JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Alterations of Flavonoid Biosynthesis in Young Grapevine (*Vitis vinifera* L.) Leaves, Flowers, and Berries Induced by the Dioxygenase Inhibitor Prohexadione-Ca

IVA PUHL, FLORIAN STADLER, AND DIETER TREUTTER*

Center of Life and Food Sciences Weihenstephan, Technische Universität München Fachgebiet für Obstbau, Alte Akademie 16, D-85350 Freising, Germany

Prohexadione-Ca is a structural mimic of 2-oxoglutarate, and according to this property, it is able to inhibit dioxygenase enzymes, which require 2-oxoglutarate as a cosubstrate. Such enzymes are involved in flavonoid biosynthesis; therefore, prohexadione-Ca treatment leads to alterations in the flavonoid metabolism in grapevine tissues. Because of the fact that phenolic compounds often are responsible for enhanced plant resistance, modification of phenylpropanoid metabolism using elicitation can be considered as a new potential strategy in plant protection. The phenolic compounds were analyzed by high-performance liquid chromatography combined with chemical reaction detection. Tissue treatment induced the accumulation of unusual flavonoids, which were identified as derivatives of pentahydroxyflavanone, eriodictyol, and luteoliflavan. Concentrations of constitutive flavonoids were also affected by the bioregulator treatment. The alterations of the flavonoid profiles are discussed with respect to substrate preferences of relevant enzymes.

KEYWORDS: Dioxygenase; pentahydroxyflavanone; eriodictyol; flavanone; flavonol; luteoliflavan

INTRODUCTION

The bioregulator Regalis (BASF Ludwigsburg, Germany) reduces fruit set and berry weight of grapevine (*1*) by inhibition of the biosynthesis of hormonally active gibberellin (*2*). Its active ingredient, prohexadione-Ca, is a structural mimic of 2-oxoglutarate, and according to this property, it is able to inhibit dioxygenase enzymes, which require 2-oxoglutarate as a cosubstrate (*2*). Such enzymes are not only involved in gibberellin but also in flavonoid biosynthesis (*3*); and hence, prohexadione-Ca is able to alter flavonoid metabolism. Thereby, novel flavonoids are formed that were previously identified as 3-deoxycatechins in young leaves of apple (*4, 5*) and pear (*6*) and in grapevine leaves (*7*). The alteration of flavonoid composition is proposed to be related to enhanced resistance of several crop plants (*5–8, 9*). Prohexadione-Ca itself does not possess any antimicrobial activity, but its application leads to a reduction of infection caused by the bacterium *Erwinia amylovora* in apples (*4, 10*) and pears (*11*). Many studies suggest that phenolic compounds can be involved in plant resistance as antimicrobial compounds (*12–15*) or as mechanical barriers (*16–19*).

All of these observations may allow one to use prohexadione-Ca as an inducer of resistance-related compounds in any plant. Because of the fact that several enzymes of the flavonoid pathway may be inhibited by the bioregulator and considering that enzymes may possess different substrate affinities (*20*), detailed knowledge on the qualitative and quantitative changes in the composition of phenolic compounds in individual plants is a prerequisite for the assessment of the bioregulator with respect to resistance induction. In this paper, we describe the alteration of flavonoid biosynthesis in grapevine leaves, flowers, and berries induced by the bioregulator prohexadione-Ca.

MATERIALS AND METHODS

Plant Material. Young shoots from 6 year old potted plants of cultivars Müller Thurgau, Regent, and Pinot Gris showing about 10 unfolded leaves were sprayed with 500 ppm prohexadione-Ca in water until dripping wet. From each plant, nine young leaves from three different shoots were harvested at day 1, day 3, and day 6 after treatment. Three leaves were combined and analyzed as one mixed sample ($n = 9$ per harvest date).

In a further experiment, 3 year old potted plants of the same cultivars were cultivated in a greenhouse without a controlled temperature and without supplementary light. The inflorescences were sprayed twice

Table 1. *P* Values of Nonparametric Mann–Whitney Test for Comparison of Leaf Control against Leaf Prohexadione-Ca Treatment

	days after treatment		
	1	3	6
hydroxycinnamic acids	14.4	0.10[a]	5.57
flavonols	42.99	0.04[a]	0.07[a]
catechin	53.65	37.57	14.47
proanthocyanidins	82.47	53.63	92.96

[a] Significant at the 95% level.

* To whom correspondence should be addressed. Tel: +49-8161713753. Fax: +49-8161715385. E-mail: dieter.treutter@wzw.tum.de.

10.1021/jf0727645 CCC: $40.75
Published on Web 03/13/2008

Table 2. *P* Values of Nonparametric Mann–Whitney Test for Comparison of Phenol Concentrations of Untreated Leaves (Controls) According to Days after Treatment

	days after treatment	
	1	3
	hydroxycinnamic acids (controls)	
3	89.46	
6	12.21	0.62[a]
	catechin (controls)	
3	72.39	
6	33.11	35.26
	flavonols (controls)	
3	9.32	
6	11.20	0.62[a]
	proanthocyanidins (controls)	
3	21.64	
6	1.92[a]	25.10

[a] Significant at the 95% level.

Table 3. *P* Values of Nonparametric Mann–Whitney Test for Comparison of Phenol Concentrations of Prohexadione-Ca Treated Leaves According to Days after Treatment

	days after treatment	
	1	3
	eriodictyol 7-glucoside	
3	0.15[a]	
6	0.08[a]	6.37
	pentahydroxyflavanone glycosides	
3	2.17[a]	
6	0.08[a]	1.04[a]
	luteoliflavan	
3	85.92	
6	3.32[a]	2.67[a]

[a] Significant at the 95% level.

Table 4. *P* Values of Nonparametric Mann–Whitney Test for Comparison of Flowers/Berries Control against Flowers/Berries Prohexadione-Ca Treatment

	days after treatment						
	flowers and berries				skin		
	0	5	22	48	48	50	80
hydroxycinnamic acids	50.00	18.31	2.64[a]	1.41[a]	4.66[a]	2.64[a]	4.66[a]
flavonols	14.98	18.31	1.41[a]	7.69	12.26	12.26	30.05
catechin	25.93	12.26	2.64[a]	36.62	18.31	34.93	12.17
proanthocyanidins	25.93	14.98	1.41[a]	25.93	2.64[a]	4.66[a]	1.41[a]

[a] Significant at the 95% level.

with 500 ppm prohexadione-Ca, before bloom and at the end of flowering. At each sampling date, inflorescences or bunches (six replicates), respectively, per treatment were harvested according to growth stages from bloom to 80 days after flowering. From already developed berries, the skins were separated from the pulp using tweezers. For both experiments, an equal set of plants was sprayed with water. All samples to be tested were frozen in liquid nitrogen directly after sampling, stored at –20 °C, and finally were lyophilized.

Extraction of Phenolic Compounds for Analytical High-Performance Liquid Chromatography (HPLC). For the extraction of phenolic compounds, lyophilized leaves, inflorescences, from which the stems were removed, and berries were ground in a ball mill. Young flowers and berries were used as a whole (0, 5, 22, and 48 days after flowering), and from 48, 50, and 80 days after flowering, the skins were analyzed. The fine powder (100 mg) was extracted with 500 μL of methanol (100%) containing 6-methoxyflavone as an internal standard for 30 min in a cooled ultrasonic water bath. This sample was then centrifuged for 10 min at 10000*g* at 4 °C, and the supernatant was directly used for analytical HPLC. The recovery of the internal standard was 90% ± 12.

Table 5. *P* Values of Nonparametric Mann–Whitney Test for Comparison of Phenol Concentrations of Untreated Flowers, Berries, and Skin (Controls) According to Days after Treatment

	flowers and berries				skin	
	days after treatment				days after treatment	
	0	5	22		48	50
	hydroxycinnamic acids (controls)					
5	57.52			50	2.02[a]	
22	3.06[a]	4.53[a]		80	0.51[a]	2.02[a]
48	9.27	3.06[a]	1.31[a]			
	flavonols (controls)					
	0	5	22		48	50
5	0.82[a]			50	17.35	
22	0.51[a]	0.82[a]		80	0.51[a]	0.51[a]
48	0.51[a]	0.51[a]	0.51[a]			
	catechin (controls)					
	0	5	22		48	50
5	37.85			5	0.82[a]	
22	81.02	9.27		22	0.51[a]	42.3
48	12.82	37.85	12.82			
	proanthocyanidins (controls)					
	0	5	22		48	50
5	100.00			5	4.53[a]	
22	93.62	93.62		22	1.29[a]	100.00
48	6.56	12.82	12.82			

[a] Significant at the 95% level.

Analytical HPLC. Phenolic compounds were separated on a column (150 mm × 4 mm) prepacked with Hyperclone ODS, 3 μm particle size, following a stepwise gradient, using mixtures of solvent A (formic acid, 5% in water) and solvent B (methanol) from 95:5 (A/B) to 10:90 (A/B) with a flow rate of 0.5 mL/min using the following gradient: 0–5 min, isocratic, 5% B; 5–15 min, 5–10% B; 15–30 min, isocratic, 10% B; 30–50 min, 10–15% B; 50–70 min, isocratic, 15% ; 70–85 min, 15–20% B; 85–95 min, isocratic, 20% B; 95–110 min, 20–25%; 110–140 min, 25–30% B; 140–160 min, 30–40% B; 160–175 min, 40–50% B; and 175–190 min, 50–90% B. Hydroxycinnamic acids and flavonols (glycosides of quercetin) were detected at 280 nm, whereas the flavanols (catechin and luteoliflavan) and proanthocyanidins were estimated at 640 nm after postcolumn derivatization with DMACA (4-dimethylaminocinnamic aldehyde) (*21*). Peak identification was conducted by their UV absorbance spectra and by comparison with authentic standards: flavonols isoquercitrin, rutin, quercitrin (Roth), flavanones eriodictyol, eriodictyol 7-glucoside (Extrasynthèse), procyanidins B_2, B_3, and B_4 (previously isolated (*22*)) hydroxycinnamic acids, caftaric and coutaric acid, flavanols catechin, luteoliflavan, and luteoliflavan 5-glucoside (formerly isolated; see ref *5*). Pentahydroxyflavanone was kindly provided by G. Forkmann.

Quantification was performed using response factors of standards. Catechin, luteoliflavan, and luteoliflavan 5-glucoside were calculated as catechin, proanthocyanidins as procyanidin B2, pentahydroxyflavanone as eriodictyol, and pentahydroxyflavanone derivatives as eriodictyol 7-glucoside. Caftaric acid and coutaric acid were quantified using the response factors of their corresponding hydroxycinnamic acids caffeic acid and *p*-coumaric acid, respectively.

Preparative Scale Extraction. Fifty-three grams of lyophilized and pulverized grapevine leaves treated with prohexadione-Ca was extracted three times with 300 mL of 80% MeOH. The combined extracts were evaporated in vacuo to 300 mL and further extracted six times each with 300 mL of petrol. The volume of the MeOH phase (1200 mL) was reduced, diluted with water, and six times extracted each with 200 mL of EtOAc. EtOAc extracts were evaporated to dryness, resuspended in water, and lyophilized yielding a residue of 2.9 g.

Gel Chromatography on a Column of Sephadex LH-20. The dry residue was dissolved in 15 mL of EtOAc and further purified by gel

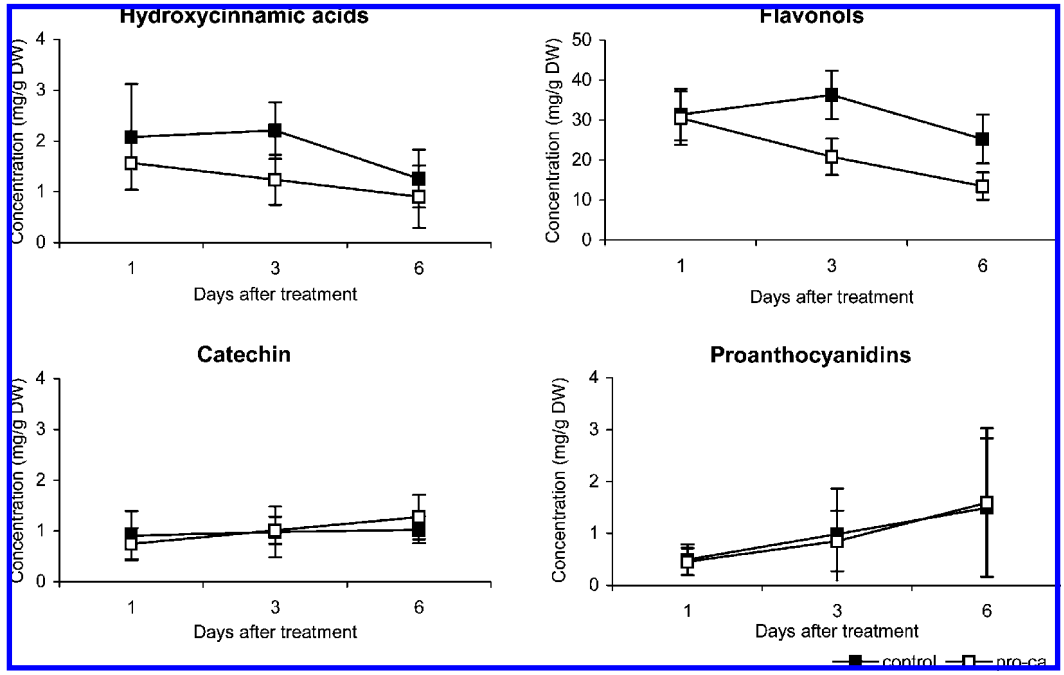

Figure 1. Changes in constitutive phenolic profiles (mg/g dry weight) in grapevine leaves after prohexadione-Ca treatment (pro-ca, 500 ppm) at days 1, 3, and 6 after treatment: hydroxycinnamic (sum of caftaric and coutaric acid), flavonols (sum of rutin, quercitrin, and isoquercitrin), catechin, and proanthocyanidins (sum of B_2, B_3, and B_4). The vertical lines represent the standard deviation from the mean of nine replicates. Significant differences and *P* values are indicated in **Tables 1** and **2**.

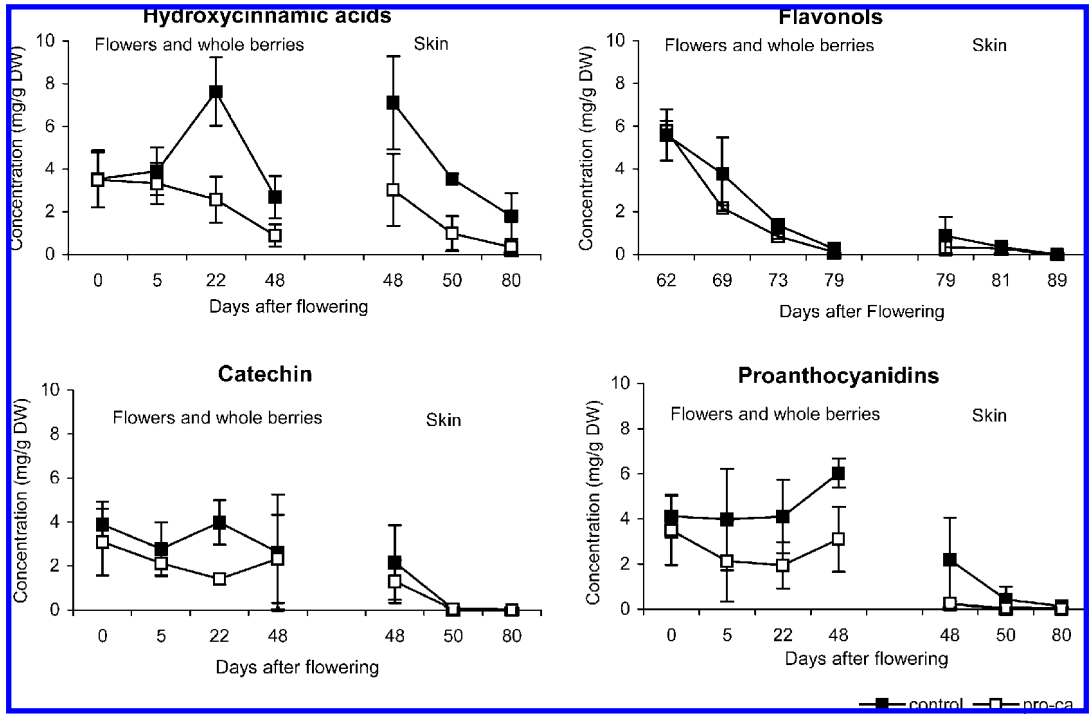

Figure 2. Changes in concentrations of constitutive phenolic compounds (mg/g dry weight) during berry development from flowering to 80 days after flowering: hydroxycinnamic (sum of caftaric and coutaric acid), flavonols (isoquercitrin), catechin, and proanthocyanidins (B_2, B_3, and B_4).

chromatography on a column of Sephadex LH-20 using ethanol as the solvent. One hundred twenty fractions were collected in portions of 10 mL and combined according to their photometric extinction and properties on thin-layer chromatography (TLC). Fractions 13−17 were combined to fraction 4 and used for the identification of compounds 2, 3, and 4. Fractions 18−23 were combined to fraction 5 and used for the identification of flavanone 1. Combined fractions were evaporated to dryness, resuspended in water, and lyophilized. Fractions 4 (31 mg) and 5 (93 mg) were redissiolved in MeOH and further purified with analytical HPLC. During five HPLC runs from each fraction, postcolumn eluates were collected within 2 min intervals. According to their retention times, HPLC fractions were combined, concentrated to dryness, and used for further identification using TLC and hydrolysis.

Enzymatic Hydrolysis. For enzymatic hydrolysis, the purified compounds were evaporated and redissolved in NaOAc buffer (pH 4.6, 0.1 M). The enzymes β-glucosidase (Sigma), Driselase (Sigma), or tannase (Sigma) were added, respectively, and the reaction mixtures were kept at 37 °C for 17 h. The reaction was stopped with MeOH and extracted three times with EtOAc. The combined EtOAc extracts

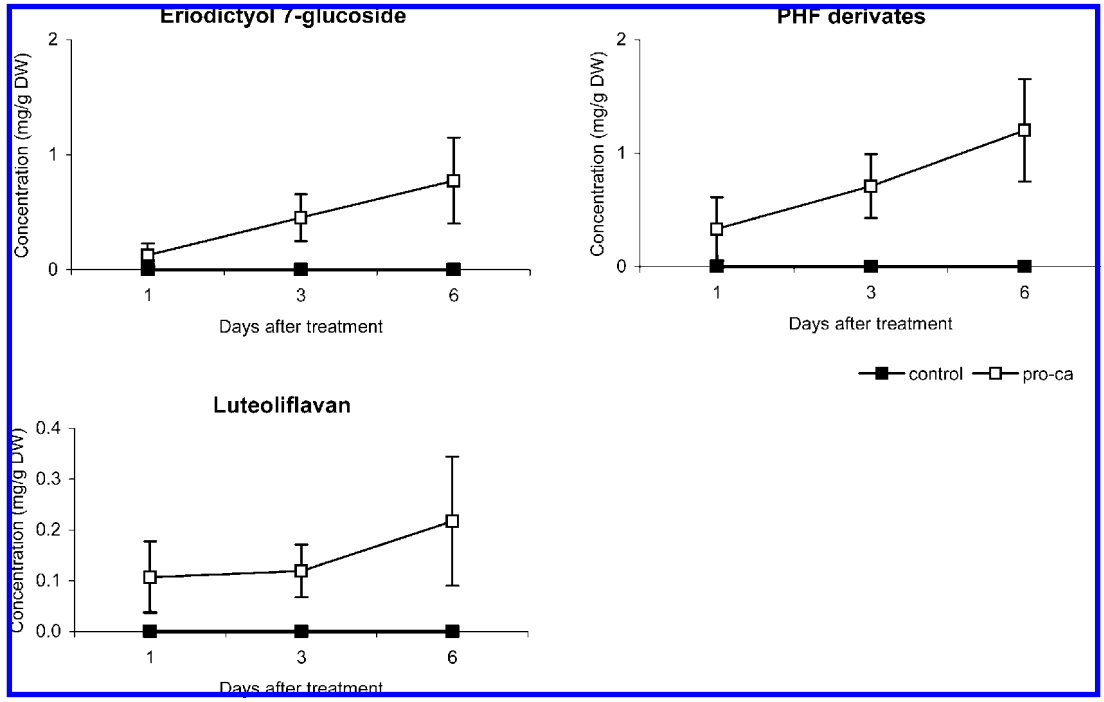

Figure 3. Changes in concentration (mg/g dry weight) in the grapevine leaves of the induced flavonoids eriodictyol 7-glucoside (flavanone 3) and pentahydroxyflavanones (PHF, sum of flavanones 1 and 2) and 3-deoxyflavonoid luteoliflavan after prohexadione-Ca treatment (pro-ca, 500 ppm) at days 1, 3, and 6 after treatment. The vertical lines represent the standard deviation from the mean of nine replicates. Significant differences and *P* values are indicated in **Table 3**.

were evaporated to dryness and redissolved in MeOH for TLC and HPLC analysis. The aqueous phases were used for the analysis of the sugar moieties.

TLC. Flavanones were separated on cellulose plates developed with *n*-BuOH:HOAc:H_2O (4:1:2.2 v/v/v). The compounds were visualized under UV light after spraying with a methanolic solution (1%) of Naturstoffreagenz A (Roth, Karlsruhe, Germany). Catechins were separated on silica gel plates developed with toluene:Me_2CO:HCO_2H; 3:6:1 (v/v/v), and were visualized by spraying with a solution of DMACA (1% in 6 M HCl:EtOH; 1:1 v/v).

Analysis of Sugar Moieties. HPLC sugar analysis was performed on a DIONEX BioLC-System equipped with a GP 40 gradient pump, an analytical column type Carbo Pac-100 (250 mm × 4 mm), and an electrochemical detector ED40 (pulsed amperometric). Sugars were eluted with 10 mM NaOH (1 mL/min) within 20 min.

Statistical Analysis. For statistical analysis, a pairwise comparison was performed by the nonparametrical Mann–Whitney test using "Minitab 14 statistical software". The *P* values are listed in **Tables 1−5**.

RESULTS

Profiles of Constitutive Phenolic Compounds in Leaves and Berries. Hydroxycinnamic acids (**Figures 1** and **2**) are mainly represented by *trans*-caffeoyltartaric and *trans*-*p*-coumaroyltartaric acid. Flavonols from the leaves were identified as the quercetin derivatives isoquercitrin, rutin, and quercitrin (**Figure 1**). Total amounts of flavonols are much lower in flowers and berries (**Figure 2**) than in leaves (**Figure 1**), where they represent the principal class of flavonoids. In flowers and berries, isoquercitrin was the only constitutive flavonol found. In flowers and green berries, proanthocyanidins are present at higher concentrations (**Figure 2**) than in leaves (**Figure 1**). A decline in the quantity of proanthocyanidins was observed in the skins of ripening berries (**Figure 2**).

Identification of Newly Formed Compounds. HPLC analyses of extracts from leaves, flowers, and berries treated with prohexadione-Ca revealed six additional compounds in total. Compounds 1−4 were stepwise purified by using Sephadex LH-20. Compounds 2−4 were eluted from Sephadex LH-20 in the fractions between 180 and 230 mL of ethanol and compound 1 with a further 50 mL. The UV spectra of these compounds indicated the presence of flavanones. After enzymatic hydrolysis with tannase, Driselase and β-glucosidase, the aglycones from flavanone 1 and flavanone 2, were identified as pentahydroxyflavanone, whereas the aglycones from flavanones 3 and 4 were identified as eriodictyol in comparison with authentic standards by UV absorbance, TLC, and HPLC. Analyses of the sugar moieties from flavanone 1 and 3 revealed glucose, whereas analysis of the aqueous phase from flavanones 2 and 4 failed. Therefore, flavanone 1 was identified as pentahydroxyflavanone glucoside. The position of the sugar remains unclear. UV, HPLC, and TLC properties of flavanone 3 were related to authentic standard eriodictyol 7-glucoside. Using analytical HPLC, flavanone 4 (eriodictyol derivate) was not detected in leaves; therefore, it was quantified only in flowers and berries. Compounds 5 and 6 were detected at 640 nm after postcolumn chemical reaction with DMACA, which classifies them as flavanols (*21*). Using TLC and HPLC, these compounds were identified as the 3-deoxycatechin luteoliflavan and luteoliflavan 5-glucoside (only in treated flowers and berries), respectively, in accordance with authentic samples previously isolated from apple leaves and identified using NMR spectroscopy (*5*).

Effect of Prohexadione-Ca Treatment on Constitutive Phenylpropanoid Composition of Grapevine Leaves and Berries. As shown in **Figures 1** and **2**, the changes in hydroxycinnamic acids and flavonols after prohexadione-Ca treatment show a similar behavior, namely, a reduction after 3 days following application onto leaves and with beginning of berry development (22 days after flowering), respectively (**Figure 2**). Herein, this effect lasted until the last stage sampled, that is, about 11 weeks after the last treatment. Catechin contents in leaves were not affected by prohexadione-Ca (**Figure 1**). During flower aging and berry development, they were slightly

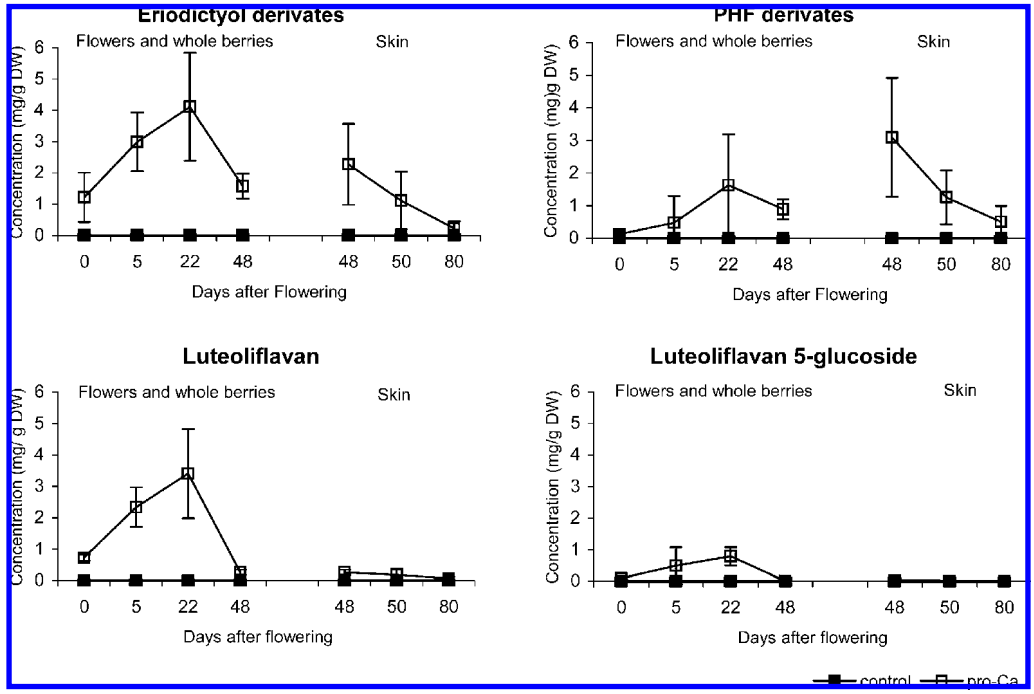

Figure 4. Changes in concentrations (mg/g dry weight) during berry development from flowering to 80 days after flowering of the induced flavonoids eriodictyol derivates (sum of flavanones 3 and 4), pentahydroxyflavanone (PHF, sum of flavanones 1 and 2), and 3-deoxyflavonoids (sum of luteoliflavan and luteoliflavan 5-glucoside).

reduced (**Figure 2**). Values strongly declined in skins of ripening berries (**Figure 2**). Proanthocyanidins tended to behave in a similar way as catechin (**Figures 1** and **2**).

Induction of Newly Formed Flavonoids after Prohexadione-Ca Treatment. In the prohexadione-Ca-treated tissues, an accumulation of flavanones and 3-deoxyflavanoids was obtained (**Figures 3** and **4**). In the leaves, the time course showed a progressive accumulation of flavanones and luteoliflavan until day 6 after treatment (**Figure 3**). The longer lasting study of the berries exhibited an increase of both flavanones and 3-deoxyflavans (luteoliflavan and luteoliflavan 5-glucoside) until day 22 after treatment, which declined afterward (**Figure 4**).

DISCUSSION

The accumulation of flavanones (**Figures 3** and **4**) and the decrease of flavonols (**Figures 1** and **2**) in treated leaves, flowers, and green berries indicated that the enzymes FHT and FLS, as 2-oxoglutarate-dependent dioxygenases (*3*), are inhibited by prohexadione-Ca (**Figure 5**). This was formerly described for apple (*5, 6, 9*) and pear (*23*). Comparing the flavonoid profiles of tested tissues, differences in flavonol contents are most conspicuous. High concentrations of flavonols in leaves (**Figure 1**) and their decrease in flowers after pollination (**Figure 2**) are consistent with literature data concerning analysis of phenolics as well as gene expression of FLS (*24*−*26*). The proposed inhibition of the FLS enzyme by prohexadione-Ca (**Figure 5**) caused a marked decrease of flavonol concentrations (**Figure 1**). As catechin is yet synthesized to a lesser extent in leaves (**Figure 1**) than in flowers and berries (**Figure 2**), we think that in the presence of a strongly active FLS, the conversion of dihydroflavonols to flavonols dominates over the DFR/LAR route of the flavonoid pathway. This may also explain why catechin as well as proanthocyanidin concentrations in leaves (**Figure 1**), unlike in berries (**Figure 2**), are scarcely affected by the prohexadione-Ca treatment. Blocking FLS (**Figure 5**) may have directed metabolites toward the synthesis of flavan 3-ols and thus may have compensated for the loss due to the partially inhibited FHT activity.

The formation of 3-deoxyflavonoids in treated grapevine tissues (**Figures 3** and **4**) is achieved by a postulated FNR enzyme, which catalyzes the reduction of the flavanone eriodictyol to the flavan-4-ol luteoforol, which is the unstable immediate precursor of luteoliflavan (*27*) (**Figure 5**). Recent work (*7, 20*) showed that the enzyme DFR, which normally catalyzes the reduction of dihydroflavonols to leucoanthocyanidins (*28*) (**Figure 5**) of apple, pear, strawberry, and grapevine, possesses FNR activity and, therefore, is able to perform the key reaction in the formation of 3-deoxyflavonoids. Furthermore, it is noteworthy that luteoliflavan was the only 3-deoxyflavanol aglycone found in prohexadione-Ca treated tissues. This observation points at the substrate affinity of the FNR since the FHT inhibition also leads to the accumulation of pentahydroxyflavanone (**Figures 3** and **4**), which, however, was not reduced to the corresponding 3-deoxyflavanol. Fischer et al. (*20*) demonstrated a substrate preference for the DFR/FNR of apple and pear. Thus, a high substrate specificity can also be assumed for the DFR/FNR of grapevine, reducing eriodictyol to 3-deoxyleucocyanidin (luteoforol) (**Figure 5**). Further reduction to luteoliflavan is catalyzed by a proposed LAR. Pfeiffer et al. (*29*) showed that grape and apple LARs are able to convert luteoforol to luteoliflavan. Under natural conditions, grapevine LAR catalyzes the conversion of leucocyanidin to catechin (*30*) (**Figure 5**).

The most recognizable accumulation of 3-deoxyflavonoids and flavanones (**Figure 4**) and decrease of catechin during grape berry development (**Figure 2**) after prohexadione-Ca treatment was observed at flowering and fruit sets. Also, the expressions of the genes encoding for LAR and DFR were both described to be highest in the same period by other authors (*31, 32*). Although the effect of prohexadione-Ca on flavanone accumulation was still noticeable in the skins of growing berries, the content of luteoliflavan decreased dramatically after the stage of bunch closure (**Figure 4**). The results obtained show that it is possible to induce an additional formation of specific phenylpropanoid compounds using the bioregulator prohexadione-Ca.

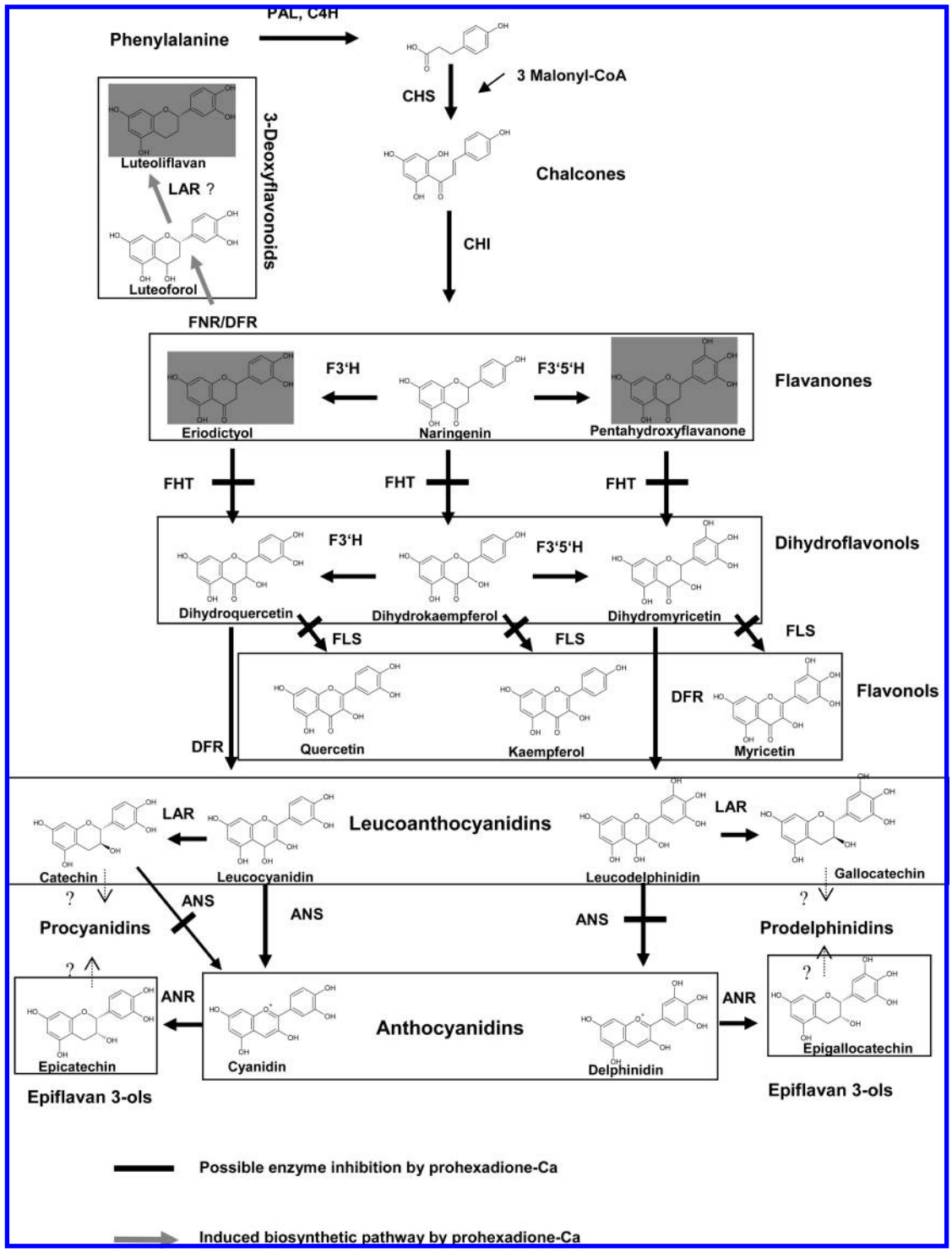

Figure 5. Simplified schematic overview of the major phenylpropanoid pathway in grapevine modified after references (*3*) and (*31*).

Even though for practical application in grape production the influence of prohexadione-Ca on the hormonal balance of the plant must be considered, which may lead, for instance, to reduced berry size and cluster compactness, the bioregulator can be applied as a tool to change the flavonoid composition of grapevine tissues. Because of the fact that phenolic compounds often are responsible for enhanced plant resistance, modification of the phenylpropanoid metabolism using elicitation can be considered as a new potential strategy in plant protection. For the 3-deoxyleucocyanidin luteoforol, antimicrobial properties were shown against, among others, *Botrytis cinerea* (*33*), and thus, susceptibility might be reduced in grapevine by prohexadione-Ca treatment. Tissues were treated with prohexadione-Ca (500 ppm) before bloom and at flowering. The vertical lines represent the standard deviation from the mean of six replicates. Significant differences and *P* values are indicated in **Tables 4** and **5**. Tissues were treated with prohexadione-Ca (500 ppm) before bloom and at flowering. The vertical lines represent the standard deviation of six replicates.

ABBREVIATIONS USED

PAL, phenyalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; CHS, chalcone synthase; CHI, chalcone isomerase;

FNR, flavanone 4-reductase; FHT, flavanone 3-hydroxylase; DFR, dihydroflavonols 4-reductase; LAR, leucoanthocyanidin 4-reductase; F3′H, flavonoid 3′-hydroxylase; F3′5′H, flavonoid 3′5′-hydroxylase; FLS, flavonol synthase; ANS, anthocyanidin synthase; ANR, anthocyanidin reductase.

LITERATURE CITED

(1) Lo Giudice, D.; Wolf, T. K.; Zoecklein, B. W. Effects of prohexadione-calcium on grape yield components and fruit and wine composition. *Am. J. Enol. Vitic.* **2004**, *55*, 73–83.

(2) Rademacher, W. Growth retardants: Effects on gibberellin biosynthesis and other metabolic pathways. *Plant Mol. Biol.* **2000**, *51*, 501–531.

(3) Forkmann, G.; Heller, W. Biosynthesis of flavonoids. In *Comprehensive Natural Products Chemistry*; Barton, D. H. R., Nakanishi, K., Eds.; Elsevier: Amsterdam, 1999; Vol. 1, pp 714–748.

(4) Römmelt, S.; Treutter, D.; Speakman, J. B.; Rademacher, W. Effects of prohexadione-Ca on the flavonoid metabolism of apple with respect to plant resistance against fire blight. *Acta Hortic.* **1999**, *489*, 359–364.

(5) Römmelt, S.; Zimmermann, N.; Rademacher, W.; Treutter, D. Formation of novel flavonoids in apple (*Malus* × *domestica*) treated with the 2-oxoglutarate-dependent dioxygenase inhibitor prohexadione-Ca. *Phytochemistry* **2003**, *64*, 709–716.

(6) Römmelt, S.; Fischer, T. C.; Halbwirth, H.; Peterek, S.; Schlangen, K.; Speakman, J.-B.; Treutter, D.; Forkmann, G.; Stich, K. Effect of dioxygenase inhibitors on the resistance-related flavonoid metabolism of apple and pears: Chemical, biochemical and molecular biological aspects. *Eur. J. Hortic. Sci.* **2003**, *68*, 129–136.

(7) Gosch, C.; Puhl, I.; Halbwirth, H.; Schlangen, K.; Römmelt, S.; Andreotti, C.; Costa, G.; Fischer, T. C.; Treutter, D.; Stich, K.; Forkmann, G. Effect of prohexadione-Ca on various fruit crops: flavonoid composition and substrate specificity of their dihydroflavonol 4-reductases. *Eur. J. Hortic. Sci.* **2003**, *68*, 144–151.

(8) Rühmann, S.; Treutter, D. Effect of N-nutrition in apple on the response of its secondary metabolism to prohexadione-Ca treatment. *Eur. J. Hortic. Sci.* **2003**, *68*, 152–159.

(9) Fischer, T. C.; Halbwirth, H.; Roemmelt, S.; Sabatini, E.; Schlangen, K.; Andreotti, C.; Spinelli, F.; Costa, G.; Forkmann, G.; Treutter, D.; Stich, K. Induction of polyphenol gene expression in apple (*Malus* × *domestica*) after the application of a dioxygenase inhibitor. *Physiol. Plant* **2006**, *128*, 604–617.

(10) Yoder, K. S.; Miller, S. S.; Byers, R. E. Suppression of fireblight in apple shoots by prohexadione-calcium following experimental and natural inoculation. *Hortic. Sci.* **1999**, *34*, 1202–1204.

(11) Costa, G.; Andreotti, C.; Bucchi, F.; Sabatini, E.; Bazzi, C.; Malaguti, S.; Rademacher, W. Prohexadione-Ca (Apogee): Growth regulation and reduced fire blight incidence in pear. *Hortic. Sci.* **2001**, *36*, 931–933.

(12) Scalbert, A. Antimicrobial properties of tannins. *Phytochemistry* **1991**, *30*, 3875–3883.

(13) Yamamoto, M.; Nakatsuka, S.; Otani, H.; Kohmoto, K.; Nishimura, S. (+)-Catechin acts as an infection-ihibiting factor in strawberry leaf. *Phytopathology* **2000**, *90*, 595–599.

(14) Del Rio, J. A.; Baidez, A. G.; Botia, J. M.; Ortuno, A. Enhancement of phenolic compounds in olive plants (*Olea europaea* L.) and their influence on resistance against *Phytophthora* sp. *Food Chem.* **2003**, *83*, 75–78.

(15) Parvez, M. M.; Tomita-Yokotani, K.; Fujii, Y.; Konishi, T.; Iwashina, T. Effects of quercetin and its seven derivatives on the growth of *Arabidopsis thaliana* and *Neurospora crassa*. *Biochem. System. Ecol.* **2004**, *32*, 631–635.

(16) Weber, B.; Hoesch, L.; Rast, D. M. Protocatechualdehyde and other phenols as cell wall components of grapevine leaves. *Phytochemisty* **1995**, *40*, 433–437.

(17) Feucht, W.; Treutter, D.; Schwalb, P. Principles of barrier formation of scab-infected apple fruits. *Z. Pflanzenkr. Pflanzenschutz* **1998**, *105*, 394–403.

(18) Schwalb, P.; Feucht, W. Affinity of flavanols to cell walls: Changes after wounding, infection, lignification, and suberization. *J. Appl. Bot.* **1998**, *72*, 157–161.

(19) Feucht, W.; Treutter, D. The role of flavan-3-ols and proanthocyanidins in plant defence. In *Principles and Practices in Plant Ecology*; Inderjit, D., Foy C. L., Eds.; CRC Press: Boca Raton, FL, 1999; pp 307–338.

(20) Fischer, T. C.; Halbwirth, H.; Meisel, B.; Stich, K.; Forkmann, G. Molecular cloning, substrate specificity of the functionally expressed dihydroflavonol 4-reductases from *Malus domestica* and *Pyrus communis* cultivars and the consequences for flavonoid metabolism. *Arch. Biochem. Biophys.* **2003**, *412*, 223–23.

(21) Treutter, D. Chemical reaction detection of catechins and proanthocyanidins with 4-dimethylaminocinnamaldehyde. *J. Chromatogr.* **1989**, *467*, 185–193.

(22) Feucht, W.; Treutter, D.; Christ, E. Flavanols in grapevine: in vitro accumuklationand defence reactions in shoots. *Vitis* **1996**, *35*, 113–118.

(23) Peterek, S. Phenolische Inhaltsstoffe der Birne und ihre Bedeutung in der Resistenz gegen den Feuerbrand. Ph.D. thesis submitted to the Technische Universität München, 2005.

(24) Downey, M. O.; Harvey, J. S.; Robinson, S. P. Synthesis of flavonols and expression of flavonol synthase genes in the developing grape berries of Shiraz and Chardonnay (*Vitis vinifera* L.). *Aust. J. Grape Wine Res.* **2003**, *9*, 110–121.

(25) Doshi, P.; Adsule, P.; Banerjee, K. Phenolic composition and antioxidant activity in grapevine parts and berries (*Vitis vinifera* L.) cv. Kishmish Chornyi (Sharad Seedless) during maturation. *Int. J. Food Sci. Technol.* **2006**, *41*, 1–9.

(26) Fujita, A.; Goto-Yamamoto, N.; Aramaki, I.; Hashizume, K. Organ-specific transcription of putative flavonol synthase genes of grapevine and effects of plant hormones and shading on flavonol biosynthesis in grape berry skins. *Biosci., Biotechnol., Biochem.* **2006**, *70*, 632–638.

(27) Stich, K.; Forkmann, G. Biosynthesis of 3-deoxyanthocyanins with flower extracts from *Siningia cardinalis*. *Phytochemistry* **1988**, *27*, 785–789.

(28) Gollop, R.; Even, S.; Colova-Tsolova, V.; Perl, A. Expression of the grape dihydroflavonol reductase gene and analysis of its promoter region. *J. Exp. Bot.* **2002**, *53*, 1397–1409.

(29) Pfeiffer, J.; Kühnel, C.; Brandt, J.; Duy, D.; Punyasiri, P. A. N.; Forkmann, G.; Fischer, T. C. Biosynthesis of flavan 3-ols by leucoanthocyanidin 4-reductases and anthocyanidin reductases in leaves of grape (*Vitis vinifera* L.), apple (*Malus* × *domestica* Borkh.) and other crops. *Plant Physiol. Biochem.* **2006**, *44*, 323–334.

(30) Tanner, G. J.; Francki, K. T.; Abrahams, S.; Watson, J. M.; Larkin, P. J.; Ashton, A. R. Proanthocyanidin biosynthesis in plants: purification of legume leucoanthocyanidin reductase and molecular cloning of its cDNA. *J. Biol. Chem.* **2003**, *278*, 31647–31656.

(31) Bogs, J.; Downey, M. O.; Harvey, J. S.; Ashton, A. R.; Tanner, G. J.; Robinson, S. P. Proanthocyanidin synthesis and expression of genes encoding leucoanthocyanidin reductase in developing grape berries and leaves. *Plant Physiol.* **2005**, *139*, 652–663.

(32) Bogs, J.; Ebadi, A.; McDavid, D.; Robinson, S. P. Identification of the flavonoid hydroxylases from grapevine and their regulation during fruit development. *Plant Physiol.* **2006**, *140*, 279–291.

(33) Spinelli, F.; Speakman, J.-B.; Rademacher, W.; Halbwirth, H.; Stich, K.; Costa, G. Luteoforol, a flavan 4-ol, is induced in pome fruits by prohexadione-calcium and shows phytoalexin-like properties against *Erwinia amylovora* and other plant pathogens. *Eur. J. Plant Pathol.* **2005**, *112*, 133–142.

Received for review September 17, 2007. Revised manuscript received January 24, 2008. Accepted February 3, 2008. We gratefully acknowledge the Bayerisches Staatsministerium für Umwelt, Gesundheit and Verbraucherschutz, for financial support of the study.

JF0727645