THE GENETICS OF *VERBENA*

II. CHEMISTRY OF THE FLOWER COLOUR VARIATIONS

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1. INTRODUCTION

FLOWER colour in the garden *Verbena* hybrids is affected by at least four pairs of mendelian factors and two groups of three multiple allelomorphs (Beale, 1940). In the present paper the chemical nature of the differences between the various colour types will be considered. For general reviews of the genetics and chemistry of flower-colour variation, reference should be made to the papers of Scott-Moncrieff (1936) and Lawrence & Price (1940).

2. METHODS

(i) *Identification of anthocyanins*

The anthocyanins were tested by the qualitative methods of Robinson & Robinson (1931, 1932, 1933). The purple and maroon anthocyanins (delphinidin 3 : 5-dimonoside and delphinidin 3-monoside respectively) have also been crystallized and fully identified by Scott-Moncrieff & Sturgess (1940).

It was impracticable to test fully some hundreds of samples, such as would occur in a large F_2 family. Consequently, when a large number of plants was to be tested, only a few of the (1 % hydrochloric acid) extracts were hydrolysed; the remainder were merely saturated with sodium chloride, amyl alcohol added and the solution shaken. When it was known that only one of the four anthocyanins—delphinidin 3 : 5-dimonoside, delphinidin 3-monoside, pelargonidin 3 : 5-dimonoside and pelargonidin 3-monoside—was likely to be present, the pigment could be readily identified by this method, because monosides are extracted by amyl alcohol while dimonosides are not, and pelargonidin derivatives give a much redder solution than those of delphinidin.

(ii) *Tests for acylation of anthocyanins*

The presence of acylated (complex) anthocyanins was suspected when the distribution number was found to be greater for fresh extracts than for those which had been kept for some weeks.

1 % aqueous hydrochloric acid solutions of *purple* and *scarlet-magenta* flowers, freshly extracted (i.e. less than 24 hr. previously), were saturated with sodium chloride and shaken with amyl alcohol; a considerable proportion of the pigment was seen to be transferred to the alcohol. After 3 weeks the same extracts were tested and the distribution numbers then found to be nil, indicating removal of the acyl groups.

The anthocyanins from *plum* and *plum-purple* flowers differed from all others in that not a trace of pigment was extracted by amyl alcohol, even when fresh extracts, saturated with sodium chloride, were used. Hence the anthocyanins in plum and plum-purple flowers are not acylated.

The anthocyanin in *scarlet* flowers is also probably acylated, since on shaking up the fresh solution with amyl alcohol, the distribution number was found to be higher than is usual for pelargonidin 3-monoside.

Further tests were made on fresh extracts of the *scarlet-magenta* and *purple* pigments. The solutions were heated to boiling-point, excess of 10 % aqueous sodium hydroxide added, and the mixtures boiled for 30 sec., cooled and made just acid. They were then saturated with sodium chloride and shaken with amyl alcohol, when the distribution numbers were found to be zero, indicating that the acyl group or groups had been removed.

(iii) p*H determinations*

For the *p*H measurements, seventy flowers were taken in each sample, the corolla-tubes removed, the petal-lobes ground up, distilled water

added and the *p*H of the solution estimated by means of a glass electrode. Three samples of plum and three of scarlet-magenta flowers were tested. The results obtained were consistent to one place of decimals, giving for plum a *p*H value of 5·8 and for scarlet-magenta 5·4.

(iv) *Tests for copigmentation*

Copigmentation of the anthocyanins was demonstrated by the following method. The samples to be tested, previously shown to contain the same anthocyanin, were extracted with 1 % hydrochloric acid and the solutions diluted to approximately the same strength. The copigmented solutions were bluer, and on heating, this increased blueness disappeared, but reappeared on cooling. The colour of the uncopigmented solutions was unaffected by heating.

The presence of anthoxanthins (which may or may not act as copigments) was demonstrated by extracting the aqueous acid solution with amyl alcohol or ethyl acetate and shaking up the extract with dilute sodium hydroxide solution. Using the copigmented solution, a deep yellow colour was produced in the sodium hydroxide; with the uncopigmented solution, only a very pale yellow was obtained. Hence the copigment is probably an anthoxanthin.

(v) *Identification of flavones*

The flavone in the *yellow* flowers was identified by the following method. The flowers were boiled for half an hour with 1 % aqueous hydrochloric acid, filtered and the treatment repeated. The combined filtrates were cooled, and a saturated aqueous solution of lead acetate added until all the chloride had been precipitated. This was removed by filtration, and more lead acetate added to precipitate the lead salt of the flavone. If necessary the *p*H was adjusted by careful addition of ammonia. The yellow lead salt was filtered and triturated with hot 5 % sulphuric acid. After removal of lead sulphate the acidity was increased and the glucoside hydrolysed by boiling for 2–3 hr. The solution was filtered while hot, and on cooling deposited a brown amorphous solid. A further quantity of this crude flavone was obtained by saturating with sodium chloride. The crude product after drying was exhaustively extracted with ether (Soxhlet), the extract evaporated to dryness, and the yellow residue acetylated with acetic anhydride containing a few drops of pyridine. On pouring the resultant liquid into water a brown solid was precipitated, and this was extracted with hot methyl alcohol, leaving a sparingly soluble white solid which constituted the bulk of the crude

acetyl derivative. This was crystallized once from aqueous acetic acid and three times from acetone-ethyl alcohol and was obtained as long colourless needles having a melting-point 227–228° C. Luteolin tetra-acetate has a melting-point of 225–227° C. (Herzig, 1896).

On analysis the acetyl derivative gave

$$C = 60{\cdot}95\ \%, \quad H = 3{\cdot}9\ \%, \quad CH_3CO = 38{\cdot}2\ \%.$$

$C_{15}H_6O_2(OOC.CH_3)_4$ requires

$$C = 60{\cdot}8\ \%, \quad H = 4{\cdot}0\ \%, \quad CH_3CO = 37{\cdot}9\ \%.$$

The pigment is therefore a tetra-hydroxy-flavone glycoside, and although mixed melting-points have not yet been carried out, the evidence strongly suggests that the flavone is luteolin.

The flavone in the *white* flowers was purified by a somewhat different method. The flowers were extracted first with ethyl alcohol and pressed out. They were then boiled in water with a drop of acid and the solution filtered while hot. The residue was boiled up and filtered a further three times, and the filtrates combined. The original alcoholic extract, which contained much chlorophyll, was distilled off and the residue boiled with water. The solution so obtained was filtered and added to the aqueous solution.

The combined aqueous extracts were concentrated, saturated with sodium chloride and extracted five times with ethyl acetate. This was distilled off and the residue boiled three times with water. The extracts were filtered while hot and hydrolysed by boiling with an equal volume of concentrated hydrochloric acid. The orange-yellow precipitate obtained was filtered off and purified by dissolving in warm 50 % aqueous ethyl alcohol. On cooling a yellowish, non-crystalline solid separated. This was dried and acetylated as with the yellow flavone. The acetyl derivative was purified by two or three crystallizations (with charcoal) from methyl alcohol. Colourless crystals were obtained having a M.P. 181–2° C. (Apigenin triacetate, M.P. 181° C.)

On analysis the acetyl derivative gave

$C = 63{\cdot}7\ \%$, $H = 4{\cdot}1\ \%$, $CH_3CO = 26{\cdot}6\ \%$ (mean of two determinations)

Apigenin triacetate, $C_{15}H_7O_2(OOC.CH_3)_3$ requires

$$C = 63{\cdot}6\ \%, \quad H = 4{\cdot}1\ \%, \quad CH_3CO = 32{\cdot}6\ \%.$$

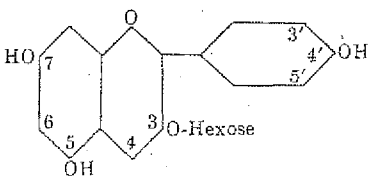
The melting-point and the carbon and hydrogen content agree remarkably well with those of apigenin triacetate, but for the present the identity of this anthoxanthin remains in doubt because of the serious discrepancy in the acetyl determination.

3. Results

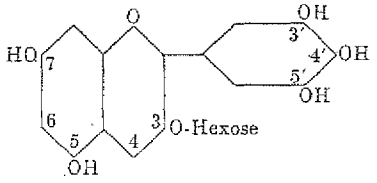
(i) *Qualitative differences in anthocyanins* (M^d–M–m; P^d–P–p)

Four main types of anthocyanin occur in the garden *Verbena*, as shown in Table 1.

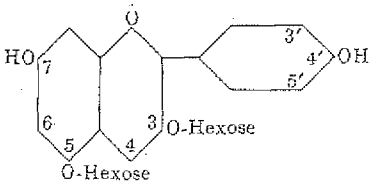
Table 1. *The principal anthocyanins occurring in* Verbena



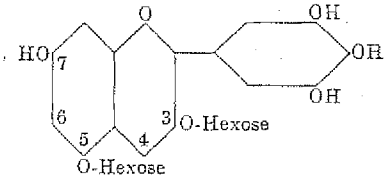
Pelargonidin 3-monoside
(scarlet)



Delphinidin 3-monoside
(maroon)



Pelargonidin 3 : 5-dimonoside
(scarlet-magenta)



Delphinidin 3:5-dimonoside
(purple)

Reference to the genetical results given earlier (Beale, 1940) will show that there are two genetical types of scarlet, the first differing from purple by two dominant allelomorphs and the second by two recessives. Both dominant and recessive scarlets are however pigmented by precisely the same anthocyanin—pelargonidin 3-monoside. Similarly, of the two kinds of maroon, one differing from purple by a single dominant factor and the other by a single recessive, both contain the same anthocyanin—delphinidin 3-monoside; and the dominant and recessive scarlet-magentas each contain pelargonidin 3 : 5-dimonoside.

According to the genetical scheme put forward, there are two series of three multiple allelomorphs: (1) M^d–M–m, corresponding to the characters dominant 3-monoside, 3 : 5-dimonoside, recessive 3-monoside and (2) P^d–P–p, corresponding to the characters dominant pelargonidin, delphinidin, recessive pelargonidin. This scheme has been confirmed by additional data obtained recently. There are, however, further complications, e.g. sometimes the dominance is definitely intermediate and a

mixture of pelargonidin and delphinidin in approximately equal quantities is obtained (see Beale, 1940, Table 5).

In addition to delphinidin and pelargonidin, anthocyanins based on cyanidin occur in some strains. The cyanidin derivatives have so far never been found pure, but always mixed with either pelargonidin or delphinidin derivatives. The inheritance of these types has not been fully worked out, but on crossing a plant containing mixed pelargonidin and cyanidin derivatives with the dominant scarlet (pelargonidin 3-monoside), the F_1 contained pure pelargonidin 3-monoside and in the F_2 the proportion of plants containing cyanidin + pelargonidin to those containing pure pelargonidin derivatives was 7 : 115. This indicates that the type containing cyanidin + pelargonidin derivatives (the variety "Crown Prince") differs from the dominant scarlet by one or more recessive factors, but the relationship between these and the factors controlling the pelargonidin-delphinidin difference is unknown.

(ii) *Variation in acylation of anthocyanins and* p*H of cell sap* (U–u)

Purple, maroon, scarlet-magenta and scarlet flowers all contain acylated anthocyanins. Plum-coloured flowers (see *J. Genet.* 40, Pl. XIII) are pigmented by non-acylated pelargonidin 3 : 5-dimonoside. The difference between the anthocyanins in plum and in scarlet-magenta flowers is therefore one of acylation. There are, however, two other associated differences: (1) In the cells of scarlet-magenta flowers the red pigment is distributed uniformly in the sap, while in the plum flowers there is a much darker, sharply delimited region in the middle of the cell where the anthocyanin is apparently more densely aggregated. Cells containing the dense structure occur chiefly towards the centre of the flower and are associated with the darker and bluer colour of this part of plum flowers. (2) The expressed sap of plum flowers is about 0·4 *p*H units more alkaline than that of scarlet-magenta flowers.

The genetical constitution of plum is $\mathbf{P^dM\,u}$ (or **p M u**), **M** and **u** being completely linked. Instead of **M** and **u** one might equally well write a single factor (say $\mathbf{M^u}$) in the $\mathbf{M^d}$, **M**, **m** series; but for reasons given previously (Beale, 1940) it has been thought preferable to postulate two factors, one controlling the difference between dimonoside and monoside, the other the difference between acylated and non-acylated anthocyanin (and associated differences). Plum contains the two factors in the repulsed condition, i.e. it is distinguished from scarlet-magenta by two effects, one dominant and one recessive.

An attempt has been made to determine the interaction of plum with other genes. The type plum-purple **P M u** is very similar in appearance to ordinary purple, though possibly a little bluer; the pigment from plum-purple is, as expected, non-acylated delphinidin 3 : 5-dimonoside. All efforts to get plum on to a scarlet or maroon background have failed, presumably owing to the tight linkage between **M** and **u**. Non-acylated 3-monoside types have therefore not been obtained.

(iii) *Copigment and flavone differences* (**D–d**; **A–a**; **Ye–ye**)

The incompletely recessive factor "dilute" (**d**) has a diluting and at the same time a blueing effect on the flower colour (see *J. Genet.* **40**, Pl. XIII). The blueing effect is caused by an increase in the degree of copigmentation; normally (i.e. in **DD** forms) there is comparatively little copigment present.

White and yellow flowers both contain flavone, not plastid pigments. The exact chemical difference between the two types is not known, but yellow flowers contain luteolin and white flowers another, unknown, flavone. The genetical difference between white and yellow is not a simple one, since in an F_2 derived from a hybrid between the two types a range of colours between the two parents is obtained. In F_1, however, yellow is completely recessive.

4. Discussion

When a comparison is made between the genetic relations of the various pigment types in *Verbena* and in other plants similarly investigated, it is apparent that *Verbena* is decidedly exceptional. Thus, in the great majority of plants (see Scott-Moncrieff, 1936) anthocyanins with more hydroxyl groups on the 2-phenyl ring are dominant to those with less (e.g. delphinidin is dominant to pelargonidin); in *Verbena*, however, delphinidin derivatives may be either dominant or recessive to pelargonidin according to which strain of scarlet is used. Secondly, in other genera there is a clear-cut segregation of anthocyanidin types and mixtures of two or more in one plant occur only rarely; in *Verbena*, on the contrary, mixtures of pelargonidin with delphinidin derivatives, and of both with cyanidin derivatives, are known. Thirdly, clear-cut segregation of differing glycosidal types, of acylated and non-acylated anthocyanins and of qualitative flavone differences, such as occurs in *Verbena*, is elsewhere uncommon or unknown.

Some at least of these irregularities are evidently due to the interspecific origin of the garden strains. The unusual dominance relations will be first considered.

Since mutants are usually recessive to their wild-type allelomorphs, it is important, when considering the dominance of a particular type, to know whether it is controlled by a wild-type or a mutant gene. The apparent regularity in the dominance of one anthocyanin type over another may be due to the fact that the direction of mutation is much more frequently from delphinidin to cyanidin or pelargonidin than in the reverse direction, and not to any fundamental cause affecting directly the dominance of one type over another. According to this view one would not expect a delphinidin type to be dominant over pelargonidin when (1) the two pigments have been derived from different species or (2) mutation has been in the unusual direction of pelargonidin to delphinidin. *Verbena* clearly is an example of class (1), and the most reasonable explanation is that the dominant pelargonidin factor has come from a wild-type allelomorph of a species containing a pelargonidin derivative—most probably the scarlet *V. peruviana*—and that the recessive pelargonidin factor has arisen by mutation. The apparent allelomorphism of the dominant and recessive pelargonidin factors needs to be confirmed before its significance can be adjudged.

A search is being made for examples of class (2). So far only one has been investigated fully, viz. *Anagallis arvensis*, in which the direction of mutation is from pelargonidin to delphinidin and in which the blue-flowered form containing a delphinidin derivative is, as expected, recessive to the red-flowered form containing a pelargonidin derivative. A similar example, *Salvia splendens*, is being studied (Beale, unpublished).

The occurrence of mixtures of different anthocyanins in the same plant, which is another unusual feature of *Verbena*, is also related to the interspecific origin of the plant, for so-called "blending inheritance" is common in species hybrids. This may be due either to the presence of modifying factors or to incomplete dominance.

As regards inheritance of glycosidal types, only one other example of a single factor controlling the difference between a 3 : 5-dimonoside and a 3-monoside has been established. This is in the China Aster, *Callistemma chinensis* (Wit, 1937), where dimonoside is dominant to monoside. This kind of variation is probably not so rare as was at first supposed, for in several other species (e.g. *Dianthus barbatus*, *D. caryophyllus*, *Phlox Drummondii*) two glycoside types occur, though their genetic relations have not been worked out. In *Verbena*, the presence of both dominant and recessive factors changing dimonoside to monoside is probably due to the same sort of cause as the presence of dominant and recessive factors changing delphinidin into pelargonidin; M^d is considered to be derived

from *V. peruviana*, which contains a 3-monoside, M from one of the other species (*V. platensis* contains a 3 : 5-dimonoside, and *V. incisa* and *V. phlogiflora* are inferred to contain 3 : 5-dimonosides from their appearance) and m by mutation. Here again, test matings with the actual wild species are required.

One other example of a gene controlling acylation of an anthocyanin is known, viz. in *Papaver Rhoeas* (Scott-Moncrieff, 1936). There, as in *Verbena*, the non-acylated type is the mutant, and recessive; it is also associated with a marked increase in alkalinity of the cell sap. Whether the increase in *p*H is the cause of the removal of acylation, or vice versa, is unknown. The correlation between non-acylation of an anthocyanin and its state of aggregation is also unexplained.

The inverse correlation between the quantity of anthocyanin and of flavone, as shown by the various phases of the factor **D**, is in accordance with the situation in numerous other plants (e.g. *Lathyrus odoratus*, *Primula sinensis*, *Dahlia variabilis*) where reduction in concentration of anthocyanin is invariably associated with increase in anthoxanthin.

There are scarcely any data on the inheritance of flavone or flavonol differences for comparison with those obtained from the white and yellow Verbenas. Wheldale & Bassett (1914) reported that in *Antirrhinum majus* there is an ivory coloured form containing apigenin and a yellow form, differing from the ivory by a single recessive factor, containing luteolin. This is apparently similar to the situation in *Verbena*, but recent work by Price (unpublished) indicates that the yellow pigment in *Antirrhinum* is not a flavone at all, but a chalkone. Hence it must be noted that there are at present no fully authenticated examples of a gene controlling flavone or flavonol differences in plants, though such examples will assuredly be discovered in the future.

5. Summary

1. The following types of variation in the flower pigments of *Verbena* (garden hybrids) have been established:

(*a*) The anthocyanidins may be pelargonidin, delphinidin, or mixtures of these with each other or with cyanidin (i.e. all combinations except cyanidin alone).

(*b*) The anthocyanins may be 3-monosides or 3 : 5-dimonosides.

(*c*) The anthocyanins may or may not be acylated.

(*d*) The non-acylated types have a more alkaline cell-sap, and in them the pigment is more densely aggregated.

(*e*) The anthocyanins may be partially or completely inhibited; if the former, there is an increase in anthoxanthin copigmentation.

(*f*) The anthoxanthins (in flowers not containing anthocyanin) may be luteolin (in yellow flowers) or an undetermined flavone (in white flowers).

2. The inheritance of these variations is contrary to rule in other plants in the following respects:

(*a*) Pelargonidin derivatives are sometimes dominant, sometimes recessive to delphinidin.

(*b*) Monosides are sometimes dominant, sometimes recessive to dimonosides.

(*c*) Mixtures of anthocyanins occur, due to incomplete dominance or to modifying factors.

These irregularities are considered to be due to the interspecific origin of the garden *Verbena*. The other variations are not abnormal as regards dominance, direction of mutation, or gene-interaction, in so far as they can be compared with homologous differences in other plants.

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