides A, C and D in white flowered plants indicates that there must be quantitative differences in the amount of quercetin pigment and that only when the concentration is above a certain level, do quercetin glycosides exhibit a phenotypic effect.

Xanthophyll.-In general, there was a very high correlation between the intensity of xanthophyll pigment and the yellow in the flowers. It appears that xanthophyll is the most important yellow pigment in determining the yellow

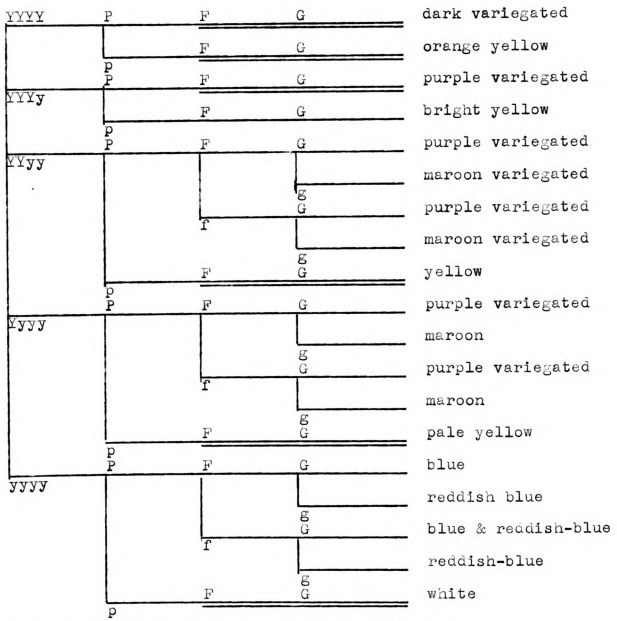


in alfalfa flowers. No white plants were found which contained xanthophyll, and all intense yellow flowers, especially M. falcata flowers contained a high intensity of xanthophyll pigment. At a low intensity of xanthophyll pigment there was some indication of a phenotypic effect from quercetin glycosides. At higher intensities however, xanthophyll may be epistatic to the effect of the quercetin pigment.

It was also evident that most of the variegated types observed were a result of xanthophyll background effects and the fading of anthocyanins. No variegated types were observed in plants which were absent in xanthophyll pigment. In a cross of M. falcata x M. sativa, the progeny were very dark to almost black in color, exhibiting the color subtraction phenomenon mentioned by Lesins (1956).

Based on the information obtained from this study, a proposed inheritance chart for flower color in diploid alfalfa is proposed (Figure 3).

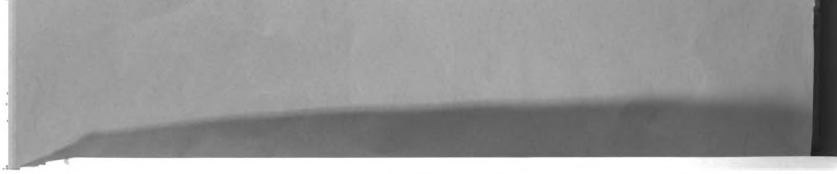
Figure 3. Flower Color Inheritance in Diploid Alfalfa

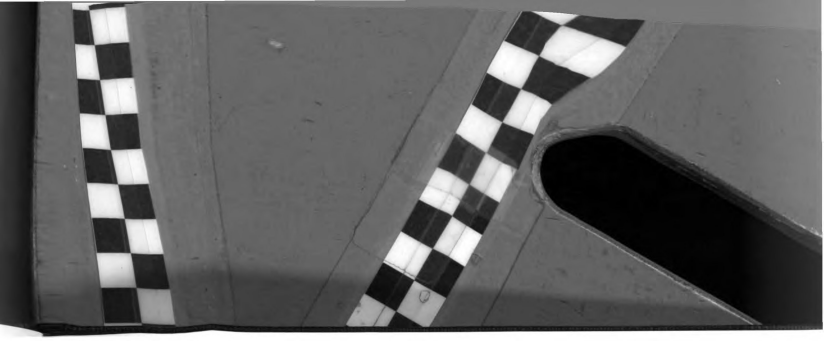


Y gives xanthophyll
P gives anthocyanin
F gives kaempferol glycosice
G gives kaempferol glycoside

In Addition:
1) diluter genes for anthocyanin
2) segregation for quercetin intensity

All variegated types have a yellow background effect from the xanthophyll and change from purple, to green, to smudgy yellow with fading of the anthocyanin.





CONCLUSIONS

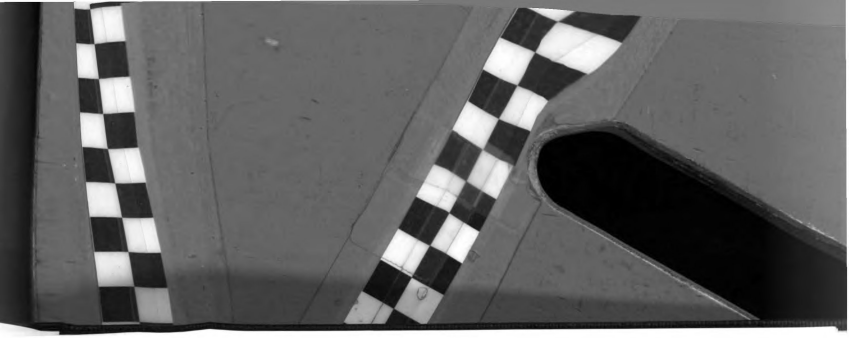
An attempt was made to determine the pattern of flower color inheritance in alfalfa by studying the inheritance of flower pigments at the diploid level.

By chromatographic techniques, three anthocyanin pigments, nine flavonol pigments, and xanthophyll esters were identified in alfalfa flowers.

The inheritance of the anthocyanin pigments, two kaempferol glycosides, and of xanthophyll pigments was determined. Two hypotheses were proposed for the inheritance of quercetin glycosides.

The segregation patterns of 5 pigment genes in 23 families, enabled the determination of genotypes for 22 parent clones.

From the inheritance patterns for these pigments and their phenotypic correlation, an inheritance chart for flower color in diploid alfalfa is proposed.



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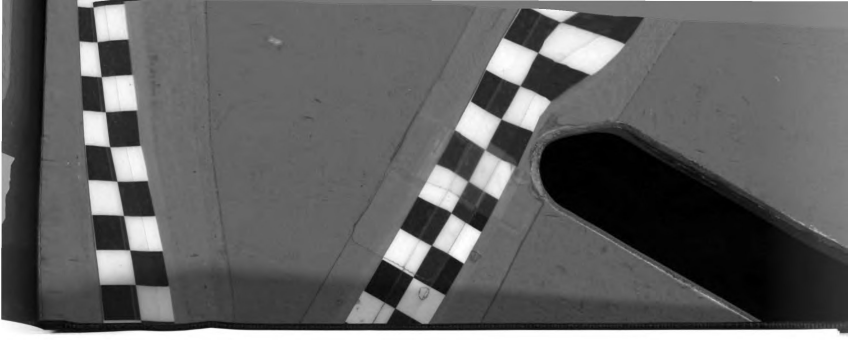

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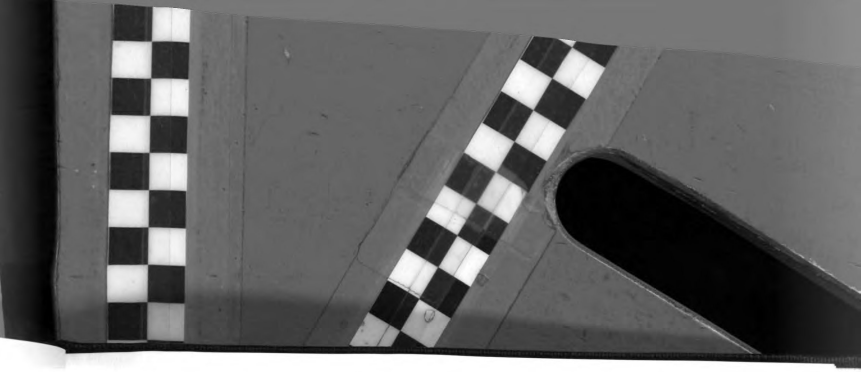

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