CHEMOGENETICAL INVESTIGATIONS OF FLOWER COLOURS IN *CYCLAMEN*

J. VAN BRAGT

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Dit proefschrift met stellingen van

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landbouwkundig ingenieur, geboren te Amsterdam, 12 oktober 1930, is goedgekeurd door de promotoren, DR. IR. S. J. WELLENSIEK, hoogleraar in de tuinbouwplantenteelt en DR. H. J. DEN HERTOG, hoogleraar in de organische scheikunde.

De Rector Magnificus der Landbouwhogeschool,
W. F. EIJSVOOGEL

Wageningen, 28 mei 1962.

CHEMOGENETICAL INVESTIGATIONS OF FLOWER COLOURS IN *CYCLAMEN*

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD
VAN DOCTOR IN DE LANDBOUWKUNDE
OP GEZAG VAN DE RECTOR MAGNIFICUS IR. W. F. EIJSVOOGEL,
HOOGLERAAR IN DE HYDRAULICA, DE BEVLOEIING, DE WEG- EN WATERBOUWKUNDE EN DE BOSBOUWARCHITECTUUR, TE VERDEDIGEN TEGEN
DE BEDENKINGEN VAN EEN COMMISSIE UIT DE SENAAT VAN
DE LANDBOUWHOGESCHOOL TE WAGENINGEN
OP VRIJDAG 29 JUNI 1962 TE 16 UUR

DOOR

JAN VAN BRAGT

H. VEENMAN & ZONEN N.V. - WAGENINGEN - 1962

STELLINGEN

I

Het geen *S* moet beschouwd worden als remfactor voor de hydroxylering van de *B*-precursor, waaruit anthocyaninen en leuco-anthocyaninen in *Cyclamen* worden gevormd.

Dit proefschrift.

II

De Horticultural Colour Chart is een juiste basis voor bloemkleurbeschrijving.

III

Als basis voor bloemkleurveredeling dient het onderzoek van bloempigmenten mede ingeschakeld te worden.

IV

Bloemverkleuring in cyclamen berust op factoren die de werking van het geen *S* beïnvloeden.

V

De gegevens, door PIETERSE bijeengebracht ter staving van de onderstelling, dat bij de inwerking van natriumamide op 3-broom-4-ethoxypyridine in vloeibare ammonia een instabiel tussenproduct met een drievoudige binding tussen de koolstofatomen 2 en 3 optreedt, sluiten de verklaring van deze omzetting volgens een ander mechanisme niet uit.

BENKESER, R. A. and SCHROLL, G.
J. Am. Chem. Soc. **75**, 1953: 3196–3197.
PIETERSE, M. J. and DEN HERTOG, H. J.
Rec. trav. chim. **80**, 1961: 1376–1386.
PIETERSE, M. J.
Proefschrift Amsterdam, 1962.

VI

De door HASEGAWA *et al.* geïntroduceerde systematiek der asporogene gisten, berustend op hun pigmentatie, is aan critiek onderhevig.

HASEGAWA, T., BANNO, I. and YAMAUCHI, S.
J. Gen. Appl. Microbiology **5**, 1960: 200–212.
J. Gen. Appl. Microbiology **6**, 1960: 196–215.

IR. J. VAN BRAGT
Wageningen, juni 1962

VII

De mening van MARAMOROSCH dat bepaalde enaties bij incarnaatklaver door een door cicaden overgebracht virus worden veroorzaakt, en zijn latere opvatting dat in het algemeen het ontstaan van enaties, na zuigen van cicaden, aan de werking van toxische speekselbestanddelen moet worden toegeschreven, zijn beide onvoldoende gefundeerd.

MARAMOROSCH, K.
Plant Dis. Rep. **37**, 1953: 612–613.
MARAMOROSCH, K., CALICA, C. A., AGATI, J. A. and PABLEO, G.
Ent. exp. & appl. **4**, 1961: 86–89.

VIII

Het is niet waarschijnlijk dat de variatie in kleurintensiteit van de *Cyclamen* cultivar 'Rosa von Zehlendorf' uitsluitend berust op dosiswerking van het geen *F.*

KESSLER, G.
Zsch. Pflanzenzücht. **42**, 1959: 250–294.

IX

De zwellingen op planten, veroorzaakt door toxische speekselbestanddelen van stekend-zuigende insekten, worden dikwijls ten onrechte gallen genoemd.

ANTOINE, R.
Rep. Mtius Sugar Ind. Res. Inst. 1959: 52–60.

Voor mijn Ouders
Voor Nel

WOORD VOORAF

Gaarne wil ik op deze plaats allen die aan het tot stand komen van dit proefschrift hebben bijgedragen, daarvoor hartelijk danken.

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NEL, jou ben ik dankbaar omdat je mij een rustig werken hebt mogelijk gemaakt door steeds naast me te staan.

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CHEMOGENETICAL INVESTIGATIONS OF FLOWER COLOURS IN *CYCLAMEN*

Met een samenvatting:

CHEMOGENETISCH BLOEMKLEURONDERZOEK IN *CYCLAMEN*

by/door

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Publication No. 223, Laboratorium voor Tuinbouwplantenteelt, Landbouwhogeschool, Wageningen

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CONTENTS

CHAPTER I

GENERAL

1. INTRODUCTION

The first studies about the chemistry of the flower colour, with reference to genetics, were carried out by WHELDALE (58) in 1909 with *Antirrhinum majus*. Since that time many similar investigations have been carried out on the flower colouring substances in other higher plants. The greater part of these investigations dealt with the sap-soluble flower pigments which belong to the group of flavonoid compounds. In plants these substances mainly are anthocyanins, that are glycosides of anthocyanidins, and glycosides of flavones and flavonols. An example of the structure of these compounds is given in fig. 1.

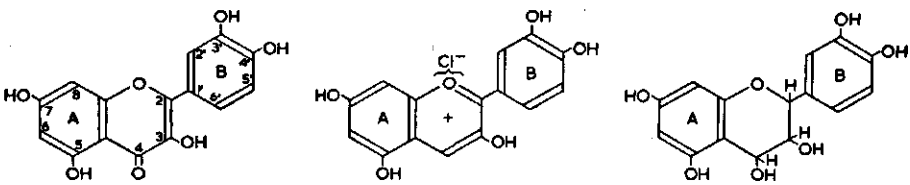

FIG. 1. Structure of some flavonoid compounds.
From left to right: quercetin (a flavonol), cyanidin (an anthocyanidin) and leuco-cyanidin (a leuco-anthocyanidin).

It shows that their molecules consist of two benzene rings, A and B, linked by a chain of three carbon atoms. This chain forms part of a third six membered ring with an oxygen atom and two carbon atoms of the A-ring. The numbering of the C-atoms is given in the formula of the flavonol.

The investigations on flower colours were stimulated by the fact that G. M. and R. ROBINSON (43, 44, 45, 46) developed a simple and rapid method for their identification. The work of BEALE (5), LAWRENCE and SCOTT-MONCRIEFF (34, 35) and SCOTT-MONCRIEFF (47, 48) showed that many genetically determined changes in flower colour were related to alterations in the structure of the anthocyanins present. This work was reviewed by SCOTT-MONCRIEFF (48).

The application of paperchromatography was introduced for the determination of flavonoid substances by BATE-SMITH (3) in 1948. This technique made possible a better analysis of the flavonoid compounds.

At present much attention is being paid to the synthesis of the flavonoid compounds in the plant. Reviews on the work on this subject have been given by BOGORAD (9) and NEISH (37).

2. SCOPE OF PREVIOUS INVESTIGATIONS IN CYCLAMEN

The flower colouring pigments in *Cyclamen* were first studied by KARRER and WIDMER (31) in 1927. From flowers of *C. persicum* MILL. they isolated an anthocyanin which was identified as a glucoside of malvidin. In 1934 G. M. and R. ROBINSON (46) demonstrated the presence of malvidin-3-glucoside in flowers of the cultivars 'Giant Crimson' and 'Vulcan'. They also showed that a peonidin-3-pentoseglycoside was present in the cultivar 'Firefly'. In 1946 WELLENSIEK (53, 54) started, at the Wageningen Horticultural Laboratory, an extensive study of *Cyclamen*. This author demonstrated the presence of a basic gene pair responsible for colour formation, and discovered the genetic conditions for the limitation of the flower colour to the base of the petal.

In 1954 WERCKMEISTER (57), using paperchromatography, analysed the pigments of several cultivars and demonstrated the presence of malvidin-3-glucoside.

The knowledge about genetics and colour formation, with reference to the chemistry of flower colouring pigments, was extended by SEYFFERT (49, 50). From chemical analysis with the aid of paperchromatography this author concluded that only sap-soluble pigments were present, both in the base of the petal, the so-called eye, and in the remaining part of the petal, the slip. He demonstrated the presence of anthocyanins, leuco-anthocyanins and flavonol glycosides. SEYFFERT (50) gave a chemical basis to the genetics as known from the investigations of WELLENSIEK and he also demonstrated the presence of new gene pairs.

Further information about the chemistry of *Cyclamen* anthocyanins has been reported by VAN BRAGT (11, 12).

KESSLER's (32) work revealed the presence of genes changing the quantity of anthocyanins present in flowers.

For convenience a more detailed discussion of previous investigations is postponed to chapter V.

3. THE AIM OF THE PRESENT INVESTIGATIONS

The investigations on the flower colours of *Cyclamen*, presented in this thesis, were carried out in order to extend the knowledge of the chemistry and the inheritance of the flavonoid compounds, as a basis for description of cultivars and for breeding work.

CHAPTER II

PLANT MATERIAL AND COLOUR SUBSTANCES

1. Wild species

As part of the studies on *Cyclamen* at the Horticultural Laboratory, a collection was made of the wild species. This material was extensively studied by Wellensiek, Doorenbos and De Haan (55), Doorenbos (14, 15) and Legro (36), and it now was used for the investigation of flavonoid compounds.

2. Cultivars

In 1955 the Horticultural Laboratory was appointed as International Registration Authority for *Cyclamen*. In the frame work of the investigations carried out on this subject a large number of West-European cultivars was collected. The collection has been studied by Wellensiek, Doorenbos, Legro and Van Bragt (56). The chromatographical data for pigmentation, obtained from the investigation of these cultivars, are included. The present investigation deals with additional data.

3. Crosses

In order to investigate the possibilities for breeding a collection of diploid *Cyclamen* cultivars with as many flower colours as possible, Wellensiek (54) made several crosses. Among them there were the crosses of the English cultivar 'Firefly' and a number of Dutch cultivars. This material, which also was promising for the investigation of genetical and chemical aspects of flower colour formation, was generously made available for the investigations presented in this paper.

4. Location of pigments and description of colours

In order to obtain data concerning the location of the flavonoid compounds investigated, cross-sections (fig. 2) were made of several flower petals.

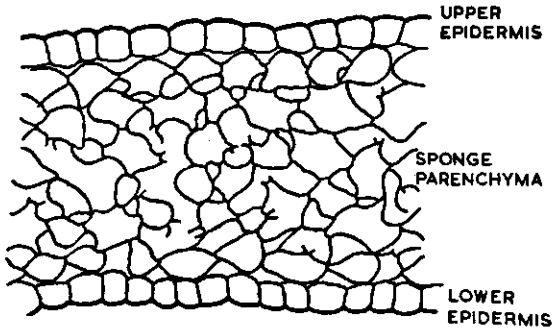

Fig. 2. Cross-section through a petal of cyclamen. Linear enlargement 340 ×.

The cross-sections of reddish, violet or bluish coloured petals showed that the majority of the anthocyanins was present in the cells of both upper and lower epidermis.

On examining the cross-sections of white petals, the yellow pigments are not visible, due to their low concentration. By treatment with ammonia, which changes these pigments into deep yellow coloured compounds, it was shown, however, that they were present in the epidermal cells also.

The presence of leuco-anthocyanins was demonstrated by treating cross-sections of white petals with an ethanolic solution of vanillin and hydrochloric acid. The reddish coloration thus obtained in the epidermal cells demonstrated the presence of leuco-anthocyanins.

From these data it was concluded that the flavonoid compounds investigated occurred chiefly in epidermal cells of the petal.

For the accurate description of flower colours the HORTICULTURAL COLOUR CHART (30) was used. The colours of the slip and of the eye were registered separately. In general the pigmentation of the slip was uniformly spred, hence it was satisfactory to register the colour in the centre of the greatest width of the slip.

CHAPTER III

METHODS OF CHEMICAL ANALYSIS

1. SELECTION OF MATERIAL

Firstly, the aim of the chemical investigations was to isolate and identify the flavonoid compounds in *Cyclamen*. In this investigation which included several hundred plants, 24 flavonoid compounds were found. It is assumed that the pigments found are the most important ones which occur in *Cyclamen*.

2. EXTRACTION

For the extraction of anthocyanins especially intensively reddish or purplish coloured flowers were used, whereas flavonol glycosides were extracted from white flowers. Leuco-anthocyanins were isolated from both types of flowers.

In order to obtain a concentrated solution of anthocyanins, 5 ml of a 1% aqueous solution of hydrochloric acid was added to about 2 g of tissue. The compounds were extracted by grinding the tissue in the liquid for five minutes with a pestle and mortar.

A concentrated solution of flavonol glycosides was obtained by treating the tissue of white flowers in the same way, using methanol as the extracting liquid. Complete extraction of the flower tissue was not necessary. As the solubility of the various coloured substances did not differ considerably, the solution obtained from a short extraction contained all flavonoid compounds present in the tissue.

In order to separate the leuco-compounds, two methods were used which are described in this chapter, see p. 7.

3. Chromatographic separation

3.1. *General*

The separation of the pigments was carried out by paperchromatography on Whatman No. 1 filter paper at about 18 °C with descending solvents of different compositions (see table 1).

Table 1. Solvents used for chromatography.

Composition of solvents	Ratio of solvents in solution volume/volume (v/v) or weight/volume (w/v)	Mobile phase	Abbreviations for solvents
water		the homogeneous liquid	
1% aqueous hydrochloric acid		ibid.	HA
5% aqueous acetic acid		ibid.	AA
25% aqueous hydrochloric acid, acetic acid and water	3/30/10 v/v	ibid.	HAW
2% aqueous boric acid		ibid.	BO
phenol and water	73/27 w/v	ibid.	PH
n-butanol, acetic acid and water	6/1/2 v/v	ibid.	BAW
n-butanol, 2 *N* aqueous hydrochloric acid	1/1 v/v	top layer	BH
m-cresol, acetic acid and water	25/1/24 v/v	bottom layer	CAW

On the chromatograms the anthocyanins ware easily localized, because they have an intensive colour.

Flavonol glycosides appear as very faint yellow spots, but in U.V. light they have a distinct brown or yellow fluorescence.

Leuco-anthocyanins have no colour, but can be converted into reddish compounds after spraying with an ethanolic solution of vanillin and hydrochloric acid (4).

3.2. *Separation of anthocyanins*

With a micropipette the extract was applied to the paper in a band of approximately 0.7 cm wide at 12 cm from the edge of the smallest side of a sheet (58 × 60 cm). The band was dried at room temperature in a current of air. For the first separation the solvent BH was used. The liquid was allowed to run down the paper and to drop from the serrated lower edge, until either distinct bands had appeared, or a band had reached the edge. The paper was allowed to dry at room temperature in a fume cupboard. Each band was cut out and eluted with BAW which gives a rapid elution and also prevents the production of sugars as an artefact from the paper (27). The eluant was allowed to evaporate at room temperature in a current of air. Since it appeared that the components of the extracts could not be separated by chromatography with only one solvent, the residues were dissolved in a 1% solution of hydrochloric acid in ethanol and applied as spots at a distance of 1.5 cm from each other, on a line at 12 cm from the edge of different filter papers, and tested for purity by treating with a second solvent (HA, AA, BH, or CAW). For the separation of each residue, a solvent was chosen that gave the best separation of the components in experiments using the various solvents mentioned. The procedure was repeated as many times as necessary to produce pure solutions. When

chromatograms were developed with BAW or CAW, they were dried, sprayed with a 1% solution of hydrochloric acid in ethanol, and dried again. By this treatment the anthocyanins are more rapidly eluted with BAW.

The anthocyanins were liberated from the sugars, which were extracted simultaneously, by the following method. The anthocyanins were applied in a streak on sheets of washed paper. These sheets were obtained by irrigating the paper with 2% aqueous acetic acid, and removing the acid by irrigating with distilled water, until the liquid which dropped from the serrated lower edge was at pH = 6.5 (measured with Universal indicator paper, Merck A.G., Darmstadt). The papers with the streaks of anthocyanins were irrigated with the solvent BO. In this solvent sugars travel faster than anthocyanins (17). The bands thus obtained were treated as described before.

3.3. *Separation of flavonol glycosides*

The extract was applied to the paper as described for the anthocyanins. For the first separation BAW was used. With a pencil the bands were marked under U.V. light, cut out and eluted with an aqueous solution containing 50% ethanol. The eluant was evaporated at room temperature in a current of air. The residues were dissolved in methanol, and tested for purity. To achieve this, spot tests were made using BAW, CAW and water as solvents. The second separation was carried out with solvents indicated by the spot tests, as described for the anthocyanins.

3.4. *Separation of leuco-anthocyanins*

In order to separate leuco-anthocyanins from anthocyanins and flavonol glycosides which were present in the same intact tissue, the method described by ALSTON and HAGEN (2) was used: The intact tissue was boiled up to 5 minutes in ethanol containing 0.04% hydrochloric acid. In this liquid only anthocyanins and flavonol glycosides were removed from the tissue. The extraction was repeated till the tissue was colourless. The leuco-anthocyanins remained in the tissue, which then was transferred to 2 *N* aqueous hydrochloric acid and heated at 100°C for 30 minutes. After this treatment the liquid contained dissolved anthocyanidins. The latter were extracted from this liquid with *iso*-amyl alcohol and used for identification.

Leuco-anthocyanins were also separated from a mixture which contained anthocyanins and flavonol glycosides with the solvent BAW. With BAW these latter compounds travelled faster on chromatograms than the leuco-compounds. The leuco-compounds as a rule could be detected at a distance not greater than 2 cm from the starting line when the paper was sprayed with vanillin and hydrochloric acid. The chromatograms were eluted with water at the places where leuco-compounds were shown to be present by orientating experiments. The eluates were acidified with 25% aqueous hydrochloric acid until they were 2 *N* HCl, and heated at 100°C for 30 minutes. With *iso*-amyl alcohol the anthocyanidins were extracted from the liquid and the aqueous residue was used for the identification of sugars.

4. IDENTIFICATION

4.1. *General*

On acid hydrolysis, the glycosides give rise to aglycones and sugars. Infor-

mation about the nature of these substances was obtained by chromatographic comparison with authentic compounds.

Further information about the identity of anthocyanins and flavonol glycosides was obtained by studying the products of partial hydrolysis.

Additional data were obtained from the spectra of the flavonoids. The anthocyanins show maximal absorption at about 520–550 mμ (24). The anthocyanins thus far detected in nature are all substituted by a sugar residue at the 3 position, or at the 3 and the 5 positions (see fig. 1, on p. 2). The substitution of the 3-hydroxyl group causes a hypsochromic shift of the wavelength of maximum absorption. The substitution of the 5-hydroxyl group by another sugar molecule does not change the wavelength of the maximum absorption (25). When two hydroxyl groups are present in the B-nucleus in ortho position, the addition of aluminium ions causes a bathochromic shift of 25–40 mμ (21, 23). The position of the sugars in flavonol glycosides is not restricted to the 3 and the 5 position. In order to get some information as to where the sugars are bound, the determination of the spectra is useful. Flavonols have two maxima, one between 250 and 280, the other between 350 and 390 mμ. When the 3-hydroxyl group is substituted by a sugar residue, this results in a hypsochromic shift of about 15 mμ of the maximum at the longer wavelength. Substitution of any other hydroxyl group results in a shift of about 5 mμ (52). The measurements of the spectra after the addition of aluminium chloride gives some further information: kaempferol-3-glucoside then gives two maxima, at 355 and 400, quercetin-3-glucoside one at 400 mμ (23, 41, 42).

By electrolytical reduction, flavonols and flavonol glycosides can be converted into anthocyanidins and anthocyanins respectively (16, 17). The identifications were further verified by reducing the flavonols and flavonol glycosides, followed by chromatographic analyses of the compounds thus obtained.

4.2. *Identification of anthocyanins*

An amount of the anthocyanin was hydrolysed by dissolving in 2 *N* aqueous hydrochloric acid, and heating at 100°C for 30 minutes. The anthocyanidin was extracted with *iso*-amyl alcohol and the aqueous mixture was used for sugar determination. An amount of the extracted anthocyanidin was co-chromatographed with authentic compounds, e.g. cyanidin from *Digitalis purpurea* (43), peonidin from *Fuchsia hybr.* (43), delphinidin from *Delphinium ssp.* (63), petunidin from *Petunia ssp.* (62), and malvidin from *Epilobium angustifolium* (43), HAW and BH were used as solvents.

The spectra were measured under standard conditions. Another aliquot of the anthocyanidin was applied to a sheet of washed filter paper, chromatographed with HAW, whereupon the fragment of the paper containing the anthocyanidin was cut out together with a strip of blank paper alongside the spot (10). Both strips were kept in a desiccator for 15 hours and after that they were stored *in vacuo* over sodium hydroxide for 24 hours. Then the strips were placed in the cuvette holder of the spectrophotometer (Unicam S.P. 500) and the spectra measured. After the measurements, the papers were sprayed with 0.1 *M* aqueous aluminium sulphate solution, dried, and again a spectral determination was made.

For the determination of sugars in the aqueous mixtures – obtained after acid hydrolysis of the anthocyanins – it was necessary to remove the acid in order to obtain good chromatograms. This was accomplished by shaking 1 ml

of the aqueous mixture with 3 ml portions of a 10% solution of methyl-di-*n*-octylamine in chloroform, which removes the acid (27). The aqueous layer, thus brought to pH = 7 (measured with Universal indicator paper, Merck A.G., Darmstadt) was applied to a sheet of paper, together with authentic samples of glucose and rhamnose. Thereupon two chromatograms were made using BAW and PH as solvents. The solvent was allowed to run down through the aper for 96 and 72 hours respectively. The paper was dried, sprayed with a solution of aniline phthalate in water-saturated *n*-butanol, and heated for 3–5 minutes at 105°C. This treatment develops the position of the sugars on the chromatogram (38).

Finally the anthocyanins were identified as such by co-chromatography and also by comparison of the spectra. For comparison authentic samples were used as follows:

pelargonidin-3-glucoside from *Pelargonium zonale* (60) and from *Callistephus chinensis ssp.* (33);

cyanidin-3,5-diglucoside from *Digitalis purpurea* (43);

cyanidin-3-glucoside from partial hydrolysis (29) of the diglucoside just mentioned and from *Callistephus chinensis ssp.* (33);

peonidin-3,5-diglucoside from *Fuchsia hybr.* (43);

peonidin-3-glucoside from partial hydrolysis of the diglucoside just mentioned;

malvidin-3,5-diglucoside from *Epilobium angustifolium* (43);

malvidin-3-glucoside from partial hydrolysis of the last mentioned preparation.

The anthocyanins from cyclamen were partially hydrolysed and the monoglucosides formed identified by co-chromatography. The hydrolysis was carried out as follows: The anthocyanins were dissolved in 1% aqueous hydrochloric acid and the solution was heated at 100°C. Samples of 0.5 ml of the liquid were removed after 0, 3, 5, 7, 10, 15 and 30 minutes, and four chromatograms were made of the compounds present in the mixture, using HA, BAW, BH and CAW as solvents.

The spectra of the authentic compounds and of the anthocyanins isolated from cyclamen were determined as described for the anthocyanidins, the chromatograms being prepared with the solvent BH.

In order to ascertain whether complex anthocyanins were present, all anthocyanins which were isolated from cyclamen were submitted to the test described by HAYASHI (28), and also chromatographed before and after treatment. From these experiments it appeared that none of the tested anthocyanins was a complex anthocyanin.

4.3. *Identification of flavonol glycosides*

An aliquot of the glycoside was dissolved in 2% aqueous hydrochloric acid and heated at 100°C for 20 minutes. The aglycone was extracted with ethyl acetate. An amount of this extract was applied to a sheet of paper, together with the authentic compounds kaempferol and quercetin [1]). BAW, CAW and HAW were used as solvents. The determination of the sugar residue in the aqueous layer was carried out as described for the anthocyanins.

Another amount of the extracted aglycone was applied in a streak to a sheet of washed paper, and a chromatogram was developed with BAW. The band was marked under U.V. and cut out, together with a blank strip of equal size and

[1]) I am indebted to Prof. Dr. H. F. LINSKENS, Nijmegen, The Netherlands for providing me with kaempferol, quercetin and rutin, and to Dr. W. J. FEENSTRA for kaempferol-3-glucoside.

in the corresponding position towards the starting line. Both strips were eluted with an aqueous solution containing 50% ethanol. The optical density of the solution of the aglycone then was measured against the blank, at different wavelengths. After the measurements, a solution of aluminium chloride in 50% aqueous ethanol was added, both to the solution of the aglycone and to the blank. The final concentration of aluminium chloride was 0.1%. The spectrum of this mixture was also measured.

The Rf values of the flavonol glycosides, and the authentic compounds rutin and kaempferol-3-glucoside were recorded from chromatograms developed with BAW, CAW and water. Partial hydrolysis of the flavonol glycosides was carried out as described for the anthocyanins.

In order to verify the conclusions drawn from these analyses, the flavonol glycosides and the aglycones were converted into anthocyanins and anthocyanidins respectively. This conversion was accomplished by electrolytical reduction in 2 *N* aqueous sulfuric acid, as indicated by EVEREST (16), using mercury and platinum electrodes, as described by FEENSTRA (17). In the experiments recorded here a continuous current of 10 mA at 6V was used. The compounds thus obtained were characterized by co-chromatography with authentic samples of anthocyanins and anthocyanidins, using HA, HAW, BH, BAW and CAW as solvents.

4.4. *Identification of leuco-anthocyanins*

The leuco-anthocyanins were converted into anthocyanidins. The latter compounds were analysed as described in 4.2. The sugar determination was only carried out when BAW was used for chromatographic separation of leuco-compounds, and was carried out according to the method used for the analysis of the anthocyanins. The eluate of a blank strip of paper was treated in the same way as the strip which contained the leuco-anthocyanins, in order to prevent errors caused by the production of sugars as an artefact from the paper.

CHAPTER IV

RESULTS OF CHEMICAL ANALYSIS

1. ANTHOCYANINS

Fourteen substances were isolated, which from their Rf values, the Rf values of the reaction products obtained by hydrolysis, and their absorption spectra, appeared to be anthocyanins or derivatives of these substances. The data observed in the measurements are given in table 2, together with those of anthocyanins of known structure which show corresponding properties. These properties were also determined in these experiments. The data concerning delphinidin-3-glucoside and petunidin-3-glucoside were obtained from literature (17).

Thus the anthocyanins A1, A2, A3, A4, A11, A12, A13 and A14 were identified. For the structure formulae of these compounds see fig. 3, on p. 13. The remaining six compounds yielded peonidin-3-glucoside when subjected to partial hydrolysis. Thusfar it has not been possible to establish the structures of this series of compounds.

In a previous publication (11) it was reported that malvidin-3-rhamnogluco-

TABLE 2. Data used for characterization and identification of anthocyanins.

Compounds	Rf values of anthocyanins in				Rf values of anthocyanidins in		λmax. abs. of anthocyanins before spraying with $Al_2(SO_4)_3$	λmax. abs. of anthocyanins after spraying with $Al_2(SO_4)_3$	Sugars detected
	HA	BAW	BH	CAW	HAW	BH			
A 1	0.06	0.32	0.30	0.42	0.52	0.80	535 mμ	560 mμ	glucose
cyanidin-3-glucoside	0.06	0.31	0.31	0.42	0.52	0.79	535 mμ	562 mμ	
A 2	0.14	0.13	0.11	0.19	0.53	0.80	535 mμ	565 mμ	glucose
cyanidin-3,5-diglucoside	0.13	0.13	0.11	0.19	0.52	0.80	535 mμ	565 mμ	
A 3	0.08	0.42	0.36	0.79	0.69	0.82	537 mμ		glucose
peonidin-3-glucoside	0.08	0.43	0.35	0.78	0.69	0.82	537 mμ		
A 4	0.17	0.22	0.13	0.50	0.69	0.81	535 mμ		glucose
peonidin-3,5-diglucoside	0.17	0.23	0.13	0.50	0.69	0.82	535 mμ		
A 5	0.17	0.34	0.37	0.66	0.69	0.82	539 mμ		glucose
A 6	0.44	0.24	0.30	0.47	0.69	0.82	537 mμ		glucose
A 7	0.30	0.30	0.38	0.60	0.69	0.82	537 mμ		glucose
A 8	0.76	0.40	0.44	0.60	0.69	0.82	535 mμ		glucose
A 9	0.60	0.40	0.55	0.60	0.69	0.82	535 mμ		glucose + trace rhamnose
A 10	0.13	0.24	0.36	0.67	0.69	0.82	537 mμ		glucose
A 11	-	0.16	0.15	0.18	0.35	0.41	550 mμ	572 mμ	?
delphinidin-3-glucoside [1])	-	0.11	-	0.14	-	-	540 mμ	580 mμ	
delphinidin					0.35	0.41			
A 12	-	0.21	0.24	0.56	0.47	0.56	543 mμ	565 mμ	?
petunidin-3-glucoside [1])	-	0.18	-	0.52	-	-	550 mμ	575 mμ	
petunidin					0.46	0.55			
A 13	0.06	0.32	0.29	0.81	0.61	0.62	545 mμ		glucose
malvidin-3-glucoside	0.06	0.33	0.29	0.82	0.60	0.63	546 mμ		
A 14	0.14	0.15	0.09	0.52	0.61	0.62	533 mμ		glucose
malvidin-3,5-diglucoside	0.14	0.15	0.09	0.52	0.61	0.62	535 mμ		

[1]) Data obtained from literature (17).

side was found. However, later analyses have shown that the compound in question is malvidin-3-glucoside instead of malvidin-3-rhamnoglucoside.

2. FLAVONOL GLYCOSIDES

Eight flavonol glycosides were isolated. They are characterized by the data listed in table 3.

A supporting contribution for the identification of these compounds was the fact that electrolytical reduction of Fl 1 yielded pelargonidin-3-glucoside, whereas Fl 5 yielded cyanidin-3-glucoside. Also the identity of Fl 6 and rutin was confirmed by the results of electrolytical reduction. After electrolytical reduction the same products were formed. All reduction products were characterized by Rf values. Thus the flavonol glycosides Fl 1, Fl 5 and Fl 6 were

TABLE 3. Data used for characterization and identification of flavonol glycosides.

Compounds	Rf values of flavonol glycosides in			Rf values of aglycones in			λmax. abs. of flavonol glycosides in		Sugars detected
	BAW	CAW	water	BAW	CAW	HAW	ethanol	0.1% ethanolic aluminium chloride	
Fl 1	0.71	0.61	0.17	0.85	0.77	0.55	267 mμ 350 mμ	271 mμ 345 mμ 389 mμ	glucose
kaempferol-3-glucoside	0.70	0.60	0.15				265 mμ 354 mμ	274 mμ 348 mμ 390 mμ	
Fl 2	0.54	0.36	0.24	0.85	0.77	0.55	265 mμ 345 mμ	268 mμ 350 mμ 392 mμ	glucose + rhamnose
Fl 3	0.36	0.21	0.72	0.85	0.77	0.55	266 mμ 350 mμ	267 mμ 345 mμ 391 mμ	glucose + rhamnose
Fl 4	0.36	0.15	0.72	0.85	0.77	0.55	266 mμ 348 mμ	269 mμ 348 mμ 390 mμ	glucose + rhamnose
kaempferol				0.85	0.77	0.55			
Fl 5	0.60	0.33	0.07	0.68	0.36	0.39	358 mμ	398 mμ	glucose
quercetin-3-glucoside [1])	0.51	0.39	-				362 mμ	410 mμ	
Fl 6	0.42	0.16	0.27	0.68	0.36	0.39	358 mμ	397 mμ	glucose + rhamnose
rutin	0.42	0.15	0.29				358 mμ	398 mμ	
Fl 7	0.41	0.28	0.22	0.68	0.36	0.39	365 mμ	400 mμ	glucose + rhamnose ?
Fl 8	0.27	0.07	0.67	0.68	0.36	0.39	357 mμ	400 mμ	glucose + rhamnose
quercetin				0.68	0.36	0.39			

[1]) Data obtained from literature (17).

identified. For the structure formulae of these compounds see fig. 3.

The characterization of the other five compounds caused severe difficulties. No Rf values were found in literature corresponding to those observed. The compounds in question had the same spectrum as flavonol-3-glucosides. Partial hydrolysis of Fl 3 and Fl 4 yielded Fl 2, which in its turn yielded kaempferol-3-glucoside. None of the compounds obtained after electrolytical reduction could be identified by its Rf values. Thusfar it has not been possible to establish the structure of these five compounds.

3. LEUCO-ANTHOCYANINS

The identification of leuco-anthocyanins was usually carried out with the liquid obtained after elution of a part of the chromatograms prepared with a mixture of leuco-anthocyanins, anthocyanins and flavonol glycosides, using

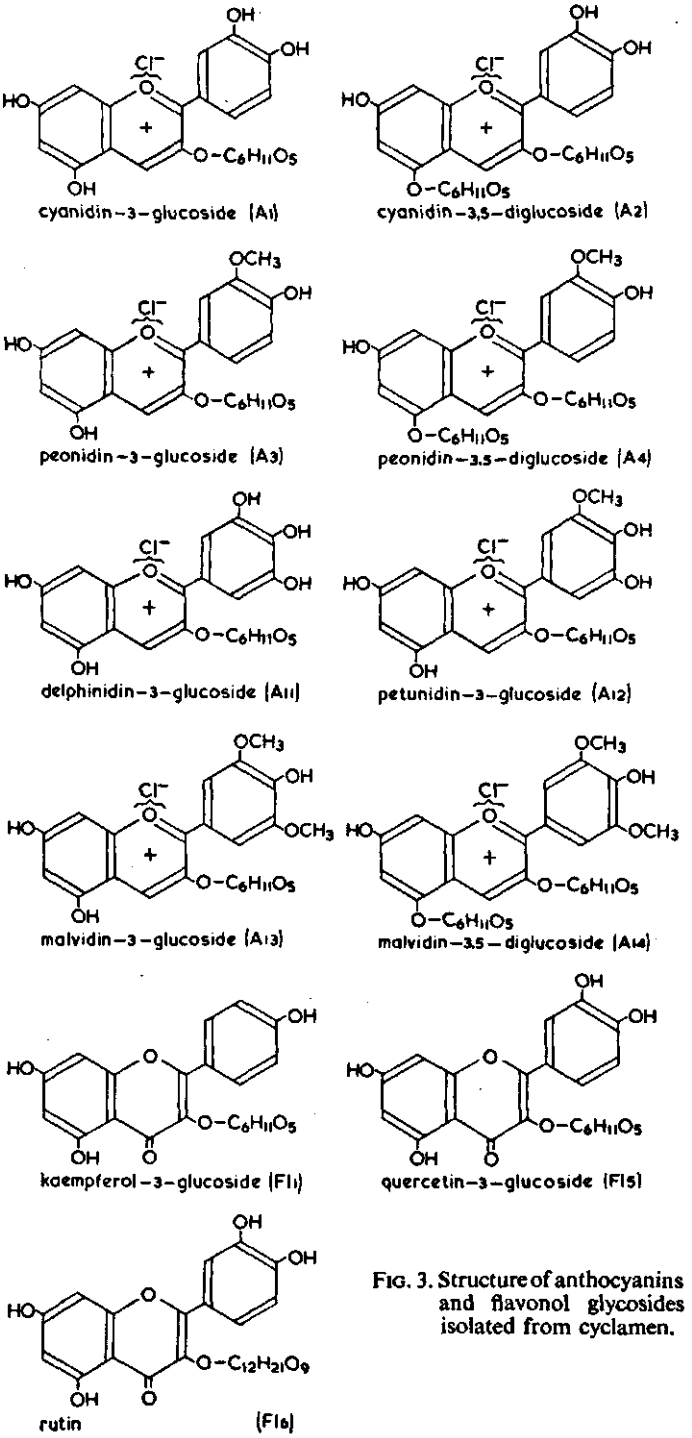

FIG. 3. Structure of anthocyanins and flavonol glycosides isolated from cyclamen.

BAW as the solvent. The method of ALSTON and HAGEN (2) was only used when the identification of sugars was not necessary. The extraction of the hydrolysate with *iso*-amyl alcohol yielded either one or two anthocyanidins. It is assumed that these compounds were obtained by conversion of leuco-compound L 1 and a mixture of leuco-compounds L 1 and L 2. The anthocyanidins were identified by their Rf values. The aqueous residues yielded only glucose. The data used for the identification of the compounds L 1 and L 2 are given in table 4.

TABLE 4. Data used for the identification of leuco-anthocyanins.

Compounds	Rf values in		Sugar detected
	BH	HAW	
anthocyanidin from leuco-compound L 1	0.41	0.35	glucose
delphinidin	0.41	0.35	
anthocyanidins from the mixture of leuco-compounds L 1 and L 2	0.42 0.80	0.36 0.52	glucose
cyanidin	0.80	0.52	

From these data it was concluded that L 1 was a leuco-delphinidin glucoside, and it was assumed that L 2 also was a glucoside, namely leuco-cyanidin glucoside. The number and the place(s) of attachment of the sugar residue(s) in the molecules were not investigated.

CHAPTER V

ANALYSIS OF THE MATERIAL

1. RESTRICTION OF IDENTIFICATION

The flavonoid compounds which were isolated from a part of the cyclamen material, and identified or characterized as described before, were used for the identification of the compounds found in flowers not previously analysed. The identification was carried out by co-chromatography, as described before.

The amount of material was so great that, as a rule, it was necessary to limit the analysis to be carried out with two chromatograms for each flower extract. BAW and BH primarily were chosen as solvents. BAW was the best solvent for the separation of flavonol glycosides and BH for the separation of anthocyanins.

With BAW, however, it is not always possible to separate the mixture of flavonol glycosides. At most five spots on the chromatograms were observed.

In these cases the first spot, Rf 0.27 (see table 3, on p. 12) was formed by the flavonol glycoside Fl 8,

the second, Rf $\sim$ 0.40, could be formed by rutin, Fl 3, Fl 4 or Fl 7,

the third, Rf 0.54, by Fl 2,

the fourth, Rf 0.60, by quercetin-3-glucoside,

the fifth, Rf 0.71, by kaempferol-3-glucoside.

With BH it was impossible to establish the presence of petunidin-3-glucoside

and the anthocyanin A 6, even though the possibility existed that, together with peonidin-3-glucoside also A 5, A 7 and A 10 were present in one spot.

Investigations with the solvents HA, AA, BAW, BH and CAW showed that, when the latter spot was observed, peonidin-3-glucoside was always present, while A 5, A 7 and A 10 were present in smaller amounts than the other anthocyanin.

2. Wild species

The results of the chromatographical analysis of *Cyclamen* species are listed in table 5.

Table 5. Results of the chromatographical analysis of the flowers of *Cyclamen* species. + = compound present, < = compound present in traces, — = compound absent.

Names of *Cyclamen* species	Compounds							
	Eye		Slip					
	Anthocyanin	Flavonol glycosides	Anthocyanin	Flavonol glycosides				
	malvidin-3,5-diglucoside		malvidin-3,5-diglucoside	Fl 8	rutin, Fl 3, Fl 4, Fl 7	Fl 2	quercetin-3-glucoside	kaempferol-3-glucoside
C. balearicum Willk.	–	–	–	+	+	–	–	–
C. repandum Sibth. et Sm.	+	–	+	–	+	–	–	–
C. creticum Hildebr.	<	–	–	–	+	+	+	+
C. cilicium Boiss. et Heldr.	+	–	+	–	–	–	–	–
C. coum Mill.	+	–	+	–	+	+	–	–
C. cyprium Ky.	+	–	+	–	–	–	–	–
C. libanoticum Hildebr.	+	–	+	–	+	+	–	–
C. pseudibericum Hildebr.	+	–	+	–	+	–	–	–
C. neapolitanum Ten.	+	<	+	+	+	–	+	+
C. purpurascens Mill.	+	–	+	+	+	+	+	+
C. persicum Mill.	+	–	+	+	+	+	–	–
C. africanum Boiss. et Reut.	+	<	+	+	+	–	+	+
C. graecum Lk.	+	–	+	+	+	–	+	+
C. rohlfsianum Asch.	+	–	+	–	–	–	–	–

The table shows that malvidin-3,5-diglucoside was the anthocyanin found in coloured flowers.

The chromatographical analyses of some wild species, e.g. *C. neapolitanum* and *C. africanum*, yielded similar results.

Wild *C. persicum*, the ancestor of the cultivated cyclamen, was investigated for the presence of leuco-anthocyanins. The presence of a leuco-delphinidin glucoside was demonstrated.

3. Cultivars

The results of the qualitative determinations of the pigments, valuable for the description of cultivars, have been published before, together with other characteristics such as chromosome number, petal size and leaf pattern (56).

In addition to the qualitative determinations, it was valuable to estimate the amounts of pigments found in the flower slips. These estimations were carried out on most of the cultivars.

With two exceptions, the eyes of the flowers were always intensively coloured; malvidin glycosides were found to be the chief flavonoid compounds in this part of the petal. Hence, the investigations usually were carried out on the flower slip only.

Malvidin glycosides were not found in the petals of 'Helvetikum rose' and 'Helvetikum lachs'. The eyes of these cultivars were as intensively coloured as the slips, and contained the same composition of the mixture of anthocyanins.

In order to compare the amounts of anthocyanins and flavonol glycosides within the flower slip of one cultivar and between the flowers of different cultivars, the following method was used.

Chromatograms were prepared under standard conditions. From each cultivar five flowers were selected. From each flower one petal was used for the determinations. With a corkborer a disc, 7 mm in diameter, was removed from the center of the greatest width of the petal. The tissue was pressed out with a glass rod on the start line of a sheet of paper, and the paper allowed to dry. With a needle the tissue was taken from the paper, wetted with 1% aqueous hydrochloric acid, and pressed out again on the same spot. The procedure was repeated until the tissue was colourless. The chromatograms were developed with BH. The results of the experiments, in which the chromatograms were prepared with known relative amounts of anthocyanins and flavonol glycosides indicate that the length of a spot is proportional to the amount of the compound which formed the spot. Hence, the amounts of anthocyanins in the flower slips of the cultivars were estimated from the lengths of the spots obtained with BH, and indicated by the numbers 1, 2, 3, 4, 5 and 6. Amount 3 is 3 × amount 1, etc. An amount smaller than 1 is indicated by tr, for "trace". With BH, however, the flavonol glycosides did not give distinct spots. Hence only the total amounts of these compounds were estimated and indicated by the numbers *1*, *2* and *3*. Amount *3* is 3 × amount *1*, etc. Amounts smaller than *1* are indicated by *tr*. The anthocyanins and flavonol glycosides found as a result of the qualitative analyses only, are indicated with +. The results of the analyses are given in table 6, which also includes the data obtained from the description of the flower slip colours with the HORTICULTURAL COLOUR CHART (30).

Some striking features are found in this table.

Reddish cultivars contain peonidin glycosides. In the most reddish cultivars A 9 was found to be the chief flavonoid compound. Purplish cultivars contain malvidin glycosides. The most purplish cultivars contain malvidin-3,5-diglucoside as the chief flavonoid compound.

Some of the white flowering cultivars contain small amounts of flavonol glycosides. The flowers of these cultivars are pure white, whereas those of cultivars containing great amounts of flavonol glycosides have a creamish colour.

Cultivars with the same name, and cultivars with the same colour do not always contain the same mixture of flavonoid compounds.

TABLE 6. Analyses of cultivars.

Column 1: The cultivars are listed in the same order as in a previous publication (56), which also gives additional data, e.g. the complete origin of the material, in the present table abbreviated between brackets. Subdivision into groups of five has been made to improve the legibility of the table.

Column 2: Colour description.

Columns 3–18: Qualitative and semi-quantitative analysis.

Qualitative analysis only: +

tr and *tr* = traces; 1–6 = relative amounts of anthocyanins, *1–3* = relative amounts of flavonol glycosides. Compound absent: —

For further explanations see text.

Names of cultivars	Slip colours (H.C.C.)	Flavonoid compounds found in the flower slip															
		Anthocyanins										Flavonol glycosides					
		delphinidin-3-glucoside	malvidin-3,5-diglucoside	malvidin-3-glucoside	cyanidin-3,5-diglucoside	cyanidin-3-glucoside	peonidin-3,5-diglucoside	peonidin-3-glucoside	A 8	A 9	Total amounts estimated	quercetin-3-glucoside	kaempferol-3-glucoside	rutin, Fl 3, Fl 4, Fl 7	Fl 2	Fl 8	Total amounts estimated
Deep purplish red																	
Afterglow (BL)	821	—	—	tr	—	tr	—	tr	tr	4	6	—	—	—	+	+	*tr*
Firebrand (Sutt)	821/1	—	—	t	—	tr	—	tr	tr	4	6	—	—	—	+	+	*tr*
Firefly (Sutt)	821/1	—	—	tr	—	tr	—	tr	tr	4	6	—	—	+	+	—	*tr*
Leuchtfeuer (Sü)	821/1	—	—	tr	—	tr	—	tr	tr	3	5	+	—	—	—	—	*tr*
Vermillon (Cl)	821/1	—	—	tr	—	tr	—	tr	tr	3	5	+	—	—	+	+	*tr*
Leuchtfeuer (OE)	821/2	—	—	tr	—	tr	—	tr	tr	3	4	+	—	—	+	+	*tr*
Leuchtfeuer (Sr)	821/2	—	—	tr	—	tr	—	tr	tr	3	4	—	—	—	+	+	*tr*
Leuchtfeuer (St)	821/2	—	—	tr	—	tr	—	tr	tr	3	4	+	—	—	—	—	*tr*
Vierbach Lachs (R)	821/2	—	—	tr	—	tr	—	tr	tr	3	4	+	—	—	+	+	*tr*
Lachsscharlach (Bi)	821/3	—	—	tr	—	tr	—	tr	tr	2	3	—	—	+	+	+	*tr*
Scarlet (D)	821/3	—	—	tr	—	tr	—	tr	tr	3	4	—	—	—	+	+	*tr*
Baardse's Wonder (B)	722	—	—	tr	—	tr	—	tr	tr	2	4	+	—	—	+	+	*tr*
Brockenfeuer (Fu)	722	—	—	tr	—	tr	—	tr	tr	2	3	+	—	+	+	+	*tr*
Carmin Salmoneum (E)	722	—	—	1	—	tr	—	tr	tr	3	4	—	—	—	+	+	*tr*
Cherry (Nu)	722	—	—	tr	—	tr	—	tr	tr	2	3	—	—	+	+	+	*tr*
Gerhard Bubeck (Bu)	722	—	—	tr	—	tr	—	tr	tr	2	3	—	—	+	+	—	*tr*
Lachsscharlach (Hü)	722	—	—	+	—	+	—	+	+	+		—	—	—	+	+	
Lachsscharlach (Sr)	722	—	—	1	—	tr	—	—	tr	2	3	—	—	—	+	+	*tr*
Lachsscharlach (Bi)	722	—	—	tr	—	tr	—	—	tr	1	2	—	—	+	+	—	*tr*
Lachsscharlachorange (H)	722	—	—	tr	—	tr	—	tr	tr	2	3	—	—	+	+	+	*tr*
Leuchtfeuer (S)	722	—	—	tr	—	tr	—	tr	tr	3	3	—	—	—	+	+	*tr*
Leuchtfeuer (May)	722	—	—	tr	—	tr	—	tr	tr	1	3	—	—	—	—	+	*tr*
Leuchtfeuer (NS)	722	—	—	tr	—	tr	—	tr	tr	2	3	+	—	—	+	+	*tr*
Leuchtfeuer (Str)	722	—	—	tr	—	tr	—	tr	tr	2	3	+	—	+	—	—	*tr*
Leuchtfeuer Silberblatt (S)	722	—	—	tr	—	tr	—	tr	tr	3	3	—	—	—	+	+	*tr*
Orange (Bi)	722	—	—	tr	—	tr	—	tr	tr	1	2	+	—	—	+	+	*tr*
Salmon Scarlet (BL)	722	—	—	tr	—	tr	—	tr	tr	2	3	+	—	—	+	+	*1*
Vermillon (He)	722	—	—	+	—	+	—	+	+	+		+	—	—	+	+	
Vuurbaak (E)	722/1	—	—	1	—	tr	—	tr	tr	1	3	+	—	+	—	—	*tr*
Dunkellachs (Sü)	722/1	—	—	tr	—	tr	—	tr	tr	1	2	+	—	—	—	+	*tr*
Flamingo (Bi)	722/1	—	—	+	—	+	—	+	+	+		—	—	+	+	+	
Lachsdunkel (Bi)	722/1	—	—	tr	—	tr	—	tr	tr	2	3	+	—	—	+	+	*tr*
Lachsdunkel (May)	722/1	—	—	tr	—	tr	—	tr	tr	2	3	—	—	+	+	+	*tr*
Lachsdunkel (S)	722/1	—	—	tr	—	tr	—	tr	tr	2	3	—	—	—	+	+	*tr*
Leuchtfeuer (Bi)	722/1	—	—	tr	—	tr	—	tr	tr	1	2	—	—	+	+	+	*tr*
Neulachsrosa (St)	722/1	—	—	tr	—	tr	—	tr	tr	2	3	+	—	—	+	+	*2*

Names of cultivars	Slip colours (H.C.C.)	Flavonoid compounds found in the flower slip															
		Anthocyanins										Flavonol glycosides					
		delphinidin-3-glucoside	malvidin-3,5-diglucoside	malvidin-3-glucoside	cyanidin-3,5-diglucoside	cyanidin-3-glucoside	peonidin-3,5-diglucoside	peonidin-3-glucoside	A 8	A 9	Total amounts estimated	quercetin-3-glucoside	kaempferol-3-glucoside	rutin, Fl 3, Fl 4, Fl 7	Fl 2	Fl 8	Total amounts estimated
Perle von Zehlendorf (D) . . .	722/1	—	—	tr	—	tr	—	tr	tr	2	3	—	—	—	+	+	*tr*
Perle von Zehlendorf (R) . . .	722/1	—	—	tr	—	tr	—	tr	tr	2	2	—	—	—	+	+	*tr*
Perle v. Zehlendorf fimbriata (E)	722/1	—	—	tr	—	tr	—	tr	tr	1	2	—	—	—	+	+	*tr*
Ruhm von Wandsbek (St) . .	722/1	—	—	tr	—	tr	—	tr	tr	1	2	—	—	—	+	+	*1*
Salmon Scarlet (Sutt)	722/1	—	—	tr	—	tr	—	tr	tr	1	2	—	—	—	+	+	*tr*
Torchlight (D)	722/1	—	—	tr	—	tr	—	tr	tr	2	3	—	—	—	+	+	*tr*
Vuurbaak (Man)	722/1	—	—	tr	—	tr	—	tr	tr	1	2	—	—	—	—	+	*tr*
Vivid purplish red																	
Leuchtfeuer mehrblütenbl. (Sü)	21	—	—	+	—	+	—	+	+	+		—	—	—	—	+	
Salmon Picotee (Sutt)	721/2	—	—	tr	—	tr	—	tr	tr	1	2	+	—	—	+	—	*1*
Saumon (He)	721/2	—	—	+	—	+	—	+	+	+		+	—	+	+	+	
Lachshell (Bi)	22/1	—	—	tr	—	tr	—	tr	tr	1	2	—	—	+	+	+	*tr*
Baardse's Wonder gefranjerd (B)	22	—	—	tr	—	tr	—	tr	tr	1	2	—	—	+	+	+	*tr*
Fleischfarben (Nu)	22	—	—	tr	—	tr	—	tr	tr	1	2	+	—	+	+	—	*tr*
Helvetikum lachs (Moll) . . .	22	—	—	—	—	tr	—	tr	tr	1	2	+	—	+	+	—	*tr*
Lachsdunkel (NS)	22	—	—	tr	—	tr	—	tr	tr	1	2	—	—	—	—	+	*tr*
Lachsdunkel (Str)	22	—	—	tr	—	tr	—	tr	tr	1	2	+	—	—	+	+	*tr*
Leuchtfeuer (Hü)	22	—	—	tr	—	tr	—	tr	tr	2	2	—	—	—	+	+	*tr*
Morgenröte (Str)	22	—	—	tr	—	tr	—	tr	tr	1	2	+	—	+	+	—	*tr*
Neuhellachs (Sü)	22	—	—	+	—	+	—	+	+	+		+	—	+	+	—	
Perle von Zehlendorf (OE) . .	22	—	—	tr	—	tr	—	tr	tr	1	2	—	—	—	+	+	*tr*
Reinlachs (S)	22	—	—	tr	—	tr	—	tr	tr	1	2	+	—	—	+	+	*tr*
Rosa von Zehlendorf (R) . . .	24/2	—	—	—	—	tr	—	tr	tr	1	1	+	—	—	+	+	*3*
Goldlachs (NS)	722/2	—	—	+	—	+	—	+	+	+		+	—	+	—	+	
Lachs mit weissem Rand (Bi) .	722/2	—	—	1	—	tr	—	tr	tr	tr	2	+	—	+	+	—	*1*
Lachsdunkel (Hü)	722/2	—	—	tr	—	tr	—	tr	tr	tr	1	+	—	—	—	—	*tr*
Lachsdunkel (Sr)	722/2	—	—	tr	—	tr	—	tr	—	1	2	—	—	—	—	+	*tr*
Lachshell (NS)	722/2	—	—	+	—	+	—	+	+	+		+	—	—	+	+	
Lachshell (Str)	722/2	—	—	+	—	+	—	+	+	+		+	—	+	—	+	
Neulachs (S)	722/2	—	—	tr	—	tr	—	tr	tr	tr	2	+	—	—	+	+	*tr*
Rose vanAalsmeer (E) . . .	722/2	—	—	tr	—	tr	—	tr	—	1	2	+	—	—	+	+	*1*
Rose foncé (He)	722/2	—	—	+	—	+	—	+	+	+		+	+	—	—	+	
Viktoria (Bi)	722/2	—	—	+	—	+	—	+	+	+		+	+	—	+	—	
Lachshell (Sr)	722/3	—	—	+	—	—	—	+	+	+		+	—	+	+	+	
Medium Salmon (OE)	722/3	—	—	tr	—	tr	—	tr	tr	1	2	+	—	—	+	+	*tr*
New Perle (dT)	722/3	—	—	tr	—	tr	—	tr	—	tr	1	+	—	+	—	+	*1*
Rose van Aalsmeer (D) . . .	722/3	—	—	tr	—	tr	—	tr	tr	1	2	—	—	+	—	+	*tr*
Salmon Pink (Sutt)	722/3	—	—	tr	—	tr	—	tr	tr	1	2	+	—	—	+	+	*tr*
Rose van Aalsmeer fimbriata (E)	23	—	—	tr	—	tr	—	tr	tr	1	1	+	—	—	+	+	*tr*
Strong purplish red																	
Lachs Silberblatt (S)	22/1	—	—	tr	—	tr	—	tr	tr	1	2	—	—	—	+	+	*tr*
Lachshell (Bi)	22/1	—	—	tr	—	tr	—	—	tr	1	2	+	—	—	+	+	*tr*
Pfirsichblüte (T)	22/1	—	—	tr	—	—	—	—	—	1	1	+	—	—	+	+	*tr*
Salmon (D)	22/1	—	—	tr	—	tr	—	tr	tr	1	2	—	—	—	+	+	*tr*
Shell Pink (BL)	22/1	—	—	+	—	—	—	+	+	+		+	—	—	+	+	
Silberlachs (NS)	22/1	—	—	+	—	—	—	+	+	+		+	—	+	—	+	

Names of cultivars	Slip colours (H.C.C.)	Flavonoid compounds found in the flower slip															
		Anthocyanins										Flavonol glycosides					
		delphinidin-3-glucoside	malvidin-3,5-diglucoside	malvidin-3-glucoside	cyanidin-3,5-diglucoside	cyanidin-3-glucoside	peonidin-3,5-diglucoside	peonidin-3-glucoside	A 8	A 9	Total amounts estimated	quercetin-3-glucoside	kaempferol-3-glucoside	rutin, Fl 3, Fl 4, Fl 7	Fl 2	Fl 8	Total amounts estimated
Barbarossa	23/1	—	—	—	—	tr	—	tr	tr	1	2	—	—	+	—	+	*tr*
Helvetikum rose (Moll)	23/1	—	—	—	—	tr	—	tr	tr	1	2	+	—	+	+	—	*tr*
Lachs gefranst (May)	23/1	—	—	tr	—	—	—	—	—	1	1	+	—	+	+	+	*tr*
Oranje As (dT)	23/1	—	—	tr	—	tr	—	tr	tr	1	2	+	+	+	+	+	*1*
Rosa von Zehlendorf (OE)	23/1	—	—	tr	—	tr	—	tr	tr	1	2	+	—	+	+	+	*2*
Brilliant purplish red																	
Bath Pink (BL)	623	—	—	+	—	+	—	+	+	+		+	—	—	+	+	
Lachshell (May)	623	—	—	+	—	+	—	+	+	+		+	—	—	+	+	
Light purplish red																	
Pink Pearl (Sutt)	23/2	—	—	tr	—	—	—	tr	tr	tr	1	+	—	+	—	+	*1*
Rosa von Wandsbek (St)	23/2	—	—	tr	—	tr	—	tr	tr	tr	2	+	+	+	+	+	*3*
Pale purplish red																	
Rosa mit Auge (S)	623/2-625/3	—	—	+	—	+	—	+	+	+		+	+	+	+	+	
Dark purplish red																	
Dunkelblutrot (Bi)	826/1	tr	—	6	—	—	—	—	—	—	6	—	—	+	+	+	*tr*
Dunkelrot (May)	826/1	—	—	6	—	—	—	—	—	—	6	—	—	+	—	—	*1*
Donkerrood Rococo (E)	826/2	tr	—	6	—	—	—	—	—	—	6	+	—	—	+	—	*tr*
Rot mit Lachsschein (St)	826/1	—	—	5	—	—	—	—	—	tr	5	—	—	—	—	—	—
Crimson (Sutt)	824/1	tr	—	6	—	—	—	—	—	—	6	—	—	—	—	+	*tr*
Dunkelrot (NS)	824/1	tr	—	6	—	—	—	—	—	tr	6	—	—	—	—	—	—
Giant Crimson (BL)	824/1	tr	—	6	—	—	—	—	—	—	6	—	—	—	—	—	—
Leuchtend blutrot (May)	824/1	tr	—	6	—	—	—	—	tr	tr	6	—	—	—	+	—	*tr*
Leuchtend dunkelrot (Hü)	824/1	tr	—	6	—	—	—	—	—	—	6	—	—	—	—	—	—
Leuchtend dunkelrot (S)	824/1	—	—	6	—	—	—	—	—	tr	6	—	—	—	—	—	—
Leuchtend rot (Sr)	824/1	tr	—	6	—	tr	—	—	tr	tr	6	—	—	—	—	—	—
Rood fimbriata (E)	824/1	tr	—	6	—	—	—	—	—	—	6	—	—	—	—	—	—
Safraninrot (Bi)	824/1	—	—	5	—	—	—	—	—	tr	5	—	—	—	—	—	—
Safraninrot (Hü)	824/1	tr	—	6	—	—	—	—	—	—	6	—	—	—	—	—	—
Deep reddish purple																	
Brilliantlachs (Bi)	724	—	1	—	tr	—	3	—	—	—	4	+	+	+	+	+	*tr*
Andenken an Gottl. Bubeck (Bu)	724/1	—	2	—	tr	—	3	—	—	—	5	—	—	+	+	+	*tr*
Rose cérise (Cl)	724/1	—	—	+	—	+	—	+	+	+		+	—	+	+	+	
Dunkelrot mit Silbersaum (NS)	824/2	tr	—	4	—	—	—	—	—	—	4	—	—	—	—	—	—
Kirschrot (OE)	824/2	tr	—	4	—	—	—	—	—	—	4	—	—	—	+	+	*tr*
Rood (E)	824/2	—	—	4	—	—	—	—	—	tr	4	—	—	—	+	—	*tr*
Safraninrot mit Silbersaum (Bi)	824/2	—	—	4	—	—	—	—	—	—	4	—	—	+	—	—	*tr*
Welriekend rood (Man)	824/2	tr	—	4	—	—	—	—	—	tr	4	—	—	—	—	—	—
Leuchtend hellrot (St)	826/2	—	—	3	—	—	—	—	—	—	3	—	—	—	—	—	—
Leuchtend rot (May)	826/3	—	—	3	—	—	—	—	—	—	3	+	—	+	—	—	*2*
Vivid reddish purple																	
Neulachsrosa (Str)	25	—	+	—	+	+	+	+	+	+		+	—	+	—	+	
Kirschlachs (Bi)	26	—	2	—	tr	tr	2	tr	tr	tr	4	+	—	+	+	—	*tr*
Strong reddish purple																	
Ridson rose (dR)	25/1	—	1	—	tr	—	3	—	—	—	4	+	+	+	+	+	*tr*
Rose Cyclamen (He)	25/1	—	+	—	+	+	+	+	+	+		+	—	+	+	+	

Names of cultivars	Slip colours (H.C.C.)	Flavonoid compounds found in the flower slip															
		Anthocyanins										Flavonol glycosides					
		delphinidin-3-glucoside	malvidin-3,5-diglucoside	malvidin-3-glucoside	cyanidin-3,5-diglucoside	cyanidin-3-glucoside	peonidin-3,5-diglucoside	peonidin-3-glucoside	A 8	A 9	Total amounts estimated	quercetin-3-glucoside	kaempferol-3-glucoside	rutin, Fl 3, Fl 4, Fl 7	Fl 2	Fl 8	Total amounts estimated
Superba (dT)	25/1	—	2	—	tr	—	2	—	—	—	4	—	—	+	+	+	*1*
Dirk Baardse (B)	025	—	—	2	—	tr	—	tr	tr	tr	3	—	—	+	+	+	*tr*
Triomf van Brussel (dT)	26/1	—	—	—	tr	—	4	—	—	—	4	+	+	+	+	+	*tr*
Light reddish purple																	
Barbarossa	24/2	—	—	1	—	—	—	tr	tr	tr	1	—	—	+	—	+	*tr*
Rosa von Zehlendorf (D)	24/2	—	—	tr	—	tr	—	tr	tr	1	1	+	+	+	—	+	*3*
Rosa von Wandsbek (May)	24/2	—	—	+	—	+	—	+	+	+		+	+	+	—	+	
Welriekend licht (Man)	24/2	—	—	1	—	tr	—	tr	tr	1	2	—	—	—	—	+	*tr*
Oculatum fimbriatum (E)	625/1-white	—	—	1	—	—	—	—	tr	tr	1	+	+	+	—	+	*3*
Rosa von Zehlendorf (E)	625/1	—	—	tr	—	—	—	tr	—	1	1	+	+	+	+	+	*2*
Rosa v. Zehlendorf fimbriata (E)	625/1	—	—	tr	—	—	—	—	—	tr	1	+	—	+	+	+	*3*
Neurosa (Bi)	25/2	—	—	2	—	—	—	tr	—	tr	2	+	—	+	+	—	*tr*
Pink Pearl (dT)	25/2	—	1	—	tr	—	2	—	—	—	3	—	+	+	+	—	*1*
Shell Pink (Sutt)	26/2	—	—	tr	—	—	—	tr	tr	1	1	+	+	+	+	+	*2*
Pale reddish purple																	
Salmoneum oculatum (E)	24/3	—	—	tr	—	—	—	—	—	tr	1	+	+	+	+	+	*3*
Aase Rosa (R)	625/2	—	—	—	—	—	—	tr	—	1	1	+	+	+	+	+	*3*
Lachshell (Hü)	625/2	—	—	tr	—	—	—	tr	tr	1	1	+	+	+	+	+	*3*
Morgenröte (Bi)	625/2	—	—	tr	—	—	—	—	—	1	1	+	+	+	+	+	*3*
Rosa mit Auge (S)	625/2	—	—	+	—	+	—	+	+	+		+	+	+	+	+	
Schöne Dresdnerin (Me)	625/2	—	—	+	—	—	—	—	+	—		+	+	+	+	+	
Seidenrosa (K)	625/2	—	—	tr	—	—	—	—	—	1	1	+	+	+	+	+	*3*
Zartrosa (Sr)	625/2	—	—	tr	—	—	—	—	tr	tr	1	+	+	+	+	+	*3*
Very pale reddish purple																	
Morgenröte (NS)	625/3	—	—	tr	—	—	—	—	—	tr	1	+	+	+	+	+	*3*
Deep purple																	
Reinrosa (NS)	827/3	—	—	3	—	—	—	—	—	—	3	+	+	+	+	+	*2*
Burgundy (BL)	828/2	—	6	—	—	—	—	—	—	—	6	—	—	—	—	—	*0*
Cattleya (S)	729	—	—	3	—	—	—	—	—	—	3	+	+	+	+	+	*2*
Leuchtendrot (NS)	729	—	—	3	—	—	—	—	—	tr	3	+	—	+	—	+	*1*
Maarse's Purper (Man)	729	—	6	—	—	—	—	—	—	—	6	+	—	+	—	+	*1*
Rose Queen (Sutt)	729	tr	—	3	—	—	—	—	—	—	3	+	—	+	—	+	*2*
Vivid purple																	
Dunkelrot (OE)	727/1-28	—	—	3	—	—	—	—	—	—	3	+	—	+	—	+	*2*
Leuchtendrot (Bi)	28	—	—	3	—	—	—	—	—	—	3	+	—	+	—	+	*1*
Leuchtendrot m. Silbersaum (NS)	28	tr	—	5	—	—	—	—	—	—	5	+	+	+	—	+	*2*
Lichtrood fimbriata (E)	29	—	—	2	—	—	—	—	—	—	2	+	+	+	+	+	*2*
Reinrosa fimbriata (E)	29	—	—	2	—	—	—	—	—	—	2	+	—	+	—	+	*2*
Lichtrood (E)	729/2	—	—	3	—	—	—	—	—	—	3	+	+	+	+	+	*2*
Reinrosa (Bi)	729/3	—	—	2	—	—	—	—	—	—	2	+	+	+	+	+	*2*
Strong purple																	
Cattleyenrosa (May)	29/1	—	—	3	—	—	—	—	—	—	3	+	+	+	—	+	*2*
Hydrangea Pink (Sutt)	29/1	—	—	2	—	—	—	—	—	—	2	+	—	+	—	+	*2*
Lichtrood fimbriata (E)	29/1	—	—	2	—	—	—	—	—	tr	2	+	+	+	—	—	*2*
Rosa von Marienthal (St)	29/1	—	—	2	—	—	—	—	—	—	2	+	+	+	—	—	*3*

Names of cultivars	Slip colours (H.C.C.)	Flavonoid compounds found in the flower slip															
		Anthocyanins											Flavonol glycosides				
		delphinidin-3-glucoside	malvidin-3,5-diglucoside	malvidin-3-glucoside	cyanidin-3,5-diglucoside	cyanidin-3-glucoside	peonidin-3,5-diglucoside	peonidin-3-glucoside	A 8	A 9	Total amounts estimated	quercetin-3-glucoside	kaempferol-3-glucoside	rutin, Fl 3, Fl 4, Fl 7	Fl 2	Fl 8	Total amounts estimated
Very pale purple																	
Blackmore's Frilled Picotee Edged (BL)	427/2	—	—	—	—	—	—	+	—	+		+	—	+	—	+	
Sutton's Unique (Sutt)	427/2	—	—	+	—	—	—	—	—	+		+	+	+	—	+	
Morgenröte (NS)	627/3	—	—	tr	—	—	—	—	—	tr	1	+	+	+	+	+	*3*
Deep violet purple																	
Violet (He)	730	—	+	—	—	—	—	—	—	—		+	—	+	+	+	
Erika (Bi)	730/1	—	+	—	—	—	—	—	—	—		—	—	—	—	—	
Maarse's Purper (Man)	730/1	—	5	—	—	—	—	—	—	—	5	+	—	+	+	+	*tr*
Vivid violet purple																	
Flieder (F)	31	—	5	—	—	—	—	—	—	—	5	+	+	+	—	+	*tr*
Strong violet purple																	
Jubileum (B)	30/1	—	—	2	—	—	—	—	—	tr	2	—	—	+	—	+	*2*
Cattleya fimbriata (E)	31/1	—	4	—	—	—	—	—	—	—	4	+	—	+	—	+	*2*
Cattleya mehrblütenblättig (Sü)	31/1	—	4	—	—	—	—	—	—	—	4	+	—	+	—	+	*2*
Fliederblau (OE)	31/1	—	3	—	—	—	—	—	—	—	3	+	+	+	—	+	*2*
Lavendel (E)	31/1	—	3	—	—	—	—	—	—	—	3	+	+	+	—	+	*3*
Mauve Queen (Sutt)	31/1	—	3	—	—	—	—	—	—	—	3	+	—	+	—	+	*2*
Medium violet purple																	
Lilac (BL)	030/1	—	—	2	—	—	—	—	—	—	2	+	—	+	—	+	*2*
Light violet purple																	
Rosa von Marienthal (E)	630/1	—	—	1	—	—	—	—	—	—	1	+	+	+	—	+	*2*
Rosa von Marienthal fimbriata (Man)	31/2	—	—	1	—	—	—	—	—	—	1	+	+	+	+	+	*2*
Strong purplish violet																	
Lavendel (Bi)	32/1	—	3	—	—	—	—	—	—	—	3	+	+	+	+	+	*tr*
Brilliant purplish violet																	
Sylphide Selecta (dT)	632	—	2	—	—	—	—	—	—	—	2	+	+	+	+	+	*2*
Light purplish violet																	
Mauve Queen (BL)	632/1	—	—	tr	—	—	—	tr	1	1	2	+	+	+	—	—	*2*
Willie (LTP)	32/2	—	+	—	—	—	—	—	—	—		+	+	+	—	+	
Cattleya (D)	34/2	—	3	—	—	—	—	—	—	—	3	+	+	+	—	—	*2*
White with purple base																	
Apfelblüte (Bi)	—	—	—	—	—	—	—	—	—	tr	tr	+	+	+	+	+	*3*
Grandiflora (BL)	—	—	—	—	—	—	—	—	—	tr	tr	+	+	+	—	+	*3*
Sonja (LTP)	—	—	—	—	—	—	tr	—	—	—		+	+	+	—	+	
Sweet scented (BL)	—	—	—	—	—	—	—	—	—	tr	tr	+	+	+	+	+	*3*
Weisz mit karmin Auge (Bi)	—	—	—	—	—	—	—	—	—	tr	tr	+	+	+	+	+	*2*
Weisz mit Auge (Hü)	—	—	—	—	—	—	—	—	—	tr	tr	+	+	+	—	+	*3*
Weisz mit Auge (May)	—	—	—	—	—	—	—	—	—	tr	tr	+	+	+	—	+	*3*
Weisz mit Auge (NS)	—	—	—	—	—	—	—	—	—	tr	tr	+	+	+	+	+	*3*
Weisz mit Auge (OE)	—	—	—	—	—	—	—	—	—	tr	tr	+	+	+	—	+	*2*
Weisz mit Auge (S)	—	—	—	—	—	—	—	—	—	tr	tr	+	+	+	+	+	*3*
Weisz mit karmin Auge (St)	—	—	—	—	—	—	—	—	—	tr	tr	+	+	+	—	+	*3*
White with crimson base (Sutt)	—	—	—	—	—	—	tr	—	—	—	tr	+	+	+	—	+	*3*

Names of cultivare	Slip colours (H.C.C.)	Flavonoid compounds found in the flower slip															
		Anthocyanins										Flavonol glycosides					
		delphinidin-3-glucoside	malvidin-3,5-diglucoside	malvidin-3-glucoside	cyanidin-3,5-diglucoside	cyanidin-3-glucoside	peonidin-3,5-diglucoside	peonidin-3-glucoside	A 8	A 9	Total amounts estimated	quercetin-3-glucoside	kaempferol-3-glucoside	rutin, Fl 3, Fl 4, Fl 7	Fl 2	Fl 8	Total amounts estimated
Wit met oog (E)	—	—	—	—	—	—	—	—	—	tr	tr	+	+	+	—	+	*3*
Wit oculatum (dT)	—	—	—	—	—	—	tr	—	—	tr	tr	+	+	+	+	+	*3*
Victoria (Man)	—	—	—	—	—	—	—	—	—	tr	tr	+	—	—	+	+	*3*
Viktoria (NS)	—	—	—	—	—	—	—	—	—	tr	tr	+	+	+	+	+	*2*
White																	
Anneke (LTP)	—	—	—	—	—	—	—	—	—	—		+	+	+	—	+	
Blanc (He)	—	—	—	—	—	—	—	—	—	—		+	+	+	+	+	
Blanc (Cl)	—	—	—	—	—	—	—	—	—	—		+	+	+	+	—	
Giant White (BL)	—	—	—	—	—	—	—	—	—	—	—	+	+	+	+	+	*3*
Käthchen Stoldt (St)	—	—	—	—	—	—	—	—	—	—	—	+	+	+	+	+	*3*
Käthchen Stoldt (Sü)	—	—	—	—	—	—	—	—	—	—	—	+	+	+	—	+	*3*
Reinweisz (Bi)	—	—	—	—	—	—	—	—	—	—	—	+	+	+	+	+	*2*
Reinweisz (Hü)	—	—	—	—	—	—	—	—	—	—	—	+	+	+	—	+	*3*
Reinweisz (May)	—	—	—	—	—	—	—	—	—	—	—	+	+	+	—	+	*3*
Reinweisz (OE)	—	—	—	—	—	—	—	—	—	—	—	+	+	+	—	+	*2*
Reinweisz frühe Stamm (OE)	—	—	—	—	—	—	—	—	—	—	—	+	+	+	—	+	*1*
Reinweisz (S)	—	—	—	—	—	—	—	—	—	—	—	+	+	+	+	+	*2*
Reinweisz (Sr)	—	—	—	—	—	—	—	—	—	—	—	+	+	+	—	+	*2*
Reinweisz lang (Sü)	—	—	—	—	—	—	—	—	—	—	—	+	+	+	—	+	*3*
White (D)	—	—	—	—	—	—	—	—	—	—	—	+	+	+	+	+	*3*
White (Ma)	—	—	—	—	—	—	—	—	—	—	—	+	+	+	+	+	*2*
White (R)	—	—	—	—	—	—	—	—	—	—	—	+	+	+	+	+	*3*
White (Sutt)	—	—	—	—	—	—	—	—	—	—	—	+	+	+	—	+	*3*
Wit (E)	—	—	—	—	—	—	—	—	—	—	—	+	+	+	+	+	*3*
Wit fimbriata (E)	—	—	—	—	—	—	—	—	—	—	—	+	+	+	+	+	*2*
Wit rococo (Man)	—	—	—	—	—	—	—	—	—	—	—	+	—	+	—	+	*1*
Zilverwit (dT)	—	—	—	—	—	—	—	—	—	—	—	+	—	+	—	—	*1*

4. Crosses

4.1. *Parent plants, crosses and F_1's*

For the chromatographical investigations of the parent plants, in addition to BAW and BH the solvents HA, AA and CAW were used.

The analyses yielded the following results:

'Firefly': Malvidin-3-glucoside was found in the eye. Cyanidin-3-glucoside, peonidin-3-glucoside, A 5, A 6, A 7, A 8, A 9 and A 10 were found in the slip plus traces of flavonol glycosides. The latter were not further investigated.

'White': Traces of flavonol glycosides were found in the eye. Great amounts of flavonol glycosides, namely kaempferol-3-glucoside, quercetin-3-glucoside, rutin, Fl 2, Fl 3, Fl 4, Fl 7 and Fl 8 were found in the slip.

'Sylphide': Malvidin-3,5-diglucoside was found in the eye and in the slip the same compound, together with a great amount of flavonol glycosides were present. The mixture of flavonol glucosides was composed of the same components as found in 'White'.

'White with Eye': Malvidin-3,5-diglucoside was found in the eye. In the slip traces of cyanidin-3,5-diglucoside and peonidin-3,5-diglucoside, together with a great amount of flavonol glycosides, namely kaempferol-3-glucoside, quercetin-3-glucoside, rutin, Fl 2, Fl 3 and Fl 8 were found.

The F_1 lines, which resulted from the crosses 'Firefly' × either 'White', 'Sylphide' or 'White with Eye', were all coloured like the cultivar 'Salmoneum oculatum'. Reciprocal crosses were identical (54). The analyses of the few F_1 plants available yielded similar results. In the eye malvidin-3,5-diglucoside, and in the slip a small amount of peonidin- and cyanidin-3,5-diglucoside, together with a great amount of flavonol glycosides, including Fl 8 were found.

Several F_1 plants were selfed.

4.2. *Literature*

Before proceeding to a description of the analysis of the F_2 lines, it is necessary to discuss the literature about chemogenetical investigations on flower colour pigmentation in cyclamen.

WELLENSIEK (53, 55) was the first to describe the two flower colour genes in cyclamen, *W* and *S*. *W* is the basic gene for flower colour, *ww* giving white flowers. Furthermore, *W.S.* represents "white with eye", whereas *W.ss* stands for purplish violet.

SEYFFERT (50) has extended these results considerably by demonstrating the existence of several additional genes and by identifying the pigments involved.

The action of the genes *W*, *F*, *S* and *M* will be described first, because these are involved in the present investigations.

W is the basic gene for the production of anthocyanins. In presence of *W*, the eyes of the flowers are always intensively coloured by malvidin glycosides, while the slip contains either malvidin glycosides or other anthocyanins. The occurrence of peonidin in the eyes of two cultivars, as described by VAN BRAGT (11), is an exception.

F is the basic gene for the production of flavonol glycosides, in presence of *ff* no flavonol glycosides are produced, and in presence of *ffff* according to KESSLER (32) only traces of them are formed. SEYFFERT (50) indicated that *F* causes the production of three flavonol glycosides, one of which was thought to be *nor*-gardenin or hibiscetin.

S, together with *W*, causes the production of peonidin glycosides. *S*, together with *F*, causes the production of *iso*-rhamnetin, while *nor*-gardenin or hibiscetin is not formed and the production of the two other flavonol glycosides (see above sub *F*) is highly suppressed. *S*, in presence of *W* and *F*, gives "white with eye" or "nearly white with eye". In the presence of *W.F.ss*, *W.ff S.* and *W.ffss* intensively coloured flowers are formed.

M causes the production of anthocyanidin-3,5-diglycosides and *mm* causes the production of anthocyanidin-3-pentoseglycosides. However, VAN BRAGT (11) found both peonidin-3,5-diglucoside and peonidin-3-glucoside in the same tissue of some cultivars.

In addition to the genes mentioned above, SEYFFERT (50) established:

E: "Elfenbeinfaktor",

C: "Aufhellungsfaktor", while causing the production of cyanidin glycosides.

I: Causes the production of delphinidin glycosides in presence of *W* and *ss*. In tetraploid material KESSLER (32) found an intensifying gene, symbolized as *H*.

On account of

(a) the co-occurrence of peonidin and *iso*-rhamnetin,

(b) the co-occurrence of delphinidin and *nor*-gardenin or hibiscetin,

(c) the occurrence of leuco-delphinidin, leuco-malvidin and leuco-cyanidin, SEYFFERT (50) concluded that the precursors of both the anthocyanins and flavonol glycosides were leuco-anthocyanins. He suggested that *W* causes the conversion of leuco-compounds into anthocyanins, whereas *F* causes the conversion of leuco-compounds into flavonol glycosides. The potency of *F* is higher than the potency of *W*.

4.3. *The F_2 lines*

In order to facilitate the description of flower characteristics in the F_2 lines, two descriptive characters are used. "Nearly white with eye" means that the flower slip is weakly coloured by anthocyanins and "full coloured" means that the flower slip it intensively coloured by anthocyanins. In a segregating progeny these two groups are always easily distinguished.

4.3.1. 'Firefly' × 'White'

In table 7 the results of the analysis of the F_2 from 'Firefly' × 'White' are given.

From this table the following conclusions can be derived:

(1) *Gene W*

The ratio of coloured flowers (with malvidin glycosides) to white flowers is 389:112, expected on a 3:1 basis (376):(125), $chi^2 = 1.80$ and $10\% < P_{(1)} < 20\%$. Hence a single gene is involved. These results confirm the conclusions of WELLENSIEK (53, 55) and SEYFFERT (50) concerning gene *W*.

(2) *Gene F*

The ratio of flowers containing a great amount of flavonol glycosides to flowers containing traces of these compounds is 394:107, expected on a 3:1 basis (376):(125), $chi^2 = 3.45$ and $5\% < P_{(1)} < 10\%$. Hence a single gene is involved.

SEYFFERT (50) found no flavonol glycosides at all in the presence of *ff*, whereas in the present work traces were found. With this modification of SEYFFERT's concept, it is concluded that *F* is also involved in the material included in this investigation. This conclusion is supported by the fact that KESSLER (32) found traces of flavonol glycosides in the presence of *ffff*.

In the present work no evidence was found that *F* causes the production of *nor*-gardenin or hibiscetin, as was suggested by SEYFFERT (50).

(3) *Gene S*

Coloured flowers were observed to segregate in the following manner.

The ratio of flowers containing peonidin glycosides in the slip to the flowers containing no peonidin glycosides in the slip is 286:103, expected on a 3:1 basis (292): (97), $chi^2 = 0.50$ and $30\% < P_{(1)} < 50\%$. A single gene is involved, which must be *S*.

It was not confirmed that *S*, in the presence of *F* causes the production of *iso*-rhamnetin, as found by SEYFFERT (50).

(4) *Genes W, F, S*

The types "white with eye" and "nearly white with eye" occur when peonidin glycosides are produced together with a great amount of flavonol glycosides. These results confirm the conclusion of SEYFFERT (50) concerning the gene com-

TABLE 7. Cross 'Firefly' × 'White'. Segregation of the F_2.

Flower colour characteristics	Flavonoid compounds		Numbers of plants (expected)	Scheme of segregation	Genotypes
	Eye	Slip			
White – or nearly white – with eye	malvidin-3,5-diglucoside	peonidin-3,5-diglucoside, flavonol glycosides in a great amount, Fl 8 present	136 (119)	3^3	*W.F.S.M.X.*
ibid.	malvidin-3,5-diglucoside	peonidin-3,5-diglucoside, flavonol glycosides in a great amount, Fl 8 absent	35 (40)	3^4	*W.F.S.M.xx*
ibid.	malvidin-3-glucoside	peonidin-3-glucoside, A 8, flavonol glycosides in a great amount, Fl 8 present	37 (40)	3^4	*W.F.S.mmX.*
ibid.	malvidin-3-glucoside	peonidin-3-glucoside, flavonol glycosides in a great amount, Fl 8 absent	17 (13)	3^3	*W.F.S.mmxx*
Full coloured	malvidin-3,5-diglucoside	malvidin-3,5-diglucoside, flavonol glycosides in a great amount, Fl 8 present	52 (40)	3^4	*W.F.ssM.X.*
ibid.	malvidin-3,5-diglucoside	malvidin-3,5-diglucoside, flavonol glycosides in a great amount, Fl 8 absent	10 (13)	3^3	*W.F.ssM.xx*
ibid.	malvidin-3-glucoside	malvidin-3-glucoside, flavonol glycosides in a great amount, Fl 8 present	18 (13)	3^3	*W.F.ssmmX.*
ibid.	malvidin-3-glucoside	malvidin-3-glucoside, flavonol glycosides in a great amount, Fl 8 absent	2 (4)	3^4	*W.F.ssmmxx*
ibid.	malvidin-3,5-diglucoside	peonidin-3,5-diglucoside, flavonol glycosides in traces	42 (53)	3^4 3^3	*W.ffS.M.X.* *W.ffS.M.xx*
ibid.	malvidin-3-glucoside	peonidin-3-glucoside, A 8, A 9, flavonol glycosides in traces	19 (17)	3^3 3^4	*W.ffS.mmX.* *W.ffS.mmxx*
ibid.	malvidin-3,5-diglucoside	malvidin-3,5-diglucoside, flavonol glycosides in traces	15 (17)	3^3 3^4	*W.ffssM.X.* *W.ffssM.xx*
ibid.	malvidin-3-glucoside	malvidin-3-glucoside, flavonol glycosides in traces	6 (6)	3^3 3^1	*W.ffssmmX.* *W.ffssmmxx*
White	flavonol glycosides in traces	flavonol glycosides in a great amount, Fl 8 present	69 (71)	$3^3 \times 4^3$	*wwF. ... X.*
White	flavonol glycosides in traces	flavonol glycosides in a great amount, Fl 8 absent	18 (24)	$3^1 \times 4^3$	*wwF. ... xx*
White	flavonol glycosides in traces	flavonol glycosides in traces	25 (31)	$4^3 \times 3^1$ 4^3	*wwff ... X.* *wwff. ... xx*
		Σ	501 (501)	$4^4 = 1024$	

$chi^2 = 15.19 \quad 30\% < P_{(14)} < 50\%$

bination *W.F.S.* In "white with eye" traces of peonidin glycosides are found in the base of the slip and in "nearly white with eye" these anthocyanins are found in low amounts in the whole slip.

The conclusions concerning the effect of the gene combination *W.F.S.* are in agreement with the data reported by WELLENSIEK (53, 55), who investigated the three crosses between the diploid cultivars 'White', 'Sylphide' and 'White with Eye'. In the present work it was found that the flower slips of these cultivars contain a great amount of flavonol glycosides. It is clear that in this material the genetical difference between "white with eye" and "full coloured" ('Sylphide') is only based on *S.*

(5) *Gene M*

On considering the data about the glycosidation pattern of anthocyanins, leaving out of consideration the compounds A 8 and A 9, the ratio of flowers containing anthocyanidin-3,5-diglucosides to the flowers containing anthocyanidin-3-glucosides is 290:99, which agrees well with a 3:1 ratio, expected (292):(97), $chi^2 = 0.05$, $80\% < P_{(1)} < 90\%$. These results point to gene *M.*

These investigations demonstrated that the anthocyanidin-3,5-diglycosides, as found by SEYFFERT (50), must have been anthocyanidin-3,5-diglucosides. In presence of *mm* SEYFFERT (50) found anthocyanidin-3-pentoseglycosides, while KESSLER (32) found anthocyanidin-3-glucosides in presence of *mmmm.*

The observations obtained in this investigation confirm the data given by KESSLER (32), but perhaps the compound A 9 is identical to SEYFFERT's peonidin-3-pentoseglycoside.

On the chromatograms prepared with extracts of flowers containing a great amount of flavonol glycosides, four or five spots were always observed, independent of the fact of whether anthocyanidin-3,5-diglucosides or anthocyanidin-3-glucosides were present. This observation confirms the conclusion of SEYFFERT (50), that *M* does not influence the glycosidation pattern of the flavonol glycosides.

It seems possible that some relation exists between *mm* and the production of A 8 and A 9, because A 8 or A 8 and A 9 only occur together with peonidin-3-glucoside. It was not possible to give a genetical interpretation of this phenomenon.

(6) *Gene X*

The plants with flowers containing a great amount of flavonol glycosides segregate as follows: 312 contain Fl 8, while 82 do not contain Fl 8, expected on a 3:1 basis (296):(98), $chi^2 = 3.45$, $5\% < P_{(1)} < 10\%$.

The dominant allele of this gene, which is provisionally assigned the symbol *X*, causes the production of Fl 8 when the gene *F* is also present. The action of *X* was not observed in the presence of *ff*, when only traces of flavonol glycosides are formed, which were not investigated.

(7) *Genotypes*

With the data thus obtained, the genotypes of the groups of plants in the F_2 from 'Firefly' × 'White' can be indicated as in the 6th column of table 7, on p. 25. The result of the chi^2-test for the whole F_2 demonstrates the independent assortment of the genes *W, F, S, M* and *X.*

In addition to the above results, it was observed that small amounts of cyanidin glycosides were always produced together with peonidin glycosides. Thus, it could not be confirmed that a gene, *C*, causes the production of cyanidin

glucosides in the presence of *W* as was found by SEYFFERT (50). Furthermore delphinidin-3-glucoside was found in the flower slips of three plants, which also contained malvidin-3-glucoside and traces of flavonol glucosides. SEYFFERT (50) also found this mixture of flavonoid compounds in the flower slips of several genotypes. However, it was not possible to confirm the presence of SEYFFERT's gene *I* which he reported to cause the production of delphinidin glycosides in presence of *W.ss*.

When the results of a paperchromatographical analysis with BAW and BH indicated the presence of delphinidin-3-glucoside in a flower slip, a third chromatogram was prepared using CAW as the solvent. This latter chromatogram always demonstrated the presence of traces of petunidin-3-glucoside.

4.3.2. 'Firefly' × 'Sylphide'

In some F_2 plants peonidin-3-glucoside, A 8 and A 9 were found in the presence of *M*. This fact does not appear to be in harmony with the action of *M*, as described earlier in this investigation, but an acceptable explanation for the discrepancy was found from the results of the analyses of the F_2 from 'Firefly' × × 'Sylphide'. In table 8 detailed information is given on the analysis of this F_2.

TABLE 8. Cross 'Firefly' × 'Sylphide'. Segregation of the F_2.

Flavonoid compounds	Numbers of plants (expected)	Scheme of segregation	Genotypes
peonidin-3,5-diglucoside, flavonol glycosides in a great amount, Fl 8 present	125 (132)	3^4 3^3	*WWF.S.MMX.Y.* *WWF.S.MMX.yy*
peonidin-3,5-diglucoside, flavonol glycosides in a great amount, Fl 8 absent	34 (44)	3^3 3^2	*WWF.S.MMxxY.* *WWF.S.MMxxyy*
malvidin-3,5-diglucoside, flavonol glycosides in a great amount, Fl 8 present	57 (44)	3^3 3^2	*WWF.ssMMX.Y.* *WWF.ssMMX.yy*
malvidin-3,5-diglucoside flavonol glycosides in a great amount, Fl 8 absent	15 (15)	3^2 3^1	*WWF.ssMMxxY.* *WWF.ssMMxxyy*
peonidin-3,5-diglucoside, flavonol glycosides in traces, peonidin-3-glucoside, A 8 and A 9	51 (44)	3^3 3^2	*WWffS.MMX.Y.* *WWffS.MMxxY.*
peonidin-3,5-diglucoside flavonol glycosides in traces	15 (15)	3^2 3^1	*WWffS.MMX.yy* *WWffS.MMxxyy*
malvidin-3,5-diglucoside, flavonol glycosides in traces	16 (19)	3^2 3^1 3^1 1	*WWffssMMX.Y.* *WWffssMMX.yy* *WWffssMMxxY.* *WWffssMMxxyy*
Σ	313 (313)	$4^4 = 256$	

$chi^2 = 9.84 \quad 10\% < P_{(6)} < 20\%$

With respect to *F*, *S* and *X* the results from 4.3.1. are confirmed. With regard to the new gene *X* the actual ratio is 182:49, expected (173):(58). The total

numbers for the crosses of tables 7 and 8 become: actual ratio 478:147, expected (469):(156).

A more detailed discussion now is given with regard to peonidin-3-glucoside, A 8 and A 9.

From table 8 it can be observed that 66 plants contain peonidin-3,5-diglucoside and traces of flavonol glycosides. Within this group of 66 plants the segregation was: 51 plants contained peonidin-3-glucoside, A 8 and A 9 whereas 15 plants did not contain these anthocyanins. The segregation is in full agreement with a 3:1 ratio, expected (50):(16), $chi^2 = 0.08$, $70\% < P_{(1)} < 80\%$. The gene in question is provisionally assigned the gene symbol *Y*.

From the available facts it seemed that *Y* causes the production of peonidin-3-glucoside, A 8 and A 9 in presence of *W.ffS.M.X.* or *W.ffS.M.xx.*

4.3.3. 'Firefly' × 'White with Eye'

In one F_2 line from the cross 'Firefly' × 'White with Eye' 5 plants were found with very pale pink flowers. The eyes of these flowers had the same colour as the slip. The colour intensity was less than indicated by H.C.C. 622/3.

In the petal peonidin glycosides and traces of cyanidin glycosides were found, together with great amounts of flavonol glycosides. One of these plants was selfed.

The F_3 segregated as follows: 55 plants were like the F_2 plant and 16 plants were found with intensively coloured eyes which contained malvidin glycosides. In the slips peonidin glycosides and great amounts of flavonol glycosides were found. The segregation 55:16 agrees with a 3:1 ratio, expected (53):(18), $chi^2 = 0.30$, $50\% < P_{(1)} < 70\%$.

The dominant allele of the gene in question is provisionally assigned the gene symbol Z. This gene evidently causes the occurrence of the pale pink coloured flowers, whereas in presence of *zz* the flowers with intensively coloured eyes are formed.

The genes *Z* and *Y* were only found in some progenies. Their effects apparently depend on other, not yet fully understood genetical constitutions.

5. Estimations of pigment contents in different genotypes

In order to obtain additional data concerning the relation between the production of the leuco-anthocyanins, anthocyanins and flavonol glycosides, two series of genotypes were investigated. The flower slips of the first series contained peonidin glycosides and those of the second series malvidin glycosides as chief anthocyanins.

Determinations were carried out by measuring the optical density of extracts at certain wavelengths, according to the method which was described previously by Feenstra (17).

Samples for analyses were obtained as follows: 15 discs, 16 mm in diameter, were cut from the flower slips, of each genotype, with a cork borer. The flavonoid compounds were extracted with 15 ml of a 1% solution of hydrochloric acid in methanol. The extract was diluted, with a sufficient quantity of the 1% solution of hydrochloric acid in methanol, so that the most favourable measuring range of the spectrophotometer was used.

A sample of 1 ml of the diluted extract was mixed with 1 ml of methanol, 1 ml of 25% aqueous hydrochloric acid added, and the optical density measured at 350 mμ for flavonol glycosides and at 500 mμ for anthocyanins.

In certain mixtures thus obtained, however, the amount of anthocyanins was extremely high compared with that of the flavonol glycosides, hence at 350 mμ the absorption of the anthocyanins interfered with that of the flavonol glycosides. Thus the measurements of the optical density of the flavonol glycosides were not reliable so are omitted from the data.

For the estimations of the amounts of leuco-anthocyanins, another 1 ml was removed from the diluted extract such as was used for the measurements of anthocyanins. To this mixture 1 ml of a methanolic solution of vanillin (3 g vanillin in 8 ml methanol) and 1 ml 25% aqueous hydrochloric acid were added. Under these conditions the leuco-anthocyanins are converted into reddish compounds having their absorption maximum at 500 mμ.

The optical density of the mixture thus obtained was measured at 500 mμ. The correction for anthocyanins was calculated from the previous determinations of the optical density of the anthocyanins. From the experiments of FEENSTRA (17) it appeared that the method gave a good estimation of the leuco-anthocyanin content.

The results of the investigations are given in table 9 on p. 30.

From this table it appears that, in general, the relative total amounts of leuco-anthocyanins is not correlated with the relative total amounts of anthocyanins or flavonol glycosides. The data also demonstrate that an increase of the amount of anthocyanins is accompanied by a decrease of the amount of flavonol glycosides.

In order to estimate the amounts of leuco-delphinidin and leuco-cyanidin glucosides in the flower slip, the flower slips were first treated according to the method of ALSTON and HAGEN (see p. 7). Thereafter the anthocyanidins were obtained from the conversion of the leuco-compounds. Then chromatograms were prepared with these anthocyanidins.

BH was used as the solvent. The relative amounts of the anthocyanidins were estimated from the length of the spots on the chromatograms. It was assumed that the original leuco-compounds in the tissue were present in the same relative amounts.

The results of the investigations demonstrated that, in the flower slips containing peonidin glycosides as the chief anthocyanins, the amounts of leuco-delphinidin and leuco-cyanidin glucosides were equal. The flower slips containing malvidin glycosides as the chief anthocyanins contained only leuco-delphinidin glucoside.

Additional investigations demonstrated that in presence of *wwS.* (white flowers) the ratio of leuco-delphinidin glucoside/leuco-cyanidin glucoside was 1, whereas in the presence of *wwss* only leuco-delphinidin glucoside was found.

CHAPTER VI

GENERAL DISCUSSION

The present chapter deals with a discussion of the biosynthesis of flavonoid compounds.

From this biosynthesis some insight could be obtained on the prospects of the production of new colours in cyclamen.

TABLE 9. Measurements of relative amounts of anthocyanins, leuco-anthocyanins and flavonol glycosides in flower slips of genotypes producing peonidin glycosides (*S.*) or malvidin glycosides (*ss*) respectively as the chief anthocyanins, and flavonol glycosides in great amounts (*F.*) or in traces (*ff*).

Compounds	Numbers of the genotypes	Anthocyanins and leuco-anthocyanins					Flavonol glycosides		
		Dilution of extracts	Optical density at 500 mμ (average of 2 measurements)		Relative total amounts of anthocyanins	Relative total amounts of leuco-anthocyanins	Dilution of extracts	Optical density at 350 mμ (average of 2 measurements)	Relative total amounts of flavonol glycosides
			without vanillin	with vanillin					
peonidin glycosides and a great amount of flavonol glycosides	1	1.00 ×	0.21	0.71	1.00	1.00	10.00	0.52	1.00
	2	1.13 ×	0.23	0.74	1.21	1.15	11.30	0.41	0.89
peonidin glycosides and traces of flavonol glycosides	3	1.00 ×	0.36	0.91	1.71	1.10	2.00	0.24	0.09
	4	1.50 ×	0.29	0.84	2.07	1.65	1.50	0.21	0.06
	5	1.50 ×	0.50	0.72	3.57	0.66	1.50	–	–
	6	2.25 ×	0.51	0.72	5.46	0.95	2.25	–	–
	7	9.00 ×	0.40	0.49	17.14	1.62	4.50	–	–
malvidin glycosides and a great amount of flavonol glycosides	8	1.00 ×	0.18	0.64	1.00	1.00	2.00	0.50	1.00
	9	1.00 ×	0.16	0.42	0.89	0.57	2.00	0.40	0.81
	10	2.00 ×	0.19	0.41	2.11	0.96	2.00	0.40	0.80
	11	2.00 ×	0.19	0.41	2.11	0.96	2.00	0.42	0.84
	12	2.00 ×	0.23	0.35	2.56	0.57	2.00	0.42	0.84
malvidin glycosides and traces of flavonol glycosides	13	2.00 ×	0.35	0.49	3.89	0.61	2.00	0.12	0.24
	14	4.00 ×	0.38	0.44	8.44	0.52	2.00	0.15	0.30

1. Synthesis of nonmethylated aglycones

In chapter V the important influences of the genes *W*, *F* and *S* have been shown. The effect of these genes on the production of flavonoids in the flower slip will now be discussed first, followed by a discussion relating to the eye.

In order to facilitate this discussion, the results are summarized in fig. 4. on p. 32.

It is clear that all flavonoid compounds have identical substitution patterns in the A-ring. Apart from any subsequent or side-reactions, a consideration of the hydroxylation pattern in the B-ring shows the following:

(a) In the presence of *W.S.* a part of the leuco-anthocyanidins, and the anthocyanidins, have two hydroxyl groups in the B-ring. For the former compounds this also occurs in presence of *wwS.*

(b) In the presence of *W.ss*, however, both the leuco-anthocyanidins and the anthocyanidins have three hydroxyl groups in the B-ring. For the leuco-anthocyanidins this was also found to be the case in the presence of *wwss.*

(c) In the presence of *S* or *ss*, flavonols have one or two hydroxyl groups in the B-ring only.

The correspondence, shown in the preceding paragraph, between the hydroxylation pattern in the B-ring of compounds only differing in the heterocyclic part of their molecules, points to an analogous course in their biosynthesis. The number of hydroxyl groups in the B-ring apparently is determined by the action of *S*. Thus, it might be possible that *S* influences the biosynthesis at a certain stage when the synthesis of the flavonoid molecule is not yet performed. In presence of *S* the dihydroxy precursor is formed, whereas in presence of *ss* the trihydroxy compound is produced. The formation of the monohydroxy compound occurs in either case.

From similar observations on *Antirrhinum majus* by Geissman *et al* (22) it was also suggested that hydroxylation of the B-ring occurred prior to the completion of the flavonoid molecules. From the investigations of other plants by several workers (8, 13, 17, 20, 22, 47, 51), it was shown that the number of hydroxyl groups in the B-ring of flavonoid compounds depends on the action of one gene. However, the dominant gene caused the introduction of a hydroxyl group. Hence the situation in *Cyclamen* is reversed, since a dominant gene causes the reduction of the number of hydroxyl groups in the B-ring. This situation was also found in *Verbena* (6), but in this species a series of multiple alleles is involved.

The phenomenon occurring in *Cyclamen* can be explained by assuming that *S* inhibits the action of a certain enzyme, probably present in all genotypes, which catalyzes the formation of the trihydroxy precursor.

As indicated earlier, all flavonoid compounds have identical substitution patterns in the A-ring. The substitution pattern in the heterocyclic part of the molecules is different for leuco-anthocyanidins, anthocyanidins and flavonols. In view of these facts the most simple assumption is that the substitution pattern in the heterocyclic part of the molecules of the anthocyanidins and the flavonols is determined by *W* and *F* respectively. (The action of a gene responsible for the formation of leuco-anthocyanidins was not demonstrated). This means that *S* acts prior to *W* and *F*. From the present investigations it was not possible to draw any conclusions as to whether *W* and *F* act prior or subsequent to the formation of the final structure of the flavonoid compounds from the B-precursor and the A-ring.

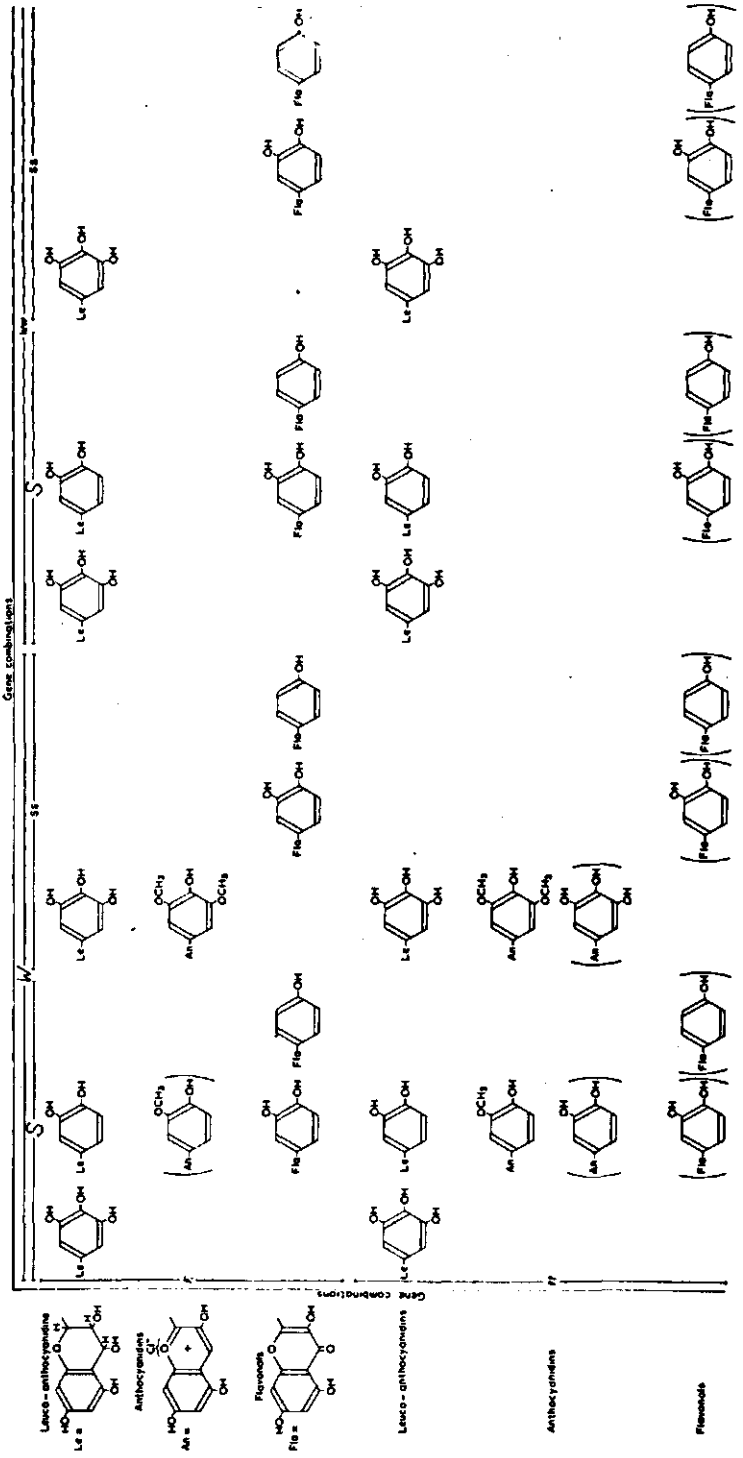

FIG. 4. Structure of flavonoid compounds formed by the action of the genes *W*, *F* and *S*.
All compounds occur as glycosides. The bonding(s) of the sugar molecules is (are) not indicated. ( ) means: found in traces.

Furthermore, it was observed that in presence of *ss*, the amount of anthocyanidins formed when *W* was also present, was greater when lower amounts of flavonols were produced (*F* present) and the greatest when only traces of flavonols were found (*ff*). Some workers (6, 59) reported similar observations on other plants. From this it is concluded that the enzymes which are produced by the action of *W* and *F* are competing for the same substrate. This conclusion is in agreement with an assumption made previously by LAWRENCE and SCOTT-MONCRIEFF (34), from similar observations on *Dahlia*. In the genotype *W.F.S.* where the dihydroxy compound is the chief precursor present, it was also observed that a greater amount of anthocyanidins was produced when a smaller amount of flavonols was formed. This fact leads to the same conclusion as before.

In the genotype *W.F.S.* the amount of anthocyanidins produced, however, is extremely reduced, thus giving rise to the phenotypes "white with eye" and "nearly white with eye". In these types mainly flavonols are formed. Here the dihydroxy compound is the sole B-precursor present and is suitable as a precursor for the syntheses catalyzed by the enzyme associated with *W* as well as by the enzyme associated with *F*.

In the formation of leuco-compounds *S.* and *ss* act similarly as in anthocyanidins.

The view of the writer on the biosynthesis of the flavonoid compounds in cyclamen is summarized in fig. 5, of which the left part refers to the synthesis of nonmethylated aglycones.

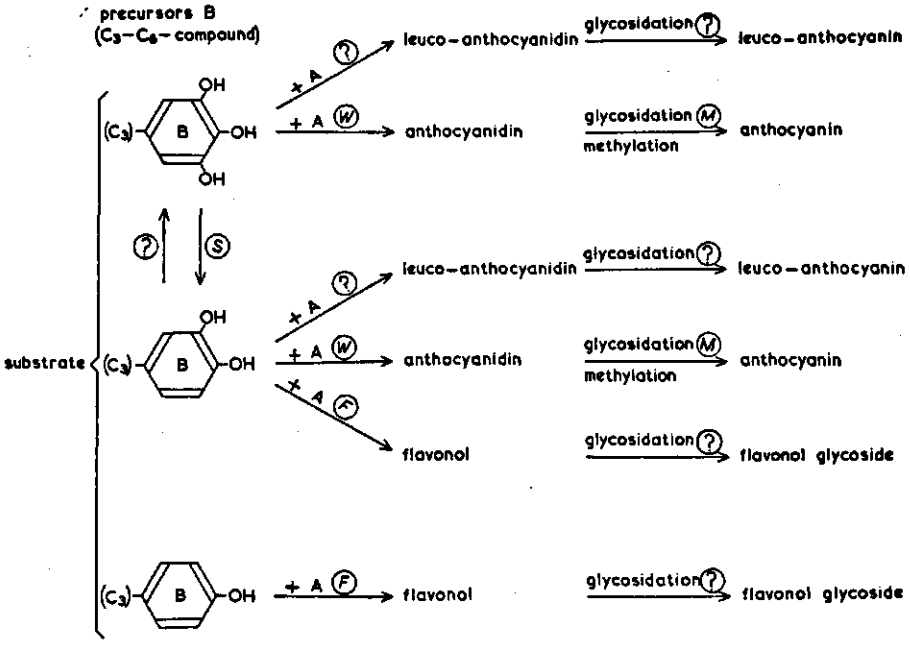

FIG. 5. Scheme of the biosynthesis of flavonoid compounds in *Cyclamen*.
A indicates A-ring. *W* indicates the action of the enzyme produced by the gene *W*, etc.
For further explanations see text.

According to this hypothesis the flavonoid compounds are produced by parallel synthesis from identical A-rings, and three B-precursors containing *one*, *two* or *three* hydroxyl groups in the benzene nuclei respectively. The B-precursors may originate from one substrate.

The proposed scheme requires the following assumptions:

(a) The enzyme produced by the action of *W* is only capable of catalyzing the syntheses of the heterocyclic structure characteristic of anthocyanidins, when the B-precursors with two or three hydroxyl groups are available. The latter B-precursor is more suitable.

(b) The enzyme produced by the action of *F* is only capable of catalyzing the syntheses of the heterocyclic structure characteristic of flavonols, when the B-precursors with one or two hydroxyl groups are available.

(c) The synthesis of the heterocyclic structure characteristic of leuco-anthocyanidins is catalyzed by an enzyme which only acts when the B-precursor with two or three hydroxyl groups is available.

Similar assumptions as outlined in the preceding paragraph have been made previously by FEENSTRA (17) for *Phaseolus vulgaris*.

(d) Furthermore, it is assumed that the enzyme produced by *F* has a greater activity than that formed by *W*. This hypothesis is in accordance with the fact that the production of a larger amount of anthocyanidins is accompanied by a lower production of flavonols, and the reverse.

The hypotheses, given above, also explain that in the genotype *W.F.S.* the production of anthocyanidins is very much decreased in favour of the production of flavonols. In this genotype only the B-precursor with two hydroxyl groups is available for the syntheses of anthocyanidins and flavonols.

It was observed that in the eye of the flower of *W.*-genotypes malvidin is the chief flavonoid. Thus, it seems that generally *S* and *F* do not act in this part of the tissue.

Some genotypes were found, however, which had petals coloured by peonidin and cyanidin only and which had the same colour intensity all over the petal, *i.e.* the two cultivars, 'Helvetikum lachs' and 'Helvetikum rose' (see table 6, on p. 17). Presumably in these genotypes *S* acts in the petal as a whole.

A similar phenomenon was observed in the flowers of certain genotypes, in one F_2 and one F_3 generation, which resulted from crosses with 'Firefly'. The petals of these genotypes were weakly coloured. The segregation ratios suggest the action of one gene, *Z*, which determines whether *S* and *F* also act in the basal part of the petal. The gene combination *W.F.S.Z.* causes a very low production of anthocyanins in the petal as a whole.

From his chemical-genetical investigations of *Cyclamen* SEYFFERT (49) concluded that anthocyanidins and flavonols were produced by parallel syntheses from leuco-anthocyanidins. However, the assumptions outlined earlier in this paper are preferred, because the amounts of leuco-anthocyanidins were not correlated with the amounts of anthocyanidins and flavonols. Also leucopelargonidin (with one hydroxyl group in the benzene nucleus) was never found in the genotype *wwff*.

The phenomena observed contradict the possibility that anthocyanidins and flavonols are synthesized sequentially, e.g. anthocyanidins from flavonols. If so, the action of *W* and *F* should be complementary, but this was found not to be the case, neither by SEYFFERT (49), nor in this work.

2. Methylation

The fact that some anthocyanidins were found to contain methoxyl groups has to be considered.

In the B-ring of the leuco-anthocyanidins and flavonols isolated only hydroxyl groups were found. In the B-ring of several anthocyanidins, however, one or two methoxyl groups at the 3′ or at the 3′ and 5′ position were found. Thusfar 4′ methylated anthocyanidins have not been isolated from plant material. It has been described in the literature that methylated anthocyanidins occur in one tissue together with unmethylated flavonols (51), and it was suggested that methylation occurs at a late stage in the biosynthesis of the flavonoid compounds. From chemogenetical investigations on *Petunia*, Bianchi (8) demonstrated the existence of a dominant gene causing hydroxylation, and also the existence of a dominant gene causing methylation, provided the dominant gene for hydroxylation was present.

It is possible that all cyclamen plants have a gene causing methylation of anthocyanidins, the unmethylated and partially methylated anthocyanidins being formed as intermediates in the syntheses of fully methylated compounds. Since no cyclamen flower was found which contained delphinidin and/or cyanidin as the only anthocyanidins, the existence of a gene for methylation could not be proven.

In view of the fact that no methylated leuco-anthocyanidins and flavonols were present in the material investigated, it is probable, that in *Cyclamen* methylation occurs in a late stage in the biosynthesis, after the action of *W* and *F*.

3. Glycosidation

All flavonoid compounds isolated were found to be glycosides and no aglycones were observed. Therefore, no gene could be demonstrated which caused glycosidation at the 3 position where a sugar residue is introduced when monoglycosidation occurs.

The action of a gene (*M*) was confirmed which causes the introduction of a sugar residue at the 5 position of the anthocyanidins. An eventual effect on the glycosidation of the leuco-anthocyanidins could not be detected as the number and the position of the sugar residue(s) in these compounds was not established. It was shown that *M* did not influence the glycosidation pattern of the flavonols. This phenomenon was found previously (19).

From the data obtained it appears that *M* acts in the biosynthesis at a stage after the action of *W* and *F*. From experiments it could not be concluded whether *M* acted before or after methylation.

The occurrence of anthocyanidin-3-glucosides together with anthocyanidin-3,5-diglucosides was found only in presence of *W*, *M* and *S* (peonidin glycosides being formed) and not in presence of *W*, *M* and *ss* (malvidin-3,5-diglucoside being formed). This points to the fact that the occurrence of peonidin-3-glucoside together with peonidin-3,5-diglucoside is not the result of incomplete glycosidation. If so, one would expect to find malvidin-3-glucoside together with malvidin-3,5-diglucoside as well.

4. Prospects for breeding

In breeding cyclamen, a special value is attached to the introduction of new, attractive flower colours. The colours are for the greater part determined by

the qualitative and quantitative production of anthocyanins and flavonol glycosides. From the fact, that both in the types "nearly white with eye" and "full coloured" divergent colour intensities exist, it is clear that the actions of several other genes than discussed in this paper are involved and that new recombinations may be valuable.

The most interesting aspect in breeding cyclamen is to extend the flower colours towards the red, and to breed a yellow flowering cyclamen. Extension towards the red does not seem to be possible when only peonidin glycosides are produced, as in the existing cultivars. The extention towards red could be rather expected by the formation of pelargonidin glycosides. For this purpose it would be necessary that *W* acts when a B-precursor with one hydroxyl group is available. Pelargonidin glycosides are often encountered in nature and there is no reason to reject the possibility that they could be formed in cyclamen, e.g. by mutation.

The breeding of a yellow flowering cyclamen depends on the possibility of promoting the production of the yellow flavonol glycosides. In nature several plants with yellow flowers occur, containing high amounts of flavonol glycosides (39, 40). Although thusfar only creamish cyclamen flowers have been found (50), there is no a priori reason to exclude the possibility for yellow flowers to arise one day. The answer remains to the future.

SUMMARY

I. General

1. The first investigations on flower colour inheritance in *Cyclamen* were carried out by Wellensiek, and extended by Seyffert. This author, using paperchromatography, demonstrated the presence of flavonoid compounds, e.g. anthocyanins, flavonol glycosides and leuco-anthocyanins.

2. The present investigations were carried out in order to extend the knowledge of the chemogenetics of flower colours in *Cyclamen*, as a basis for the description of cultivars and for breeding work.

II. Plant material and colour substances

1. The plant material consisted of wild species, cultivars and crosses.

2. The chief amounts of flavonoid compounds occur in the upper and lower epidermis of the petal.

III. Methods of chemical analysis

Paperchromatography was used for separation. Paperchromatography and absorption spectrophotometry were used for identification.

IV. Results of chemical analysis

1. From a part of the bulk of available flowers 14 anthocyanins were isolated, of which 8 were identified, and 8 flavonol glycosides, of which 3 were identified. The structures of these compounds are given in fig. 3, on p. 13. In addition leuco-cyanidin and leuco-delphinidin glucoside were found.

2. It was assumed that the isolated substances were the chief flavonoid compounds occurring in *Cyclamen* flowers.

V. Analysis of the material

1. The identification of the flavonoid compounds occurring in the other part of the available flowers, was based on a comparison of their Rf values with the Rf values of the substances described in IV.

2. The results of the analysis of wild species are given in table 5, on p. 15.

3. The results of the analysis of cultivars are given in table 6, on p. 17, which also gives quantitative data about anthocyanins and flavonol glycosides.

4. The chemogenetical analyses of the crosses confirmed the presence of the genes *W*, *F*, *S* and *M*, with the following details on their action.

W is the basic gene for anthocyanin production, *ww* giving white flowers.

F causes the production of a great amount of flavonol glycosides. It was not confirmed that *nor*-gardenin or hibiscetin occurred among them, as was suggested by Seyffert. In presence of *ff* traces of flavonol glycosides were found. Seyffert did not found these compounds at all in presence of *ff*, while Kessler found traces in presence of *ffff*.

S causes the production of peonidin glycosides in the flower slip, which is the non basal part of the petal. In presence of *ss*, malvidin glycosides are formed in the flower slip. These compounds usually are produced in the basal part of the petal, the eye, both in presence of *S.* and *ss*. In the slip *S* causes the production of leuco-cyanidin and leuco-delphinidin glucoside, *ss* causing the production of leuco-delphinidin glucoside.

It was not confirmed that *S.F.* causes the production of *iso*-rhamnetin, as was concluded by Seyffert.

The effects of *W*, *F* and *S* are schematically represented in fig. 4, on p. 32.

M causes the production of anthocyanidin-3,5-diglucosides, *mm* giving anthocyanidin-3-glucosides. The anthocyanidin-3,5-diglycosides, as found by Seyffert, are anthocyanidin-3,5-diglucosides. Instead of anthocyanidin-3-pentoseglycosides, as found by the same author, anthocyanidin-3-glucosides were found. *M* has no effect on the glycosidation pattern of flavonol glycosides.

Three new genes were found. They were provisionally named *X*, *Y* and *Z*.

X, in presence of *F*, causes the production of the unidentified flavonol glycoside Fl 8, which is not formed in presence of *xx*.

Y, in presence of *W.ffS.M.*, causes the production of peonidin-3-glucoside and two unidentified peonidin glycosides A 8 and A 9. These compounds are not formed in presence of *yy*.

Z enables the effect of *F* and *S* to be perceived in the petal. In presence of *zz* their effect is not perceived.

Cyanidin glycosides are exclusively produced in presence of *S*, and the existence of the gene *C*, which, according to Seyffert, causes the production of cyanidin glycosides, could not be confirmed.

Furthermore, the existence of a gene I, which, according to Seyffert, causes the production of delphinidin glycosides, was not confirmed.

5. Different genotypes were used for quantitative estimations of flavonoid compounds. The results are given in table 9, on p. 30.

VI. General discussion

1. The phenomena observed are explained on the assumption that the biosynthesis of the flavonoid compounds in *Cyclamen* occurs as is schematically represented in fig. 5, on p. 33.

According to this hypothesis, the compounds are synthesized from an A-ring and a B-precursor. The number of hydroxyl groups in the B-ring of this precursor is influenced by *S* which gives two hydroxyl groups, *ss* giving three hydroxyl groups in the B-ring. It is assumed that *S* inhibits the action of a gene which causes the introduction of the third hydroxyl group. The precursor with one hydroxyl group in the B-ring is always produced.

W and *F* determine the structure of the heterocyclic part of the flavonoid compounds, *W* causing the production of anthocyanins, *F* causing the production of flavonol glycosides. The genetic condition for the production of leuco-anthocyanins was not found.

The B-precursors with two or three hydroxyl groups in the B-ring are suitable for the synthesis of anthocyanins. The B-precursors with one or two hydroxyl groups are suitable for the synthesis of flavonol glycosides.

M causes the introduction of a glucose residue at the 5-position of the anthocyanins.

It is assumed that methylated anthocyanins are produced as the result of the action of a gene, which is present in all cyclamen. Nonmethylated or partially methylated anthocyanins are formed by incomplete methylation. This hypothesis is preferred to the assumption of SEYFFERT, who suggested that anthocyanins and flavonol glycosides are formed by parallel synthesis from leuco-anthocyanins.

2. Red coloured flowers might be expected when pelargonidin glycosides could be produced. Yellow flowering cyclamen might be expected when the production of flavonol glycosides could be increased.

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SAMENVATTING

CHEMOGENETISCH BLOEMKLEURONDERZOEK IN *CYCLAMEN*

I. ALGEMEEN

1. Het eerste genetische bloemkleuronderzoek bij *Cyclamen* werd uitgevoerd door WELLENSIEK, het eerste onderzoek naar het verband tussen genetica van de bloemkleuring en chemie van de daarbij betrokken kleurstoffen door SEYFFERT. Door toepassing van papierchromatografie werden anthocyaninen, flavonol glycosiden en leuco-anthocyaninen gevonden. Deze stoffen behoren tot de groep van de flavonoide verbindingen.

2. Het in deze publikatie beschreven onderzoek beoogt een uitbreiding te geven aan de kennis van de chemogenetica der bloemkleuren in *Cyclamen*, als basis voor rasbeschrijving en veredeling.

II. Plantenmateriaal en kleurstoffen

1. Het onderzochte materiaal bestond uit *Cyclamen*-soorten, *Cyclamen*-cultivars en kruisingen van diploide cultivars.
2. De flavonoide verbindingen bevinden zich voornamelijk in boven- en onderepidermis van het bloemblad.

III. Methodiek van de chemische analyse

De flavonoide verbindingen werden geïsoleerd met behulp van papierchromatografie. De identificatie werd uitgevoerd met behulp van papierchromatografie en absorptie-spectrofotometrie.

IV. Resultaten van de chemische analyse

1. Uit een gedeelte van de beschikbare bloemen werden geïsoleerd 14 anthocyaninen, waarvan 8 werden geïdentificeerd, en 8 flavonol glycosiden, waarvan 3 werden geïdentificeerd. De structuren van deze verbindingen zijn weergegeven in fig. 3 op p. 13. Tevens werden leuco-cyanidine en leuco-delphinidine glucoside gevonden.
2. Aangenomen werd dat de geïsoleerde stoffen de belangrijkste flavonoide verbindingen zijn, die in cyclamenbloemen voorkomen.

V. Analyse van het materiaal

1. De identificatie van de flavonoide verbindingen, geëxtraheerd uit bloemen, die niet onderzocht waren in het kader van IV, berustte op vergelijking – op éénzelfde chromatogram – van de Rf waarden van deze verbindingen met die van de onder IV beschreven stoffen.
2. De resultaten van de analyse van de *Cyclamen*-soorten zijn weergegeven in tabel 5 op p. 15.
3. De resultaten van de analyse van cultivars, zoals reeds gegeven in een vroegere publikatie (56), werden aangevuld met kwalitatieve en kwantitatieve gegevens over anthocyaninen en flavonol glycosiden, zie tabel 6 op p. 17.
4. De chemogenetische analyse van de kruisingen 'Firefly' × 'Wit', 'Firefly' × 'Sylphide' en 'Firefly' × 'Wit met Oog' bevestigde de aanwezigheid van de reeds door Wellensiek gevonden genen *W* en *S* en de door Seyffert gevonden genen *F* en *M*. Over de werking van *W*, *F*, *S* en *M* werd het volgende gevonden:

W is grondgeen voor de vorming van anthocyaninen, *ww* geeft witte bloemen.

F veroorzaakt de vorming van grote hoeveelheden flavonol glycosiden. Niet bevestigd werd de veronderstelling van Seyffert, dat dit mengsel bestaat uit slechts drie verbindingen waarvan één *nor*-gardenine of hibiscetine zou zijn. Bij aanwezigheid van *ff* werden sporen flavonol glycosiden gevonden. Seyffert vond deze verbindingen niet bij *ff*, Kessler vond sporen bij *ffff*.

S veroorzaakt de vorming van peonidine glycosiden in de bloemslip, d.i. het niet basale deel van het bloemblad. Bij aanwezigheid van *ss* ontstaan hierin malvidine glycosiden. In het basale deel van het bloemblad, het oog, worden meestal alleen malvidine glycosiden gevormd, zowel bij *S.* als bij *ss*. In de slip geeft *S* een leuco-cyanidine en leuco-delphinidine glucoside, *ss* leuco-delphinidine glucoside. Niet bevestigd werd de conclusie van Seyffert, dat *S.F.* de vorming

van *iso*-rhamnetine veroorzaakt. Een overzicht van het effect van *W*, *F* en *S* is gegeven in fig. 4, op p. 32.

M veroorzaakt de productie van anthocyanidine-3,5-diglucosiden, *mm* geeft anthocyanidine-3-glucosiden. De door SEYFFERT gevonden anthocyanidine-3,5-diglycosiden zijn anthocyanidine-3,5-diglucosiden. In plaats van de door SEYFFERT gevonden anthocyanidine-3-pentoseglycosiden werden anthocyanidine-3-glucosiden gevonden. Er is geen effect van *Mm* op het glycosideringspatroon van flavonol glycosiden.

Drie nieuwe genen werden gevonden, voorlopig genoemd *X*, *Y* en *Z*.

X veroorzaakt, bij aanwezigheid van *F*, de vorming van een niet geïdentificeerd quercetine glycoside, Fl 8. Deze verbinding wordt niet gevormd bij aanwezigheid van *xx*.

Y veroorzaakt, bij aanwezigheid van *W.ffS.M.*, de vorming van peonidine-3-glucoside en twee niet geïdentificeerde peonidine glycosiden, A 8 en A 9. Deze verbindingen worden niet gevormd bij aanwezigheid van *yy*.

Z veroorzaakt omstandigheden, waardoor in gekleurde bloemen het effect van *S* en *F* in het gehele bloemblad waarneembaar is. Bij aanwezigheid van *zz* treden deze omstandigheden niet op.

Cyanidine glycosiden werden uitsluitend geproduceerd bij aanwezigheid van *S*. en het bestaan van het geen *C*, dat volgens SEYFFERT de productie van cyanidine glycosiden bewerkstelligt, kon niet bevestigd worden.

Voorts werd geen bevestiging gevonden van het bestaan van het geen *I*, dat volgens SEYFFERT de produktie van delphinidine glycosiden bewerkstelligt.

5. Kwantitatieve bepalingen van anthocyaninen, leuco-anthocyaninen en flavonol glycosiden in verschillende genotypen gaven een indruk van de onderlinge verhoudingen van de hoeveelheden, waarin deze stoffen geproduceerd werden. De resultaten zijn weergegeven in tabel 9, op p. 30.

VI. ALGEMENE DISCUSSIE

1. Om de waargenomen verschijnselen te verklaren werd een hypothese opgesteld over de biosynthese van flavonoide verbindingen in *Cyclamen*. Deze is schematisch weergegeven in fig. 5, op p. 33, en betreft voornamelijk het effect van *W*, *F*, *S* en *M*. De verbindingen, alle van het C_6-C_3-C_6 type, worden gesynthetiseerd uit een C_6 deel (A-ring) en een C_3-C_6 deel (B-precursor). Het aantal hydroxylgroepen in de B-ring van deze precursor wordt beïnvloed door *S*, opgevat als remfactor van een geen dat de introductie van een derde hydroxyl groep in de B-ring bewerkstelligt. De precursor met één hydroxyl groep in de B-ring wordt altijd gevormd.

W en *F* bepalen de structuur van het heterocyclische deel van de gevonden verbindingen, waardoor bij aanwezigheid van *W* anthocyaninen, bij *F* flavonol glycosiden in de bloem ontstaan. De genetische voorwaarde voor het ontstaan van leuco-anthocyaninen werd niet gevonden.

Voor de synthese van anthocyaninen door *W* zijn alleen de B-precursor met twee en met drie hydroxyl-groepen in de B-ring geschikt, voor de synthese van flavonol glycosiden door *F* alleen B-precursors met twee en met één hydroxyl groep in de B-ring.

M veroorzaakt de introductie van een glucose-rest op de 5-plaats van het anthocyanine-molecuul.

Aangenomen wordt dat in *Cyclamen* een geen werkt, waardoor gemethy-

leerde anthocyaninen worden gevormd.

Deze hypothese lijkt aannemelijker dan de veronderstelling van SEYFFERT, waarin wordt aangenomen dat leuco-anthocyaninen de precursors zijn van anthocyaninen en flavonol glycosiden.

2. Rode bloemen kunnen alleen verwacht worden, wanneer pelargonidine glycosiden geproduceerd zouden worden. Gele bloemen zouden gevormd kunnen worden, wanneer de hoeveelheid geproduceerde flavonol glucosiden toeneemt.

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