FOLIA
HORTICULTURAE
Ann. 17/1, 2005, 61-72

Changes in flower colour among Lady Group of *Chrysanthemum* × *grandiflorum* /Ramat./Kitam. as a result of mutation breeding

Justyna Lema-Rumińska, Małgorzata Zalewska

Department of Ornamental Plants and Vegetable Crops,
Laboratory of Biotechnology
University of Technology and Agriculture
Bernardyńska 6, 85-029 Bydgoszcz, Poland
e-mail: lem-rum@atr.bydgoszcz.pl

Key words: ionising radiation, mutants, anthocyanins, carotenoids, flavones and flavonols, spectrophotometry, absorbance

ABSTRACT

Ten mutants and one original cultivar of chrysanthemum (*Chrysanthemum* x *grandiflorum* /Ramat./Kitam., syn. *Dendranthema grandiflora* Tzvelev) representing the Lady Group were analysed for pigment content in the inflorescence by the spectrophotometric method. The respective mutants obtained by ionising radiation differed in their quality and quantity of flavonoids and carotenoids in the inflorescence as compared with the original 'Richmond'. Each mutant studied exhibited its own permanent repetitive profile of the occurrence of pigments, which allows the possibility of showing the distinctness of the cultivars analysed and may be useful in their identification.

INTRODUCTION

Chrysanthemum (*Chrysanthemum* × *grandiflorum* /Ramat./Kitam., syn. *Dendranthema grandiflora* Tzvelev) is a species, the breeding of which began approximately 2500 years ago in China (Datta 1998). Chrysanthemums (*Asteraceae*) have inflorescence in the form of calathidum. The colour, or decorative value of a given cultivar, is determined by pigments contained mainly in ray (ligulate) florets. The largest group of pigments is formed by flavonoids, which occur in the vacuole represented by anthocyanins, flavones and flavonols (Harborne 1988, Hattori 1992). Anthocyanins are responsible for the following colours: red, blue and violet, depending on pH of the cell sap and co-pigments of metals and flavonoids (Jurd and Asen 1966, Takeda et al. 1994). The most frequent anthocyanidin in ornamental plant flowers is cyanidin (Harborne 1988). Cyanidin, as the main anthocyanidin pigment of chrysanthemums, shows the absorption maximum at the wavelength of λ = 530 nm, which can constitute a basis for pigment identification (Kawase et al. 1970, Saito et al. 1988).

Flavones and flavonols are responsible for the following flower colours: white, cream and yellow (Williams et al. 1981). The most frequent flavones and flavonols in chrysanthemums include apigenin, acacetin, luteolin, diosmethin and eriodictyol (Schwinn et al. 1994).

The other big and very important group of colour compounds are carotenoids. They are the pigments which, occurring in plastids, are responsible for the following plant colours: yellow, orange and even red. The molecule is composed of numerous double bonds, playing the role of chromophores absorbing light, frequently double-ended with rings. The basic pigment of that group, which occurs in chrysanthemums, is 5,8-epoxy α-carotene.

In horticulture, there is frequently a need for objective defining of flower colour. The traditional method of comparing the colour is with colour models, e.g. the Royal Horticultural Society Colour Chart (RHSCC). The colour evaluation with that method is, however, highly subjective as it depends on the optical skills of the observer as well as the source of light and its intensity. An alternative method involves the measurement of pigment absorbance with a spectrophotometer, which is easy, universal and cheap and, additionally, meets the most essential criterion – objectiveness and accuracy of the measurements, however it has not found a wider application in horticultural practice to evaluate the flower colour.

The aim of the present study was to define changes in the pigments of chrysanthemum inflorescence obtained as a result of mutagenesis induced *in vitro.*

MATERIAL AND METHODS

The Lady Group of chrysanthemum was analysed to define the pigments (anthocyanins, carotenoids, flavones and flavonols) in ray florets. The group was composed of ten mutants and the original cultivar, 'Richmond', which has a medium-size full type purple-pink inflorescence. The cultivars studied show the following inflorescence: golden-brown* - 'Lady Amber', golden-beet - 'Lady Apricot', reddish-brown* - 'Lady Bronze', orange-red - 'Lady Orange', pink* - 'Lady Pink', purple-gold* - 'Lady Rosy', salmon* - 'Lady Salmon', white* -'Lady White', yellow - 'Lady Yellow' and heather-pink - 'Lady Vitroflora' with ray florets forming tubes. All the above mutants were developed by Jerzy and Zalewska as a result of exposure to X or gamma radiation of leaf explants of the original cultivar *in vitro* applying regeneration with the adventitious bud techniques (Jerzy et al. 1991).

The analysis involved sampling fresh fragments of ray florets (about 2 cm from the apex) of a cultivar- and mutant-specific colour.

The shoot cuttings of the chrysanthemum cultivars studied were planted in a permanent place on benches, with the some professional substrate for chrysanthemum cultivation ("Hollas" from Pasłęk), in outdoor glasshouse on May 21 1999, and in glasshouse on July 10 2000. The cultivation took place under conditions of natural photoperiod with a standard method. The ray florets were sampled starting from October 27 to November 10, 1999 and from October 23 to November 17, 2000 over full flowering. From the five random inflorescences sampled, 4 weighed amounts were prepared each time, two of 100 mg were used for carotenoids analysis and the other two of 200 mg for anthocyanins analysis. A total of ten 100 mg samples and ten 200 mg samples were collected from each cultivar (ten repetitions).

The tissues were crushed in a porcelain mortar with the addition of a few mg of quartz sand. Extracting carotenoids followed the Wettstein method (1957), in which 100% acetone was used with the addition of a few mg of $CaCO_3$, while for anthocyanins - the Harborne method (1967) was used with methanol containing 1% of HCl. The extracts obtained were filtered through funnel with filter paper into 10 cm^{-3} volumetric flask.

The spectrophotometric analysis of extracts was carried out in the two-beam spectrophotometer (UV-VIS 1601-PC SHIMADZU). The absorbance measurements were carried out in 1 cm-wide quartz cuvettes within the wavelength range from 300 to 800 nm. Adequate solvents were applied as control solutions applied to extract a given group of pigments. The results were analysed with standard spectrophotometer software for spectral analysis.

* introduced into the Polish Cultivar Register in 1992

Absorption maxima were defined for pigment-specific wavelengths (λ_{max}) for flavones and flavonols from 330 to 340 nm, carotenoids at 440 nm and anthocyanins at 530 nm. A mean absorbance was calculated at the absorption maximum and the content of carotenoids and anthocyanins per 1 g of fresh matter of ray florets was determined. A statistical analysis of the results was carried out with the Student t-test at the level of significance of p = 0.05.

The quantitative determination of anthocyanins was possible with the algebraic method following Harborne (1967) at the wavelength λ_{max}= 530 nm. Total anthocyanin concentration was calculated based on cyanidin 3-glucoside molar absorptivity 26 900 L $mol^{-1}cm^{-1}$ (Jurd and Asen 1966).

The concentration of the total carotenoids was calculated with the coefficient obtained from the Wettstein equation (1957) at the wavelength λ_{max}= 440 nm.

RESULTS

Lady mutants come from the 'Richmond' original cultivar. The composition of pigments for respective cultivars expressed in absorbance units over successive study years is given in Table 1, and mean absorbance values for two analysis years in Fig. 1. In chrysanthemum ray florets, depending on the cultivar, there occurred three groups of pigments: anthocyanins, carotenoids, flavones and flavonols.

Table 1. Absorbance of extracts from ray florets of chrysanthemum Lady Group over 1999 and 2000

Cultivar	Code	Absorption maximum					
		λ = 330 - 340 nm Flavones and Flavonols		λ = 440 nm Carotenoids		λ = 530 nm Anthocyanins	
		Years					
		1999	2000	1999	2000	1999	2000
'Richmond'	R	0.45 b	0.37 a	-	-	2.80 c	0.53 c
'Lady Amber'	LAM	0.64 c	0.37 a	0.78 d	0.56 d	-	-
'Lady Apricot'	LAP	0.62 c	0.55 b	0.31 b	0.23 b	0.15 b	0.08 b
'Lady Bronze'	LB	0.92 d	0.59 b	0.76 d	0.63 e	0.18 b	0.08 b
'Lady Orange'	LO	1.15 d	0.61 b	0.53 c	0.46 c	0.19 b	0.07 b
'Lady Pink'	LP	0.31 a	0.40 ac	-	-	-	-
'Lady Rosy'	LR	0.43 b	0.65 b	-	-	-	-
'Lady Salmon'	LS	1.41 de	0.51 bc	0.27 a	0.18 a	-	-
'Lady White'	LW	1.36 de	0.56 b	-	-	-	-
'Lady Vitroflora'	LV	1.40 e	1.11 d	-	-	0.09 a	0.06 a
'Lady Yellow'	LY	1.28 e	0.63 b	0.74 d	0.58 d	-	-

Means in columns for respective study years marked with the same letters do not differ significantly (p = 0.05)

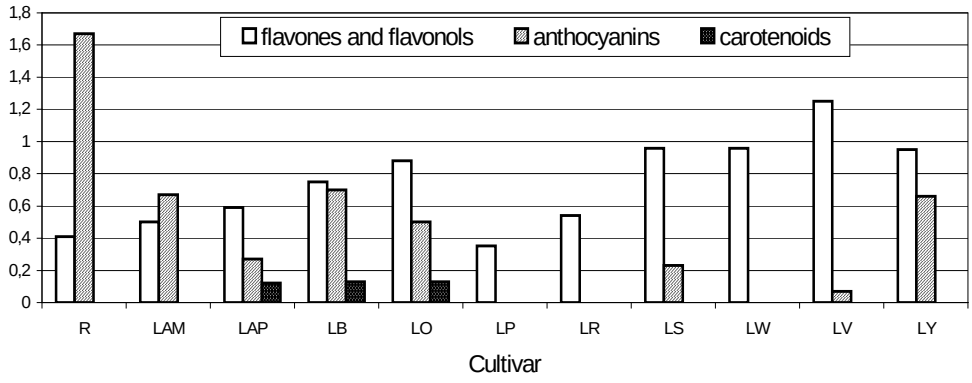

Figure 1. Occurrence of pigments in ray florets of chrysanthemum Lady Group: R -'Richmond', LAM - 'Lady Amber', LAP - 'Lady Apricot', LB - 'Lady Bronze', LO - 'Lady Orange', LP - 'Lady Pink', LR - 'Lady Rosy', LS - 'Lady Salmon', LW - 'Lady White', LV - 'Lady Vitroflora', LY- 'Lady Yellow' (average absorbance of two-year study)

'Richmond' showed only flavonoids (anthocyanins, flavones and flavonols), however no carotenoids. Carotenoids occurred in six out of ten mutants obtained from that cultivar ('Lady Amber', 'Lady Apricot', 'Lady Bronze', 'Lady Orange', 'Lady Salmon' and 'Lady Yellow'). In six other mutants anthocyanins disappeared ('Lady Amber', 'Lady Pink', 'Lady Rosy', 'Lady Salmon', 'Lady White' and 'Lady Yellow'). In the other four, in which anthocyanins occurred, there was a considerable decrease in the value of absorbance as compared with the original cultivar. Three mutants, namely 'Lady Apricot', 'Lady Bronze' and 'Lady Orange', did not differ in their value of absorbance for anthocyanins. All cultivars showed flavones and flavonols, but higest level of absorbance was observed in mutants: 'Lady Salmon', 'Lady White', 'Lady Vitroflora' and 'Lady Yellow'.

Values of concentrations of carotenoids and anthocyanins in ray florets of the chrysanthemum cultivars studied per 1 g of fresh matter are presented in Table 2. The lowest concentration of carotenoids over the two study years was recorded in 'Lady Salmon', while the highest value in 1999 in 'Lady Amber', 'Lady Bronze' and 'Lady Yellow', while in 2000 'Lady Bronze' showed a slightly higher level of carotenoids than 'Lady Amber' and 'Lady Yellow'. Out of all the cultivars in which anthocyanins occurred, the lowest level was recorded over two study years in 'Lady Vitroflora', while the highest in the original cultivar 'Richmond'.

Table 2. Concentrations of carotenoids and anthocyanins [mg] in ray florets of chrysanthemum Lady Group per 1g of fresh matter over 1999 and 2000

Cultivar	Carotenoids		Anthocyanins	
	Years			
	1999	2000	1999	2000
'Richmond'	-	-	228.0 c	43.5 c
'Lady Amber'	36.5 d	26.4 d	-	-
'Lady Apricot'	14.5 b	11.0 b	12.5 b	6.5 b
,Lady Bronze'	35.7 d	29.8 e	15.0 b	6.5 b
'Lady Orange'	25.1 c	21.6 c	15.5 b	5.5 b
'Lady Pink'	-	-	-	-
'Lady Rosy'	-	-	-	-
'Lady Salmon'	12.7 a	8.6 a	-	-
'Lady White'	-	-	-	-
'Lady Vitroflora'	-	-	7.0 a	4.5 a
'Lady Yellow'	34.5 d	27.3 d	-	-

Means in columns for respective study years marked with the same letters do not differ significantly ($p = 0.05$)

However, in 2000 it was over five-fold lower than in 1999. In the other cultivars the concentration of anthocyanins was also lower in 2000, but it was two- or three times lower.

Each of the chrysanthemum cultivars studied showed its permanent repetitive profile of the occurrence of specific pigments at characteristic wavelengths, which was confirmed over two-year study. A change was recorded only in the general level of absorbance values; in 2000 it was generally lower than in 1999. Even though in some cultivars the same pigments were recorded (e.g. mutants 'Lady Pink' and 'Lady Rosy' showed flavonols only), their content in inflorescence differed significantly in each study year, which allowed for differentiating the cultivars from one another.

DISCUSSION AND CONCLUSIONS

The present study, which investigated the pigments in the inflorescence of eleven chrysanthemum cultivars of the Lady Group, applied the spectrophotometric method first developed by Shibata (1958). An extensive work into the flower colour based on the spectral analysis of intact florets of 68 chrysanthemum cultivars was carried out by Kawase and Tsukamoto (1974, 1976). The authors divided the cultivars studied into four groups, depending on the occurrence of characteristic absorption maxima showing the presence of specific pigments as well as measured both their quality and quantity.

The main anthocyanidin pigment, which occurs in chrysanthemum, is cyanidin 3-glucoside, also known as chrysanthemin, identified for the first time by Willstätter and Bolton (1916, cited in Kawase et al. 1970). Later study (Saito et al. 1988) showed that the pigment in question was cyanidin 3-malonyloglucoside. The analysis with the modern apparatus using the nuclear magnetic resonance (NMR) spectra and mass spectrometry showed that the main anthocyanidin of all the purple pink chrysanthemums is cyanidin 3-dimalonyloglucoside in its native form (Nakayama et al. 1997). In five out of eleven chrysanthemum cultivars studied in the Lady Group at present there was an absorption maximum, which indicates the presence of this anthocyanin in ray florets.

The biosynthesis of flavonoids (anthocyanins, flavones and flavonols) depends on the presence of the key enzyme, namely flavonoid 3'-hydroxylase, which conditions whether cyanidin and quercetin or pelargonidin and kaempferol are produced (Biolley et al. 1994). In chrysanthemums flavonoid 3'-hydroxylase enzyme is active and, as a result, the dihydrokaempferol produced is completely transformed into dihydroquercetin and further into cyanidin. There exists, therefore, no possibility for pelargonidin to be produced. Only by blocking the activity of the gene responsible for the production of flavonoid 3'-hydroxylase enzyme is there a possibility of producing pelargonidin - a new anthocyanin for chrysanthemums. A genetic modification of the biosynthesis pathway by the inhibition of the activity of this hydroxylase makes it possible to produce new cultivars of another inflorescence colour (Schwinn et al. 1994). The goal can be reached with the application of genetic engineering (Mol et al. 1999, Aida et al. 2000, Cadic and Widehem 2001, Zaccai et al. 2001) or mutation breeding (Broertjes et al. 1976, Jerzy et al. 1991, Banerji and Datta 1992, Zalewska and Jerzy 1997).

The mutants studied were obtained as a result of ionising radiation, which is the second method mentioned. A colour change in mutants obtained in that way involved both the qualitative and the quantitative pigments modifications. The original cultivar, 'Richmond', that the Lady Group originated from, showed a high level of anthocyanins. Of the mutants obtained in that group, in six there was observed a disappearance of anthocyanins, in the other four a considerable decrease in the value of absorbance was recorded, which shows a decrease in the content of this pigment in inflorescence. It can be assumed that ionising radiation in most mutants resulted in a partial or complete inactivation of genes participating in the biosynthesis of these pigments.

There are known genes responsible for the production of enzymes of biosynthesis of anthocyanins. In many cases a single gene mutation results in the accumulation of indirect compounds, which leads to a change in flower or seed colour (Onozaki et al. 1999, Selinger and Chandler 1999, Kobayashi et al. 2001).

Mutation can also involve proteins (GS-X) transporting anthocyanins through membranes into the vacuole where they accumulate.

The other essential plant pigments are carotenoids. In chrysanthemums they are found in ray florets in a form of 5,8-epoxides. The present study shows that in six Lady Group mutants carotenoids which were not present in the original cultivar 'Richmond' were recorded.

A study into colour inheritance in chrysanthemum was carried out by Langton (1980, 1989), Teynor et al. (1989 a and b) and by Hattori (1991, 1992). Langton (1989) suggests that white- and pink-flowering chrysanthemum cultivars which exhibited or did not exhibit anthocyanins have a dominant gene I – carotenoids biosynthesis inhibitor. In that simple way the author explains a genetic control of the carotenoids pigments expression in epidermis of ray florets in chrysanthemums. The blockade of gene I activity makes the production of carotenoids possible in yellow, orange or brown cultivars. Hattori (1991) adds that, besides the carotenoids-biosynthesis-controlling inhibitor gene there must exist another dominant gene responsible for their biosynthesis, which shows that it is enough to block the inhibitor gene in order to obtain cultivars showing a capacity for biosynthesis of carotenoids. Therefore it cannot be excluded that a change in colour in the chrysanthemum mutants obtained was due to the inhibitor gene mutation, which led to an appearance of carotenoids absent in the original cultivar, 'Richmond', in six out of the ten mutants obtained in the Lady Group. It is therefore very likely that in the original cultivar 'Richmond' carotenoids biosynthesis genes were blocked by inhibitor gene.

In all the cultivars studied there were different flavones and flavonols. According to Stich et al. (1997) in chrysanthemum (*Chrysanthemum segetum* L.) inflorescence there occur quercetin, kaempferol and gossypetin glucoside, while Hu et al. (1994) reports on acacetin, apigenin, luteolin and quercetin glucoside in *Chrysanthemum morifolium* Ramat ray florets. All that suggests that the mutation in the mutants obtained resulted in changes in genes of flavonoids biosynthesis involving an inactivation of any of the enzymes on the biosynthesis pathway.

Obtaining new inflorescence colours with the use of mutation seems to be related to a genetic material destruction. It can be assumed that in original cultivars genes of biosynthesis of respective pigments are blocked. Blocking genes, the so-called inhibitors, if occurring in a dominant form, effectively block pigments biosynthesis pathways. A destruction of these genes, by the application of e.g. ionising radiation, shows a possibility of obtaining a given pigment in mutant.

The contemporary new cultivar breeding especially values the traits related to a change in plant colour or habit. Owing to the application of induced mutagenesis one can obtain a wide spectrum of inflorescence colour, which has a great importance in breeding new cultivars. The present data confirm the applicability of

the spectrophotometric method to define the changes recorded in mutants exposed to ionising radiation and showing their distinctness as compared with the cultivar they originated from. All the mutants of the Lady Group analysed differed from one another, as well as from the original cultivar over successive study years. The absorption maxima at specific wavelengths remained unchanged, unlike the general level of absorbance, which can be due to different light conditions over successive study years.

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ZMIANY BARWY KWIATÓW U CHRYZANTEMY WIELKOKWIATOWEJ *CHRYSANTHEMUM* × *GRANDIFLORUM* /RAMAT./KITAM. W GRUPIE LADY JAKO WYNIK HODOWLI MUTACYJNEJ

Streszczenie: Dziesięć radiomutantów i jedna odmiana wyjściowa chryzantemy wielkokwiatowej (*Chrysanthemum* x *grandiflorum* /Ramat./Kitam., syn. *Dendranthema grandiflora* Tzvelev) tworzące grupę odmianową Lady były analizowane pod względem zawartości barwników w kwiatostanie metodą spektrofotometryczną. Stwierdzono, że odmiany uzyskane w wyniku działania promieniowania jonizującego różniły się jakością i ilością flawonoidów i karotenoidów w kwiatostanie w stosunku do odmiany 'Richmond', z której powstały. Każda z badanych odmian chryzantem posiadała swój stały i powtarzalny profil występowania określonych barwników, co daje możliwość wykazania odrębności analizowanych odmian oraz ich identyfikację.

Received September 24, 2004; accepted June 15, 2005