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Elicitor-induced resveratrol production in cell cultures of different grape genotypes (*Vitis* spp.)

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Summary

Higher plants synthesize secondary metabolites in response to abiotic and biotic stress. Liquid cell culture may be a useful tool for studying phytoalexin biosynthesis during plant interaction with pathogens. Dimethyl-β-cyclodextrin (DIMEB), an oligosaccharide consisting of 2,6-methylated cyclic α(1→4)-linked glucopyranose moieties, was shown to be capable of inducing stilbene biosynthesis in liquid grape cell cultures, also in the absence of pathogenic organisms. DIMEB is capable of inducing a response in the liquid cell cultures of the *Vitis vinifera* cultivars Pinot Noir and Merzling and furthermore in *V. amurensis* and a cross between *V. riparia* and *V. berlandieri*. More detailed attention was focused on different responses in cell suspensions of Pinot Noir and the cross of *V. riparia* and *V. berlandieri* after DIMEB treatment.

K e y w o r d s : cell culture, *trans*-resveratrol, *trans*-piceid, DIMEB.

A b b r e v i a t i o n s : *2,4 D* 2,4-Dichlorophenoxyacetic acid; *DIMEB* heptakis (2,6-di-O-methyl)-β-cyclodextrin.

Introduction

Higher plants are capable of synthesizing a wide range of low molecular weight compounds, secondary metabolites. These compounds are known to play an important role in the adaptation of plants to the environment. The production of secondary metabolites is influenced by physiological and ambient conditions. They belong to large families, e.g. phenolics, terpenes and steroids, alkaloids.

Among the polyphenols, resveratrol and its oligomers, the viniferins, can play a phytoalexin role in grapevine. A positive correlation between the ability to synthesize resveratrol both in berries and leaves and the resistance to *Botrytis cinerea* infection in different *Vitaceae* has been found by Stein and Blaich (1985). Resveratrol is indeed capable to control the germination of conidia and mycelial growth in *Phomopsis viticola* and *Botrytis cinerea* (Hoos and Blaich 1990). Resveratrol has also an effect on *Plasmopara viticola* zoospore mobility and disease development (Pezet *et al*. 2004). The production of resveratrol and viniferins can also be induced in grapevines through abiotic factors such as ultraviolet radiation (Langcake and Pryce 1977). ε-viniferins seem to play a role in the defence response after pathogen infection. Berries of cv. Castor, resistant to *Botrytis cinerea*, synthesized more *trans*-resveratrol and ε-viniferin than berries of cv. Huxelrebe, a susceptible variety (Bavaresco *et al*. 1997, 1999).

Plant cell cultures are capable to produce secondary metabolites. Grape cell suspension culture can be a useful tool for studying the biosynthesis of stilbenes induced by abiotic and biotic factors. In parsley liquid cell cultures, treatments with a crude elicitor preparation from mycelium of *Phytophthora sojae* (Dangl *et al*. 1987, Somssich *et al*. 1989) and with Pep-13 (Hahlbrock and Scheel 1989) stimulated production of the furanocumarin phytoalexins. Furthermore, soluble glucan elicitors, prepared from acid hydrolysis of *Botrytis cinerea* cell walls, can induce biosynthesis of both, stilbene synthase and L-phenylalanine ammonia-lyase (Liswidowati *et al*. 1991), while treatment of the cell wall of *Phytophthora cambivora* leads to accumulation of mRNA of both, stilbene synthase and L-phenylalanine ammonia-lyase in cell cultures of *Vitis vinifera* cv. Optima (Melchior and Kindl 1991).

Metal ions (lanthanum, europium, calcium, silver, cadmium) act as abiotic elicitors and induce biosynthesis of phytoalexins in plant cell cultures (Radman *et al*. 2003). In berries of *Vitis vinifera* cv. Corvina UV-light treatment increased the level of mRNA of stilbene synthase at veraison (Versari *et al*. 2001). Irradiation of cvs Corvina and Corvinone grapes with UV-light doubled the concentration of resveratrols in their wines (Paronetto and Mattivi 1999).

Stilbene synthase mRNA accumulates in Scots pine seedlings treated with ozone (Zinser 1996). 5'-deletion analysis of the promoter of *Vst*1, which is a coding stilbene synthase in cv. Optima, showed a minimal pathogen-responsive region, an ozone-responsive region (Schubert *et al*. 1997) and a minimal ethylene-responsive region (Grimmig *et al*. 2002). The results of this gene analysis can explain the synthesis of phytoalexins in plants after exposure to stress.

Other abiotic elicitors which induce biosynthesis of phytoalexins are cyclodextrins. Cyclodextrins are cyclic oligosaccharides of 6, 7 or 8 α-D-glucopyranoside residues linked by α 1→4 glucosidic bonds, with hydrophilic external surface and hydrophobic central cavity that can trap apolar compounds (López-Nicolás *et al*. 1995). β-Cyclodextrin is formed from 7 glucose residues. In cell cultures of *V. vinifera* cv. Gamay DIMEB (heptakis(2,6-di-O-methyl)-β-cyclodextrin) can also induce biosynthesis of resveratrol without infection with *Xylophilus ampelinus*

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(Morales *et al*. 1998). The glucose ring of β-cyclodextrin has free hydroxyl groups in 2, 3 and 6 positions. Effects of various substitutions on the ability of modified cyclodextrins to induce defence responses were studied in the liquid cell cultures of three different varieties of *V. vinifera* (Bru Martinez and Pedreno Garcia 2003, Bru *et al*. 2006). It was shown that uncharged, non-bulky, alkyl groups are the most effective in allowing these cyclic oligosaccharides to act as elicitors (Bru Martinez and Pedreno Garcia 2003, Bru *et al*. 2006).

The aim of our study was to investigate the effect of DIMEB on the resveratrol metabolism in liquid cell cultures of *Vitis vinifera* (cvs Pinot Noir and Merzling), *V. amurensis* and of an interspecific cross between *V. riparia* and *V. berlandieri*.

Material and Methods

Cell culture: Calli were established from the stems of 4 different grape genotypes: *V. vinifera* cvs Pinot Noir and Merzling, *V. amurensis* and a cross between *V. riparia* and *V. berlandieri*. Pieces of stem were sterilized with 80 % ethanol for 3 min and afterwards with a 3 % NaClO solution for 3 min. The pieces were then rinsed with sterile water and incubated on plates with a solid growth MS medium (Murashige and Skoog, mod. 1B, Duchefa, Netherlands), containing microelements, macroelements, vitamins and supplemented with 20 g l^{-1} sucrose (Duchefa) and 1 mg l^{-1} 2,4-D (Duchefa). Calli were grown at 24 °C in the dark and were maintained by changing the growth medium every month. Liquid cell cultures were established by growing 2-3 g of grape callus in a 250 ml Erlenmeyer flask with 50 ml of a liquid CP medium (Chèe and Pool Vitis medium, Duchefa) containing microelements, macroelements, vitamins and supplemented with 20 g l^{-1} sucrose (Duchefa) and 1 mg l^{-1} 2,4-D (Duchefa). These liquid cell cultures were subcultured every week with 1:2 (v:v) ratio and maintained at 24 °C on an orbital shaker (120 rpm) (UNIMAX 2010, Heidolph Instruments, Germany). After three months stable liquid cell cultures were obtained.

Induction experiments: Seven-d-old grape liquid cell cultures were used to induce resveratrol biosynthesis *in vitro* as previously described by Morales *et al*. (1998). Four g of washed cells, recovered by partial vacuum, were inoculated in 10 ml of liquid CP medium in a 50 ml Erlenmeyer flask with 50 mM DIMEB (CYCLOLAB, Hungary) for the treated sample and without DIMEB for the control sample. The cell cultures were incubated at 24 °C, in the dark with orbital shaking at 100 rpm. Induction experiments were repeated at different times and therefore with different number of subcultures.

Sampling: In a preliminary screening of all 4 genotypes (*V. vinifera* cvs Pinot Noir and Merzling, *V. amurensis* and a cross between *V. riparia* and *V. berlandieri)* one ml of suspension culture of control and treated samples was collected after 48 h.

Pinot Noir and the cross between *V. riparia* and *V. berlandieri* were chosen for further experiments in which one ml of the cell liquid culture of control and treated samples was collected at 30 min, 2 h, 6 h and 24 h after induction. The sampling times were also chosen for a future characterization of gene expression of cell suspensions. Four repeated, independent, experiments were performed for both genotypes, together with a fifth experiment in which only 24 h after induction samples were taken. Cells and medium were separated by centrifugation at 12.000 rpm for 10 min at room temperature. Samples of medium were filtered through 0.45 µm, 13 mm PTFE syringe-tip filters (Millipore, Bedford, Massachusetts) into LC vials before injection into HPLC.

Extraction of resveratrol from cells: Methanol (1.8 ml) was added to 250 mg of cells to induce cell lysis and the extraction of stilbenes. Extraction took place 12 h in the dark at room temperature. Afterwards the extracts were centrifuged and the supernatant was directly filtered through 0.45 µm, 13 mm PTFE syringe-tip filters (Millipore) into LC vials before injection into HPLC.

HPLC analysis: All chromatographic solvents (acetonitrile, acetic acid) were HPLC grade and were purchased from Carlo Erba (Italy). *trans*-Resveratrol was purchased from Sigma (Steinheim, Germany). The *trans*-piceid was isolated from the methanolic extract of dried roots of *Polygonum cuspidatum* by sequentially applying the following purification techniques: (1) flash-chromatography on Isolute ENV+ (International Sorbent Technology, United Kingdom); (2) flash-chromatography on Isolute Flash-Si (International Sorbent Technology); (3) preparative HPLC on reversed-phase column Lichrospher 100 RP 18, 10 µm (Merck, Germany) and (4) crystallization from ethyl acetate with n-hexane. The *trans*-piceid had a chromatographic purity of > 98 % (HPLC); the structure was confirmed by ^{1}H NMR and found to agree with data published by Mattivi *et al*. (1995).

Analysis of resveratrols was carried out on a Waters 2695 HPLC system with a Waters 2996 DAD (Waters Corp., Milford, Massachusetts) and the Empower Software (Waters). Separation was performed using a Zorbax column (SB-Aq, 5 µm, 2.1 mm x 150 mm, Agilent Technologies, Palo Alto, California) and a Zorbax precolumn (SB-Aq, 5 µm, 2.1 mm x 12.5 mm, Agilent Technologies).

The mobile phases consisted of 0.1 % acetic acid in H_2O (A) and acetonitrile (B). Separation was carried out at 40 °C in 27 min, under the following conditions: linear gradients starting at 5 % B, to 70 % B in 25 min, to 95 % B in 0.1 min, 95 % B for 2 min, back to 5 % B in 0.1 min. The column was equilibrated for 7 min prior to each analysis. The flow rate was 0.25 ml min^{-1} and the injection volume 6 µl. The UV-VIS spectra were recorded from 220 to 400 nm, with detection at 310 nm. *trans*-Resveratrol and *trans*-piceid were quantified and identified using the external standard method on the basis of the retention time and UV-VIS spectra. Quantitative data were calculated on the basis of a 5 point calibration curve in the range of 2.5 mg l^{-1} to 25 mg l^{-1} for *trans*-resveratrol, with a correlation coefficient of 0.9984 and in the range of 1 mg l^{-1} to 20 mg l^{-1} for *trans*-piceid, with a correlation coefficient of 0.9975. The limits of detection (LOD) were 0.024 mg l^{-1} for *trans*-piceid and 0.016 mg l^{-1} for *trans*-resveratrol with a signal-to-noise ratio (S/N) ≥ 3. The limits of quantifica-

tion (S/N ≥ 10) were 0.078 mg l^{-1} for *trans*-piceid and 0.053 for *trans*-resveratrol.

Results and Discussion

In a preliminary screening we were able to detect mainly free *trans*-resveratrol in spent media of treated samples (Tab. 1). Fig. 1 shows chromatograms with *trans*-resveratrol and *trans*-piceid of media and cells of control and treated samples (48 h after DIMEB treatment) for the cross between *V. riparia* and *V. berlandieri*. The lowest *trans*-resveratrol concentrations in media were detected in both *V. vinifera* cultivars: 0.51 mg l^{-1} for Pinot Noir and 4.31 mg l^{-1} for Merzling. Much higher concentrations were found in spent media of treated *V. amurensis* cell cultures (225.2 mg l^{-1}) and the *V. riparia* and *V. berlandieri* cross (911.3 mg l^{-1}) (Tab. 1).

The concentration of *trans*-resveratrol in the cells agreed with the amount released into the relevant culture media (Tab. 1). Samples of *V. amurensis* had higher concentrations of *trans*-piceid than the other genotypes in both, cells and culture medium (Tab. 1). Subsequently we focused our research on two out of 4 genotypes which were included in the preliminary screening: Cv. Pinot Noir and the cross between *V. riparia* and *V. berlandieri*. The aim was to characterize the kinetics of resveratrol production after induction with DIMEB. The experiments with Pinot Noir showed that the *trans*-piceid content in cells was similar in control and treated samples; it remained more or less constant during sampling (Tab. 2). The same tendency was observed for the cross between *V. riparia* and *V. berlandieri* (Tab. 3). Previously similar results have been presented for liquid cell cultures cv. Gamay treated with DIMEB (Morales *et al.* 1998). It is assumed that (1) *trans*-piceid is a stocked form of *trans*-resveratrol during the response to infections or stress, (2) it could also play a role as a highly hydrophilic, mobile form of resveratrol in transport from the intracellular space to apoplastic space and (3) *trans*-piceid could prevent *trans*-resveratrol degradation by peroxidase activity (Morales *et al*. 1998). The *trans*-resveratrol content in cells of inducted samples tended to increase

Table 1

trans-resveratrol and *trans*-piceid levels in medium and cells of control and treated samples (48 h after treatment with DIMEB)

	control	treated
V. vinifera cv. Pinot Noir		
t-resveratrol in medium	n.d.	0.51 mg l^{-1}
t-piceid in medium	n.d.	0.15 mg l^{-1}
t-resveratrol in cells	5.59 µg g^{-1}	5.80 µg g^{-1}
t-piceid in cells	0.80 µg g^{-1}	1.85 µg g^{-1}
V. vinifera cv. Merzling		
t-resveratrol in medium	0.49 mg l^{-1}	4.31 mg l^{-1}
t-piceid in medium	n.d.	0.17 mg l^{-1}
t-resveratrol in cells	2.64 µg g^{-1}	3.91 µg g^{-1}
t-piceid in cells	1.09 µg g^{-1}	1.24 µg g^{-1}
V. amurensis		
t-resveratrol in medium	0.31 mg l^{-1}	225.22 mg l^{-1}
t-piceid in medium	0.17 mg l^{-1}	3.11 mg l^{-1}
t-resveratrol in cells	6.10 µg g^{-1}	187.35 µg g^{-1}
t-piceid in cells	47.89 µg g^{-1}	39.82 µg g^{-1}
V. riparia x *V. berlandieri*		
t-resveratrol in medium	0.37 mg l^{-1}	911.25 mg l^{-1}
t-piceid in medium	n.d.	1.51 mg l^{-1}
t-resveratrol in cells	2.75 µg g^{-1}	622.90 µg g^{-1}
t-piceid in cells	1.43 µg g^{-1}	1.44 µg g^{-1}

n.d. = not detected

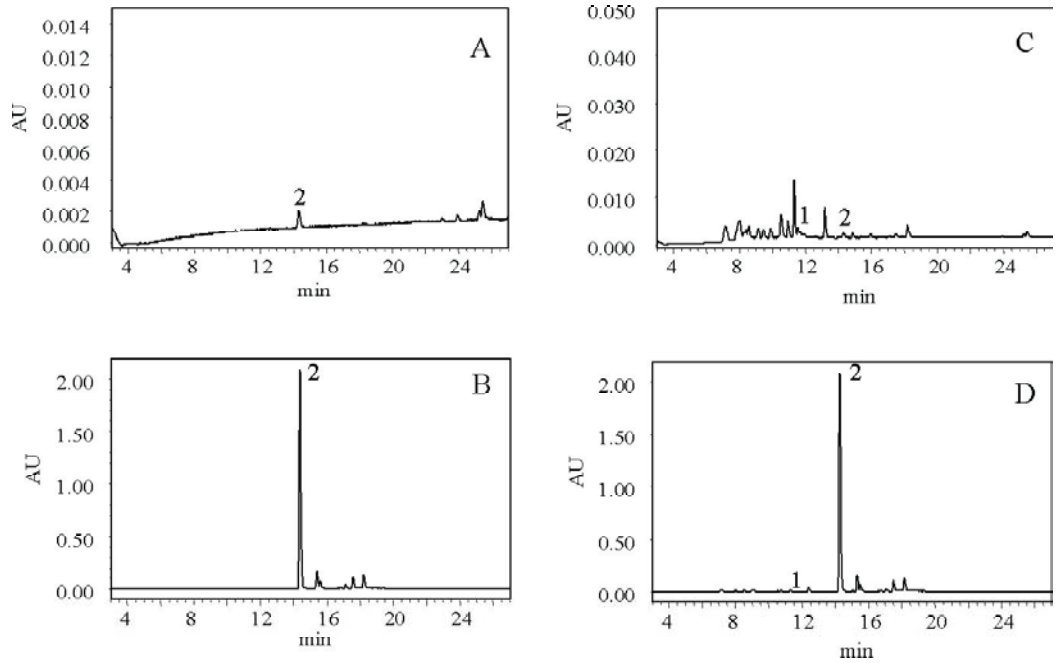

Fig. 1: HPLC chromatograms of *V. riparia* x *V. berlandieri* suspension cultures (48 h after DIMEB treatment). **A**: Medium of control sample, **B**: Medium of treated sample, **C**: Cells of control sample, **D**: Cells of treated sample. **1**: *trans*-piceid, **2**: *trans*-resveratrol.

Table 2

Accumulation of *trans*-resveratrol and *trans*-piceid in medium and cells of control and of samples treated with DIMEB (cv. Pinot Noir). Means and standard deviations (s.d.) are calculated from positive results

sampling times	control		treated	
	number of positives	mean and s.d. of positives	number of positives	mean and s.d. of positives
t-resveratrol in medium, mg l^{-1}				
0.5 h[a]	1	n.d.	2	0.32
2 h[a]	n.d.	n.d.	4	0.35 ± 0.02
6 h[a]	n.d.	n.d.	4	0.44 ± 0.14
24 h[b]	1	n.d.	5	1.83 ± 0.97
t-piceid in medium, mg l^{-1}				
0.5 h[a]	n.d.	n.d.	2	0.15
2 h[a]	n.d.	n.d.	4	0.16 ± 0.01
6 h[a]	n.d.	n.d.	2	0.15
24 h[b]	n.d.	n.d.	4	0.16 ± 0.01
t-resveratrol in cells, µg g^{-1}				
0.5 h[a]	1	n.d.	1	n.d.
2 h[a]	1	n.d.	3	2.81 ± 0.43
6 h[a]	2	3.73	4	3.19 ± 0.83
24 h[b]	2	2.35	5	3.71 ± 2.38
t-piceid in cells, µg g^{-1}				
0.5 h[a]	4	1.55 ± 0.90	4	1.78 ± 0.84
2 h[a]	4	2.12 ± 0.87	4	2.21 ± 0.63
6 h[a]	4	2.04 ± 0.90	4	2.28 ± 0.94
24 h[b]	4	2.07 ± 0.72	5	2.26 ± 1.23

n.d. = not detected; [a] 4 repeated experiments; [b] 5 repeated experiments.

mainly for the cross between *V. riparia* and *V. berlandieri*, especially in the period between 6 and 24 h after treatment with DIMEB while the amount of *trans*-resveratrol in the cells of control samples remained constant (Tab. 3). Differences in *trans*-resveratrol in cells of cv. Pinot noir were less evident (Tab. 2). For both grapevine genotypes *trans*-piceid was never detected in the spent medium of control samples, while low levels remained constant during the whole sampling time for treated samples (Tab. 2, Tab. 3). The concentration of *trans*-resveratrol was higher in the spent media of induced samples and its concentration increased over time.

β-Cyclodextrins have already been reported to act as genuine elicitors due to their oligosaccharide nature for the culture of cv. Gamay (Morales *et al*. 1998) and other *V. vinifera* cell lines (Bru-Martinez and Pedreno Garcia 2003, Bru *et al*. 2006). In this study it has, again, been shown that DIMEB is capable to induce defence responses, in particular due to its ability to induce phytoalexin biosynthesis, both in cv. Pinot Noir and in the cross between *V. riparia* and *V. berlandieri*. The differences in the resveratrol levels between the two genotypes might be the reason for their different levels of defence response. Differences in stilbene production in leaves and berries were reported to be correlated with susceptibility to *Botrytis cinerea* (Stein and Hoos 1984, Hoos and Blaich 1990), and *Plasmopara viticola* (Dercks and Creasy 1989, Adrian *et al*. 1997). More evidence supporting the role of resveratrol in resistance to pathogen infection was supplied by the transfer of stilbene synthase genes in plants that do not produce stilbenes, such as tobacco and alfalfa (Hain *et al*. 1993, Hipskind and Paiva 2000). In some cases, however, the expression of the STS gene in transgenic plants leads to the biosynthesis of stilbene compounds but not to an increase in resistance to pathogens, as in the case of transgenic kiwi plants (Kobayashi *et al*. 2000) and in transgenic poplar (Giorcelli *et al*. 2004).

Since *trans*-resveratrol mainly accumulates in media, the relevant compound can be recovered directly from the spent medium without biomass destruction. Apparently, the very high levels of *trans*-resveratrol which accumulate in the spent medium (up to 4 M) had no toxic effect on the cell lines, allowing successful subcultures.

High values of standard deviation were observed especially for *trans*-resveratrol in media and cells for the *V. riparia* and *V. berlandieri* cross (Tab. 3). The wide variability of *trans*-resveratrol and *trans*-piceid levels obtained during the 5 repeated, independent experiments can be explained in the light of a decreasing tendency to *trans*-resveratrol accumulation during subsequent subcultures *in vitro* for *V. riparia* x *V. berlandieri* (Fig. 2). A similar behaviour was described for solid and submerged cultures of *Daucus carota* in batch cultivation, where decreasing anthocyanin production during subsequent subcultures using

Table 3

Accumulation of *trans*-resveratrol and *trans*-piceid in medium and cells of control and of samples treated with DIMEB (*V. riparia* x *V. berlandieri*). Means and standard deviations (s.d.) are calculated from positive results

sampling times	control		treated	
	number of positives	mean and s.d. of positives	number of positives	mean and s.d. of positives
t-resveratrol in medium, mg l^{-1}				
0.5 h[a]	n.d.	n.d.	1	n.d.
2 h[a]	n.d.	n.d.	3	0.60 ± 0.34
6 h[a]	1	n.d.	4	3.68 ± 3.23
24 h[b]	3	0.37 ± 0.07	5	136.22 ± 107.75
t-piceid in medium, mg l^{-1}				
0.5 h[a]	n.d.	n.d.	1	n.d.
2 h[a]	n.d.	n.d.	4	0.16 ± 0.01
6 h[a]	n.d.	n.d.	3	0.17 ± 0.02
24 h[b]	n.d.	n.d.	4	0.16 ± 0.01
t-resveratrol in cells, µg g^{-1}				
0.5 h[a]	2	2.26	2	2.60
2 h[a]	2	2.13	4	3.18 ± 0.81
6 h[a]	4	2.34 ± 0.51	4	5.60 ± 2.18
24 h[b]	5	2.95 ± 0.74	5	76.46 ± 61.45
t-piceid in cells, µg g^{-1}				
0.5 h[a]	4	2.20 ± 1.49	4	2.80 ± 1.90
2 h[a]	4	218 ± 1.47	4	2.85 ± 1.69
6 h[a]	4	2.60 ± 2.03	4	3.42 ± 2.69
24 h[b]	5	160 ± 0.37	5	2.18 ± 0.69

n.d. = not detected; [a] 4 repeated experiments; [b] 5 repeated experiments.

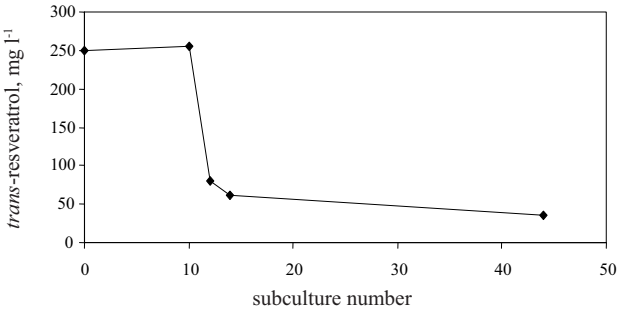

Fig. 2: Concentration of *trans*-resveratrol in medium of *V. riparia* x *V. berlandieri* culture 24 h after DIMEB treatment as a function of subsequent subcultures.

MS supplemented with IAA was reported (Narayan *et al*. 2005). Calli of *V. vinifera* cv. Bailey Alicant A., cultivated in liquid medium, were used to study anthocyanin production in long-term repeated batch culture (Nagamori *et al*. 2001). Here again, anthocyanin biosynthesis decreased during subsequent subcultures. The unstable biosynthesis of secondary metabolites could be caused by hydrodynamic stress during agitation. In this case, anthocyanin biosynthetic capacity could be retained for a higher number of subsequent subcultures by using a viscous additive in liquid medium rather than biosynthesis in a normal medium (Nagamori *et al*. 2001).

Considering the variability of the response to DIMEB elicitation in the 4 grape genotypes included in this study under the same conditions, it was demonstrated that it is important to choose genotypes which have the ability to produce higher levels of the relevant secondary metabolites. In our case, suspension cultures of the cross between *V. riparia* and *V. berlandieri*, and the *V. amurensis* cell line were shown to produce more *trans*-resveratrol *in vitro* than cultures of Pinot Noir or Merzling. The experimental protocols established in this study are useful for future characterization of gene expression for the cell suspensions of these genotypes.

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