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Specific Poly-phenolic Compounds in Cell Culture of *Vitis vinifera* L. cv. Gamay Fréaux

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Abstract Cell cultures established from plants represent an attractive alternative to whole plants for effective production of bioactive secondary metabolites. Cell culture from *Vitis vinifera* L. cv. Gamay Fréaux accumulated high amounts of hydroxycinnamic acid derivatives and anthocyanins. Two new compounds were identified: 3-*O*-glucosylresveratrol, a stilbene derivative, abundant in cell suspension culture, and a hydroxyphenol, 4-(3,5-dihydroxyphenyl)-phenol, abundant in callus culture. The major anthocyanin monoglucosides present in cell suspension culture were cyanidin 3-*O*-glucoside and peonidin 3-*O*-glucoside, and the major cinnamoyl derivatives were cyanidin 3-*O*-*p*-coumaryl glucoside and peonidin 3-*O*-*p*-coumaryl glucoside. Three minor anthocyanin compounds were found in *V. vinifera* cell culture: delphinidin 3-*O*-glucoside, petunidin 3-*O*-glucoside, and delphinidin 3-*O*-*p*-coumaryl glucoside. Anthocyanin levels of cell suspension cultures increased significantly—about eight fold—after 4-day cultivation in new medium. Salicylic acid at a concentration of 50 μM did not enhance anthocyanin accumulation in cell suspension culture, and similar levels of jasmonic acid significantly reduced the anthocyanin content.

Keywords Resveratrol derivative · Anthocyanin · Cell culture · *Vitis vinifera* · Elicitation · Wine

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Introduction

Terrestrial plants, comprising about 250,000 living species [1], are an extremely diverse source of chemicals with bioactive properties that are in particular valuable to the pharmaceutical and food industry. Plant-derived products are expected to play an increasingly significant role in the commercial development of new drugs, dietary supplements, and functional food products in the near future [2, 3]. Recently, compounds such as taxol, vinblastine, vincristine, podophyllotoxin, and camptothecin, which are all plant secondary metabolites or at least based on them, have significantly improved the success and effectiveness of chemotherapy against some serious cancer types such as breast, lung ovarian, renal, colon, and gastric cancer [4, 12].

However, plants themselves may not be an ideal source of bioactive natural compounds due to a number of reasons such as (1) slow plant growth, (2) endangered plant species, and (3) low in vivo productivity. Cell cultures established from plants represent an attractive alternative to whole plants for effective production of specific secondary metabolites, and they have the potential to accumulate novel natural compounds [5, 6]. Utilization of cell culture and bioreactor technology has long been considered as an attractive alternative to the problematic process of extracting the compounds from the whole plants [5] since cells can be cultured in large quantities under controlled conditions and since secondary metabolites are easier to isolate. Plant cell culture is considered as an optimal method for continual production of secondary metabolites and is not influenced by environmental factors. However, chemical and physical factors such as media component, phytohormones supplements, pH, temperature, and light are affecting secondary metabolite accumulation [7]. The production of secondary metabolites can be enhanced by (1) feeding cultures with precursor substances, e.g., ferulic acid increased vanillin accumulation in cell culture of *Vanilla planifolia* [30]; (2) potentially, synthesis of entirely novel substances which can be done through biotransformation or by taking advantage of somaclonal variation [6]; (3) selection of highly producing cell lines, e.g., a sevenfold increase of anthocyanin compared with parent cultures was obtained after 24 cell line selections in *Euphorbia milii* [9]; and (4) elicitation of valuable secondary metabolites by using biotic and/or abiotic elicitors, e.g., increased amounts of paclitaxel and baccatin III were observed in cell cultures off *Taxus* species after treatment with methyl jasmonate [8, 11]. In the present study, some of the measures were investigated to achieve high levels of poly-phenolic compounds in cell culture of *Vitis vinifera* L. cv. Gamay Fréaux.

Phenolics of wine and grapes usually include derivatives of hydroxybenzoic and hydroxycinnamic acids, trihydroxystilbenes such as *cis*- and *trans*-resveratrol (stilbene-3,5,4′-triol) as well as *cis*- and *trans*-piceid (resveratrol-3-*O*-β-D-glucoside), flavonoids (e.g., flavan-3-ols, flavones, anthocyanins, and anthocyanidins), and condensed tannins [13, 14]. White wines tend to be low in their content of phenolic compounds compared with red wine. Malvidine-3-glucoside is the major anthocyanin of most cultivars such as Cabernet Sauvignon, Merlot, Pinot Noir, Monastrell, and Temparillo [14, 15]. In addition, these wines also contain 3-monoglucosides of delphinidin, cyanidin, petunidin, and peonidin. Resveratrol is the parent molecule of a family of polymers called viniferins, and this wine constitute occurs freely in glycoside [19]. Earlier phytochemical studies revealed that grape cell cultures biosynthesize resveratrol monomer derivatives, and this resulted in research for novel natural cancer chemopreventive products and antioxidative agents [16]. Numerous studies have described the cancer-preventive mechanisms of resveratrol [20, 31]. However, derivatives of resveratrol could be even more effective than resveratrol in treating cancer development in humans, like it has been reported for the synthetic 3,5,3′,4′,5′-

pentamethoxystilbene [17]. Cell suspension culture of *V. vinifera* Gamay Fréaux has also been shown to accumulate bioactive compounds such as anthocyanin monoglucosides and *p*-coumaryl glucosides with different cell lines and culture conditions exhibiting differences in anthocyanin composition [18]. The aim of the present study was to identify abundant poly-phenolic compounds such as stilbene derivatives and anthocyanins in a cell culture of *V. vinifera* under different culture conditions. In principle, secondary metabolites can be derived from callus, cell, and suspension cultures, but the biosynthesis of compounds may differ depending on cultivation conditions applied [32]. The present study gives preliminary insides in qualitative composition of phenolic compounds present in callus and—after selection process—high-yielding cell suspension cultures of *V. vinifera*. A key factor for feasible economic production is the induction of respective secondary metabolites by using chemical and physical elicitors. This factor is exemplarily addressed in this study by applying signaling molecules to cell culture to enhance the production of anthocyanins. Further options for improving the efficiency of cell factories are discussed.

Material and Methods

Cultivation of Cell Culture

The cell culture of *Vitis vinifera* L. cv. Gamay Fréaux was originally obtained 15 years ago from Francois Cormier (Food Research and Development Center, Agriculture Canada). Since then, it has been continuously maintained at the Department of Food Biotechnology of Berlin University of Technology. The cell culture was cultivated on B5 medium [10] with 0.1 mg/L NAA, 0.2 mg/L kinetin, 0.25 casein hydrolysate, 3% sucrose, and 0.8% agar. Callus cultures were transferred every 28 days to fresh solidified sterile medium. Here, red pigmented cell aggregates of calli were selected preferably for further cultivation. During the continuous cultivation process, light red or occasionally occurring white cells were discarded. Cell suspension cultures were established by transferring red pigmented cell aggregates of the agar culture into 50 mL of liquid B5 medium in 200-mL Erlenmeyer flasks and continuously agitating the flasks on a rotary shaker at 110 rpm. The cell cultures were transferred to new medium every 3 weeks. *V. vinifera* cell cultures were maintained at 25 °C and at a L 12/D 12 h. Samples for chemical analysis of hydroxycinnamic acid derivatives were taken from 2-week-old callus and cell suspension cultures. In cell suspension cultures, the medium was removed from cell culture by applying vacuum to culture in a suction filter. Harvested calli were immediately flash frozen in liquid nitrogen and freeze-dried.

Chemical Analysis of Hydroxycinnamic Acid Derivatives and Preparative HPLC

A total of 20 mg grounded samples from callus and cell suspension culture were extracted for 15 min using 750 μL 70% methanol (*v*/*v*, pH 4, phosphoric acid) in an ultrasonic water bath on ice. Samples were centrifuged for 5 min at 6,000 rpm. The supernatants were collected, and the pellets were re-extracted twice more with 500 μL 70% methanol. Coumaric acid or cinnamic acid (40 μL of 3 mM solution) was added as internal standard to the first extraction. The combined supernatants from each sample were reduced to near dryness in a centrifugation evaporator (SpeedVac, SC 110) at 25 °C. Samples were then made up to 1 mL with 40% acetonitrile. Extracts (10 μL) were analyzed on a Dionex Summit P680A high-performance liquid chromatography (HPLC) system, equipped with

an ASI-100 auto-sampler and a PDA-100 photodiode array detector. Hydroxycinnamic acid derivatives were separated on a narrow bore Acclaim PA C16-column (150×2.1 mm, 3 μm, Dionex) at a flow rate of 0.4 mLmin^{-1} and a column temperature of 35 °C. A 30-min gradient program was used with 1% (*v*/*v*) phosphoric acid in ultrapure water (eluent A) and of 40% (*v*/*v*) acetonitrile in ultrapure water (eluent B) as follows: 0–1 min,: 0.5% B; 1–10 min, 0–40% B; 10–12 min, 40% B; 12–18 min, 40–80% B; 18–20 min, 80% B; 20–24 min, 80–99% B; 24–30 min, 99–100% B. The gradient program was followed by a 4-min period to return to 0.5% B and a 5-min equilibration period, resulting in a total duration of 39 min. The eluent was monitored at 290, 330, and 254 nm. Phenolic acid quantity was calculated from HPLC peak areas at 290 nm against the internal standard.

Collection of Fractions and LC–MS Analysis

Fractions of the most abundant hydroxycinnamic acid compounds, which eluted at 18.0 and 19.0 min, were collected on an Agilent 1100 HPLC system equipped with the fractionator over a Supelcosil LC-18DB column (250×10 mm, 5 μm) by using acidified ultrapure water, 0.2% (*v*/*v*) formic acid, and acetonitrile. Fractions were reduced to dryness as previously described for extracts in a vacuum concentrator. Peak identity of concentrated fractions was proved on the Dionex HPLC system Acclaim equipped with PA C16 column by using retention times and UV spectra. Liquid chromatography–mass spectrometry (LC–MS) analyses of concentrated extracts were carried out on an HPLC-1100 series chromatograph (Agilent Technologies, Böblingen, Germany). Chromatograph was coupled to an Esquire 6000 electrospray ionization (ESI)–ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany) operated in negative ion mode in the range *m*/*z* 50–700. The MS was operated with a capillary voltage of −4,000 V and a nebulizer pressure of 35 psi; the drying gas flow was 10 Lmin^{-1}, and the drying gas temperature was 330 °C. Samples were analyzed over LiChrospher 100 RP18E column (25 cm×4.6 mm, 5 μm, Wicom, Germany) with a gradient of 0.2% (*v*/*v*) formic acid (solvent A) and acetonitrile (solvent B) at a flow rate of 1.0 mLmin^{-1} at 25 °C with a gradient described above for HPLC analysis. The flow coming from the column was diverted in a ratio of 4:1 before reaching the ESI unit. Mass spectrometer was operated in full scan mode as well as in AutoMS mode in order to produce MS^2 and MS^3 spectra.

NMR Analysis

Aqueous samples were freeze-dried (Modulyo, Edwards) and dissolved in DMSO (100 At. % D, Aldrich). Proton nuclear magnetic resonance (NMR), ^{13}C NMR, distortionless enhancement by polarization transfer (DEPT), COSY, and heteronuclear single quantum coherence (HSQC) spectra were recorded on a Bruker 400-MHz NMR against tetramethylsilane (TMS) as standard. Spectra were processed using iNMR (Mestrelab Research); estimates of chemical shifts were calculated using ChemDraw Ultra (7.0.1, Cambridge Software).

Chemical Analysis of Anthocyanins

Anthocyanins were extracted from 100 mg of *V. vinifera* cell suspension cultures, after removing the medium with a suction filter. To each sample, 750 μL of 79% (*v*/*v*) ethanol with 1% (*v*/*v*) glacial acetic acid was added, and samples were incubated in a heat block for 20 min at 85 °C. After centrifugation for 5 min at 13,000 rpm, the supernatants were

collected, and the pellets were re-extracted twice more with the extraction solvent. Supernatants were combined, and 50 μL of 37% hydrochloric acid for stabilizing of anthocyanins was added. After 15-min incubation at room temperature, the extracts were analyzed with HPLC for identification of compounds. For total anthocyanin determination, the absorbance at 535 was recorded photometrically by using ε=98.2 (dilution 1:2).

HPLC and LC–MS Analysis of Anthocyanins

An amount of 20 μL of anthocyanin extract was injected into the Dionex HPLC system as described previously using a PA C16 column. The eluent was monitored at 518 nm at a flow of 0.4 $mL\,min^{-1}$ and an oven temperature of 45 °C. The following 26-min gradient program was used with eluent A, 10 mM potassium dihydrogen phosphate in ultrapure water (pH=2, phosphoric acid), and eluent B, 90% acetonitrile (*v*/*v*, pH=2, phosphoric acid): 0–1 min, 0.5% B; 1–12 min, 0.5–30% B; 12–13 min, 30% B; 13–20 min, 30–50% B; 20–25 min, 50–100% B; 25–26 min, 100% B, followed by 3 min return to 0.5% B and equilibration for 6 min (35 min total). Main anthocyanins were collected by peak at 11.5, 12.5, 14.4, and 15.0 min using a fractionator (Foxy Jr., Dionex). Fractions were concentrated in a vacuum concentrator. After concentration, the samples were analyzed with LC–MS, as described above, with conditions resembling those for hydroxycinnamic acids.

Treatment of Cell Suspension Cultures with Signaling Molecules

V. vinifera cell suspension cultures were pooled in a sterile suction filter, and the medium passed the filter dropwise (15 min). From this pool, 4 g (drain weight) of calli was transferred to 100-mL flasks containing 25-mL sterile B5 medium (control), B5 medium with 50 μM jasmonic acid or 50 μM salicylic acid. The cell suspension culture used to obtain the pool was 18 days old. Cell cultures were kept on a rotary shaker at 110 rpm at 25 °C and at a LD 12/D 12 h for 4 days. The experiment was conducted using six replications per treatment. Fresh weight of culture was measured after applying vacuum to cell cultures in the suction filter. The dry weight of the cell culture was determined by drying culture samples for 24 h at 105 °C. After incubation of 4 g (drain weight) cell culture each for 15 min in B5 medium (refers to day 0), samples for anthocyanin analysis were taken from the pool (three replicates). After 4 days, the fresh and dry weights of cell cultures in the treatments were determined, and samples for anthocyanin analysis were extracted as described previously. The statistical significance of variation in fresh weight as well as anthocyanin content in the three treatments after 4 days of cultivation was determined using analysis of variance (ANOVA) followed by the post hoc test Tukey's honestly significant difference (HSD) in the statistic program SYSTAT 11.0.

Results

Hydroxycinnamic Acid Derivatives in *Vitis vinifera* Cell Culture

HPLC analysis of phenolic acid derivatives in *V. vinifera* cell culture revealed the occurrence of one major compound in cultures grown on agar and two major hydroxycinnamic acid derivatives in cell suspension cultures (Fig. 1a, b). Fractions by peak were collected using HPLC and analyzed with LC–MS with the following mass

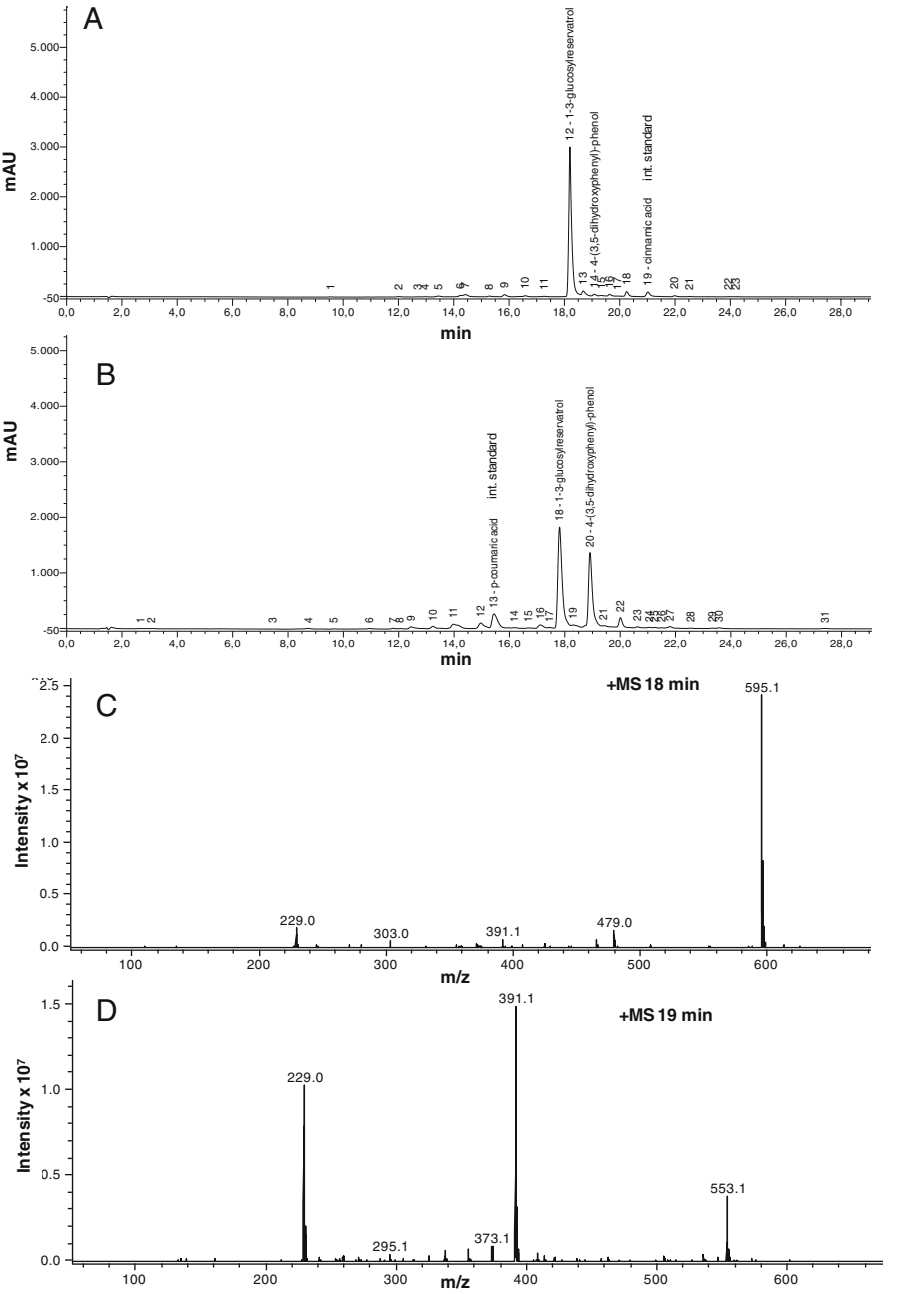

Fig. 1 HPLC chromatogram of cinnamic acid derivatives in cell culture (**a**) and cell suspension culture (**b**) at 290 nm; LC–MS results of peaks fractions at 18 min (**c**) and 19 min (**d**)

spectra ESI–MS (negative mode): (1) fraction 1 at 18 min, *m/z* 595 [M(594) $-H^+$], *m/z* 391 [M(390) $-H^+$], and a resveratrol fragment *m/z* 229 [M(228) $-H^+$]; and (2) fraction 2 at 19 min, *m/z* 553 [M(552) $-H^+$], *m/z* 391 [M(390) $-H^+$], and a fragment *m/z* 229 [M(228) $-H^+$] (Fig. 1c, d). The NMR measurements of both fractions did not yield any direct observation of ^{13}C resonances in either ^{13}C or DEPT experiments. The proton spectrum of both showed a complex signal in the spectrum between ∂ 3.0 and 3.6 ppm and clearly resolved signals in a lower field from 3.7 to 7.5 ppm, indicating an aromatic moiety in both fractions. A complete assignment of the structures was, however, not possible because of the complex unresolved signal in the aliphatic region of the spectra. Values of chemical shifts of carbon nuclei were obtainable for most carbon nuclei from HSQC data for both fractions. The available data on chemical shifts of proton and carbon, observed coupling and coupling constants for fraction 1, supported resveratrol as an aromatic moiety of fraction 1 and suggested a hexose in position 3 of resveratrol, as in 3-*O*-gucosylresveratrol (Fig. 2a, Table 1). This corresponds with the observed *m/z* of 391. The spectral data for fraction 2 allowed only the interpretation of the aromatic part of the compound and supported the structure of a 4-(3.5-dihydroxyphenyl)-phenol (Fig. 2b, Table 1). The HSQC data indicated the presence of six carbon nuclei in the unidentified residue, which shows chemical shifts comparable to carbon 1″ to 6″ of fraction 1 but did not allow unambiguous assignment.

The abundance of the two major phenolic acid derivatives, identified as 3-*O*-glucosylresveratrol and 4-(3,5-dihydroxyphenyl)-phenol, differed in cell suspension and agar cultures of *V. vinifera* (Fig. 1). The total content of these compounds was approximately 83.9±1.07 $\mu mol\,g^{-1}$ dry weight in 2-week *V. vinifera* calli and 100.7± 19.03 $\mu mol\,g^{-1}$ dry weight in cell suspension culture. The stilbene derivative was more abundant in cultures grown on agar, with 81.4 μmol/g dry weight compared to 68.3 $\mu mol\,g^{-1}$ dry weight in cell suspension cultures. 4-(3,5-dihydroxyphenol)-phenol accumulated in higher amounts in the cell suspension culture, with approximately 32.4 μmol/g dry weight compared with 2.6 $\mu mol\,g^{-1}$ dry weight in cell culture cultivated on agar.

Anthocyanins in *Vitis vinifera* Cell Suspension Culture

Figure 3a displays a chromatogram of anthocyanin extract from *V. vinifera* cell suspension culture recorded at 518 nm. Seven anthocyanins belonging to the group of monoglucosides or *p*-coumaryl glucosides were identified by their spectroscopic characteristics and/or fragmentation pattern. Peak fractions of main peaks were collected and analyzed by LC–MS. The following spectroscopic characteristics and fragments were found: fraction 1, 11.5 min λ_{max} 514, *m/z* 449 [M(448) $-H^+$]; fraction 2, 12.5 min λ_{max}

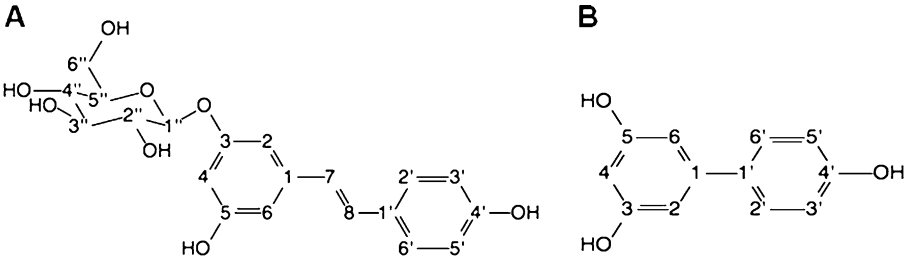

Fig. 2 Structure and annotation of fraction 1 (**a**) 3-*O*-glucosyl-resveratrol and fraction 2 (**b**) *R*-4-(2,3-dihydroxyphenyl)-phenol

Table 1 $^{1}H/^{13}C$ NMR spectral data of phenolic acid derivatives (500 MHz, coupling constants in Hertz, δ relative to TMS)

Fraction 1							Fraction 2						
No	Carbon δ	Proton Δ	Integral	Proton pattern	*J*	COSY coupling	No	Carbon δ	Proton δ	Integral	Proton pattern	*J*	COSY couplings
1	130.4						2′, 6′	131.1	7.08	2	D	–	3′, 5′
1′	130.0						3′, 5′	116.2	6.65	2	D	–	2′, 6′
7	129.0	7.03	1	d	16.4	8	2	108.4	6.38	1	M	–	4. 6
8	125.7	6.87	1	d	16.4	7	6	110.3	6.33	1 or 2	M	–	2, 4
2′, 6′	128.4	7.40	2	d	8.6	3′,5′	4	103.7	6.30	1	M	–	2, 6
3′,5′	115.9	6.76	2	d	8.6	2′, 6′	R1	101.8	4.65	1	M	–	R4, R5, R6
6	107.6	6.56	1	t/dd	1.9	2, 4	R2	56.8	3.83	1	M	=	
2	105.3	6.73	1	t/dd	1.9	6, 4	R3	61.7	3.47	1	M	=	R4, R5, R6
4	103.2	6.34	1	t/dd	1.9	2, 6	′R4	78.0	3.15	1 or 2	M	=	R1, R3
1″	101.1	4.80	1	d	7.6	2″	R5	74.2	3.15	1	M	=	R1, R3
2″	73.7	3.20	1	d	8.9	1″,3″	R6	70.5	3.15	1	M	=	R1, R3
4″	77.6	3.31	1 or 2	d		5″, 3″							
3″	70.2	3.16	1 or 2	t		2″, 4″							
6″	61.1	3.72	1	dd?	11.5	5′							
5″	61.1	3.46	1 or 2	dd?	11.9	4″, 6″							

515, *m/z* 463 [M(462) –H$^+$]; fraction 3, 14.4 min λ_{max} 308/522, *m/z* 595 [M(594) –H$^+$]; and fraction 4, 15.0 min λ_{max} 311/522, *m/z* 609 [M(608) –H$^+$] (Fig. 3b, c). The major monoglucosides present in the cell culture were identified as cyanidin 3-*O*-glucoside (fraction 1) and peonidin 3-*O*-glucoside (fraction 2), and major cinnamoyl derivatives were cyanidin 3-*O*-*p*-coumaryl glucoside (fraction 3) and peonidin 3-*O*-*p*-coumaryl glucoside (fraction 4). Furthermore, three minor compounds were present in *V. vinifera* cell culture delphinidin 3-*O*-glucoside (λ_{max} 522), petunidin 3-*O*-glucoside (λ_{max} 522), and delphinidin 3-*O*-*p*-coumaryl glucoside (λ_{max} 312,527).

Influence of Signaling Molecules on Anthocyanins Accumulation

The effects of signaling molecules on anthocyanin accumulation and cell culture growth were evaluated in a short-term experiment. The effects of jasmonic acid and salicylic acid on anthocyanin content and fresh weight increase in cell cultures were found to differ significantly (ANOVA: $p<0.001$). The fresh weights of cell cultures determined after 4 days of cultivation in 50 μM salicylic acid were significantly higher compared to the controls (Fig. 4). On the contrary, treatment with 50 μM jasmonic acid resulted in significantly lower fresh weight increases—virtually no growth—compared with the control

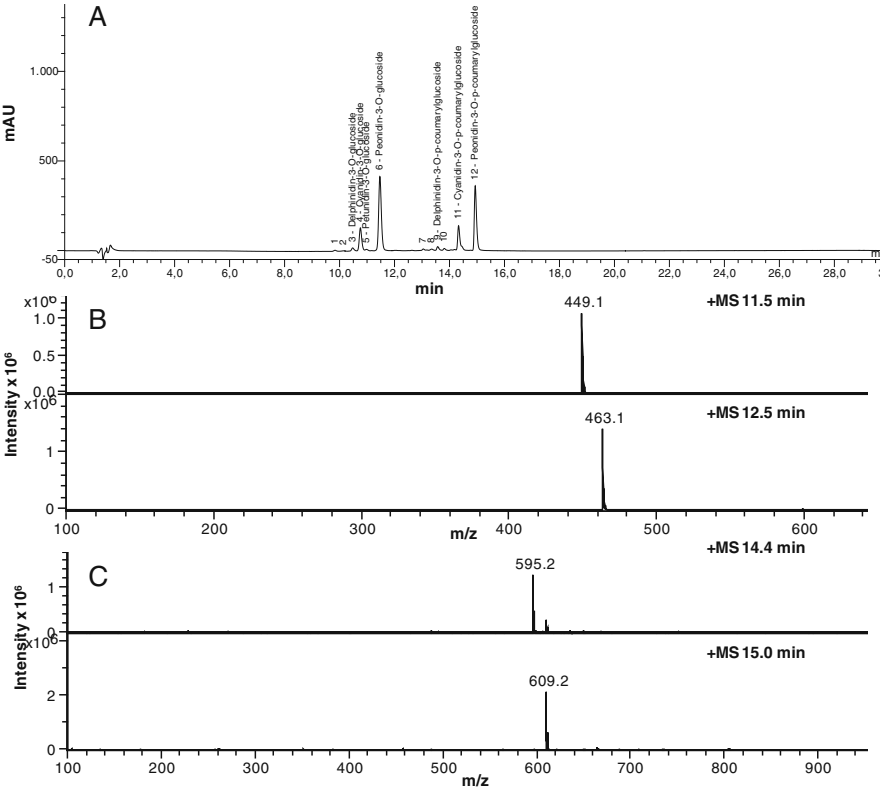

Fig. 3 HPLC chromatogram of anthocyanins in *V. vinifera* cell culture (**a**) at 518 nm; LC–MS results of peaks fractions of monoglucosides at 11.5 and 12.5 min (**b**) and *p*-coumaryl glucosides at 14.4 and 15.0 min (**c**)

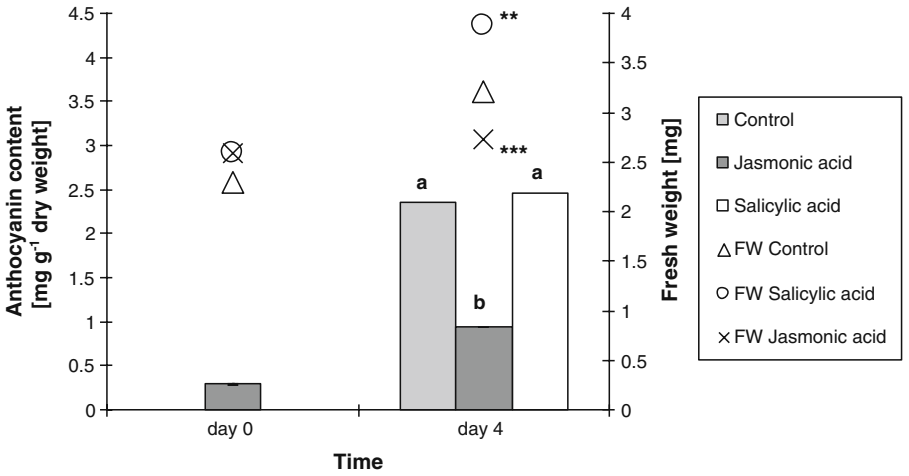

Fig. 4 Anthocyanin contents and fresh weights (*FW*) of *V. vinifera* cell culture in the pool (day 0) and in the treatments (day 4) with 50 μM jasmonic acid and 50 μM salicylic acid compared with the control (different *small letters* indicate significant differences between anthocyanin contents (*bars*) of treatments on day 4 Tukey's HSD test $p<0.001$; the different numbers of *asterisks* indicate significant differences in fresh weight in treatments on day 4 Tukey's HSD test $p<0.001$)

(Fig. 4). After transferring the cell cultures to a new media, the anthocyanin content increased from initial levels of 0.29 mg g^{-1} dry weight in the pool (day 0) in all treatments, albeit to a different extent. After 4 days, the anthocyanin content in the jasmonic acid treatment with 0.94 mg g^{-1} dry weight was significantly lower than contents in the control and salicylic acid treatments (Fig. 4). The anthocyanin content increased similarly in the control and salicylic acid treatments, with 2.58 and 2.59 mg g^{-1} dry weight, respectively.

Discussion

Cell cultures are an attractive source for obtaining specific and novel natural compounds [5, 6]. The present study demonstrates the capability of *V. vinifera* cell culture from Gamay Fréaux to produce hydroxycinnamic acid derivatives and anthocyanins in sufficient amounts for a possible supply of bioactive compounds. It is evident that the class of wine stilbenes comprises more compounds than described to date. Monohydroxystilbenes, dihydroxystilbenes, and tetrahydroxystilbenes were found to be more abundant in red wine than in white wine [19, 21]. Besides already known stilbenes, such as *trans*-resveratrol and isomeric piceids, seven additional stilbene derivatives like monostilbene, isomeric resveratrol-2-*C*-glucosides, and dimeric stilbenes were isolated from commercial Riesling wine [19]. Cell cultures of *V. vinifera* (L.) cv. Gamay Fréaux var. Tenturier were found to produce a dimeric (*E*)-dehydro-resveratrol and pallidol, as well as two dimeric glycosides: resveratrol (*E*)-dehydrodimer 11-*O*-β-D-glucopyranoside and resveratrol (*E*)-dehydrodimer 11′-*O*-β-D-glucopyranoside [16]. More than 400 new naturally occurring stilbenes were isolated and identified from wine from January 1995 to the end of 2008 [22]. To the best of our knowledge, the present study describes, based on NMR results, two new compounds for the first time: a stilbene derivative, 3-*O*-glucosylresveratrol, abundant in cell suspension culture, and a hydroxyphenol, 4-(3,5-dihydroxyphenyl)-phenol, abundant in callus culture.

The MS analysis of the phenolic fraction 1 of *V. vinifera* showed a molecular ion M^+ at *m*/*z* 595, which can indicate an attached acetyl glucoside group on the 3-*O*-glucosyl-resveratrol. Such coupling is supported by the NMR results. Also, fraction 2 shows a molecular ion M^+ at *m*/z 553, which may indicate binding to a glucoside group 4-(3,5-dihydroxyphenyl)-phenol. The NMR and LC–MS results will enable identification of the found compounds in cell cultures from wine. However, based on the data obtained, the absolute configuration (e.g., geometry, glucosylation) of the two compounds still needs to be clarified.

Resveratrol was synthesized in *V. vinifera* in response to biotic and abiotic stress, such as fungal infection, UV light exposure, ozone stress, anoxic treatment, and wounding [33, 34]. Since the dietary value of the compound resveratrol accumulated mainly in grape berry skin and seeds [34], much attention had been paid on changes of resveratrol during grape development and vinification process. In *V. vinifera*, resveratrol is synthesized from phenylalanine precursor by catalysis of stilbene synthase (4-coumaroyl-CoA malonyltransferase) using one molecule of 4-coumaroyl-CoA and three molecules of malonyl-CoA [35]. Grape cell culture can be a useful tool for studying biosynthesis of stilbenes to reveal factors which influence their production within a plant. Treatment of *V. vinifera* cv. Optima cell cultures with cell wall of *Phytophtera cambivora* (Petri) lead to accumulations of mRNA from stilbene synthase and L-phenylalanine ammonia lyase [36]. Dimethyl-β-cyclodextrin was capable of inducing stilbene biosynthesis in liquid grape cell culture also in the absence of a pathogenic organism [37]. It would be interesting to study the effects of abiotic elicitors, such as dimethyl-β-cyclodextrin or UV light, and precursors on the biosynthesis of the two phenolic compounds identified in the present study. In our study, we found that the stilbene derivative, 3-*O*-glucosylresveratrol, had higher levels in *V. vinifera* callus cultures cultivated on agar compared with cell suspension cultures. The opposite was true for the red 4-(3,5-dihydroxyphenyl-phenol). According to the literature, satisfactory in vitro biosynthesis of selected secondary metabolites and intensive tissue growth can be extremely difficult [38, 39]. Cultivation in suspension culture can accelerate cell culture growth compared with those of callus culture. For example, the fresh weight of *V. vinifera* culture increased about 1.5-fold within 4 days in the present study, which was faster than the growth of the callus culture. However, culture cells in liquid medium may not produce the same secondary metabolites, such as that shown for cell cultures of *Lithospermum erythrorhizon* Sieb. et Zucc. [40]. The high-pigment-producing callus strain 18 of *L. erythrorhizon* did not produced naphthoquinone (shikonin) in liquid medium without agar, but if a small amount of agar was added, the cell suspension cultures resume synthesizing of shikonin derivatives. The most important differences between the two basal media (solid versus liquid B5) used in this study are the presence and absence of ammonium which can lead to a different phenolic profile.

Anthocyanin composition is cultivar specific, and some authors believe that this can be used as an analytical tool to certify their identity [23]. Five different anthocyanidin-3-glucosides are generally present in red grapes with various ratios, depending on the varieties, which are usually absent in white gapes [24]. Cell culture lines of *V. vinifera* Gamay Fréaux have been shown to produce the monoglucosides peonidin 3-*O*-glucoside and cyanidin 3-*O*-glucoside as well as the cinnamoyl derivative peonidin 3-*O*-*p*-coumarylglucoside in cell suspension cultures [18]. The present study confirms the abundant occurrence of these anthocyanins in *V. vinifera* cell culture. However, we have identified a further cinnamoyl derivative—cyanidin 3-*O*-*p*-coumaryl glucoside—as a major compound in this cell culture and three minor compounds: the monoglucosides delphinidin 3-*O*-glucoside, petunidin 3-*O*-glucoside, and delphinidin 3-*O*-*p*-coumaryl glucoside. The fact that additional anthocyanins were detected in *V. vinifera* cell culture in the present

study can be explained by the detection method, which was more sensitive than those used in the previous study such as uses of narrow bore columns, smaller flow cells, and higher detector sensitivity. Anthocyanin composition may also have changed during the long period of continuous cultivation. Interestingly, the major monoglucosides present in wine—malvidine 3-*O*-glucoside—was not even detected in traces, while peonidin 3-*O*-glucoside, another major anthocyanin, was detected in both wine and cell culture [15].

V. vinifera cell culture is a good model system to investigate the factors that influence anthocyanin and resveratrol derivative metabolism. Anthocyanin pigments are widely used in the food market and medicine, but only a few in vitro cultures produced biomass with a high anthocyanin content equal to the plant in nature [41]. The established cell culture of *V. vinifera* has good potential of anthocyanin production, and we conducted preliminary experiments to enhance anthocyanin production in cell suspension cultures by applying signaling molecules. Anthocyanin levels of cell suspension cultures (18 days old) increased about eightfold after transferring the culture to new B5 medium with and without supplementing of signaling molecules and cultivation for 4 days. In vitro, the synthesis of secondary metabolites is typically considered as non-growth-associated and occurs when the division of cells in the culture decreases or stops or when the culture passes from the logarithmic developmental stage to the static phase [11]. Corresponding to our results, Cormier et al. [18] found a peak anthocyanin accumulation on days 5 to 9 (a fourfold increases) and decreases down to initial levels as early as on day 12.

Signaling molecules such as jasmonic acid and salicylic acid are believed to trigger the accumulation of secondary metabolites in cell suspension culture like it was shown for the production of taxol [25, 28]. These molecules are involved in the multiple signaling cascades, which mediate plant responses to various stressors. The wound hormone jasmonic acid and related compounds activate the octadecanoid signaling pathway and are responses to mechanical wounding or herbivory [26]. Salicylic acid induces the phenylpropanoid signaling and is crucial for local hypersensitive response and systemic acquired resistance against many pathogens [27]. D'Onofrio et al. [29] reported a significantly higher amount of proanthocyanidins compared with controls in *V. vinifera* cell cultures that had been exposed to jasmonic acid and salicylic acid. Nevertheless, stilbene biosynthesis was induced only by jasmonic acid and not by salicylic acid. In our study, however, salicylic acid did not induce anthocyanin accumulation in cell culture, and jasmonic acid reduced anthocyanin levels significantly compared with the control. The cell culture cultivated at jasmonic acid concentration of 50 μM in B5 medium stopped growing and turned from violet to brownish—indicating cell death from oxidized exudates—which suggests that the chosen concentration in the present study was too high. On the other hand, salicylic acid was found to promote cell culture growth significantly at the same concentration, and the anthocyanin content was at least as high as in the control. The present study identified salicylic acid as a suitable factor to upregulate biomass production.

Further studies are needed to find the optimum culturing conditions for anthocyanin and/or resveratrol derivative production. To enhance metabolite production in the cell culture, the following method developments are applicable: (1) chemical elicitor application such as signaling molecules and phytohormones (jasmonic acid at lower concentrations than 50 μM), (2) precursor treatment with, e.g., shikimic acid, cinnamic acid, and phenylalanine, (3) usage of physical elicitors such as UV-B irradiation, and (4) biological elicitor application such as inoculates of plant pathogenic bacteria and fungi. Alternative extraction techniques of phenolic compound from the medium are equally of special interest, whereby such studies are currently being prepared for publication.

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