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ACCUMULATION OF TOTAL ANTHOCYANINS IN WHEAT GRAIN

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In the recent years, for specific goals of utilization, winter wheat breeding has been aimed on increasing total anthocyanins concentration in winter wheat grains considering their high antioxidant activity. The aim of research was to evaluate grain colour development in four wheat genotypes (ANK 28A and 62/0 purple pericarp, UC 66049 blue aleurone and Ilona red pericarp) during grain filling period. Grain samples from two replications of field experiment, established in the vegetation 2010/11, were taken in five to six sampling times. Total anthocyanins concentration was determined by spectrophotometer. The genotypes responded differently to the dynamics of total anthocyanins accumulation during grain filling. The process was described by linear and also by polynomial regression on the number of days post anthesis. Genotypes with purple pericarp reached the highest total anthocyanins concentration on the 22nd day post anthesis with increasing and decreasing before and after this sampling time, respectively. At maturity the highest total anthocyanins had UC 66049 (193.38 mg/kg). Newly bred genotype 62/0 had similar concentration (34.50 mg/kg) as its parent ANK 28A (37.80 mg/kg). At maturity, registered cultivar Ilona was about 93.7% lower in total anthocyanins concentration compared to ANK 28A. Significant variability in total anthocyanins concentration indicated that breeding for their increasing is possible.

Key words: wheat, grain color, anthocyanins, breeding

Anthocyanins are generally specified as bioactive, non-nutritional compounds, responsible for antioxidant (Kong *et al.* 2003) and UV/photoprotective functions (Ryan *et al.* 2001). They have been shown to have some beneficial health effects on oxidative damage (Prior & Wu 2006) and dispose of higher antioxidant activity than vitamins C and E and appear to have a synergic effect on vitamin C and other flavonoids (Duthie *et al.* 2006). Beneficial effect of antioxidants on health promoting is believed to be achieved through several possible mechanisms, such as directly reacting with and quenching free radicals, chelating transition metals, reducing peroxides, as well as stimulating the antioxidative defense enzyme system (Kong *et al.* 2003).

Epidemiological studies reveal that number of diseases including cancer, diabetes, Alzheimer's disease, coronary heart diseases, and aging have been found to be associated with oxidative stress (Baublis *et al.* 2000). Anthocyanins have gained attention due to their anti-inflammatory (Bowen-Forbes *et al.* 2010), antimutagenic and anticarcinogenic properties (Wang & Stoner 2008). Other biological activities are antibacterial, hepatotoxicity and induction of apoptosis (Mazza 2007).

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Wheat cultivars with high level of anthocyanins have potential for using as a dietary source of bioactive material to prevent diseases and promote health in the functional food industry (Revanappa *et al.* 2011). Anthocyanins participate in the formation of non-specific disease resistance in plants (Treutter 2006) and plant protection against biotic and abiotic stress factors (Khlestkina *et al.* 2011). The environment, such as high light intensity, low temperature, high salinity and/or drought stress, affects their production (Chalker-Scott 1999).

Blue, purple, red, white, or orange coloration of plant tissues and organs in wheat and other cereals is done by anthocyanins (Abdel-Aal *et al*. 2006). Anthocyanins are flavonoids classified within phenolic phytochemicals (Liu 2004). They represent a big group of water soluble natural colorants and from the structural point of view anthocyanins are glycosides composed of polyhydroxyled or polymethoxyled 2-phenylbenzopyrilium skeleton with hydroxyl and methoxyl groups in the B-ring. The structural variation extends bounded sugars such as pentoses-xylose, arabinose, rhamnose, fructose and hexoses-galactose, glucose (Hosseinian *et al.* 2008). The most occurred anthocyanins in wheat grain are: cyanidin (responsible for the red color), delphinidin (blue color), peonidin (blue color), pelargonidin (orange and red color), petunidin (purple color), and malvidin (purple color) (Oomah & Mazza 1999). The amount of anthocyanins is rapidly increased during maturing; nevertheless in maturity the amount drops (Knievel *et al.* 2009).

The aim of research was to evaluate the development of the grain color during grain filling in selected wheat genotypes of different grain color.

MATERIAL AND METHODS

Selected winter wheat genotypes were evaluated in the field experiment established in the Plant Production Research Center Piestany by randomized complete block design in two replications in the vegetation 2010/11. Evaluated set consisted of ANK 28A, spring form, purple pericarp, which is the isogenic line of Novosibirskaya 67; UC 66049, spring form, blue aleurone, that originated from USA (Qualset *et al.* 2005); newly bred genotype 62/0, winter form, purple pericarp, that originated from breeding of color wheat on the Research and Breeding Station at Viglas-Pstrusa and control winter wheat cultivar Ilona with standard red grain color. From milk to grain physiological maturity according to procedure published by Knievel *et al.* (2009) (in the interval from 4 to 10 days) at least eight spikes (from each replication) were harvested and only kernels from the middle part were hand threshed and stored. The first sampling time was expressed by the number of days from anthesis to the beginning of milk maturity. Due to torrential rains at the beginning of July sampling time was not carried out in the regular intervals. Harvested grain samples were dried at the temperature 40°C. Selected grain yield formation traits were evaluated during vegetation and maturity – stand of height, one thousand grain weight (TGW), portion of grain over the sieve > 2.5 mm (PGS) and volume weight.

Extraction of anthocyanins from wheat grain bran was accomplished according to the method described by Hosseinian *et al.* (2008). A ratio of wheat bran and solvent (methanol:1M HCl, 85:15, v/v) of 1:8 was used to extract anthocyanins. The samples were shaken at 300 rpm for 45 min at room temperature and extracted twice. After extraction, the supernatants were collected. The crude wheat bran extract was separated by centrifuging at 5,000 rpm for 15 min. The supernatant was concentrated under vacuum to dryness at 45˚C, resolved in methanol (15 mL) and stored at 4˚C.

Determination of total anthocyanins using the pH differential method. Total anthocyanins were measured by the method adapted from Fuleki and Francis (1968). Two dilutions of samples were prepared: one for pH = 1.0 using a potassium chloride buffer, and the other for pH = 4.5 using a sodium acetate buffer. The absorbance of each sample was measured at 520 and 700 nm against distilled water as blank. The total anthocyanin concentration was expressed as cyanidin-3-glucoside equivalents (mg/dm^3):

$$\frac{A \times MW \times DF \times 10^3}{\varepsilon \times l}$$

where: A (the absorbance) = $(A_{520}–A_{700})_{pH1} – (A_{520}–A_{700})_{pH4.5}$, MW = 449.2 g/mol for cyanidin-3-glucoside, DF = dilution factor, ε = 26,900 molar extinction coefficient in L/mol/cm, l = pathlength in cm^{-1}, and 10^3 = factor for conversion from g to mg.

The concentration of total anthocyanins was re-calculated to mg/kg.

The data were analyzed by Statgraphics plus for Windows. Analysis of variance was conducted on the total anthocyanins concentration with sampling time as the factor and on the grain yield formation traits with genotype as the factor. Simple linear and polynomial regression response equations were determined for total anthocyanins concentration on the number of days post anthesis for each genotype.

RESULTS AND DISCUSSION

Genotype 62/0 was 8 days later in heading and anthesis compared to cultivar Ilona; however, milk maturity was recorded one day earlier (Table 1). Genotypes of winter forms reached the physiological maturity in the same number of days from anthesis (42), during which spikes were sampled six times to determine total anthocyanins accumulation in the grain. Out of spring forms, the physiological maturity was earlier reached in genotype ANK 28A (10 days earlier compared to winter forms) and two days later in genotype UC 66049.

By visual observation we found out that blue pigmentation of genotype UC 66049 started in the basal part of grain, near by the embryo; and the whole grain was blue on the 24th day post anthesis (Table 1). Intensity of blue coloring had an increasing trend until its maturity. In genotypes with purple color of the seed, coloring was firstly expressed in apical part of the seeds on the 12th and 17th day post anthesis for genotypes ANK 28A and 62/0, respectively. Genotype ANK 28A had on the third sampling time (22 days post anthesis) purple pericarp, but in genotype 62/0 the purple color of grain was observed only on the 27th day post anthesis (Table 2).

As expected, the highest total anthocyanins concentration in mature grain was observed in blue wheat UC 66049 (193.38 mg/kg) (Table 2), which is in accordance with the finding of Abdel-Aal *et al.* (2006), who indicated higher level of anthocyanins extracted from

T a b l e 1

Dates of spikes sampling time of spring* and winter wheat genotypes expressed by number of days post anthesis

Genotype/cultivar	Date		Sampling time					
	heading	anthesis	1	2	3	4	5	6
ANK 28A*	June, 5, 2011	June, 12, 2011	8	12	22	26	32	–
UC 66049*	June, 3, 2011	June, 10, 2011	6	10	14	24	28	34
62/0	May, 24, 2011	May, 31, 2011	13	17	22	27	34	42
Ilona	May, 16, 2011	May, 23, 2011	14	18	25	30	35	42

T a b l e 2

Mean concentration of total anthocyanins [mg/kg] of spring* and winter wheat genotypes according to sampling time

Genotype/ cultivar	Sampling time**						$\bar{x}$	$LSD_{0.05}$
	1	2	3	4	5	6		
ANK 28A*	40.47	95.33	291.07	55.75	37.80	–	104.08	42.14
UC 66049*	14.61	117.88	50.18	171.37	173.29	193.38	120.12	28.74
62/0	124.09	91.83	179.53	81.00	56.32	34.50	94.54	28.19
Ilona	8.74	13.36	6.28	6.73	3.10	2.37	6.76	1.90

**see Table 1

wholemeal flour or bran of blue wheat compared to purple. The second highest total anthocyanins in mature grain was detected in genotype ANK 28A (37.80 mg/kg). The success of our local breeding was also by the fact that genotype 62/0 reached the high total anthocyanins (34.50 mg/kg), which is comparable with parental line ANK 28A. Notable traits and properties of new genotype are similar to productivity of winter genotypes (Table 4), corresponding to the current cultivars registered and grown in the Slovak conditions. Ilona had in its maturity about 93.1% lower amounts of total anthocyanins compared to genotype 62/0 (Table 2). Significant variability in total anthocyanins in mature grains indicates that breeding for increasing its concentration is possible, which was also confirmed on a broader set of colored wheat by Knievel *et al.* (2009).

Both genotypes with purple color of grain had the highest anthocyanins on the 22nd day post anthesis with a gradual increase and decrease before and after this sampling time (Figure 1, Table 2) mainly observed by genotype ANK 28A. Unexpectedly the second highest total anthocyanins was observed during the grain filling (124.09 mg/kg) in genotype 62/0 on the 13th day post anthesis (beginning of milk maturity) (Table 2). Genotypes with purple color of grain had approximately the

T a b l e 3

Simple linear and polynomial regression of total anthocyanins (y) on the number of days post anthesis (x) of spring* and winter wheat genotypes

Genotype/cultivar	Simple linear response equation	R^2
ANK 28A*	$y = 97.85 + 0.31x$	0.08
UC 66049*	$y = 5.93 + 5.90x$	77.62^{++}
62/0	$y = 174.52 – 3.01x$	42.84^{++}
Ilona	$y = 15.66 – 0.32x$	69.62^{++}
	Polynomial response equation	
ANK 28A*	$y = 290.55 + 50.00x – 1.26x^2$	55.18^{++}
UC 66049*	$y = –26.7323 + 10.3498x – 0.11x^2$	79.05^{++}
62/0	$y = 106.18 + 2.73x – 0.10x^2$	46.57^{++}
Ilona	$y = 13.91 – 0.18x – 0.002x^2$	69.91^{++}

$^{++}P < 0.01$

T a b l e 4

Average values of selected traits of spring* and winter wheat genotypes in 2011

Genotype/cultivar	Stand of height [cm]	TGW [g]	PGS [%]	Volume weight [%]
ANK 28A*	110^{d}	46.44	90.56^{b}	95.06^{d}
UC 66049*	100^{b}	44.65	95.04^{c}	88.36^{a}
62/0	106^{c}	47.75	93.93^{c}	90.62^{b}
Ilona	88^{a}	44.84	87.75^{a}	92.84^{c}
$\bar{x}$	101	45.92	91.82	91.72
$LSD_{0.05}$	2.384	–	2.275	2.080

TGW – one thousand grain weight, PGS – portion of grain over the sieve > 2.5 mm
Different letters within the same column of each trait indicate significant differences at $P < 0.05$

same concentration of total anthocyanins in the second sampling time; however, they differed in grain development (12 and 17 days post anthesis, respectively). Although the difference in total anthocyanins between ANK 28A and 62/0 in the third sampling time was 111.54 mg/kg against genotype 62/0, in the maturity it was minimized (only 3.3 mg/kg). In both genotypes, the highest reduction in total anthocyanins was on the 26th and 27th day post anthesis compared to the former sampling time and it was of 80.8% for ANK 28A and 54.9% for 62/0. Knievel *et al.* (2009) reported that reduction of anthocyanins may be a result of dry matter accumulation in endosperm which is much faster than anthocyanins storage in aleurone and pericarp. In the second sampling time, which was 10 days post anthesis for blue grain genotype UC 66049, we unexpectedly found about eight times higher concentration of anthocyanins (117.88 mg/kg) compared to the first sampling time (6 days post anthesis). On the third sampling time (14 days post anthesis), the concentration was reduced by 42% (Figure 2, Table 2), too. The reason of these differences is not clear yet; it is possible to explain it by subjective estimation of beginning of milk ripeness or variation of plants within a genotype. Bustos *et al.* (2012), for example, found out that grain position (in purple wheat genotype) affected grain weight and anthocyanin concentration, decreasing at distal position.

In the evaluated set, each genotype as well as cultivar Ilona responded differently to the dynamics of total anthocyanins accumulation during grain filling. Process of anthocyanins accumulation was described by linear and also by polynomial regression on the number of days post anthesis (next number of days) (Table 3).

At ANK 28A, dynamics of total anthocyanins accumulation described by linear regression on the number of days reached very low determination ($R^2 = 0.08$); however, by polynomial regression the de-

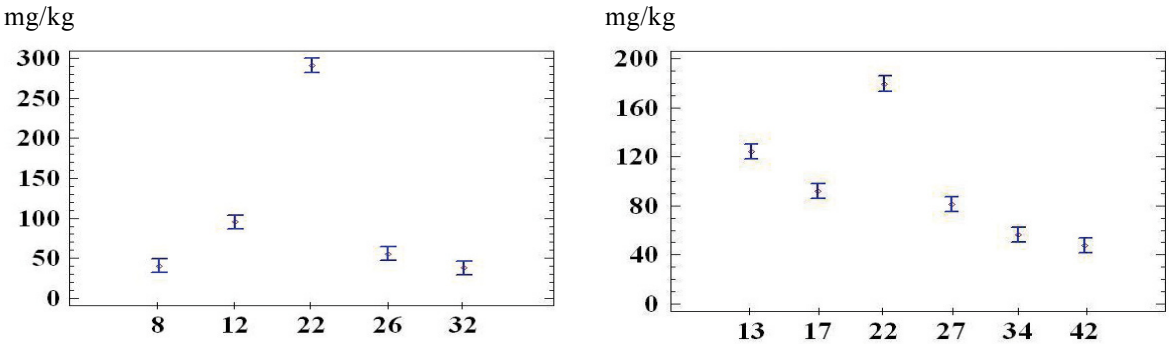

Figure 1. Trend of anthocyanins accumulation from the milk to physiological maturity (x = days post anthesis) in genotypes ANK 28A (left) and 62/0 (right) of spring and winter wheat, respectively

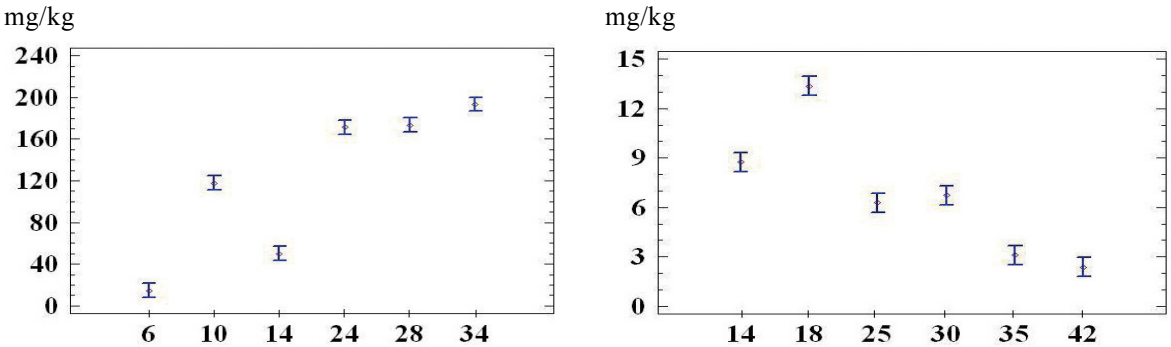

Figure 2. Trend of anthocyanins accumulation from the milk to the physiological maturity (x = days post anthesis) in genotype UC 66049 (left) and cultivar Ilona (right) of spring and winter wheat, respectively

termination was increased on the average ($R^2 = 55.18^{++}$) and after adjustation was particularized only on $R^2_{adj} = 49.91^{++}$ (not shown). Medium degree of determination was caused by high data dispersion from polynomial curve. On analysis it resulted that except number of days post anthesis another unknown factors affected dynamics of total anthocyanins accumulation. At UC 66049 genotype total anthocyanins accumulation was possible almost equally described by linear and polynomial regression on the number of days. Deviations of individual anthocyanins data from linear curve were relatively low ($r = 0.881^{++}$) with the highest determination coefficient from evaluated set ($R^2 = 77.62^{++}$). According to the linear regression in UC 66049, total anthocyanins concentration was increased from the beginning of milk maturity by 5.906 mg/kg per day and maximum value reached in grain physiological maturity. Although process of total anthocyanins accumulation is possibly deceptive to be described by linear and also polynomial regression on the number of days, parameter x^2 was not significant and therefore linear model was valid. Similar process of total anthocyanins accumulation was at genotype 62/0, where high total anthocyanins were detected at the beginning of milk maturity with consecutive reduction $–3.01^{++}$ mg/kg per day. At cultivar Ilona, determination of degree of total anthocyanins accumulation was equally described by linear and polynomial regression on the number of days, but linear one is valid as neither linear nor quadratic component was significant. Decreasing of anthocyanins concentration per one day at cultivar Ilona was 10 times lower than at genotype 62/0 ($b = –0.32^{++}$).

Generally, different dynamics of total anthocyanins accumulation during grain filling can be also caused by different anthocyanins concentration in mature grain. For example at Ilona, average total anthocyanins concentration during grain filling was only 6.76 mg/kg (Table 2) and the maximum value was only two times higher in the second sampling time, which explained low value of regression coefficient (Table 3). On the contrary, genotype 62/0 had 14 times higher anthocyanin concentration in the first sampling time and equally in average during grain filling compared to cultivar Ilona. Effect of weather conditions during grain formation is also considerable. Heimler *et al.* (2010) found out in the set of old and modern durum and soft cultivars that temperature conditions 30 days before harvesting were the principal factor which differentiated the quantitative profile of polyphenols, and high temperatures caused a drop in their concentration. Our one-year observation on the narrow set of evaluated cultivars and an atypical course of weather in 2011 during grain filling period, however, does not entitle us to similar conclusions. Further research will concentrate on the analysis of weather factors.

CONCLUSIONS

At four colors of wheat genotypes, different dynamics of total anthocyanins accumulation during grain filling were caused by different anthocyanins concentration in mature grain. In purple pericarp wheats, during grain filling, the anthocyanin concentration increased and subsequently decreased prior maturity. In blue aleurone wheat, total anthocyanins concentration was reached in grain physiological maturity

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