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Full Length Research Paper

Elicitation and precursor feeding influence phenolic acids composition in *Vitis vinifera* suspension culture

Heidi Riedel[1]*#, Divine N. Akumo[3#], Nay Min Min Thaw Saw[1], Onur Kütük[1], Peter Neubauer[3] and Iryna Smetanska[1, 2]

[1]Department of Methods in Food Biotechnology, Institute of Food Technology and Food Chemistry, Berlin University of Technology, Königin-Luise Str 22, 14195 Berlin, Germany.
[2]Department of Plant Food Processing, Agricultural Faculty, University of Applied Science Weihenstephan-Triesdorf, Steingruber Str 2, 91746 Weidenbach, Germany.
[3]Laboratory of Bioprocessing Engineering, Department of Biotechnology, Berlin University of Technology, Ackerstr 71-76, 13355 Berlin, Germany.

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Plant cell cultures have been industrially used for the synthesis of secondary metabolites. Different elicitors, [jasmonic acid (JA), salicylic acid (SA) and ethephone (E)] and precursors [shicimic acid (SH) and phenylalanine (PHE)] were independently used to enhance the synthesis of phenolics in suspension cultures of *Vitis vinifera* c.v. Muscat de Frontignan followed by chemical analysis with high-performance liquid chromatography (HPLC). Differences between the treated and untreated *in vitro* grape cultures were confirmed statistically. On day 2, JA, SA and SH significantly increased phenolic acid contents with E on day 4 ($p < 0.05$). The grape cells treated with SA, E, SH, and PHE increased biomass whereas JA led to decreases over time. From the chemical analysis of *V. vinifera* suspension cell cultures, two major resveratrol derivatives; 3-*O*-glucosyl-resveratrol and 4-(3,5-dihydroxyphenyl)-phenol were in high concentrations after treatment most especially with JA. These results show that elicitors (JA, SA, and E) and the precursor SH can be economically used to enhance the synthesis of phenolic compounds in *V. vinifera* within a very short time lapse. Furthermore, these enhancers could be used to target the rapid extraction of medicinally important resveratrols for pharmaceutical purposes.

Key words: *Vitis vinifera*, phenolic acids, resveratrol, elicitors, precursors

INTRODUCTION

Vitis vinifera (grapevine) is one of the major fruit crop worldwide and is of high economic interest (Kammerer et al., 2004). Grape plants cultivated on the field are very susceptible to microbial and fungal attacks. Wine grapes are a rich source of potentially bioactive secondary metabolites which include most especially polyphenolics such as phenolic acids, anthocyanins, flavonols and catechins (Thimothe et al., 2007). In recent years, special attention has been focused on polyphenolics because of their taste, color, antioxidative potential, flavor and their role in food preservation (Zhong, 2001). Many food manufactures' have in the last decade shown a high interest in phenolic compounds because of their; antioxidant properties, abundance in our diet and for their different preventive role against diseases associated with oxidative stress such as cancer, cardiovascular and neurodegenerative diseases (Riedel et al., 2010; Scalbert and Williamson, 2000).

Suspension cultures of grape have been reported to accumulate a wide range of catechins, anthocyanins, proanthocyanins and stilbenes like *E*- and *Z*- Resveratrol with multiple constituents of double bonds (Krisa et al., 1999). The resveratrol (3,5,4`-trihydroxytransstilbene) and related components are the major compounds found in grapes (Yadav et al., 2009). The most common pigments in grape berries are 3-O-glucosides of delphinidin, cyanidin,

*Corresponding author. E-mail: heidemarie.riedel@tu-berlin.de Tel: +49 30 314 71848. Fax: +49 30 31471492.

#Both authors contributed equally in this work.

petunidin, peonidin and malvidin as well as their related compounds; 3-acetylglucosides and 3-p-coumaryl-glucosides (Ghiselli et al., 1998). Phenolic compounds exhibits many different biological properties such as antibacterial, anticancer, estrogenic and heart protecting activities (Guerrero et al., 2009). Reservatrol and its related com-pounds have been investigated for the biological activity in a wide range of biological assays including breast, lung and central nervous system cancer cell lines (Nassiri-Asl and Hosseinzadeh, 2009; Springob et al., 2003).

In most cases, the production of secondary metabolites can be enhanced by treating the undifferentiated cells with elicitors (Raskin et al., 2002). For example jasmonates has been reported to have the ability to induce many secondary metabolic processes to enhance the *in vitro* production of many compounds of interest (Creelman and Mullet, 1997). In nature, the process of elicitation induces the synthesis of plant secondary metabolites to ensure their survival, persistence and competitiveness (Wu et al., 2003). Another successful strategy used in influencing the biosynthetic pathways to activate and increase the production of secon-dary metabolites is by feeding cell cultures with precursors (Smetanska, 2008). Precursors such as amino acids have been successful used when they are cheaper than the desired end products (Tumova et al., 2004). Nonetheless, the induction of exogenous precursors to a plant *in vitro* culture causes an increase in the production of secondary metabolites (Namdeo, 2007).

Previous studies have focused on investigating the single effects of elicitors or precursors on anthocyanin and resveratrol contents. This is one of the first studies aimed at simultaneous evaluating the effects of different elicitors and precursors on the phenolic acid contents in suspen-sion cultures of *V. vinifera*. In this study, we pointed out the individual effect of some elicitors (JA, SA, and E) and precursors (SH and PHE) on the phenolic compounds synthesis in *V. vinifera* suspension cell cultures; followed by chemical analysis with HPLC for the identification of phenolic acids.

MATERIAL AND METHODS

Cultivation and maintenance of *V. vinifera*

One of a high productive cell line of grape is *Vitis vinifera* c.v. Muscat de Frontignan. The cell suspensions of *V. vinifera* c.v. Muscat de Frontignan were maintained in a culture medium containing B5 basal medium (Gamborg B5 Medium B5VIT, Duchefa B.V. Netherlands) supplemented with 250 mg/l casein hydrolysate (Merck, Darmstadt), 0.1 mg/l α- naphthalene acetic acid, 0.2 mg/l kinetin and 30 g/l sucrose. The medium for the callus culture contained additionally 8 g/l agar. The plant cell callus and the suspension culture were grown at 25°C in 24 h photoperiods under a fluorescent lamp (approx. 3,000 lx). The transfer interval for the suspension culture into new medium was 14 days and the callus was replanted on agar every 28 days. The suspension cultures were agitated on an orbital shaker operating at 100 rpm.

Experimental design

Culture preparation

For the experimental design, 100 ml Erlenmeyer flasks containing 25 ml of B5VIT basal medium were used. After sterilization at 121°C for 25 min, 4 g fresh weight plant cells of *V. vinifera* were inoculated to each flask. The flasks (triplicate) were harvested on day 2, 4, 7, 10, 15 and 18 respectively after treatment with elicitors and precursors. On every harvesting day, the following parameters were measured: fresh weight, dry weight, pH, conductivity, and the chemical analysis for phenolic acids were performed as described below.

Treatment with elicitors and precursors

In 100 ml Erlenmeyer flasks containing 25 ml *V. vinifera* media each, 4 g of fresh weight plant cells from *V. vinifera* (without using vacuum) were inoculated into each flask. For the stimulation of the plant cells to optimize the synthesis of phenolic compounds, valuable polyphenolics elicitors including: jasmonic acid (JA), salicylic acid (SA), ethephone (E) and precursors such as shicimic acid (SH) and phenylalanine (PHE) (all substances from SIGMA ALDRICH, GERMANY) were added to the media at day 0 and standardized to a final concentrations of 0.1 mM per treatment. The stock solutions of each substance were sterilized by passing them through a 0.22 µm filter. Our experiment was made up of triplicates of every treatment (JA, SA, E, SH and PHE) and control (no treatment) across day 2, 4, 7, 10, 15 and 18 respectively. Samples from each triplicate flask with and without treatments were harvested for the determination of fresh and dry weight, pH, conductivity, phenolic acids and anthocyanin on day 2, 4, 7, 10, 15 and 18 after stimulation.

Estimation of experimental parameters and harvesting procedure

The following experimental parameters were measured; pH, conductivity, fresh and dry weight. The pH and conductivity were tested to estimate the metabolic end products and the nutrient contents in the medium. For every sample, the pH and conductivity of the nutrient medium were measured within a time labs of 30 s to stabilize both parameters at room temperature. The ratio of dry and fresh weight was estimated as the index of water content (%) in the cells and the state of the vacuole respectively. The plant cells were filtered using suction filter in a vacuum for a minute and were later weighted. 1 g of fresh plant material was dried in a prepared alumi-num box and kept at 105°C in an oven for 24 h. After the drying process, the samples were transferred for one hour in to a desiccator. Thereafter, the dry weights were measured. Plant cells of *V. vinifera* were harvested using vacuum filtration flask. At each day of harvest, the fresh and dry weights were estimated and the chemical components were analyzed. The harvested plant cells for the phenolic acid extraction were immediately flash frozen in liquid nitrogen and transferred for the lyophilization.

Chemical analysis of phenolic acids with HPLC and LC-MS

For chemical analysis, about 40 mg powdered callus samples (freeze-dried) were extracted for 15 min with 750 µl of 70%

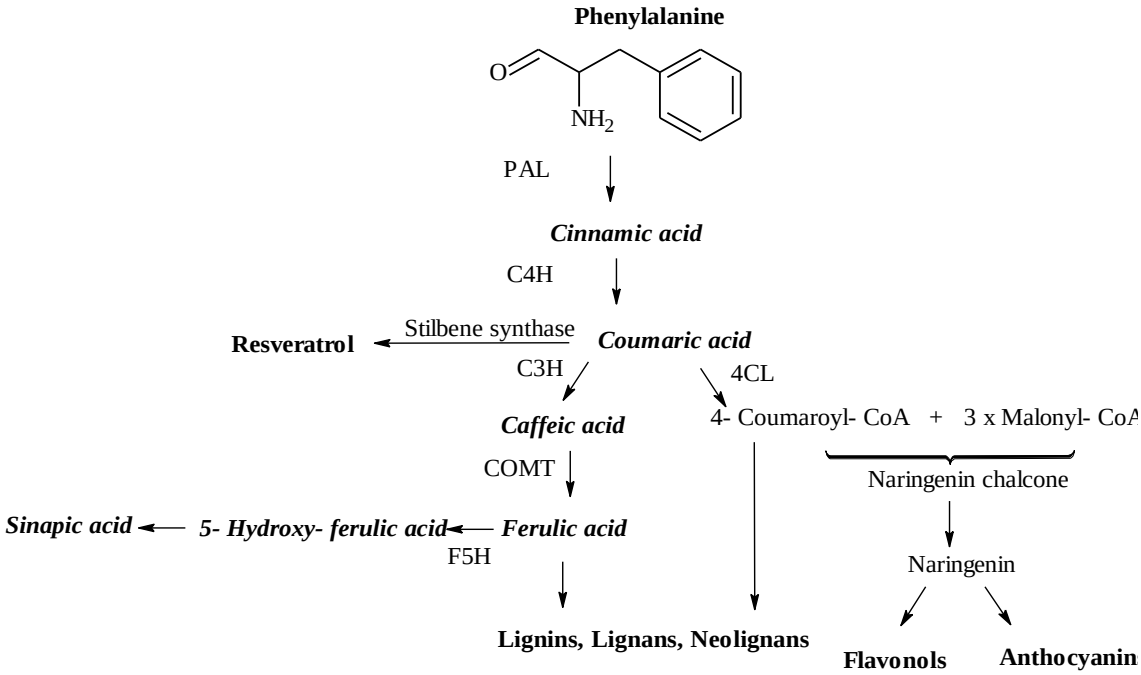


Figure 1: Biosynthetic pathway of phenolic compounds

methanol (v/v; pH 4; 0.1% phosphoric acid) containing 40 µl of the internal standard *p*- coumaric acid (3 mmol) in an ultrasonic water bath filled with ice. All samples were centrifuged at 4,500 rpm and 4°C for 5 min. The supernatants were collected in new tubes and the pellets were re-extracted with 500 µl of 70% methanol (twice). After the extraction, aliquots of the samples were collected and the solvent was completely removed using a rotary evaporator (Speed Vac, SC 110) under vacuum at room temperature (25°C). The residues were filtered using centrifuge tubes (SpinX) and the extracts were dissolved with 40% acetonitrile to reach the 1 ml mark. The chromatography was performed using a Dionex Summit P680A HPLC system 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 x 2.1 mm, 3 µm, Dionex) with an injection volume of 40 µl and the temperature of the column oven was 35°C. The eluent flow rate used was 0.4 ml/ min. A 39 min gradient program was used with 1% (v/v) phosphoric acid in ultrapure water (eluent A) and 40% (v/v) acetonitrile in ultrapure water (eluent B) as followed: 1 min 0.5% (v/v) B, a gradient from 0 to 40% (v/v) B for 9 min, a 2 min hold, a gradient from 40 to 80% (v/v) B for 6 min, a 2 min hold, gradient from 80 to 99% (v/v) B for 4 min, a gradient from 99 to 100% (v/v) B for 6 min, a gradient from 100 to 0.5% (v/v) B for 4 min, and a final step at 0.5 % B for 5 min. Peaks were monitored at 290, 330, and 254 nm. The phenolic acid quantity was calculated from HPLC peak areas at 290 nm. The retention times in the HPLC for our experiments were 12.13 min for vanillic acid, 12.72 min for chlorogenic acid, 13.29 min for caffeic acid, 15.98 min for the internal standard p-coumaric acid and 21.59 min for cinnamic acid. For the identification of the unknown phenolic compounds, a semi-quantitative analysis was performed using HPLC coupled with mass spectrometric detection (LC/MS). Chromatography was performed using a Finnigan MAT95S (EI samples) and Orbitrap LTQ XL (Thermo Scientific) for the ESI samples. The spray voltage of the electro-spray ionization was 5 kV with the source temperature 275°C. The solvent was a mixture of methanol with 0.1% formic acid and at a flow rate of 200µl/ min. The flow rate of the syringe pump was 5µl/ min. Gradient elution solvent A was water mixed with 0.1% formic acid and solvent B was methanol with 0.1% formic acid. The flow rate in the HPLC gradient program was 1 ml/min and the elution started at time 0 min with 95% of solvent A and 5% of solvent B. After 25 min, the solvent composition was 0% and 100% for solvents A and B respectively which remain the same until the 38 min. At the terminal phase, between 38.01 min and 40 min, the solvent composition was 95% of solvent A and 5% of solvent B.

Statistical analysis

Data sets were made up of triplicates of suspension culture of *V. vinifera* subjected to five different treatments and one control group across the harvesting days (2, 4, 7, 10, 15 and 18). The estimated experiment parameters for phenolic acid production did include: anthocyanin and phenolic acids content, pH, conductivity, fresh and dry weight. In order to investigate the effects of the different treatments on polyphenolic production in cell cultures of *V. vinifera*, the mixed procedure in the statistical package SAS (2003) PC version 9.1 (SAS Institute, Cary, NC, USA) was used. The effects of the fixed factors on phenolic acid production were tested using the maximum likelihood (ML). All statistically significant factors (treatment, day, dry weight and anthocyanin) were included in the final model. The mixed procedure was used because it allows for the estimation of the unbiased estimates with the approximation of the missing values (Littell et al, 1996). The selection of the best model was based on testing for different covariant structure comparing between the Akaike's information criterions (AIC) values of every mixed model tested with the restricted maximum likelihood (REML). The model with the least AIC value was the best model and the most appropriate covariance structure was the unstructured (UN). For all analyses, the level of statistical significance was assigned at p-values ≤ 0.05. For multiple comparison tests, the Tukey-Kramer was used with adjusted p-values. Correlation

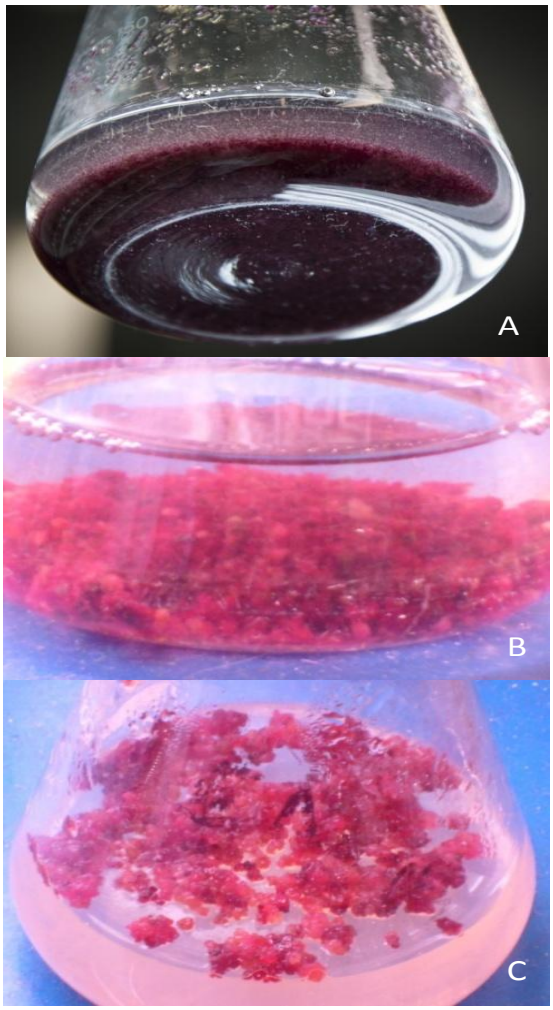


Figure 2. A:Suspension culture of treated grape cells with JA; B: suspension culture of untreated grape; C: Grape callus aggregates.

analyses between the estimated parameters were done using the "ProcCorr" procedure in SAS. The following statistical models were used in estimating the effects of the different factors (based on ML) and the differences between the treatments and days of treatment (using the REML) on phenolic acid production as shown in Model 1 and Model 2 respectively

$$Y_{ijklm} = \mu + Treat_i + Day_j + DW_k + Anth_l + e_{ijklm}$$

Model 1

$$Y_{ijklm} = \mu + Treat_i + Day_j + Treat_i * Day_j + e_{ijklm}$$

Model 2

Where;

Y_{ijklm} = Phenolic acid content,

μ = Overall mean of phenolic acid content,
$Treat_i$ = fixed effect of Treatment (i = JA, SA, E, SH, PHE and control),
Day_j = fixed and random effect day (j = 2, 4, 7, 10, 15 and 18),
DW_k = fixed effect of dry weight (k = sample),
$Anth_l$ = fixed effect of anthocyanin content (l = sample),
$Treat_i*Day_j$ = interaction between treatment and day (i = treatment, j = day)
e_{ijklm} = residual error.

RESULTS

Suspension culture of *V. vinifera*

In this study, we investigated for the concentration of secondary metabolites (phenolic acids) in suspension cultures of grape after independent treatment with elicitors (JA, SA, and E) and precursors (SH and PHE). Figures 2; A, B and C show the characteristic coloration of *in vitro* cell cultures of treated (JA), and untreated callus aggregate and sus-pension cultures of *V. vinifera* respectively.

HPLC Chromatogram of phenolic compounds in *V. vinifera*

HPLC chromatogram was carried out on extract of suspen-sion cell cultures (*V. vinifera)* for further chemical analysis. Two major phenolic compounds; 3-*O*-glucosyl-resveratrol and 4-(3, 5-dihydroxyphenyl)-phenol as well as the internal standard *p*- coumaric acid were identified. The retention times for the peaks were 12.13 min for vanillic acid, 12.72 min for chlorogenic acid, 13.29 min for caffeic acid, 15.98 min for the internal standard p-coumaric acid and 21.59 min for cinnamic acid. The HPLC chromatogram (Figure 3) showed the identified phenolic compounds at their respec-tive retention time.

Two very important phenolic compounds (resveratrol derivatives) were identified in our grape cell cultures; 3-O-glucosyl-resveratrol and 4-(3, 5-dihydroxyphenyl)-phenol. Their chemical structures are shown in Figure 4.

Growth kinetics and phenolic acid concentration per treatment

The growth kinetics and the phenolic acids concentrations of the suspension cell cultures of *V. vinifera* after treatment with elicitors (SA, E and JA), precursors (SH and PHE) and the control group are shown in Figure 5.

The cell culture growth trend was similar in almost all cases with a steady increasing in biomass from day zero until day 15; then after further increases was only noticed in the control samples. Meanwhile, the biomass in *V. vinifera* suspension cells treated with jasmonic acid was decreasing very slightly with increases in days throughout the experimental period. On the other hand, the total phenolic acid content increased sharply from day zero until day 2 in all treated *in vitro* cultures and control group

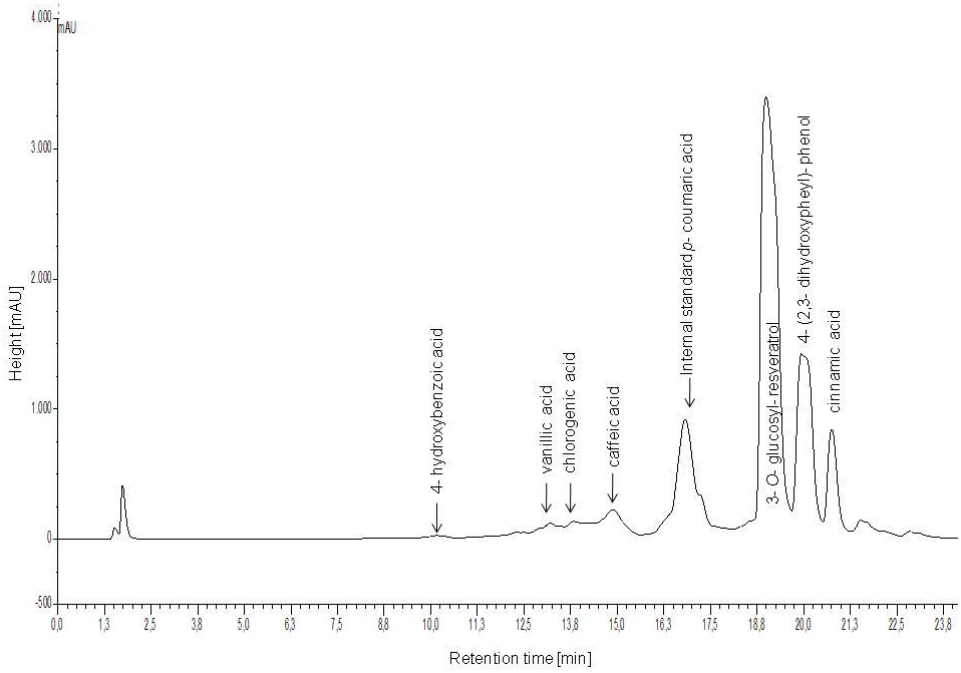
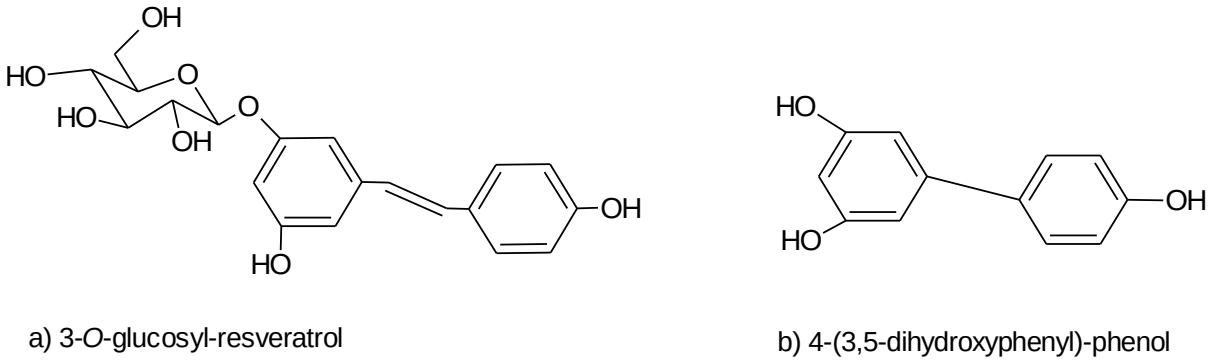


Figure 3. HPLC chromatogram of phenolic acids in *V. vinifera.*



a) 3-*O*-glucosyl-resveratrol

b) 4-(3,5-dihydroxyphenyl)-phenol

Figure 4. Chemical structures of major resveratrol derivatives identified with the HPLC.

before gradually dropping until day 7. Day 4 was optimum for phenolic acid synthesis for grape suspension cells treated with ethe-phonee.

Test of fixed factors on phenolic acids content

Based on Model 1, the maximum likelihood (ML) was used to test for the influence of experimental parameters (anthocyanin content, pH, conductivity, fresh weight and dry weight) and their interactions on phenolic acid content. The type treatment, day of harvesting, the dry weight and the anthocyanin content showed statistically significant influences ($p < 0.01$) on the production of phenolic acids in cell cultures of *V. vinifera* (Table 1).

Phenolic acids concentration after cell culture treatment

From our statistical analysis, day two and four showed significant influence on the phenolic acids content in *V. vinifera* cell cultures. A multiple comparison test was carried out between the treatments by day and the control group. Because the phenolic acid content was highest on day 2 and 4, the differences between the phenolic acid content

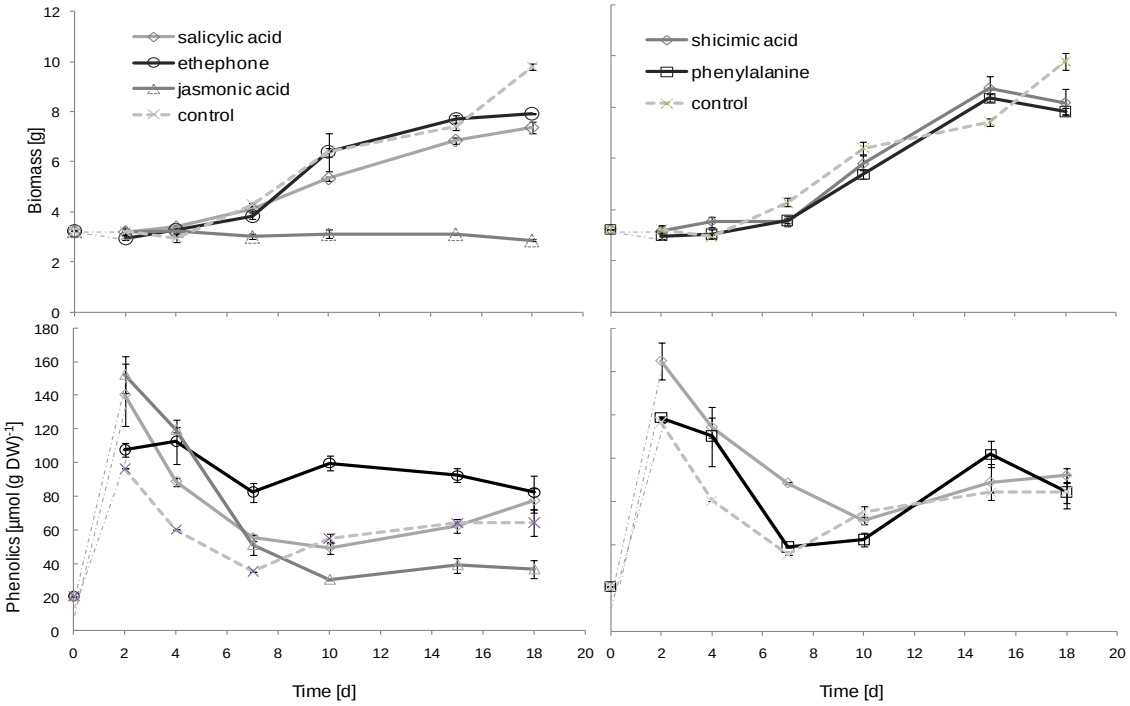


Figure 5. The growth kinetics and phenolic acid after treatment with elicitors and precursors.

Table 1. Tests of factors influencing phenolic acid production.

	Tested factors			
	Treatment	**Day**	**Dry weight**	**Anthocyanin**
p-value	<.0001	<.0001	0.0264	0.0162

Level of significance $p \leq 0.05$

Table 2. Differences in phenolic acids between treatment and control group.

Type	Day	Treatment	Phenolic acids	Standard error	p-value
Elicitor	2	JA – Control	55.71	13.32	<0.0001*
	2	SA – Control	43.98	13.32	0.0017*
	4	E – Control	52.55	11.51	<0.0001*
Precursor	2	SH – Control	28.22	12.16	0.0243*
	2	PHE – Control	2.07	12.16	0.8653

Level of significance $p < 0.05$, *adjusted p-value remains significant.

in the treated and control group are shown in Table 2.

The phenolic acid content in the suspension cell cultures treated with the elicitors (JA, SA and E) were about half a fold higher than that of control group on day 2 and 4 respectively. From the two precursors SH and PHE, SH was statistically different from the control group.

Further comparison between the experimental trials on day 2 was carried out for the suspension cell cultures treated with elicitors and precursors respectively (Table 3). There were no statistically significant differences between the grape cell cultures treated with JA and SA. Nevertheless, there were strong statistically significant differences between the elicitors (JA to E) and (SA to E) respectively and between the precursor SH and PHE.

Table 3. Differences in phenolic acids between treatments with elicitors and precursors.

Type	Day	Treatment	Phenolic acids	Standard Error	p-value
	2	JA – SA	11.73	13.32	0.382
Elicitor	2	JA – E	44.56	13.32	0.002*
	2	SA – E	32.82	13.32	0.017*
Precursor	2	SH – PHE	26.14	10.87	0.020*
Elicitors Vs Precursor	2	JA – SH	27.49	12.16	0.028*

Level of significance $p < 0.05$, *adjusted p-value remains significant.

Table 4. Pearson correlation coefficients between experimental parameters and phenolic acid.

N = 87	Phenolic acid	Day	Fresh weight	Dry weight	Anthocyanin
Phenolic acid	1	<.0001	0.0864	0.4457	0.0156
Day	-0.5066	1	<.0001	<.0001	0.0874
Fresh weight	-0.1850	0.7748	1	<.0001	<.0001
Dry weight	-0.0828	-0.4626	-0.6104	1	0.0002
Anthocyanin	0.2585	0.1843	0.4820	-0.3937	1

The top right and bottom left diagonals respectively represent the p-values and correlation coefficients

Table 5. Correlation tests between parameters for grape cells treated with jasmonic acid.

N = 15	Phenolic acid	Day	pH	Fresh weight	Dry weight	Anthocyanin
Phenolic acid	1	-0.0004	<.0001	*0.0562*	0.0733	<.0001
Day	-0.7969	1	<.0001	0.0273	0.2608	0.0001
pH	0.8481	-0.8430	1	*0.0581*	0.3614	<.0001
Fresh weight	0.5026	-0.5677	0.4994	1	0.8970	0.1926
Dry weight	-0.4754	0.3100	-0.2538	0.0366	1	0.2125
Anthocyanin	0.8597	-0.8364	0.9148	0.3561	-0.3417	1

The top right and bottom left diagonals respectively represent the p-values and correlation coefficients.

The best enhancing elicitor JA was better than the precursor SH in phenolic acid content. The differences in the phenolic acid contents for the *V. vinifera* suspension cultures treated with elicitors and precursors are shown in Table 3.

Correlation test between experimental parameters

A correlation test was performed between the experimental parameters (phenolic acid, day, pH, fresh weight and anthocyanin). Based on the correlation analysis, there was a strong statistically significant ($p < 0.0001$) between the phenolic acid content in treated and untreated *V. vinifera* and the day of harvest, fresh weight, pH, dry weight and anthocyanin respectively. The Pearson correlation coefficients and p-values for the experimental parameters significantly influencing phenolic acids production are shown in Table 4.

For the grape cell cultures treated with the elicitor jasmonic acid (JA), the correlation coefficient between phenolic acid content, day of harvesting, anthocyanin content and pH were 79.7, 85.97 and 84.81% respectively. Furthermore, the days of harvesting were positively correlated with anthocyanin content, pH and fresh weight (p-values; <0.0001, <0.0001 and 0.027) with correlation coefficients of 83.64, 84.3 and 56.77% respectively (Table 5). There were similar correlation trend between the parameters (phenolic content, day, fresh weight, pH and anthocyanin content) of *V. vinifera* suspension cell cultures treated with the elicitors E, SA, and the precursor SH respectively.

DISCUSSION

Our main objective was to investigate the independent effects of some elicitors (JA, SA and E) and the

precursors (SH and PHE) on the synthesis of phenolic acids in *in vitro* cell cultures of *V. vinifera*. The grape cell cultures expres-sed the characteristic wine red and pink colors after treatment (Figure 2), evidence of the presence of phenolic compounds in *V. vinifera*. After chromatographically analyzing the grape cell cultures with HPLC, we identified many phenolic compounds and most especially two major resveratrol derivates (3-O-glucosyl-resveratrol and 4-(3, 5-dihydroxyphenyl)-phenol) were present in very high concentrations (Figure 3). We suggest that the synthesis of *trans*-resveratrol is dependent on the beginning of the exponential cell growth phase (Figure 5, on days 2^{th} and 4^{th}). This suggestion corresponds with previous findings by Waffo; who hypothesized that stilbene production is coupled to cell growth (Waffo Teguo et al., 1998) with similar demonstrations for anthocyanins and condensed tannins (Decendit and Merillon, 1996). Furthermore, resveratrol accumulation in *V. vinifera* has been previously reported in other studies (Cai et al., 2011; Mewis et al., 2011; Righetti, 2007). Resveratrol have been reported to be of pharmaceutical importance because of cardioprotective and anticarcinogenic activity (Guerrero et al., 2009).

From our statistically analysis, we found that the treatment, day of harvesting, dry weight and the anthocyanin content did significantly influence the rate of phenolic acid synthesis in *V. vinifera*. The multiple comparison test between grape cell cultures treated with the elicitors' jasmonic acid (JA), salicylic acid (SA) and ethephone (E), compared to the untreated (control group) independently lead to higher synthesis in phenolic acid content on day 2 (JA and SA) and day 4 (E) respectively. On the other hand, only the grape cells treated with the precursor shicimic acid (SH) had the highest content in phenolic acid on day 2 and was statistically different from the control group ($p < 0.05$). Similar results suggest JA to be an elicitor signal trans-ducer which plays an important role in inducing the synthesis of many secondary metabolites phenolic com-pounds inclusive (Brader et al., 2001) and could inhibited cell growth (Tassoni et al., 2005).

Salicylic acid (SA) has also been reported to positively influence the biosynthetic pathway of phenolic acids when used at 20 and 100 µM (Cortell et al., 2007). Some other studies have equally confirmed ethephone (E) to have lead to several fold increase in enzymatic activity of pheny-lalanine ammonia-lyase thus a significant accumulation in phenolic acids (Cvikrová et al, 2007). Furthermore, all the tested elicitors (JA, SA, and E) and the precursor (SH) seemed to promote the accumulation of reservatrol in *V. vinifera* cells. Righetti et al. (2007) suggest that some of them also activate the release of phenolic compounds in the culture medium without significantly affecting cell growth and viability.

From the correlation analysis between the experimental parameters (phenolic acid content, treatments, day of harvesting, pH, fresh weight and anthocyanin contents) in *V. vinifera* suspension cell cultures treated with the elicitors (JA, SA and E) and the precursor (SH and PHE); there were statistically significant correlation between phenolic acid content and the treatments, days of harvesting, pH, fresh weight and anthocyanin content ($p < 0.001$). These results suggest that increases in the synthesis of phenolic compounds were dependent on the type of treatment and the day of harvesting was very crucial in increasing the phenolic acids contents in suspension cell cultures in *V. vinifera*. Phenolic acids are intermediary products in the biosynthetic pathways of anthocyanin (Rao and Ravishankar, 2002) thus the later the day of harvesting, the less the phenolic acid content. Furthermore, the days of harvest were positively correlated with anthocyanin content, pH and fresh weight (p-values; <0.0001, <0.0001 and 0.027) respectively.

Conclusion

In this study, we have shown through chemical analysis with HPLC that *V. vinifera* has a wide variety of phenolic compounds most especially for the reservatrol derivative 3-O-glucosyl-resveratrol and 4-(3, 5-dihydroxyphenyl)-phenol. Our results confirm the fact that elicitors or precur-sors can be economically used to enhance the synthesis of phenolic acids in grape suspension cell cultures. Day 2 and 4 were the best days for harvesting the grape cells and furthermore the extraction of phenolic compounds was at their magnitude on day 2 after treatment with JA, SA, SH and on day 4 for E. Nevertheless, JA was the most efficient enhancer for the synthesis of phenolic acids, thus JA could be industrially used to induce the synthesis and extraction of phenolic compounds in *V. vinifera*. A combine use of elicitor and precursor to treat grape cells could exponen-tially increase the synthesis of phenolic compounds in *V. vinifera.* Nonetheless, this hypothesis needs to be further investigated.

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REFERENCES

Brader G, Tas E, Palva E (2001). Jasmonate-dependent induction of indole glucosinolates in Arabidopsis by culture filtrates of the nonspecific pathogen Erwinia carotovora. Plant Physiol. 126(2): 849-860.

Cai Z, Riedel H, Thaw Saw NM, Kutuk O, Mewis I, Jager H, Knorr D,

Smetanska I (2011). Effects of Pulsed Electric Field on Secondary Metabolism of *Vitis vinifera* L. cv. Gamay Freaux Suspension Culture and Exudates. Appl. Biochem. Biotechnol. 164(4): 443-453
Cortell JM, Halbleib M, Gallagher AV, Righetti TL, Kennedy JA (2007). Influence of vine vigor on grape (*Vitis vinifera* L. Cv. Pinot Noir) anthocyanins. 1. Anthocyanin concentration and composition in fruit. J. Agric. Food Chem. 55(16): 6575-84.
Creelman RA, Mullet JE (1997). Biosynthesis and Action of Jasmonates in Plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. 48: 355-381.
Cvikrová M, Malá J, Hrubcová M, Eder J, Foretová S (2007). Induced changes in phenolic acids and stilbenes in embryogenic cell cultures of Norway spruce by culture filtrate of Ascocalyx abietina. J. Plant Diseases and Prot. 115(2): 57-62.
Decendit A, Merillon JM (1996). Condensed tannin and anthocyanin production in *Vitis vinifera* cell suspension cultures. Plant Cell Rep 15: 762-765.
Ghiselli A, Nardini M, Baldi A, Scaccini C (1998). Antioxidant Activity of Different Phenolic Fractions Separated from an Italian Red Wine. J. Agric. Food Chem. 46(2): 361-367.
Guerrero RF, Garcia-Parrilla MC, Puertas B, Cantos-Villar E (2009). Wine, resveratrol and health: a review. Nat. Prod. Commun. 4(5): 635-58.
Kammerer D, Claus A, Carle R, Schieber A (2004). Polyphenol screening of pomace from red and white grape varieties (*Vitis vinifera* L.) by HPLC-DAD-MS/MS. J. Agric. Food Chem. 52(14): 4360-4367.
Krisa S, Teguo PW, Decendit A, Deffieux G, Vercauteren J, Merillon JM. 1999. Production of 13C-labelled anthocyanins by *Vitis vinifera* cell suspension cultures. Phytochemistry, 51(5) :651-656.
Littell RC, Milliken GA, Stroup WW, Wolfinger R (1996). SAS System for Mixed Models. SAS Institute Inc. Cary, NC, USA.
Mewis I, Smetanska IM, Muller CT, Ulrichs C (2011). Specific Polyphenolic Compounds in Cell Culture of *Vitis vinifera* L. cv. Gamay Freaux. Appl. Biochem. Biotechnol. 164(2):148-161
Namdeo AG (2007). Plant cell elicitation for production of secondary metabolites. Pharmacognosy, 1: 69-79.
Nassiri-Asl M, Hosseinzadeh H (2009). Review of the pharmacological effects of *Vitis vinifera* (Grape) and its bioactive compounds. Phytother. Res. 23(9): 1197-204.
Rao SR, Ravishankar GA (2002). Plant cell cultures: Chemical factories of secondary metabolites. Biotechnol. Adv. 20(2): 101-153.
Raskin I, Ribnicky DM, Komarnytsky S, Ilic N, Poulev A, Borisjuk N, Brinker A, Moreno DA, Ripoll C, Yakoby N, O'Neal JM, Cornwell T, Pastor I, Fridlender B (2002). Plants and human health in the twenty-first century. Trends Biotechnol. 20(12): 522-531.
Riedel H, Cai Z, Kütük O, Smetanska I (2010). Obtaining phenolic acids from cell cultures of various Artemisia species. Afr. J. Biotechnol. 9(51): 8805-8809.
Righetti LM, Franceschetti, Maura F, Annalisa T, Nello B (2007). Resveratrol production in *Vitis vinifera* cell suspensions treated with several elicitors. Caryologia, 60(1-2): 169-171.
Scalbert A, Williamson G (2000). Dietary intake and bioavailability of polyphenols. J. Nutr. 130(8): 2073-2085.
Smetanska I (2008). Production of secondary metabolites using plant cell cultures. Adv. Biochem. Eng. Biotechnol. 111: 187-228.
Springob K, Nakajima J, Yamazaki M, Saito K (2003). Recent advances in the biosynthesis and accumulation of anthocyanins. Nat. Prod. Rep. 20(3): 288-303.
Tassoni A, Fornale S, Franceschetti M, Musiani F, Michael AJ, Perry B, Bagni N (2005). Jasmonates and Na-orthovanadate promote resveratrol production in *Vitis vinifera* cv. Barbera cell cultures. New Phytol. 166(3): 895-905.
Thimothe J, Bonsi IA, Padilla-Zakour OI, Koo H (2007). Chemical characterization of red wine grape (*Vitis vinifera* and Vitis interspecific hybrids) and pomace phenolic extracts and their biological activity against *Streptococcus mutans*. J. Agric. Food Chem. 55(25): 10200-10207.
Tumova L, Gallova K, Rimakova J (2004). [*Silybum marianum in vitro*]. Ceska Slov. Farm, 53(3): 135-140.
Waffo Teguo P, Fauconneau B, Deffieux G, Huguet F, Vercauteren J, Merillon JM (1998). Isolation, identification, and antioxidant activity of three stilbene glucosides newly extracted from *vitis vinifera* cell cultures. J. Nat. Prod. 61(5): 655-657.
Wu CT, Mulabagal V, Nalawade SM, Chen CL, Yang TF, Tsay HS (2003). Isolation and quantitative analysis of cryptotanshinone, an active quinoid diterpene formed in callus of Salvia miltiorrhiza BUNGE. Biol Pharm. Bull. 26(6): 845-848.
Yadav M, Jain S, Bhardwaj A, Nagpal R, Puniya M, Tomar R, Singh V, Parkash O, Prasad GB, Marotta F, Yadav H (2009). Biological and medicinal properties of grapes and their bioactive constituents: an update. J. Med. Food, 12(3): 473-484.
Zhong JJ (2001). Biochemical engineering of the production of plant-specific secondary metabolites by cell suspension cultures. Adv. Biochem. Eng. Biotechnol. 72: 1-26.