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STUDIES ON FLOWER PIGMENTS OF CHRYSANTHEMUM MUTANTS: NERO AND WONDER GROUPS

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Abstract. Two groups of chrysanthemum (*Dendranthema grandiflora* Tzvelev) representing the five cultivars were analysed to define their content of pigments in the inflorescence with the spectrophotometric method.
It was observed that respective cultivars obtained as a result of the ionising radiation differed in their quality and quantity of flavonoids and carotenoids in the inflorescence as compared with original cultivars they originated from. Each of the chrysanthemum cultivars studied showed its own permanent repetitive profile of the occurrence of specific pigments, which gives a possibility of showing the distinctness of the cultivars analysed and their identification.

Keywords: *Dendranthema grandiflora*, flower pigments, radiomutants, spectrophotometry

INTRODUCTION

Chrysanthemum – *Dendranthema grandiflora* Tzvelev (syn. *Chrysanthemum* × *grandiflorum* /Ramat./ Kitam.) is one of the more economically essential ornamental plant species cultivated in the world. Chrysanthemum flowers are arranged into characteristic calathidum whose colour is determined by pigments contained mainly in ligulate florets which can have a varied shape and size. Apart from ligulate florets in inflorescence there are also complete disc flowers which can occur on all of the calathidum area or be concentrated in its middle part forming a characteristic 'eye'. As for the colour of the inflorescence, however, ligulate florets show more important. It is there where, first of all, you can find all the richness of pigments showing the decorative value of a given cultivar.

The greatest group of pigments is composed by flavonoids occurring in vacuoles, including both anthocyanins, flavones and flavonols [Harborne 1988; Hattori 1992]. Anthocyanins are responsible for the following colours: red, blue and violet, depending on

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pH of the cell sap and co-pigments of metals and flavonoids [Jurd and Asen 1966; Takeda et al. 1994]. Flavones and flavonols, however, are responsible for the following flower colours: white, cream and yellow [Williams et al., 1981].

Next to flavonoids, a large very important group of pigments is made up of carotenoids. It is these pigments which occur in plastids, give plants the following colours: yellow, orange and even red [Goodwin 1980].

Today's breeding values especially a development of cultivar groups formed from cultivars of a varied plant colour and habit but with similar growing requirements. The aim can be obtained today with mutation methods which involve inducing mutation in genes and in and frequently give expected results [Broertjes et al. 1976; Jerzy et al. 1991; Jerzy and Zalewska 1997]. An alternative method uses the achievements of genetic engineering, making it possible to introduce a given gene with a vector into the recipient genome. In that way transgenic plants can be obtained with changed features required [Mol et al. 1998, 1999; Griesbach 1998].

Horticulture frequently requires an objective definition of the flower colour and identifying the cultivar, breeding line or mutant. So far the traditional method has been used which compares to the catalogue R.H.S. Colour Chart. An alternative method measures the pigment absorbance with spectrophotometer. The method is easy, universal and cheap as well as complies with the most essential criterion of measurement objectiveness and preciseness.

The study aimed at defining changes in the composition of pigments in inflorescence of two chrysanthemum groups, Nero and Wonder, developed due to induced mutagenesis.

MATERIAL AND METHODS

The Nero and Wonder *Dendranthema grandiflora* Tzvelev cultivar groups were analysed to define the content of pigments in ligulate florets. The Nero group was composed of one dwarf mutant 'Mini Nero'[1] with semifull, miniature orange-red inflorescence and the original cultivar, 'Red Nero' of vigorous growth and semifull, small dark-red inflorescence. The Wonder group was characterised by a medium-size type inflorescence and it was composed of two mutants 'Bronze Wonder'[2] (reddish-brown) and 'Red Wonder'[2] (red) and the original cultivar – 'Lilac Wonder' (violet-pink). All the above mutants were developed as a result of exposure to X or gamma radiation of leaf explants of the original cultivar *in vivo* or *in vitro* applying regeneration with the adventitious shoots method [Zalewska 1995; Jerzy and Zalewska 1997].

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

The shoot cuttings of the chrysanthemums studied were planted in a permanent place on benches in outdoor glasshouse on May 21 1999, and in 2000 in glasshouse on July 10. The cultivation took place under conditions of natural photoperiod with a standard

[1]entered into the Polish Cultivar Register in 1995
[2]entered into the Polish Cultivar Register in 1996

method. The ligulate florets were sampled starting from October 27 to November 10, 1999 and from October

23 to November 17, 2000 over full flowering. Out of the five inflorescences sampled 4 weighed amounts each were prepared; 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.

The tissues were crushed in porcelain mortar with the addition of a ~24 mg of quartz sand. Extracting carotenoids followed the Wettstein method [1957] in which 100% acetone was used with the addition of a ~24 mg of $CaCO_3$, while for anthocyanins – the Harborne method was used [1967] with methanol containing 1% of HCl. The extracts obtained were filtered through funnel with filter paper into 10 ml 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.

Absorption maximums were defined for pigment-specific wavelengths (λ_{max}) for flavones and flavonols from 332 to 344 nm, carotenoids at 440 nm and anthocyanins at 530 nm. There were calculated a mean absorbance at the absorption maximum and the content of carotenoids and anthocyanins per 1 g of fresh matter of ligulate florets. A statistical analysis of the results was carried out with the Student t-test at the level of significance of $\alpha = 0.05$.

The quantitative determination of anthocyanins was possible with the algebraic method following Harborne [1967] at the wavelength $\lambda_{max} = 530$ 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 $\lambda_{max} = 440$ nm.

RESULTS

The qualitative composition of pigments over respective study years expressed in absorbance units for Nero and Wonder group is given in table 1, while mean absorption spectra with characteristic absorption maximums are given in figures 1–4. The presence of specific pigments was observed by the absorption maximum at the characteristic wavelength of λ_{max}, marked with an arrow.

Table 1 presents that ligulate florets of the chrysanthemum cultivars studied show four kinds of pigments: flavones, flavonols, carotenoids and anthocyanins.

The Nero group was formed by the original cultivar 'Red Nero' and its mutant 'Mini Nero'. In this group was observed flavones and flavonols at $\lambda = 332–344$ nm, but level of absorbance was similar in original cultivar and its mutant. The level of absorbance for carotenoids was identical in these cultivars in 1999, while in 2000 it was slightly lower as compared with the previous year. However no significant differences across

cultivars were recorded. There decreased, however, considerably, the value of absorbance for anthocyanins in 'Mini Nero' as compared with the original cultivar 'Red Nero', in 1999 an over four-fold decrease, while in 2000 – threefold.

Table 1. Absorbance of extracts from ligulate florets of *Dendranthema grandiflora* over 1999 and 2000

Tabela 1. Absorbancja ekstraktów z kwiatów języczkowatych chryzantemy wielkokwiatowej w latach 1999 i 2000

Cultivar Odmiana	Code Oznaczenie	Absorption maximum – Maksimum absorpcji					
		λ = 332–344 nm flavones and flavonols flawony i flawonole		λ = 440 nm carotenoids karotenoidy		λ = 530 nm anthocyanins antocyjany	
		years – lata					
		1999	2000	1999	2000	1999	2000
Red Nero	RN	1.29 a	0.52 a	1.07 a	0.89 a	3.36 b	0.77 b
Mini Nero	MN	1.02 a	0.57 a	1.07 a	0.82 a	0.76 a	0.26 a
Lilac Wonder	LIW	0.96 b	0.42 b	-	-	0.77 b	0.28 b
Bronze Wonder	BW	0.69 a	0.36 a	0.84 b	0.67 b	1.35 c	0.57 c
Red Wonder	RW	0.87 b	0.40 b	0.38 a	0.23 a	0.35 a	0.18 a

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

Średnie w kolumnach w poszczególnych latach badań oznaczone tymi samymi literami nie różnią się istotnie ($\alpha = 0,05$)

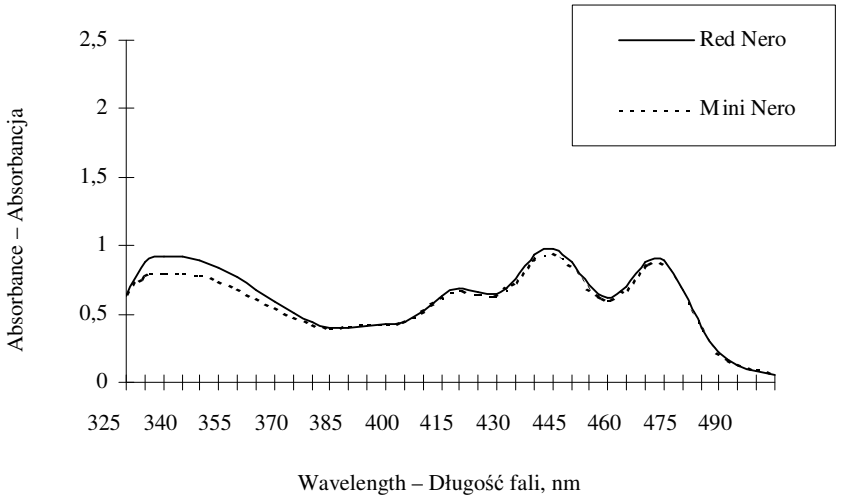

Fig. 1. Absorption spectra with characteristic absorption maxima for flavones and flavonols (λ = 332–344 nm) and carotenoids (λ = 440 nm) of Nero group (mean two-year)

Ryc. 1. Widmo absorpcyjne z charakterystycznymi maksimami absorpcji dla flawonów i flawonoli (λ = 332–344 nm) i karotenoidów (λ = 440 nm) w grupie Nero (średnia z dwóch lat)

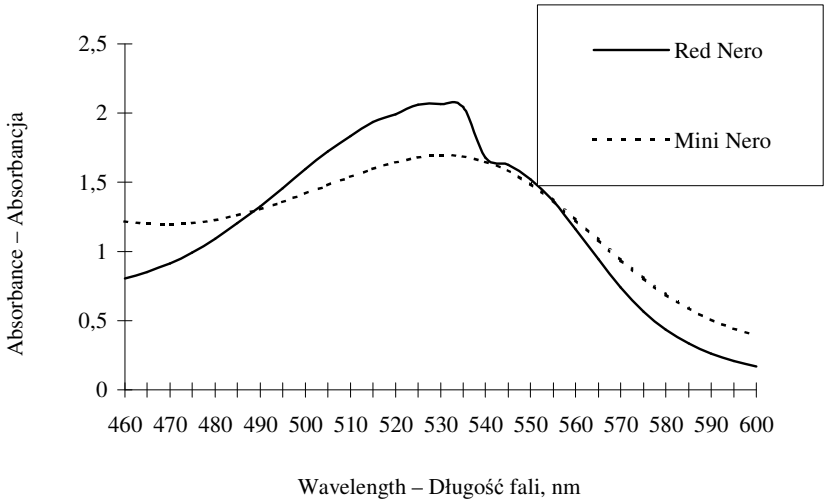

Fig. 2. Absorption spectra with characteristic absorption maximum for anthocyanins (λ = 530 nm) of Nero group (mean two-year)

Ryc. 2. Widmo absorpcyjne z charakterystycznym maksimum absorpcji dla antocyjanów (λ = 530 nm) w grupie Nero (średnia z dwóch lat)

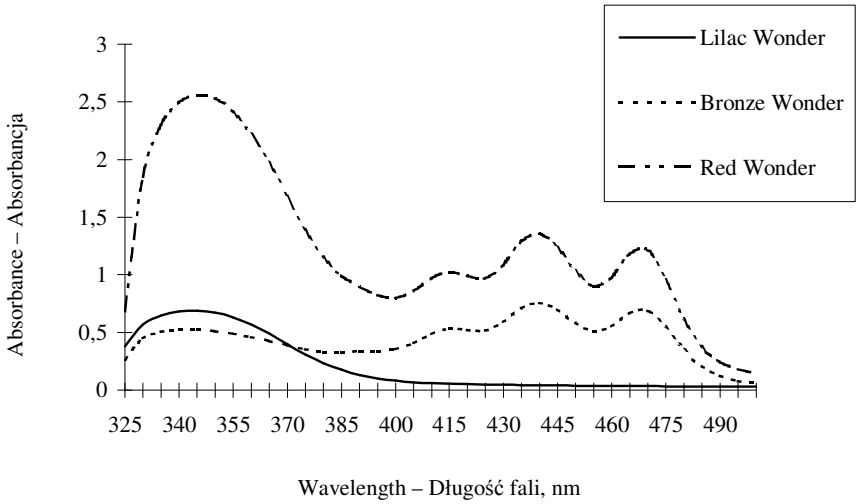

Fig. 3. Absorption spectra with characteristic absorption maxima for flavones and flavonols (λ = 332–344 nm) and carotenoids (λ = 440 nm) of Wonder group (mean two-year)

Ryc. 3. Widmo absorpcyjne z charakterystycznymi maksimami absorpcji dla flawonów i flawonoli (λ = 332–344 nm) i karotenoidów (λ = 440 nm) w grupie Wonder (średnia z dwóch lat)

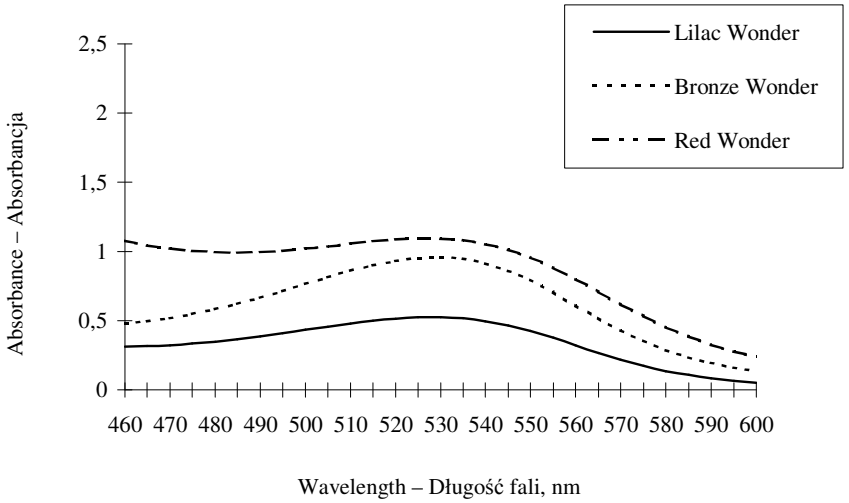

Fig. 4. Absorption spectra with characteristic absorption maximum for anthocyanins (λ = 530 nm) of Wonder group (mean two-year)
Ryc. 4. Widmo absorpcyjne z charakterystycznym maksimum absorpcji dla antocyjanów (λ = 530 nm) w grupie Wonder (średnia z dwóch lat)

The Wonder group is made up of two mutants obtained from the original cultivar 'Lilac Wonder' which did not include carotenoids. All the cultivars of that group showed flavones and flavonols at a characteristic maximum but only 'Bronze Wonder' differed from the other cultivars across the group, the absorbance value. Both the 'Red Wonder' mutant and the 'Bronze Wonder' showed varied absorbance value showing the presence of carotenoids. However the level of anthocyanins increased in 'Bronze Wonder', however decreased in 'Red Wonder' compared with the original cultivar.

Table 2. Concentrations of carotenoids and anthocyanins (mg) in ligulate florets of *Dendranthema grandiflora* per 1 g of fresh matter over 1999 and 2000
Tabela 2. Stężenie karotenoidów i antocyjanów, wyrażone (mg) na 1 g świeżej masy, w kwiatach języczkowatych chryzantemy wielkokwiatowej w latach 1999 i 2000

Cultivar Odmiana	Carotenoids – Karotenoidy		Anthocyanins – Antocyjany	
	years – lata			
	1999	2000	1999	2000
Red Nero	50.4 a	41.8 a	273.0 b	62.5 b
Mini Nero	50.1 a	38.7 a	61.5 a	21.5 a
Lilac Wonder	-	-	64.0 b	22.5 b
Bronze Wonder	39.6 b	31.6 b	109.0 c	46.0 c
Red Wonder	17.8 a	11.0 a	28.5 a	14.5 a

Means in columns for respective study years marked with the same letters do no differ significantly (α = 0.05)
Średnie w kolumnach w poszczególnych latach badań oznaczone tymi samymi literami nie różnią się istotnie (α = 0,05)

The relationship between the values of concentrations and anthocyanins in ligulate florets of the chrysanthemums are given in table 2. The values of concentration exactly reflect the absorbance values at the wavelength of $\lambda = 440$ nm for carotenoids and $\lambda = 530$ nm for anthocyanins.

All the mutants obtained from original cultivars differed from one another and as compared with the original cultivar in their absorbance value and in the wavelength at which the maximum occurred. It was only the total level of the absorbance value which changed; in 2000 it was generally lower than in 1999. Even if in some cultivars there was recorded the same group of pigments, the values of absorbance at the same wavelengths differed significantly.

DISCUSSION

The present study confirm the applicability of the spectrophotometric method to indicate the uniqueness of mutants as compared with the original cultivars and their identification. All the mutants obtained from original cultivars differed from one another and against the original cultivar. Each cultivar of the original cultivars and their mutants showed its fixed and repetitive profile of occurrence of specific pigments at characteristic wavelengths. Over successive study years it was only the total level of absorbance which was changed, while absorption maximums at specific wavelengths remained unchanged, which can be due to different light conditions over respective-year cultivation.

The main anthocyanidin pigment which occurs in chrysanthemums is cyanidin 3-glucoside, isolated amongst others by Kawase et al. [1970]. Further research showed that all the purple – pink chrysanthemums have in their native form cyanidin 3-dimalonyloglucoside [Nakayama et al. 1997].

Depending on the presence of the key enzyme of biosynthesis of flavonoids – flavonoid 3'-hydroxylase, cyanidin and quercetin or pelargonidin and kaempferol will be obtained [Biolley 1994]. In chrysanthemums the enzyme of flavonoid 3'-hydroxylase is active and so the dihydrokaempferol produced is completely transformed into dihydroquercetin and further into cyanidin. There are therefore no possibilities for the pelargonidin to be obtained. Only by blocking the activity of the gene responsible for the production of responsible for obtaining the enzyme of flavonoid 3'-hydroxylase is it possible to produce pelargonidin – a new anthocyanin for chrysanthemums. A genetic modification of the biosynthesis path by the inhibition of the activity of this hydroxylase makes it possible to produce new cultivars of a different colour of inflorescence [Schwinn et al. 1994]. The aim can be achieved by applying 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; Zalewska 1995].

The mutants studied were obtained due to the exposure to ionising radiation, which is the second of the methods mentioned. A change in colour in the mutants obtained that way involves both the qualitative and the quantitative modifications of the pigments. The Nero group observed a considerable decrease in the content of anthocyanins in the mutant obtained, while in the Wonder group in 'Bronze Wonder' mutant there occurred

an increase in the content of anthocyanins, while in 'Red Wonder' – a decrease. One can assume that ionising irradiation in 'Mini Nero' and 'Red Wonder' mutants resulted in a partial inactivation of the genes participating in the biosynthesis of these pigments. What seems interesting, however, is an almost twofold increase in the content of anthocyanins in 'Bronze Wonder' as compared with the original cultivar, which can be related with an increase in the number of chromosomes on this mutant by nine [Lema-Rumińska and Zalewska 2002].

Currently there are known genes which are responsible for the production of enzymes of biosynthesis of anthocyanins. In many cases the mutation in a single gene causes an accumulation of intermediary compounds, which results in a change in the flower or seed colour [Onozaki et al. 1999; Kobayashi et al. 2001]. The mutation can also concern proteins acting as intermediary (GS-X) in the transport of anthocyanins through membranes to vacuole where they are accumulated.

In *Saintpaulia ionantha* there were identified as many as eight genes controlling the flower colour. Two of them (I and S) are responsible for the inhibition of genes of biosynthesis of anthocyanins and are present as dominant allele in white flowers. The other genes are responsible for the colour intensity [Griesbach 1998].

Obviously we do not know all the factors controlling genes of biosynthesis of anthocyanins. Similarly the mechanism of the activity of regulatory proteins has not been yet well known and calls for further numerous study. However today it is possible to modify the flower colour due to the application of genetic engineering. It is becoming feasible to develop in that way blue-flower roses due to genetic manipulation by introducing genes of synthesis of flavonoids or genes responsible for the value of vacuolar pH [Mol et al. 1998]. Blue flowers have already been obtained in transgenic *Torenia fournieri* Lind. by inactivating the gene of dihydroflavonol 4-reductase, which resulted in the accumulation of indirect metabolites in a form of flavonols [Aida et al. 2000]. It has been known for a long time that the colour depends not only the pigments but also other factors, including pH [Griesbach 1992; Dangles et al. 1993], co-pigments of flavones, flavonols and metals [Griesbach 1992]. Light and temperature which can stimulate the biosynthesis of anthocyanins also affect the plant colour [Griesbach 1992; Dangles et al. 1993]. There have been already known genes responsible for these factors. *Petunia hybrida*, for instance, was identified with as many as seven genes responsible for the value of osmotic pH (genes ph-1 – ph-7), gene encoding the synthesis of flavonol (Fl), or gene regulator of light LRU-1 [Mol et al. 1998]. Thanks to that knowledge there have already been developed yellow-flowering petunias in which the gene responsible for the synthesis of the enzyme of chalcon isomerase was not functional [Mol et al. 1999].

Another important group of pigments which occur in plants are carotenoids which occur in ligulate florets in a form of 5,8-epoxides. Carotenoids are synthesised from a specific precursor – melavonic acid from which by numerous transformations of pyrophosphates a compound of four carbon atoms – phytoene is obtained. The compound gives rise to the basic red colour – lycopene which undergoes cyclising which results in a production of specific rings. Most frequently in plants a few different carotenoids occur (α, β, λ), creating a specific mixture [Goodwin 1980].

The present studies show that the Wonder group mutants identified carotenoids which were not observed in 'Lilac Wonder' original cultivar. In the Nero group

a change in colour did not cover carotenoids as in the original cultivar and its mutant there was recorded the same content of the pigment. Numerous enzymes have already been isolated which are responsible for successive stages of the carotenoids biosynthesis [Giuliano et al. 1993; Bouvier et al. 1998]. Similarly there have been clonned numerous genes responsible for the biosynthesis of these important pigments; most frequently genetic manipulations concern the gene of synthesis of phytoene which catalyses the key stage of their production [Hirschberg 2001].

The studies into the inheritance of the colour in chrysanthemums was carried out by Langton [1980, 1989], Teynor et al. [1989a, 1989b] and by Hattori [1991, 1992]. Langton [1989] suggests that white- and pink-flowering chrysanthemum cultivars with or without anthocyanins have dominant gene I – inhibitor of carotenoids biosynthesis. In that simple way the author explains a genetic control of carotenoid pigment expression in ligulate floret epidermis in chrysanthemums. Blocking the activity of gene I makes it possible to produce carotenoids in yellow, orange or brown cultivars. Hattorii [1991] adds that except for the gene-inhibitor controlling biosynthesis of carotenoids there must exist the other dominant gene responsible for their biosynthesis. All of that shows that it is enough to block gene-inhibitor to obtain cultivars capable of carotenoids biosynthesis. A change in colour in the chrysanthemum mutants obtained could have been due to the mutation of gene-inhibitor which in the mutants obtained in the Wonder group there were observed carotenoids which were absent in 'Lilac Wonder', the original cultivar. It is, therefore, highly probable that 'Lilac Wonder' original cultivar showed genes of biosynthesis of carotenoids blocked by gene-inhibitor. One can assume, that in cultivars in which there was recorded a decrease in the content of anthocyanins in ligulate florets mutation occurred in the gene responsible for the production of any of the enzymes of biosynthesis of anthocyanins.

The development of new colours of inflorescence due to mutation seems to be related with a destruction of the new genetic material. It can be assumed that in original cultivars covered by the present study the genes of biosynthesis of respective pigments are blocked. Blocking genes the so called inhibitors, if they occur in a dominant form effectively block paths of pigments biosynthesis. A destruction of these genes by the application of, e.g. ionising radiation, shows a possibility of developing a given pigment in the mutant.

The colour inheritance in chrysanthemums is additionally complicated by the fact that the colour is determined by two layers of cells L1 and L2, out of which ligulate florets are made up of. Carotenoids occurring in chromoplasts can be present in two layers, however the occurrence of anthocyanins is only limited to vacuoles of epidermis [Langton 1980; Hattori 1992]. It is possible that in most chrysanthemums are periclinal chimeras [Hattori 1991; Malaure et al. 1991; Hattori 1992; Shibata et al. 1998], which makes it more difficult to understand the mechanisms of transferring genetic information on inflorescence colour.

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BADANIA NAD WYSTĘPOWANIEM BARWNIKÓW W KWIATACH U MUTANTÓW CHRYZANTEMY W GRUPACH: NERO I WONDER

Streszczenie. Dwie grupy chryzantemy (*Dendranthema grandiflora* Tzvelev) reprezentowane przez pięć odmian były analizowane pod względem zawartości barwników w kwiatostanie metodą spektrofotometryczną.

Stwierdzono, że poszczególne odmiany uzyskane w wyniku działania promieniowania jonizującego różniły się jakością i ilością flawonoidów i karotenoidów w kwiatostanie w stosunku do odmian wyjściowych, z których 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.

Słowa kluczowe: *Dendranthema grandiflora,* barwniki kwiatowe, radiomutanty, spektrofotometria

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