Planta (2017) 246:217–226
DOI 10.1007/s00425-017-2670-3

ORIGINAL ARTICLE

Polyphenol metabolism in differently colored cultivars of red currant (*Ribes rubrum* L.) through fruit ripening

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Received: 3 January 2017 / Accepted: 25 February 2017 / Published online: 17 March 2017

Abstract

Main conclusion **Rare red currants colors caused by low anthocyanin content in the pink and a lack of anthocyanins in the white cultivar correlated with low *ANS* gene expression, enzyme activity, and increased sugar/acid ratios.**

Changes in the contents of polyphenols, sugars, and organic acids in berries of the three differently colored *Ribes rubrum* L. cultivars ('Jonkheer van Tets', 'Pink Champagne', and 'Zitavia') were determined by LC–MS and HPLC at 4 sampling times during the last month of fruit ripening. The activities of the main flavonoid enzymes, chalcone synthase/chalcone isomerase (CHS/CHI), flavanone 3-hydroxylase (FHT), and dihydroflavonol 4-reductase (DFR), and the expression of anthocyanidin synthase (*ANS*) were additionally measured. Despite many attempts, activities of flavonol synthase and glycosyltransferase did not show reliable results, the reason of which they could not be demonstrated in this study. The pink fruited cultivar 'Pink Champagne' showed generally lower enzyme activity than the red cultivar 'Jonkheer van Tets'. The white cultivar 'Zitavia' showed very low CHS/CHI activity and *ANS* expression and no FHT and DFR activities were detected. The DFR of *R. rubrum* L. clearly preferred dihydromyricetin as substrate although no 3′,4′,5′-hydroxylated anthocyanins were present. The anthocyanin content of the red cultivar slightly increased during the last three weeks of ripening and reached a maximum of 890 mg kg^{-1} FW. Contrary to this, the pink cultivar showed low accumulation of anthocyanins; however, in the last three weeks of ripening, their content increased from 14 to 105 mg kg^{-1} FW. Simultaneously, the content of polyphenols slightly decreased in all 3 cultivars, while the sugar/acid ratio increased. The white cultivar had no anthocyanins, but the sugar/acid ratios were the highest. In the white and pink cultivars, reduction/lack of anthocyanins was mainly compensated by increased relative concentrations of hydroxycinnamic acids and flavonols.

Keywords Anthocyanins · Enzyme activity · Gene expression · Phenolic compounds · Sugar/organic acid ratio

Abbreviations

ANS	Anthocyanidin synthase
CHS/CHI	Chalcone synthase/chalcone isomerase
DFR	Dihydroflavonol 4-reductase
DHK	Dihydrokaempferol
DHM	Dihydromyricetin
DHQ	Dihydroquercetin
FHT	Flavanone 3-hydroxylase
TPC	Total phenolic content

Special topic: Polyphenols II: biosynthesis and function in plants and ecosystems. Guest editor: Stefan Martens.

Electronic supplementary material The online version of this article (doi:10.1007/s00425-017-2670-3) contains supplementary material, which is available to authorized users.

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Introduction

The *Ribes* genus consists of many different species and is important in the world production of berry fruits. Currants are highly appreciated for their sour taste, high nutritional value, and also because of the well-known health-promoting properties of their polyphenols: anthocyanins, hydroxycinnamic acids, flavonols, and flavanols (Veberic et al. 2015; Mikulic-Petkovsek et al. 2015, 2016). Anthocyanins, the main pigments responsible for fruit color of black currant (*Ribes nigrum* L.), are mainly found in the epidermis and in the tissue directly under the skin (Milivojevic et al. 2012). Although, in some red currant (*R. rubrum* L.) cultivars, anthocyanins can also be found in the pulp to some extent, similarly to few grape cultivars (He et al. 2010), bilberry (Jaakola et al. 2002), or some other berries. In intensely colored berries of black currant, high levels of anthocyanins are typically present, while smaller amounts are accumulated in red currant (Gavrilova et al. 2011; Mattila et al. 2016). In addition, pink and acyanic colored white currants which are color variants of *R. rubrum* L. could be found, while green currant (*R. nigrum* L.) additionally lacks anthocyanins (Määttä et al. 2001, 2003). This indicates that the biosynthetic routes of anthocyanins vary between different varieties and also cultivars (Mattila et al. 2016). Due to differently pigmented phenotypes, regulation of anthocyanin biosynthesis has been studied across a number of plants: bilberry (Jaakola et al. 2002; Primetta et al. 2015), grapevine (Castellarin and Gaspero 2007), pear (Yang et al. 2015), cherry (Wei et al. 2015), kiwifruit (Halbwirth et al. 2009), pomegranate (Ben-Simhon et al. 2015; Zhao et al. 2015), etc.

Anthocyanins and other phenolic compounds are synthesized via the phenylpropanoid/flavonoid pathway, which is driven by the structural genes encoding the enzymes that directly participate in the formation of pigments and other flavonoids, and the regulatory genes that control the transcription of structural genes. Differences in the pattern of anthocyanin accumulation and fruit color intensity are, therefore, attributed to variations in the expression of these two types of genes (Zhao et al. 2015).

Biosynthesis starts from phenylalanine to produce phenylpropanoids, which are channeled into the flavonoid pathway by chalcone synthase (CHS), which is one of the key enzymes initializing the pathway. Further steps are catalyzed by chalcone isomerase (CHI), flavanone 3-hydroxylase (FHT), and dihydroflavonol 4-reductase (DFR), which converts dihydroflavonols into leucoanthocyanidins. DFR is a key enzyme in anthocyanin biosynthesis and also affects the biosynthesis of other flavonoids. DFR competes with flavonol synthase for dihydroflavonolos as a common substrate and thus interferes with flavonol production (Davies et al. 2003; Wang et al. 2013). Finally, anthocyanidin synthase (ANS) leads to the synthesis of anthocyanidin pigments, while different glycosyltransferases and other transferases determine the substitution pattern of phenylpropanoid and flavonoid end-products (Tanaka et al. 2008). Although flavonoid metabolism is generally influenced by genetic predisposition, environmental and developmental factors also play an important role in its regulation (Jaakola 2013; Yang et al. 2013; Mikulic-Petkovsek et al. 2015).

The polyphenol composition of black, red, and white currants is generally well investigated (during ripening and among different varieties/cultivars) (Gavrilova et al. 2011; Mikulic-Petkovsek et al. 2015; Mattila et al. 2016). However, to the best of our knowledge, pink colored currants have not previously been studied. Furthermore, flavonoid enzyme activities have also not been investigated in currant berries; only the expression of the *ANS* gene has previously been studied in *R. nigrum* L. cv. 'Broad' (Li et al. 2015). Polyphenol metabolism in fruits has become an interesting object of study in terms of the regulation and organization of the formation of different flavonoid classes in a well-defined time schedule.

The aim of the present study was to analyze, for the first time, the activities of the main flavonoid enzymes (CHS/CHI, FHT, and DFR) and *ANS* gene expression through fruit ripening in differently colored *R. rubrum* L. cultivars. The last few weeks of fruit ripening, as the essential period for fruit quality (firmness, flavor, color, etc.), particularly draw our attention, but visible changes were negligible. Since pink colored cultivar in our study accumulated anthocyanins only in the skin, and according to reports which indicated that anthocyanin biosynthesis key enzymes were expressed predominantly in the skin of grape berries (Grimplet et al. 2007; Mu et al. 2014), we decided to study berry skins only. In addition, berry color parameters, the composition and content of primary metabolites, anthocyanins, and other polyphenolics were determined.

Materials and methods

Plant material

Ribes rubrum L. berries of red ('Jonkheer van Tets'), pink ('Pink Champagne'), and white ('Zitavia') color cultivars were hand-harvested weekly at the experimental station of the Agricultural Institute of Slovenia in Brdo pri Lukovici (46°10′N, 14°41′E). During the last period of fruit ripening, four samplings were performed (each contained approx. 500 g of fruits, collected from different bushes of each cultivar). The first sampling date was on the 8th (S1), the second on the 15th (S2), the third on the 22nd (S3), and the

fourth on the 29th (S4) of June 2015. Color parameters were measured after each harvest. The berry skin was separated from the pulp, shock-frozen in liquid nitrogen, and stored at −80 °C until enzyme analysis. For primary and secondary metabolites analysis, whole berries were stored at −20 °C.

Chemicals

The following standards were used for the determination of sugars and organic acids: fructose, glucose, and sucrose; citric and malic acid from Fluka Chemie (Buchs, Switzerland); and shikimic and fumaric from Sigma-Aldrich Chemie (Steinheim, Germany). The following standards were used for the quantification of phenolic compounds: cyanidin-3-*O*-glucoside, cyanidin-3-*O*-rutinoside, and quercetin-3-*O*-rutinoside from Sigma-Aldrich Chemie; caffeic, (+)-catechin from Roth (Karlsruhe, Germany), (−)-epicatechin, quercetin-3-*O*-rhamnoside, quercetin-3-*O*-glucoside, *p*-coumaric acid, procyanidin B1, and kaempferol-3-*O*-glucoside from Fluka Chemie; myricetin-3-rhamnoside from Apin Chemicals (Abingdon, UK). Methanol for the extraction of phenolics was acquired from Sigma-Aldrich Chemie. The chemicals for the mobile phases were HPLC–MS grade acetonitrile and formic acid from Fluka Chemie. Water for the mobile phase was double distilled and purified with the Milli-Q system (Millipore, Bedford, MA, USA). For the total phenolics, Folin-Ciocalteu phenol reagent (Fluka Chemie), sodium carbonate (Merck, Darmstadt, Germany), gallic acid (Sigma-Aldrich Chemie), and ethanol (Sigma-Aldrich Chemie) were used. (2-^{14}C)-Malonyl-coenzyme A was obtained from Amersham International (Freiburg, Germany). (^{14}C)-Labeled flavonoids naringenin, dihydrokaempferol (DHK), dihydromyricetin (DHM), and dihydroquercetin (DHQ) were prepared as described previously (Fischer et al. 2003).

Fruit color measurements

Fruit color was evaluated using a portable colorimeter (Konica Minolta, Tokyo, Japan) on 30 fruits for each sampling date and individual cultivar. The data were expressed in lightness (L^*), chroma (C^*), and hue angle ($h°$) values, calculated as $\tan^{-1}$ (b^*/a^*) in degrees from 0° to 360°. As C^* increases, color becomes more intense. The L^* value corresponds to a dark-bright scale and represents the relative lightness with a range from 0 to 100 (0 = black, 100 = white) (McGuire 1992).

Extraction and determination of sugars and organic acids

Primary metabolites (sugars and organic acids) were analyzed in the whole berry fruit. For the extraction of primary metabolites, 1.4 g of fruit was homogenized with 7 ml of double distilled water and left for 30 min at room temperature with continuous stirring. After the extraction, the homogenate was centrifuged and the supernatant was filtered into a vial. Sugars and organic acids were measured using a Thermo Finnigan Surveyor HPLC system (Thermo Scientific, Waltham, MA, USA). Further analysis was performed as reported by Zorenc et al. (2016), where detailed method conditions are also reported. For each currant cultivar and sampling date, five replicates were prepared and analyzed. Contents of individual and total sugars and organic acids were expressed in mg g^{-1} of FW.

Extraction and determination of individual phenolic compounds using HPLC–DAD–MS^n analysis

One gram of berry skin was extracted with 4 ml methanol containing 3% (v/v) formic acid in a cooled ultrasonic bath for 1 h and then the extract was centrifuged and filtered into a vial. Phenolic compounds were analyzed on a Thermo Finnigan Accela HPLC system (Thermo Scientific) and identified using a mass spectrometer LCQ Deca XP MAX (Thermo Scientific) with ESI operating in negative (all phenolic groups except anthocyanins) and positive (anthocyanins) ion mode according to Mikulic-Petkovsek et al. (2015). For individual, as well as total phenolic content, five replicates were carried for each cultivar and sampling date. For compounds lacking standards, quantification was carried out using similar compounds as standards. Contents of phenolic compounds were expressed in mg kg^{-1} of FW.

Determination of total phenolic content (TPC)

The extraction of total phenolics was made according to the same protocol as for individual phenolic compounds. TPC of extracts was assessed by the Folin–Ciocalteu phenol reagent method (Singleton et al. 1999) and was expressed as gallic acid equivalents (GAE) in mg kg^{-1} of FW.

Extraction and assay of enzymes

Shock-frozen currant skin was ground to powder with liquid nitrogen. A total of 0.20 g fine skin powder, 0.20 g quartz sand, 0.20 g Polyclar AT, and 3 ml extraction buffer (described by Thill et al. 2012b) was homogenized in a mortar. The homogenate was centrifuged for 10 min at 4 °C and 13,000*g*. To remove low molecular compounds, 400 µl of supernatant were passed through a gel chromatography column (Sephadex G25 medium). The protein solution eluted in the excluded volume of the column (crude extract) was used for enzyme assays.

Table 1 Color parameters (L^*, C^*, h°), content of total sugars, organic acids (mg g^{-1} FW), and sugar/organic acid ratio of red ('Jonkheer van Tets'), pink ('Pink Champagne'), and white ('Zitavia') currant cultivars in different ripening stages (S1–S4)

Cultivar	Stage	L^*	C^*	h°	Total sugars	Total organic acids	Sugar/acid ratio
'Jonkheer van Tets'	S1	29.90 ± 0.94a	37.44 ± 1.21a	11.0 ± 0.70a	39.40 ± 0.94c	28.18 ± 1.51	1.40 ± 0.05b
	S2	23.97 ± 0.35b	31.55 ± 0.96b	6.67 ± 0.39b	48.20 ± 0.68b	31.59 ± 1.45	1.53 ± 0.05ab
	S3	24.03 ± 0.31b	22.31 ± 0.84c	4.25 ± 0.32c	52.06 ± 0.51a	30.32 ± 1.90	1.72 ± 0.11a
	S4	25.21 ± 0.23b	19.95 ± 0.73c	4.64 ± 0.27c	53.43 ± 1.11a	30.16 ± 3.16	1.77 ± 0.16a
'Pink Champagne'	S1	63.15 ± 0.96a	28.67 ± 0.63a	79.2 ± 1.90a	35.01 ± 1.85d	32.65 ± 2.84a	1.07 ± 0.13d
	S2	54.87 ± 1.15b	21.53 ± 0.67bc	47.9 ± 2.91b	47.22 ± 0.75c	25.63 ± 1.79b	1.84 ± 0.11c
	S3	47.10 ± 0.85c	23.61 ± 0.82b	42.3 ± 1.84b	65.59 ± 0.87b	19.49 ± 1.44c	3.37 ± 0.22b
	S4	45.09 ± 0.94c	21.29 ± 0.83c	30.1 ± 3.02c	73.42 ± 0.94a	18.55 ± 1.36c	3.96 ± 0.24a
'Zitavia'	S1	57.62 ± 0.44a	25.49 ± 0.65a	73.6 ± 1.06a	42.26 ± 0.87d	23.08 ± 0.59a	1.83 ± 0.07c
	S2	53.16 ± 0.54b	22.44 ± 0.99b	63.9 ± 1.18b	56.11 ± 0.58c	18.67 ± 0.50b	3.01 ± 0.11b
	S3	51.41 ± 0.50c	21.09 ± 0.70b	59.9 ± 1.46c	68.26 ± 0.85b	17.74 ± 0.28b	3.85 ± 0.07a
	S4	50.96 ± 0.59c	16.33 ± 0.79c	60.0 ± 1.01c	71.75 ± 0.81a	18.06 ± 0.17b	3.98 ± 0.08a
	'Jonkheer van Tets'	25.78 ± 0.38B	27.81 ± 0.93A	6.63 ± 0.37C	48.27 ± 1.41C	30.06 ± 1.00A	1.61 ± 0.07C
	'Pink Champagne'	52.55 ± 0.94A	23.78 ± 0.50B	49.9 ± 2.37B	55.31 ± 3.93B	24.08 ± 1.70B	2.30 ± 0.31B
	'Zitavia'	53.29 ± 0.38A	21.34 ± 0.55C	64.3 ± 0.84A	59.60 ± 3.09A	19.39 ± 0.59C	3.07 ± 0.22A

Mean and standard errors are presented

Different letters (a–d) in columns denote significant differences among ripening stages (S1–S4) within the cultivar (Duncan's test $P < 0.05$). Different letters (A–C) in columns denote significant differences among cultivars (HSD test $P < 0.05$)

Enzyme assays were performed as described previously (Slatnar et al. 2012) using the assay conditions optimized for currant skin (Suppl. Table S1). The assays were incubated for 15 min at 30 °C. Values on each sampling date represent an average of three independent biological replicates for each treatment. All assays were run in duplicate at least. To determine the specific enzymatic activity, a modified Lowry method for protein determination (Sandermann and Strominger 1972) with BSA as a standard was used. Activities of CHS/CHI, FHT, and DFR were calculated and expressed as nkat g^{-1} protein.

Gene expression studies

Expression of *ANS* was analyzed by qPCR using a StepOnePlus system and the SYBRW Green PCR Master Mix (Applied Biosystems, Darmstadt, Germany) according to the supplier's instruction. The analysis was carried out in triplicates, and the *ANS* expression was normalized against *actin* as control gene. Primers for *ANS* (RibANS_f: ATGGTGACAGTATCAGAGGCGGC; RibANS_r: TCATTTAGGTAGGAGTTCATCTTGGG) and *actin* (RibAct_f: TGTTCCCTGGTATT-GCTGAC, RibAct_r: CTGGAAGGTGCTAAGGGATG) were designed on the base of the sequences published in the NCBI database (LN736332, LN736331, and LN736321). Differences between the cycle threshold (*Ct*) of the target gene and the *Actin* gene were used to obtain relative transcript levels of the target gene, and calculated as 2 exp-($Ct_{target} - Ct_{actin}$).

Statistical analysis

Results were evaluated with the Statgraphics Centurion XV.II program (Statpoint Technologies Inc., Warrenton, VA, USA). Data were tested for any differences among sampling dates within each cultivar and among cultivars (in average data calculated from all harvest dates) using the one-way analysis of variance. The differences among ripening stages were tested using the Duncan's test and among cultivars using the HSD test with the significance level of 0.05.

Results

Fruit color and primary metabolites content

The fruit color and content of total sugars, organic acids, and sugar/organic acid ratio of the three currant cultivars are presented in Table 1. Significant differences in all color parameters were observed during fruit ripening in all cultivars. The highest color (L^*, C^*, h°) values were measured at the first sampling date and decreased afterwards in all cultivars, meaning ripening resulted in darker,

Table 2 Content of total anthocyanins, hydroxycinnamic acid derivatives, flavanols, and flavonol glycosides obtained by HPLC–MS analysis and total phenolic content (TPC) (mg kg^{-1} FW) obtained with the Folin–Ciocalteu method of red ('Jonkheer van Tets'), pink ('Pink Champagne'), and white ('Zitavia') currant cultivars in different ripening stages (S1–S4)

Cultivar	Stage	Total anthocyanins	Total hydroxycinnamic acids	Total flavanols	Total flavonols	TPC
'Jonkheer van Tets'	S1	709.6 ± 15.9b	14.34 ± 0.73a	45.66 ± 1.87a	123.9 ± 12.5a	1115 ± 58.4a
	S2	802.7 ± 30.3ab	12.73 ± 0.37b	31.14 ± 1.56b	115.5 ± 9.81ab	1020 ± 35.3ab
	S3	817.3 ± 15.1ab	9.75 ± 0.28c	25.78 ± 1.32b	79.28 ± 3.18bc	924.1 ± 17.1b
	S4	889.8 ± 22.7a	10.72 ± 0.49c	29.33 ± 1.10b	71.87 ± 3.24c	964.2 ± 49.0b
'Pink Champagne'	S1	14.11 ± 1.33d	58.90 ± 5.01c	18.78 ± 1.35ab	118.1 ± 6.67a	713.9 ± 16.5a
	S2	47.14 ± 2.88c	95.67 ± 5.96a	21.51 ± 1.62a	105.2 ± 5.28b	644.3 ± 11.1b
	S3	79.25 ± 1.22b	78.21 ± 3.50b	16.66 ± 0.71b	96.71 ± 2.11b	610.9 ± 10.2b
	S4	104.7 ± 1.91a	69.03 ± 2.66bc	16.72 ± 0.75b	125.8 ± 2.27a	618.5 ± 21.0b
'Zitavia'	S1	nd	69.39 ± 0.01	14.72 ± 0.31a	120.2 ± 10.1	568.1 ± 19.6a
	S2	nd	72.18 ± 3.24	10.04 ± 0.60b	106.9 ± 7.13	425.1 ± 8.83b
	S3	nd	75.72 ± 4.28	11.27 ± 0.24b	95.93 ± 7.66	420.1 ± 7.18b
	S4	nd	76.62 ± 5.84	11.65 ± 0.94b	99.17 ± 8.73	457.2 ± 30.5b
	'Jonkheer van Tets'	804.9 ± 20.6A	11.89 ± 0.49B	32.98 ± 2.38A	97.64 ± 9.20	1006 ± 24.4A
	'Pink Champagne'	61.30 ± 8.83B	75.45 ± 4.51A	18.42 ± 0.79B	111.5 ± 4.22	646.9 ± 12.2B
	'Zitavia'	nd	73.48 ± 3.37A	11.92 ± 1.01C	105.5 ± 4.08	467.6 ± 20.2C

Mean and standard errors are presented. Different letters (a–d) in columns denote significant differences among ripening stages (S1–S4) within the cultivar (Duncan's test $P < 0.05$). Different letters (A–C) in columns denote significant differences among cultivars (HSD test $P < 0.05$)

nd not detected

less intense and redder ('Jonkheer van Tets'), pinker ('Pink Champagne'), or more light yellow ('Zitavia') berry skin color.

Fructose and glucose were the prevalent sugars in all three currant cultivars (sucrose was detected only in traces), while citric acid was the prevalent organic acid in berries, together with smaller amounts of malic, shikimic, and fumaric acids (data not shown). The lowest content of total sugars was measured in the fruits of all cultivars on the first harvest date (Table 1). Conversely, total organic acid contents were the highest at the beginning of ripening in 'Pink Champagne' and 'Zitavia'. The highest content of total sugars was measured on the last harvest date in 'Pink Champagne' and 'Zitavia' and on to the third sampling date in 'Jonkheer van Tets'. White currant 'Zitavia' had the highest sugar/organic acid ratio, while red currant 'Jonkheer van Tets' was characterized by the lowest.

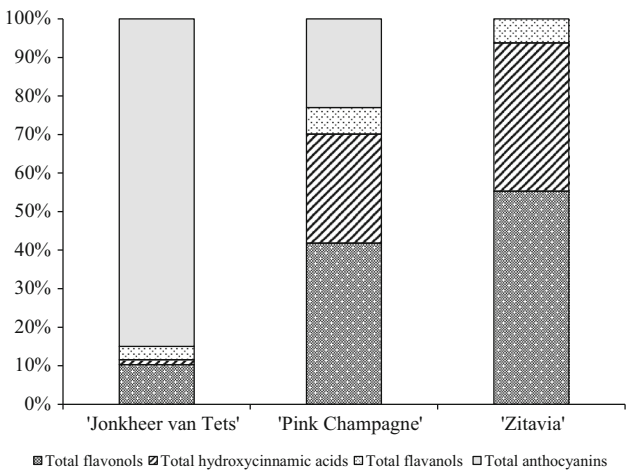

Fig. 1 Share levels of different phenolic groups in red ('Jonkheer van Tets'), pink ('Pink Champagne'), and white ('Zitavia') currant cultivars

Phenolic compound composition

The detailed phenolic compositions and the relative levels of individual phenolics of the red, pink, and white fruiting currant cultivars are presented in Suppl. Tables S2 and S3, while total amounts of the four polyphenol classes (anthocyanins, hydroxycinnamic acids, flavanols, flavonols) and TPC detected in berry skins are presented in Table 2. Figure 1 additionally visualizes the differences in the spectrum of phenolic compounds present in the 3 cultivars.

The highest total anthocyanins content was detected in the red cultivar (Table 2). The pink cultivar contained a 13.1-fold lower content than the previous, while anthocyanins were not detected in the white cultivar. The same

four cyanidin glycosides (glucosylrutinoside, sambubioside, xylosylrutinoside, and rutinoside) were found in the red and pink cultivars, but cyanidin-3-*O*-glucosylrutinoside and cyanidin-3-*O*-sambubioside were present only in traces (Suppl. Table S2 and S3). Total anthocyanin contents increased during fruit ripening, with the highest increase, of 642%, in the pink cultivar, while only 25% increase was detected in the red cultivar, which was characterized by generally high total anthocyanins in all ripening stages.

The concentration and spectrum of hydroxycinnamic acid derivatives significantly varied in the red and pink cultivars during fruit ripening. The pink and white cultivars showed more than 6-fold higher hydroxycinnamic acids concentration than the red cultivar (Table 2). The latter accumulated only *p*-coumaric acid derivatives (Suppl. Table S3) and the content decreased during fruit ripening. Contrary to the red cultivar, the two other cultivars accumulated caffeic acid derivatives in addition to *p*-coumaric acid derivatives, while the total content remained unchanged in the white cultivar and even slightly increased in the pink cultivar during ripening. Relative hydroxycinnamic acid concentration showed strong variation among the three cultivars (Fig. 1), and was higher in the pink and the white cultivars in comparison to the red (23 and 31 times, respectively).

The red cultivar accumulated the highest total flavanols (catechin, epicatechin, and procyanidin dimers), reaching up to 1.8- and 2.8-fold higher levels than the pink and the white cultivars, respectively (Table 2). Total flavanols were highest in the red and white cultivars at the first sampling date and decreased thereafter, while the pink cultivar showed a maximum at the second sampling. In the white and pink cultivars, the relative flavanol concentrations were increased by a factor of 1.8 and 2.0, respectively (Fig. 1). Interestingly, catechin was predominant in the pink and white cultivars, whereas in the red cultivar, catechin, epicatechin, and procyanidin dimers 1 and 2 were almost equally distributed.

Total flavonol glycosides (quercetin, myricetin, and kaempferol glycosides) showed no variation among the 3 cultivars, although the pink and white cultivars contained 8–14% higher contents than the red cultivar (Table 2). Sixteen differently glycosylated flavonols were detected, most of them present in all 3 cultivars (Suppl. Tables S2 and S3), with the exception of kaempferol-3-*O*-galactoside, which was detected only in the pink cultivar, and quercetin-rhamnosyl hexoside 3 and quercetin-rhamnosyl hexoside pentoside, which were found only in the berry skin of the red cultivar. However, the majority of the flavonols were found to be present in minor concentrations and quercetin dirhamnosyl hexoside 1 and quercetin-3-*O*-rutinoside were predominant in all 3 cultivars. Whereas the total flavonol content decreased during fruit ripening in the red cultivar, it remained quite stable in the two other cultivars. Relative flavonol concentrations, however, varied markedly between the three cultivars and were higher by a factor of 4 in the pink and by a factor of 5 in the white compared to the red cultivar.

The red cultivar showed the highest TPC, which was clearly due to the high anthocyanin content (Fig. 1 and Table 2). The pink and white cultivars showed significantly lower contents (1.6- and 2.2-fold lower, respectively) and only half of the contents were due to the presence of the main flavonoid classes or hydroxycinnamic acids. In all 3 cultivars, TPC slightly decreased during fruit ripening.

Activities of main flavonoid enzymes and *ANS* gene expression in currant skin

All enzyme assays had to be developed *de novo*, due to the absence of protocols for the determination of flavonoid enzymes in the literature. Various methods for enzyme preparations were tested (data not shown) and that by Thill et al. (2012b) provided the best results. The three key enzymes in the pathway leading to anthocyanin formation, CHS/CHI, FHT, and DFR, were included in the study. Since ANS activity cannot be tested from tissues so far, we also included *ANS* gene expression studies. DFR was tested with DHK, DHM, and DHQ as substrates. DFR from *Ribes* did not convert DHK and clearly preferred DHM over DHQ (tenfold lower conversion rates compared to DHM, on the average). Despite many attempts, activities of flavonol synthase and glycosyltransferase did not show reliable results, the reason of which they could not be demonstrated in this study.

Changes in the tested specific enzyme activities (CHS/CHI, FHT, and DFR) and *ANS* expression in the berry skin of red, pink, and white fruiting cultivars during ripening are presented in Fig. 2. In the red cultivar, the flavonoid pathway was highly induced, as indicated by generally high activities of all tested enzymes (Fig. 2a–c) and an *ANS*/*actin* ratio above 1 (Fig. 2d). The white cultivar was characterized by low CHS/CHI activity, very low *ANS*/*actin* ratio, and a lack of FHT and DFR activity. The *ANS*/*actin* ratio in the pink cultivar was below 1, but higher by a factor of 200 than that of the white cultivar. CHS/CHI activities were comparable to those detected with enzyme preparations of the white cultivar, but FHT and DFR activities were clearly present, although they were generally lower than in preparations from berry skins of the red cultivar.

During fruit ripening, similar patterns were observed in the red cultivar for CHS/CHI and DFR activity, with a maximum at the third stage, whereas FHT activity remained constantly high during the last three weeks of fruit ripening (Fig. 2a–c). No significant change in CHS/CHI activity was observed in the pink and white cultivars

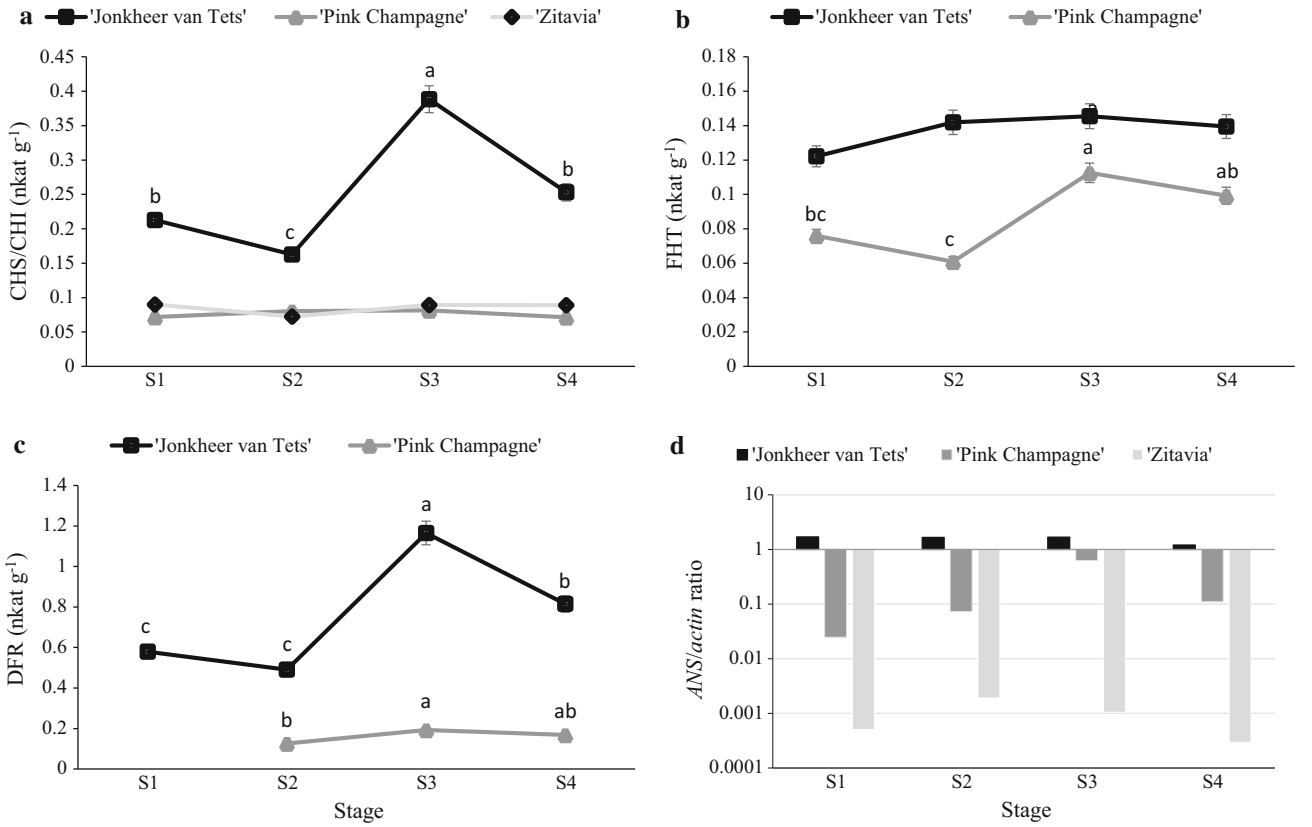

Fig. 2 Changes in specific enzyme activity (nkat g^{-1} protein) of CHS/CHI (**a**), FHT (**b**), DFR (**c**), and *ANS/actin* expression ratio (**d**) of red ('Jonkheer van Tets'), pink ('Pink Champagne') and white ('Zitavia') currant cultivars in different ripening stages (S1–S4). *Different letters* (*a–c*) above the lines denote significant differences among ripening stages (S1–S4) within the cultivar (Duncan's test $P < 0.05$)

during the sampling period. In the pink cultivar FHT showed a high activity and DFR a much lower maximum at the third sampling date, which correlated with the highest *ANS/actin* expression ratio (Fig. 2d).

Discussion

Ripening is a complex physiological process in fruit crops. Changes in flavonoid patterns are driven by changing physiological requirements, switching from protection against pathogens and herbivores to a timely attraction of seed dispersers. Fruit color is an important evolutionary trait and one of the few important ripening indicators for currants contributing to berry quality and subsequent market value. We, therefore, studied red, pink and white fruiting currant cultivars for changes in their color, different metabolites (primary and secondary), and selected enzyme activity in fruits.

All color parameters in our study decreased during fruit ripening, as reported earlier (Mikulic-Petkovsek et al. 2015). During ripening, all cultivars developed their specific color—red for cv. 'Jonkheer van Tets', translucent pink for 'Pink Champagne' and pale yellow for 'Zitavia' (Suppl. Fig. S1). Fruit color is mostly affected by anthocyanins, which in our study increased with ripening and improved fruit color, while the actual hue also depends on the presence of co-pigments and vacuolar pH (Dixon et al. 2013). We also measured juice pH (data not shown). However, during the fruit ripening of each cultivar, pH values in our study did not show obvious patterns, and therefore, no connection with total anthocyanins was observed.

Sugars and other carbohydrates are common regulators for the expression of genes encoding proteins involved in various processes, including anthocyanin biosynthesis (Solfanelli et al. 2006; Zheng et al. 2009; Shi et al. 2014). In our study, the content of total sugars increased significantly (32–70%) with advanced maturity and could thus influence anthocyanin accumulation. Conversely, total organic acids generally decreased somewhat, contributing to increased sugar/organic acid ratios during ripening. Interestingly, the sugar/organic acid ratio showed a negative correlation with the anthocyanin content of the three tested cultivars, but it remains open whether this is a general phenomenon in currants, since the number of cultivars tested was not high enough.

Some white cultivars showed complete lack of anthocyanins (Määttä et al. 2001, 2003; Mikulic-Petkovsek et al. 2015; Mattila et al. 2016), while a few others had detectable amounts (Pantelidis et al. 2007; Lugasi et al. 2011). No anthocyanins could be detected in our white cv. 'Zitavia'. Our data suggest that the flavonoid pathway in the white cultivar generally has low performance. The high absolute and relative content of total hydroxycinnamic acids in the white compared to the red cultivar confirms the presence of an early bottleneck in the flavonoid pathway. The higher relative flavonol content of berries of the white cultivar, however, indicates that at some stage, some of the intermediates not processed by the anthocyanin route are redirected towards flavonol formation. Since we could not detect FHT and DFR activity and the flavonol concentration did not further increase in the last three weeks of fruit ripening, we assume that the enzymes upstream DFR should be active in earlier fruit developmental stages. It has been shown for several strawberry varieties that flavonoid metabolism occurs following different gene and enzyme expression patterns in a two-stage process, resulting in the biosynthesis of flavonoid classes at distinct stages during early and late fruit development (Halbwirth et al. 2006; Carbone et al. 2009). The lack of anthocyanin formation in the white cultivar is probably due to the absence of DFR and ANS activity in the last three weeks of fruit ripening, in which the formation of anthocyanins is usually initiated to provide fruit pigmentation as a ripening indicator for animal seed dispersal. The absence of DFR and/or FHT activities in white cultivars of otherwise colored fruits has previously been reported for other species, such as ripe white bilberry (Jaakola et al. 2002; Primetta et al. 2015), strawberry (Thill et al. 2012a), or kiwi fruit (Halbwirth et al. 2009).

The pink cultivar in our study was characterized by a less active flavonoid pathway than with the red cultivar, which results in generally lower concentrations of TPC and particularly of total anthocyanins. The pink coloration is primarily caused by quantitative effects, since the anthocyanin composition showed moderate change and relative increase of cyanidin-3-*O*-glucosylrutinoside and cyanidin-3-*O*-rutinoside at the expense of cyanidin-3-*O*-xylosylrutinoside. The bottleneck in CHS/CHI (Fig. 2a) is correlated with the 23-fold increase of relative hydroxycinnamic acid concentrations. Interestingly, the white and pink cultivars also showed qualitative changes and accumulated up to 16% of glycosylated caffeic acid derivatives, in addition to glycosylated coumaric acids (Suppl. Table S3). We assume that hydroxylation in position 3 of coumaric acid occurs as a result of the tailback of hydroxycinnamic acid intermediates and could be part of a detoxification process. Hydroxylation is often the first step of increasing solubility and enabling storage of phenolic compounds in glycosylated and, frequently, acylated forms in the vacuoles to avoid damage of cell structures (Zhao and Dixon 2010). The occurrence of caffeic acid derivatives in the white cultivar also indicates that the hydroxylation step is favored in the competition of coumaric acid converting enzymes in an increasing pool of hydroxycinnamic acids.

A second important bottleneck in the pink compared to the red cultivar is caused by low DFR activity. This is demonstrated by the relatively high flavonol concentrations in comparison with anthocyanins and flavanols, indicating that the flux is primarily directed towards flavonol biosynthesis, particularly in earlier fruit developmental stages. In the investigated time period, there was a relatively high increase of anthocyanins, which correlates well with a peak of FHT activity and, to a lesser extent, DFR at the third sampling date. In addition to the low DFR activity, the observed substrate specificity could have a strong impact on anthocyanin formation. No delphinidin-based anthocyanins were detected, although DHM was the preferred substrate for DFR, and myricetin derivatives were present to a very low extent (Suppl. Table S3). We assume that the absence of F3′5′H is a key difference between red currants accumulating cyanidin glycosides and black currants accumulating delphinidin glycosides in the late stages of fruit development (Mikulic-Petkovsek et al. 2015). In the absence of 3′,4′,5′-hydroxylated dihydroflavonol precursor DHM, 3′,4′-hydroxylated dihydroflavonol DHQ will probably also be converted but lower contents of anthocyanins in red than in black currants can be expected, due to the low substrate specificity for DHQ. Although myricetin glycosides were present to a very low extent in all 3 cultivars, F3′5′H must have been active at some stage, although we assume that F3′5′H was expressed only at earlier developmental stages and not when anthocyanidins are mainly formed.

Intensely (mostly black) colored berries usually accumulate higher TCP levels than red or white ones (Lugasi et al. 2011; Mikulic-Petkovsek et al. 2015). In our study, the red cultivar contained the highest TPC, mostly due to high anthocyanin content (Fig. 1) and the white cultivar the lowest, probably due to lower or lack of activity of main flavonoid enzymes. These results obtained by the Folin–Ciocalteu method correlated positively with the sum of the concentrations of individual polyphenol classes obtained by HPLC–MS. Contrary to this, calculated sum of polyphenol classes detected by HPLC–MS in tested white and pink cultivars was only half of the amount measured with the Folin-Ciocalteu method.

The present study provides insight into the regulation of the flavonoid metabolism in differently pigmented currant cultivars. Genotype profoundly affected all tested enzymes activities and *ANS* gene expression, which determined different polyphenol composition and differences in

accumulation patterns of all phenolic groups, especially anthocyanins.

Author contributions statement DK, RV, MMP, and HH designed the study; SM, OSH, MMP, and ZZ performed research; ZZ and HH wrote the manuscript.

Acknowledgements The research is part of program Horticulture No. P4-0013-0481 funded by the Slovenian Research Agency (ARRS). Olly S. Hutabarat gratefully acknowledges Hasanuddin University (Agricultural Engineering Department, Makassar, South Sulawesi, Indonesia), the Ministry of Education and Culture of the Republic of Indonesia (DIKTI), and Austrian Agency for International Cooperation in Education and Research (OeAD-GmbH) for enabling the performance of the PhD studies abroad.

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