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Content of Phenolic Compounds and Free Polyamines in Black Chokeberry (*Aronia melanocarpa*) after Application of Polyamine Biosynthesis Regulators

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The total contents of anthocyanins, flavonoids, and phenolics in 60 samples of black chokeberries (*Aronia melanocarpa*), after treating with catabolites of polyamine biosynthesis (KPAb) and ornithine decarboxylase inhibitor, were analyzed spectrophotometrically, and quercetin and free polyamine contents were analyzed by RP-HPLC with UV detection. The average total contents of the individual substances and phenolic subgroups in control berries were as follows (mg·kg^{-1}): anthocyanines, 6408; flavonoids, 664; phenolics, 37 600; quercetin, 349. KPAb decreased total contents of anthocyanines and phenolics only slightly but significantly increased the content of flavonoids. This caused an important change in the abundance of flavonoids in the pigment complex. The absolute content of quercetin was increased, but its ratio to flavonoids content was decreased. Ornithine decarboxylase inhibitor had a markedly different effect as it significantly increased total content of anthocyanins and total phenolics, inhibited the total content of free polyamines, and stimulated the processes of saccharides transformation to phenolic pigments.

KEYWORDS: Black chokeberry; *Aronia melanocarpa*; anthocyanins; flavonoids; total phenolics; free polyamines; polyamine inhibitor and stimulators application; photospectrometry; HPLC analysis

INTRODUCTION

Black chokeberry (*Aronia melanocarpa*, *A. melanocarpa*) is native in North America. It is grown as an ornamental plant but also for berry production in northern Europe. In North America the compact shrub grows up to 2−3 m high, and in our country (Slovakia) from 1.5 to 2 m (*1*). The shrub is very fruitful, and the fruits are a very rich source of biologically active substances (*2*). Fruits have a high content of vitamins P, C, PP, B2, B9, E, and provitamin A. They also contain mineral substances including microelements (boron, fluorine, iron, copper, zinc, manganese, molybdenum, and cobalt), including iodine (up to 400 mg·kg^{-1}). A distinguishing feature of black chokeberry fruits is a high content of rutin. They are used as a source of natural coloring agents. They contain high levels of phenolic compounds, tannins, and particularly anthocyanins, as well as lower levels of flavonoids, substances with antioxidative, radical scavenger, and anticarcinomatoid effects (*3*−*7*). The total content of pigment is a function of the variety, its origin, agroclimatic conditions, and harvest time, etc. The total content of anthocyanins in the varieties "Nero", "Rubina", and "Viking" ranged from 6500 to 8500 mg·kg^{-1} dry weight (*8*) and flavonoids from 100 to 250 mg·kg^{-1} fresh weight. Quercetin is the main component of flavonoids with an average content of 89 mg·kg^{-1} fresh weight (*3*, *4*). Higher dosages of NPK fertilizers stimulate vegetative growth, increase berry yields, but decrease contents of anthocyanins and total acids. The composition of anthocyanin pigments was not influenced by fertilizer dosage (*9*). Fruits of black chokeberry of Russian origin have a lower content of polyphenols (7487 mg·kg^{-1}) than fruits of North American origin (*10*, *11*).

Less coloration of fruits is directly related to lower pigment content and indirectly related to saccharides level. The relationship between polyamine metabolism and phenolic compounds is indirect. The first alternative way originates from the antagonistic physiological effects of the ethylene and polyamine pathway end products which have been well-documented: the role of ethylene in promotion of ripening, coloring, and senescence (*12*) vs the antisenescent properties of polyamines (*13*). Ethylene may negatively affect enzymes involved in

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Table 1. Treatment of Variants

treatment	regulator	rate, a.i.[a] (g·ha^{-1})
control	water[b]	
GABA	γ- aminobutyric acid	5.6
PDA-1	1,3- propanediamine·2HCl	5.6
PDA-5	1,3- propanediamine·2HCl	28.0
KF	ethanolamine phosphate	5.6

[a] Active ingredient. [b] 1240 mL of H_2O/(4 shrubs)/treatment.

polyamine biosynthesis (*14*). The second alternative way originates from the catabolic pathway for polyamines in higher plants; putrescine, the basic compound for polyamines biosynthesis, is oxidized in two successive reactions to form γ-aminobutyric acid (GABA). GABA is converted to succinic semialdehyde by a transaminace reaction, and then to succinate, Krebs cycle intermediate, via an oxidation step (*15*). The decrease of the Krebs cycle rate connected with deficiency some of the intermediates (decreases in putrescine level effects the lower level of succinate) cause the redundance of acetyl CoA, which could form aromatic rings of phenols. The relation between glycolysis, the Krebs cycle, and polyamines biosynthesis is controlled by the catabolic products of polyamines, particularly putrescine (*14*, *16*). The third alternative way results from one of the polyamine properties. Polyamines may protect the plant against environmental stress. Various stresses were reported to increase either phenylalanine ammonia-lyase (PAL) synthesis or activity in plants (*17*). PAL is the key enzyme for the metabolism of phenols (*18*).

In this paper, we present the study results of the effect of ornithine decarboxylase inhibitor (inhibitor of putrescine formation) and the two catabolites of polyamines synthesis (γ-aminobutyric acid and 1,3-propanediamine) on the total content of anthocyanins, flavonoids, phenolics, quercetin, free polyamines (putrescine, spermidine, and spermine), and refractometric dry matter.

MATERIALS AND METHODS

Apparatus. Gradient elution was performed with the HPLC system (ECOM, Prague, Czech Republic) consisting of the Model GP-3 solvent programmer, Model LCP 4100.2 pump, and Type C injector. Quercetin was monitored with the LCD 2040 UV detector at 340 nm; free polyamines were at 254 nm. The CSW-2 chromatography station for Windows was used to record chromatograms. The column RP-18 (5 μm particles), 12,5 cm × 3 mm i.d. (Merck, Germany), was used at ambient temperature.

The total anthocyanins, flavonoids, and phenolics were determined using the UV−vis spectrophotometer (Model Mini 1240, Shimadzu Corp., Japan).

Reagents and Standard Solutions. Quercetin dihydrate standard, methanol, ethyl acetate (all spectrophotometric grade), γ-aminobutyric acid (99%; GABA), and 1,3- propanediamine dihydrochloride (PDA) were obtained from Sigma-Aldrich (Germany). A standard solution of quercetin was prepared in 96% ethanol. Putrescine, spermidine, and spermine were obtained from Merck (Darmstad, Germany). Ethanol (96%), hydrochloric acid, kalium hydroxide, natrium carbonate, and kalium chloride (all p.a. grade) were obtained from Microchem Ltd. (Pezinok, Slovakia). Ethanolamine phosphate, 98% (KF), was synthesized in the Petrochemistry Research Institute (Prievidza, Slovakia). All aqueous solutions and dilutions were prepared with redistilled water.

Samples. Twenty shrubs of black chokeberry of the variety "Nero" were 10 years old at the beginning of the experiment. The plants were chosen by random, and the same plants were treated by the same compound all three years and cultivated on the grounds of the Slovak Agricultural University in Nitra. Foliar treating was used for shrubs (4 shrubs/treatment/(1240 mL of water solution); see **Table 1**) between July 10 and July 15 (green berries) in 2002−2004. A 1 kg amount of berries from each shrub was harvested on September 3−7 of each year (colored berries), stored in a freezer at −25 °C, and processed within a week.

Analytical Procedures. *Total Anthocyanins.* After homogenization of 1 kg of berries in a blender a 5 g sample was taken and transferred to a 50 mL beaker with 10 mL of acidified methanol (28 mL of 36% HCL in 1 L of methanolic solution). After 5 min of heating in a water bath (maximum temperature, 70 °C) the contents were filtered through an S4 frit to a 100 mL flask. The residue was extracted by titrating with small portions (10 mL) of acidified methanol until it was colored. The extract in the flask was filled to 100 mL. A 5 mL aliquot of this solution was pipetted into the two 50 mL volumetric flasks. A 35 mL aliquot of buffered solution with pH 1.0 was added to the first flask and 35 mL of buffered solution of pH 4.5 to the second flask. In the first flask, the pH of the solution was adjusted to pH 1.0 by adding HCl (1:1) and filled up to the mark with buffered solution (pH 1.0). In the second flask, pH was adjusted to the required pH of 4.5 with 30% KCl solution and filled up to the mark with buffered solution (pH 4.5). After mixing, the flasks were kept in the dark for 2 h. Consequently, absorbance of the extract adjusted to pH 1.0 was measured at 570 nm, and the extract adjusted to pH 4.5 was measured at 520 nm in the spectrum maximum.

The total anthocyanin content was calculated using the relative molecular weight of cyanidin-3-rutinosid (595) and its molar absorption coefficient of 28 800. The result was recalculated to earlier analyzed dry matter and expressed in miligrams per kilogram.

Total Flavonoids. A 5 g amount from the homogenized sample was mixed at 0 °C with 30 mL of 1% HCl. The extract was centrifuged at 10000*g*. The solid part of the extract was washed several times with 1% HCl and centrifuged until the solution was pink colored. Consequently, the extract was shaken two times with 50 mL of ethyl acetate. The acetate extract was evaporated in the vacuum evaporator at 65−67 °C. After adding 20 mL of 96% ethanol, 5 mL of this solution was pipetted to the 25 mL volumetric flask and filled up to the mark with ethanol (96%). Three drops of 1% KOH colored the solution to yellow. Absorbance was measured at 520 nm. A calibration curve was constructed from the standard solution of quercetin (0.025 g in 25 mL of 96% ethanol). The total content of flavonoids was expressed as quercetin equivalents in miligrams per kilogram dry matter.

Quercetin. Quercetin was analyzed from the ethanolic solution prepared by the previous technique (for total flavonoids detection) in the HPLC system after filtration through a 0.22 μm GP filter unit. Separation was accomplished by gradient elution according to Tamma and Miller (*19*): solvent A, acetic acid−water (1:99, v/v); solvent B, acetonitrile. The gradient profile was linear from 20 to 90% solvent B in 20 min at the flow rate of 1.5 mL·min^{-1} and a column pressure of 23 MPa. The retention time of quercetin was 5.90 min.

Total Phenolics. Frozen samples were thawed and then homogenized for 1 min at maximum speed in a blender, and 3 g of the homogenized sample was extracted with 40 mL of a mixture containing methanol and water (70:30, v/v). Samples were vortexed and allowed to stand for 1 h at room temperature and centrifuged at 5000*g* for 15 min at 20 °C. The residue was reextracted twice with 70% methanol, and supernatants were combined and taken to dryness. The solid residue was dissolved in methanol. Extractions were repeated on three independent samples from the initial homogenate, the same as for anthocyanins, flavonoids, and quercetin. Total phenolics were measured using the Folin-Ciocalteu method (*20*). Briefly, 5 mL of water, 0.5−1.0 mL of sample, and 1.0 mL of Folin-Ciocalteu reagent were added to a 25 mL volumetric flask. The contents were mixed and allowed to stand for 5−8 min at room temperature. Next, 10 mL of a 7% of sodium carbonate solution and water filled to volume was added. After 30 min standing at room temperature, absorption at 675 nm was measured. The total phenolic content was expressed as gallic acid equivalents in miligrams per kilogram dry matter.

Free Polyamines and Analysis. From 1 kg of homogenized berries 1 g was taken and homogenized in 5 mL of 5% (w/v) trichloracetic acid in an ice bath (*21*). After 1 h, the extract was centrifuged for 15 min at 26000*g*. The supernatant fraction containing unconjugated (free) polyamines was benzoylated according to Redmond and Tseng (*22*)

Table 2. Total Pigment Contents (mg·kg^{-1} of dw and % vs Control) and Refractometric Dry Mass (RDM (%)) of Black Chokeberry var. Nero[a]

treatment	anthocyanins		flavonoids		phenolics × 10^3 (as GAE)[b]		quercetin		RDM[c]
control	6408 a	100	664 a	100	37.6 a	100	349 a	100	16.5 ab
GABA	6007 a	93.7	924 b	139.2	35.2 b	93.6	412 ab	118.1	17.3 a
PDA-1	6425 a	100.3	743 a	112.0	37.2 a	99.0	353 a	101.2	15.0 c
PDA-5	6216 a	97.0	1105 c	166.5	35.9 ab	95.5	458 b	131.3	15.5 bc
KF	7922 b	123.6	1008 bc	151.9	43.2 c	114.9	496 b	142.2	16.5 ab

[a] Means followed by the same letter are not significantly different by LSD test at 5% level. [b] Concentration based upon gallic acid as standard. [c] Refractometric dry mass, hand refractometer.

Table 3. Ratio of Anthocyanins/Flavonoids Content and Percent Portion of Quercetin from Flavonoids in Chokeberry[a]

treatment	anthocyanins/ flavonoids	% vs control	quercetine × 100/flavonoids	% vs control
control	9.7 a	100	52.6 a	100
GABA	6.5 b	67.0	44.6 a	84.8
PDA-1	8.7 ab	89.7	47.5 a	90.3
PDA-5	5.6 bc	57.7	41.4 a	78.7
KF	7.9 abc	81.4	49.2 a	93.5

[a] Means followed by the same letter are not significantly different by LSD test at 5% level.

Table 4. Accumulation of Free Polyamines (μmol·g^{-1} Fresh Weight) in Black Chokeberry[a]

treatment	putrescine	spermidine	spermine	total
control	4.23 a	3.78 a	0.28 a	8.29
GABA	7.49 b	12.44 b	0.32 a	20.25
PDA-1	5.01 a	12.85 b	0.37 a	18.23
PDA-5	5.46 ab	13.12 b	0.39 a	18.97
KF	4.28 a	1.40 a	0.29 a	5.97

[a] Means followed by the same letter are not significantly different by LSD test at 5% level.

and separated by HPLC using methanol/water (58:42) mixture as a mobile phase at a flow rate of 1 mL·min^{-1}. The results were compared with the standards (putrescine, spermidine, and spermine) benzoylated in the same way.

Data Analysis. Except for polyamines, all analyses were repeated three times (3 years) in four repetitive experiments for each sample from four shrubs in each treatment. Polyamines were analyzed similarly except that instead of three there were two repetitions for each sample. The results were evaluated using general statistical methods. The LSD (least significant deviation) method was used to determine the differences between the treatments.

RESULTS AND DISCUSSION

Pigments Content. The total contents of anthocyanins, flavonoids, phenolics, and flavonoid quercetin were determined in 60 samples of black chokeberry *(A.)* of the variety "Nero" (3 years × 4 repetitions of the treatment, 4 shrubs, in 5 treatments). The results expressed in milligrams to 1 kg of dry matter are given in **Table 2**, as well as the contents of the refractometric dry matter of harvested berries. Differences in pigment content between specific years and specific treatments, even in the very dry year 2003, were not significant, although according to Macheix et al. (*23*) an increase in solar radiation but also low temperatures generally yield a higher content of phenolics, especially anthocyanins, in fruits. The average of the total content of anthocyanins, flavonoids, phenolics, and quercetine in berries of the control variant was 6408, 664, 37 600, and 349 mg·kg^{-1}, respectively. The catabolic products of polyamine biosynthesis, γ-aminobutyric acid (GABA) and 1,3-propanediamine (PDA), in the applied doses, did not decrease the total contents of anthocyanins and flavonoids with any statistical significance. On the other hand, they significantly increased the total content of flavonoids, and 1,3-propanediamine alone, using a higher applied dose, significantly increased quercetine (on average to 458 mg·kg^{-1}). This caused a significant change in the ratio of anthocyanins to flavonoids. The value of this ratio in the control was 9.7 (**Table 3**). This ratio was 5.6 in berries treated with 1,3-propanediamine. This represents a significant shift in the representation of flavonoids in the pigment complex. All applied regulators decreased, but not with statistical significance, the ratio of quercetine to the total content of flavonoids, but most significantly after application of a higher dose of 1,3-propanediamine. The ornithine decarboxylase (ODC) inhibitor ethanolamine phosphate had distinctly diffrerent effects on total anthocyanins and phenolics. In contrast to polyamine catabolites (GABA, PDA), ethanolamine phosphate statistically very significantly increased the total content of anthocyanins to 7922 mg·kg^{-1}, which is an increase of 23.6%, and the total content of phenolics to 43200 mg·kg^{-1} which is an increase of 15.0% compared to the control (**Table 2**). The increase of flavonoids content after treating with ethanolamine phosphate did not change the ratio of anthocyanins to flavonoids (**Table 3**) as dramatically as it did after treatment with polyamine catabolites (*6*, *8*). Berry quercetin content was most strongly increased by ODC inhibitor. These changes could change berry properties including antioxidant activity. This result could influence the health value and the utilization of black chokeberry in the food or medicine industry. The classic inverse relation between saccharides content (partially represented by refractometric dry matter) and the content of evaluated pigments can be seen with the evaluation of total anthocyanins and phenolics, respectively, and refractometric dry matter after treating with GABA. The same can be said of the evaluation of the content of flavonoids and refractometric dry matter after treating with PDA (**Table 2**). Polyamine catabolites evaluated as stimulants of polyamines biosynthesis (*14*, *16*, *19*) decreased the total content of phenolics in berries of black chokeberry while a polyamine biosynthesis inhibitor (inhibitor ODC) increased these phenolics in *A. melanocarpa* berries.

Free Polyamines Content. Results, expressed in μmol·g^{-1} of fresh matter, are given in **Table 4**.

The dynamics of polyamine biosynthesis regulator effects on endogenous free polyamine (PA) levels in berries of black chokeberry are not documented. These dynamics would show more clearly the direct effects of the structure of the used compounds on the instant changes in PA levels. Nevertheless, their regulatory effect on this biosynthesis can be seen from the results. The level of free putrescine increased the most (by 77% vs control) after treating with GABA. The character of regulators used is most clearly reflected in the changes in the level of free spermidine. After treating with PA catabolites the level of free spermidine increased, and increased the most after

treating with PDA-5 (by 247% vs control). On the other hand, ODC inhibitor reduced its content to 37% of its level in the control sample of berries. Spermine levels were the least influenced by regulators used in this study. The level of total free PAs is in an inverse relation to the level of total anthocyanins and phenolics. The increased level of total free PAs suggests inhibition of the process of berry maturation and coloring. On the other hand, by reducing free PAs, ODC inhibitor stimulated the transformation of saccharides to phenolic pigments. This result refers to the mediated relation between the polyamines and saccharides synthesis. It is in accordance with Turano et al. (*14*) and Tiburcio et al. (*16*).

ABBREVIATIONS USED

KPAb, catabolites of polyamine biosynthesis; GABA, γ-aminobutyric acid; PDA, 1,3- propanediamine dihydrochloride; KF, ethanolamine phosphate; ODC, ornithine decarboxylase; PAs, polyamines; a.i., active ingredient; RDM- refractometric dry mass.

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