

Inheritance of Determinants of Flower Colour in Tetraploid Roses

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Abstract

The choice of selection breeding for crop improvement in cut roses requires a better understanding of biological mechanisms and knowledge of the inheritance of the major target traits which can lead to new or improved screening methods. Colour is still the most important trait in cut roses. A tetraploid mapping population will be characterized for flower colour, by using colour charts such as the official chart of the Royal Horticultural Society, and additionally, by image analysis and measuring reflectance using a spectrophotometer. The genetics of flower colour will be studied. In addition, flower petals of all genotypes will be analysed by HPLC to characterize secondary metabolic components that determine flower colour, such as anthocyanins. The inheritance of these components will also be assessed and compared to that of flower colour. Preliminary results show that the most effective method to quantify colour is by HPLC analysis of the extracted anthocyanins. The highest pelargonidin concentrations occur at relatively low cyanidin concentrations. Absorbance and reflectance measurements illustrate the accumulated effect of all the individual anthocyanins present in the petal.

INTRODUCTION

Rosa is the major economically important genus of ornamental horticulture. The turnover in 1996 was Euro 484 million, which had increased to Euro 758 million in 2006 (VBN, The Netherlands). The genus *Rosa* belongs to the family of the Rosaceae. While the genus *Rosa* comprises more than 150 species and thousands of cultivars (Gudin, 2000), only 11 species of them were used to create the modern rose we know today (Crespel et al., 2003). The rose industry thrives on novelty and the production of novel flower colour has been extensively studied. Flower colour is predominantly due to three types of pigments: flavonoids, carotenoids and betalains. The flavonoids are the most common of the three types of pigment and contribute to a range of colours from yellow to red to blue. They are water soluble compounds. The flavonoid pathway leading to the first coloured anthocyanins, i.e. anthocyanidin 3-O-glucosides, is generally conserved among plant species and is well established. In rose, primarily 3,5-O-diglucosides are present.

In order to study the inheritance of anthocyanins in roses a concise objective and effective measure of colour is required. Studies on the chemical and colorimetric features in relation to the colour range have been carried out by Biolley and Jay (1993).

MATERIALS AND METHODS

Plant Material

A tetraploid (K5) population of 149 hybrids from a cross between two tetraploid rose genotypes, P867 (light pink) and P540 (dark red) were used. The F₁ population shows segregation for many morphological traits including colour. Cuttings from each of the individuals and the parents were made from mother plants of the same age and rooted in plastic trays with commercial potting soil. Rose flowers were plucked at development stage two (early stage of anthesis).

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Colorimetric Measurements

1. Colour Chart. Three methods were used to take colorimetric measurements. The first method of visual evaluation was with the use of the Royal Horticultural Society (RHS) colour standards 2001. The system comprises a printed set of standard colours, each colour patch being marked with a unique number or code, to act as a standard reference against which the petals could be viewed for matching. To avoid the influence of fluctuating light conditions the petals were measured in an artificial daylight simulation light cabinet. The first three petals were discarded and the code that best described the outer epidermis of the subsequent petals was recorded.

2. Reflectometry. Two petals were randomly selected from the remaining petals and the inner and the outer epidermis were measured using a spectrophotometer Spectrophotometer: Ocean Optics, Inc. SD2000 Lightsource: Top Sensor Systems, with halogen light-source: HL 2000 FSHA Fitted: Bifurcated fiber: FCR-74V200-2-ME-S1 White reference: Top Sensor Systems WS-2. The reflectance curves (between 400 and 700 nm) were further translated into the CIELab system developed by the *Commission Internationale d'Eclairage* (CIE $L^*a^*b^*$ colour space). The spectral curves were reduced to three values (L^* , a^* and b^*) that describe the position in the 3-dimensional colour space.

3. UV/VIS Spectrometry. Depending on the size of flower head 6 to 9 petals were selected and immediately frozen in liquid nitrogen and kept at -80°C in a freezer for anthocyanin analysis. The petals were further dried in a freeze dryer for 24 h and subsequently ground up using a bead miller type MM2 from Retsch. Set at two minutes at 50% max RPM. 100 mg for dark and 200 mg for light coloured rose samples were suspended in 5 ml of 1% (v/v) hydrochloric acid (HCl) and 0.1% (w/v) butyl hydroxyl anisol (BHA) in methanol for 30 min at room temperature with shaking on 150 RPM in a shaker type RS500 from LaboTech. The extracts were centrifuged and the process repeated until all the anthocyanin was extracted from the samples. The supernatant was filtered through a micro-filter (0.45 μm) and used for spectrometry or HPLC. Spectroscopic analyses of the non-hydrolyzed anthocyanin extracts were carried out on a wavelength-scanning UV/Vis spectrophotometer (Ultrospec 2000, Pharmacia Biotech) controlled by SWIFT WAVESCAN II applications software. Extracts were diluted in order to avoid an absorbance above the maximum possible sensor response. Cuvets with a light path of 1 cm were obtained from Brand (Wertheim, Germany). The reference solution was 1% (v/v) hydrochloric acid (HCl) and 0.1% (w/v) butyl hydroxyl anisol (BHA) in methanol. The absorbance was recorded at 1 nm steps over the range 350-750 nm at a scan rate of 2500 nm min^{-1} .

4. HPLC. Complete acid hydrolysis was obtained from 2 ml volume of the sample solution with 2 ml of 2N HCl at 100° after 2 h. Isolated compounds were characterized by HPLC.

Ten micro-liters of the filtrated supernatant were injected into a C18 reversed-phase column (Mightysil RP-18GP (ODS), 5 μm , $250\times 4.6\text{ mm}$). The separation of anthocyanins was done by using 10 min elution with 1.5% (v/v) phosphoric acid in water, followed by 20 min linear gradient increase elution from 30 to 50% with 1.5% (v/v) phosphoric acid and 20% (v/v) acetic acid and 25% (v/v) acetonitrile in water. The column chromatography was performed at 35°C and samples were eluted at a flow rate of 1 ml min^{-1} . Anthocyanidins were monitored at 520 nm using a Photodiode Array Detector. Anthocyanidins detected in HPLC profiles were identified by matching their retention times to those of anthocyanidin standards obtained from Sigma-Aldrich Chemie B.V. (Zwijndrecht, the Netherlands).

RESULTS AND DISCUSSION

The colour codes of the K5 individuals were in the range of 27 to 67 (orange, orange-red, red and red-purple) and 184 to 187 (grayed colours of red, blackish red). Assigning single colours, or even two colours, frequently was an oversimplification, as the petals displayed many more, depending on the area of the petal and location in the flower.

There was variation in the reflection curves of flowers from different genotypes. The colours obtained from the calculated L^* , a^* and b^* coordinates were generally in good agreement with the perceived flower colour. As the pigment distribution in flowers was not completely homogeneous, the reflection measurement was a spot measurement. The perceived colour of the complete flower did not always match the colour obtained from the calculated $L^*A^*b^*$.

After extraction of the anthocyanins, absorbance curves of the non-hydrolyzed extracts were obtained. The individuals containing predominantly pelargonidin derivatives exhibit a λ_{\max} ranging from 510 to 513 nm and the cyanidin individuals have a λ_{\max} of 524 nm. The pelargonidin individuals have a higher absorbance in the blue region and a lower absorbance in the yellow/orange region of the spectrum. These results are indicative for pelargonidin derivatives (Marshall et al., 1983).

De Vries et al. (1974) showed that pelargonidin is always found together with cyanidin. In the K5 population pelargonidin- and cyanidin derivatives are the major pigments, together they represent 96.4% of all pigment ratings, the absorbance graphs of the K5 individuals are predominantly build from the combined absorbance graphs of cyanidin-based and pelargonidin-based pigments. According to Hong and Wrolstad (1990) pelargonidin derivatives exhibit a lower λ_{\max} (about 505 nm) compared to cyanidin derivatives (520-526 nm).

The four different anthocyanidin standards used in this study in HPLC were cyanidin -, pelargonidin -, peonidin - and delphinidin chloride. These compounds elute at 15.3-15.5, 18.3-18.5, 19.7-19.9 and 12.1-12.2 min respectively.

After analysis 98% of the individuals contained cyanidin in varying measurable concentrations, 2% of the progeny contained only traces of cyanidin. Pelargonidin and an unidentified anthocyanidin were found in varying concentrations in 34.0 and 40.1% of the flowers respectively. Traces of pelargonidin and the unidentified anthocyanidin were found in 24.5 and 44.2% of the individuals respectively. Figure 1 shows an example on the chromatograms obtained from the K5 population. These observations are in accordance with Marshall et al. (1983) who found cyanidin in 99% of the seedlings, and Yokoi (1974) and Arisumi (1963) who found cyanidin in all of the roses examined. Dominance of cyanidin over pelargonidin synthesis is reported in a great number of plant species. This common dominance maybe explained by the activity of the enzyme flavonoid 3'-hydroxylase (F3'H) which in *Gerbera* is controlled by the dominant allele $f3'h^+$ (Tyrach et al., 1997)

Correlation was calculated for all the different measurements (Table 1). Each K5 individual has been defined by the RHS colour codes (RHS), the colorimetric values ($L^*A^*b^*$), the λ_{\max} from the absorbance measurements and five chemical parameters: the total content of anthocyanidins (Total anth.), cyanidin, pelargonidin, unidentified anthocyanidin and the relative pelargonidin concentration.

When comparing the chemical data from the HPLC with the RHS colour codes assigned to the flowers, the colour code was positively correlated (0.652) with the cyanidin concentration and concentration of the unidentified anthocyanidin (correlation =0.545). With increasing amounts of cyanidin and the unidentified anthocyanidin, a higher colour code is assigned to the flowers. The individuals characterized by colour codes in the range of 184-187, contained relatively high amounts of both cyanidin and the unidentified anthocyanidin (respectively 9.78-20.00 and 0.33-1.17 mg g⁻¹ petal dry weight). Yokoi and Saito (1973) showed a higher code number of the RHS colour chart with an increase in the amount of cyanidin. The correlation matrix showed a significant negative correlation of -0.224 between the relative pelargonidin concentration and the RHS colour code. Yokoi and Saito (1973) also showed that a lower code number of the RHS colour chart agrees with an increase in the amount of pelargonidin (Yokoi and Saito, 1973). Thus the colour code obtained by visual comparison of the colour sheets with the flower colour would give some idea about the anthocyanin composition of the flower.

There were strong negative correlations between highly positively correlated chemical variables, these are; total anthocyanidin amount, cyanidin amount and amount

of unidentified anthocyanidin, and lightness (L^*) of the petal colour. With an increase in total anthocyanidin concentration, the colour of the flower becomes darker. A very high concentration of cyanidin as seen in individual K167 resulted in a weak lightness ($L^*=19.4$). Thus, individuals K210 and K167, characterized by the darkest ($L^*=18.1$) and the second darkest colour ($L^*=19.4$) contained a high amount (>18 and >12 mg g⁻¹ petal dry wt. respectively) of anthocyanidins. This was also found by Jia et al. (2008) and Biolley and Jay (1993) who found a negative correlation between total anthocyanins and the L^* value. The L^* value decreased with increasing anthocyanin concentrations.

There was a high significant negative correlation of -0.756 between the relative pelargonidin content and the wavelength of maximum absorption. An increase in the relative pelargonidin concentration lowers the λ_{\max} in the absorbance measurements. Similar observations were reported by Prodanov et al. (2005).

Quantification of anthocyanin pigments was possible using UV/Vis spectrophotometry, by running a series of four concentrations (10, 20, 40 and 100 mg L⁻¹) of the different anthocyanidin standards. The resulting absorbance graphs showed a perfect linear relationship between the anthocyanidin concentration and the height of the curve. For cyanidin, pelargonidin, peonidin and delphinidin the R^2 's are respectively 0.999, 0.997, 1 and 1. The resulting corrected absorbance values showed a good exponential correlation with the anthocyanidin concentration with a R^2 of 0.813. Since the standard error of the observations was rather high (s.e.=1.89), the absorbance measurements gave only a rough estimation of the anthocyanidin concentration in the flowers.

Inheritance of flower colour can only be described in the individual flower colour. De Vries et al. (1980) showed that inheritance of each pigment was mainly controlled by additive gene action. The method most useful when studying the inheritance of individual pigments proved to be the HPLC method. HPLC provided both qualitative data on the anthocyanidin composition as well as quantitative data on the amount of each individual anthocyanidin. In this study, the salmon parent P867 contained low levels of pelargonidin (~ 0.4 mg g⁻¹ petal dry wt.) and traces of cyanidin. The dark red parent P540 contained high levels of cyanidin (~ 17 mg g⁻¹ petal dry wt.), a relatively high level of the unidentified anthocyanidin (~ 1 mg g⁻¹ petal dry wt.) but no pelargonidin.

CONCLUSIONS

When studying the inheritance of colour, one must focus on the inheritance of the individual pigments since a flower colour is not inherited as such but results from a specific combination of pigments. RP-HPLC yields precise information on the exact anthocyanidin composition in rose flowers. Using anthocyanidin standards, the compounds can be identified and quantified resulting in information on the type and concentration of anthocyanidin pigments present in the flower.

The RHS colour chart is excellent for recording flower colour in the field as they are quick and handy to use, and there is a large range of colours suitable for flower description. Being referenced by numbers or codes, they are not so dependent on language and can be used internationally. However they would not be an effective tool for measuring colour in roses and subsequently the mode of inheritance of this colour as they are not accurate enough and are subject to many variables daylight lighting conditions, the immediate surroundings, viewing angle and area of the petal.

Absorbance measurements yield information on the λ_{\max} of the anthocyanin extracts. The absorbance curve is build up from the combined absorbencies of the different pigments and thus can not be used to calculate the mode of inheritance of the individual pigments.

Reflectance measurements resulted in spectral curves describing the colour of each individual. The flower colours could be very precisely quantified using the CIELab, however, the variation in calculated values was large. There is evidence that, in general, there are good correlations between chemical components and colorimetric indices so that one method could suggest the outcome of the other one.

Currently the map of the K5 population is under construction and QTL analysis will be carried out on all the anthocyanins. Also further chemical analysis will be carried out to determine the unidentified anthocyanin. The results described in this paper are only preliminary as only one set of data was used. Further tests will be carried out to confirm the observed trends.

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Tables

Table 1. Correlation matrix on combined data from the RHS, reflectance/absorbance measurements and chemical data from HPLC.

Cyanidin											
Pelargonidin	-0.047 ^{NS}										
Unidentified	0.878**	-0.028 ^{NS}									
Total anth.	0.926**	0.334**	0.831**								
% pelargonidin	-0.275**	0.660**	-0.205*	-0.008 ^{NS}							
RHS code	0.652**	-0.167*	0.545**	0.551**	-0.224**						
λ_{\max} absor	0.432**	-0.491**	0.327**	0.220**	-0.756**	0.226**					
Lightness (L*)	-0.871**	0.020 ^{NS}	-0.715**	-0.811**	0.293**	-0.577**	-0.535**				
Chroma (C*)	0.039 ^{NS}	0.376**	-0.005 ^{NS}	0.176*	0.173*	-0.287**	0.151 ^{NS}	-0.298**			
Hue angle (h°)	0.134 ^{NS}	0.365**	0.110 ^{NS}	0.264**	0.398**	-0.068 ^{NS}	-0.341**	-0.178*	0.290**		
	Cya.	Pelar.	Unk.	Total anth.	% pelar.	RHS	λ_{\max} abs.	L*	C*	h°	

** Correlation is significant at the 0.01 level (2-tailed).

* Correlation is significant at the 0.05 level (2-tailed).

^{NS} Correlation is not significant.

Figures

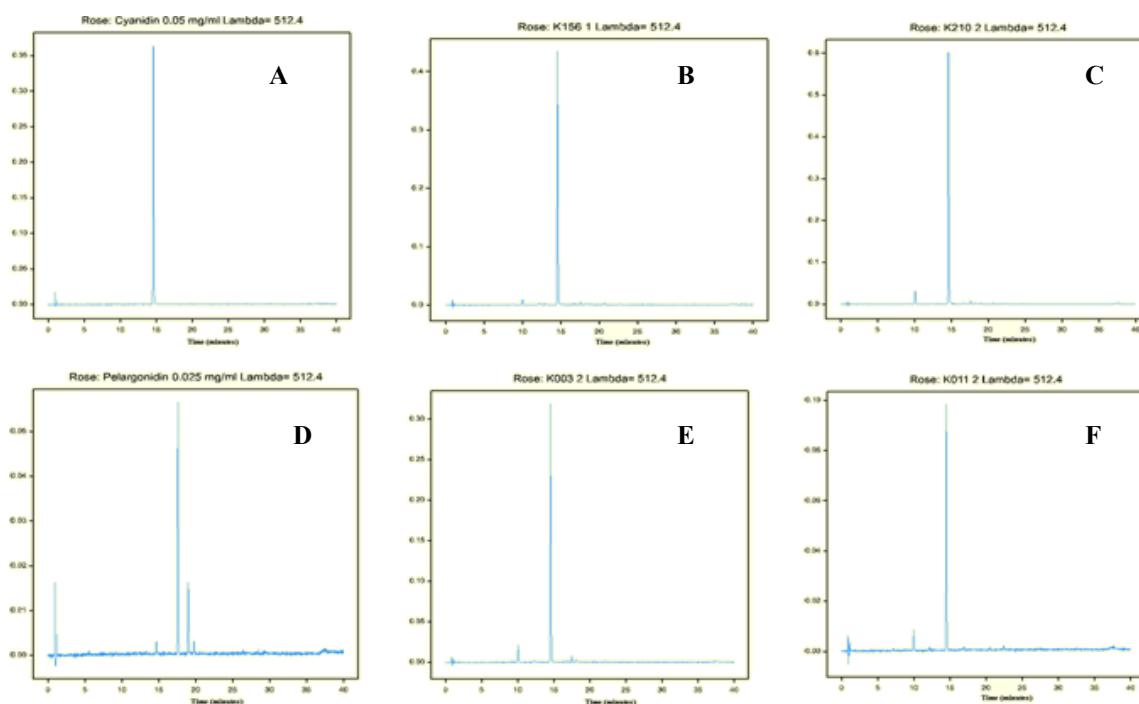


Fig. 1. HPLC chromatograms with detection at 512 nm: A) cyanidin chloride (Retention time (T_r)=15.3-15.5 min); B & C) Chromatogram of the purified extracts of K156 and K210 containing cyaniding; D) pelargonidin chloride (T_r =18.3-18.5 min); E & F) chromatograms of the purified extracts of K003 and K011 containing both cyanidin and pelargonidin.