Advances in data analysis in Metabolomics: towards a dynamic view of responses in plant cell cultures

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This book is dedicated with greatest love and affection to:

My beloved parents,

U Saw Maung and Daw Khin Mya Nwet

Erklärung

Ich versichere, dass ich meine Dissertation

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Singapore, den 08.08.2018

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Nay Min Min Thaw Saw

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Publications and presentations

Publication	Nay Min Min Thaw Saw, Claudio Moser, Stefan Martens and Pietro Franceschi. "Applying generalized additive models to unravel dynamic changes in anthocyanin biosynthesis in methyl jasmonate elicited grapevine (*Vitis vinifera* cv. Gamay) cell cultures" Horticulture Research 4 (July-2017): 17038
Oral presentations	Time-course targeted metabolite profiling in *Vitis vinifera* cell suspension cultures Merlion Metabolomics Workshop 2014 *(Singapore)*
	Growth kinetics and targeted metabolite profiling of *Vitis vinifera* suspension cultures First Eduard Strasburger-Workshop of the German Botanical Society 2014 *(Nuremberg, Germany)*
Poster presentation	Time-course changes of anthocyanin synthesis in MeJA-induced grapevine (*Vitis vinifera* cv. Gamay) cell suspension cultures 8th International Workshop on Anthocyanins 2015 *(Montpellier, France)*

List of abbreviation

4CL	-	4-coumarate-CoA ligase
AAT	-	anthocyanin acyltransferases
ANOVA	-	analysis of variance
ANR	-	anthocyanidin reductase
ANS syn. LDOX	-	anthocyanidin synthase syn. leucoanthocyanidin dioxygenase
B5 medium	-	Gamborg B5 Medium
C3H	-	4-coumarate 3-hydroxylase
C4H	-	cinnamate 4-hydroxylase
CCoAOMT	-	caffeoyl CoA 3-*O*-methyltransferase
CE	-	capillary electrophoresis
CHI	-	chalcone isomerase
CHS	-	chalcone synthase
COMT	-	caffeic acid/5-hydroxyferulic acid–*O*-methyltransferase
Cy	-	cyanidin
Cy-ac-glu,	-	cyanidin acetyl-3-*O*-glucoside
Cy-glu	-	cyanidin-3-*O*-glucoside
Cy-pc-glu,	-	cyanidin *p*-coumaroyl-3-*O*-glucoside
DFR	-	dihydroflavonol 4-reductase
DHK	-	dihydrokaempferol
DHM	-	dihydromyricetin
DHQ	-	dihydroquercetin
Dp	-	delphinidin
Dp-ac-glu,	-	delphinidin acetyl-3-*O*-glucoside
Dp-glu	-	delphinidin-3-*O*-glucoside
Dp-pc-glu,	-	delphinidin *p*-coumaroyl-3-glucoside
DW	-	dry cell weight
ERI	-	eriodictyol
F100C	-	100 mg fresh frozen cells extracted by chloroform/methanol/water
F100M	-	100 mg fresh frozen cells extracted by cold absolute methanol
F3’5’H	-	flavonoid-3’5’-hydroxylase
F3’H	-	flavonoid-3’-hydroxylase
F300C	-	300 mg fresh frozen cells extracted by chloroform/methanol/water
F300M	-	300 mg fresh frozen cells extracted by cold absolute methanol
F3H syn. FHT	-	flavanone 3ß-hydroxylase
F5H	-	ferulate 5-hydroxylase/coniferaldehyde 5-hydroxylase
FFD30C	-	30 mg freeze-dried cells extracted by chloroform/methanol/water

FFD30M	-	30 mg freeze-dried cells extracted by cold absolute methanol
FFD50C	-	50 mg freeze-dried cells extracted by chloroform/methanol/water
FFD50M	-	50 mg freeze-dried cells extracted by cold absolute methanol
FLS	-	flavonol synthase
FNSs	-	flavone synthases
FW	-	fresh cell weight
g	-	gram
GAMs	-	generalized additive models
GC	-	gas chromatography
GLM	-	generalized linear models
GT	-	*V. vinifera* cv. Gamay Fréaux var. Teinturier
HSD	-	honestly significant difference
JA	-	jasmonic acid
Km	-	kaempferol
LAR	-	leucoanthocyanidin reductase
LC	-	liquid chromatography
LC-MS	-	liquid chromatography followed by mass spectrometry
LCy	-	leucocyanidin
LDp	-	leucodelphinidin
LMs	-	linear models
LOQ	-	limit of quantification
LPg	-	leucopelargonidin
MeJA	-	methyl jasmonate
mg	-	milligram
MRM	-	multiple reaction monitoring
MS	-	mass spectrometry
Mv	-	malvidin
Mv-ac-glu,	-	malvidin acetyl-3-*O*-glucoside
Mv-ac-glu,	-	malvidin p-coumaroyl-3-*O*-glucoside
Mv-glu	-	malvidin-3-*O*-glucoside
My	-	myricetin
NAR	-	naringenin
NMR	-	nuclear magnetic resonance
OMT	-	*O*-methyltransferase
PAL	-	phenylalanine ammonia-lyase
PAs	-	proanthocyanidins
PCA	-	principal component analysis

Pn	-	peonidin
Pn-ac-glu	-	peonidin acetyl-3-O-glucoside
Pn-ac-glu	-	peonidin p-coumaroyl-3-O-glucoside
Pn-glu	-	peonidin-3-O-glucoside
Pt	-	petunidin
Pt-ac-glu,	-	petunidin acetyl-3-*O*-glucoside
Pt-ac-glu,	-	petunidin p-coumaroyl-3-*O*-glucoside
Pt-glu	-	petunidin-3-*O*-glucoside
Pz	-	phloridzin
QqQ	-	quadrupole mass spectrometry
QTOF	-	quadrupole time-of-flight
Qu	-	quercetin
REML	-	restricted maximum likelihood
RSD	-	relative standard deviation
SFC	-	supercritical fluid chromatography
SNP	-	sodium nitroprusside
STS	-	stilbene synthase
TMP	-	total mycelial phenolic
UFGT	-	UDP glucose: flavonoid-3-*O*-glucosyltransferase
UPLC	-	ultra performance liquid chromatography
VGR	-	*Vitis vinifera* cv. Gamay Fréaux
VPN	-	*Vitis vinifera* cv. Pinot Noir

Summary

Metabolite profiling of plant cell cultures under stress is an important approach towards the understanding of the biology of stress responses in plants by identifying by-products of the stress metabolism, either stress signal transduction molecules or molecules that are involved in adaptive responses. To model in a comprehensive way such complex biological phenomena, it is important to analyse the time-dependent behaviour of the cell metabolism in response to external stimuli. A single point measurement indeed gives only a snapshot of the cellular state in which all the dynamical information is not captured. In this respect, high resolution time-course experiments are expected to provide more information in the phase of data analysis than a series of measurements collected only at a few time-points. The main objective of this thesis is to study the metabolic response of grapevine cell cultures subjected to an external stimulus, designed to mimic the interaction of the plant with environment factors. The PhD thesis firstly describes the optimization of the sample preparation method, extraction protocol and targeted metabolomics analysis of compounds isolated from the grape cells. This optimized metabolomics protocol was then applied to study the long-term dynamic response of elicited grapevine (*Vitis vinifera* cv. Gamay) cell suspension cultures treated by methyl jasmonate or sodium nitroprusside. Generalized additive models were applied to study the time dependent profile of secondary metabolites in grapevine cell suspension cultures. The results suggested that this class of non-parametric statistical models in combination with metabolomics could be an effective tool to explore the complete time-dependent changes of secondary metabolites and their responses to external stimuli in plant cells and foundation for further integrative omics studies.

Zusammenfassung

Inhaltsstoffanalysen (metabolite profiling) in pflanzlichen Zellkulturen, die sich unter Stress befinden, tragen nicht unerheblich zum Verständnis der biologischen Mechanismen der Stressantworten der Pflanzen durch die Identifizierung von Produkten des Stressstoffwechsels, der Signaltransduktion oder von Molekülen, die an adaptiven Reaktionen beteiligt sind, bei. Darüber hinaus kann die zielgerichtete (targeted) und nicht zielgerichtete (non-targeted) Inhaltsstoffanalyse heutzutage eine komplexe Sicht auf die Reaktion des pflanzlichen Stoffwechsels auf einen bestimmten Stimulus liefern. Um solche komplexen biologischen Phänomene umfassend zu modellieren, ist es wichtig das zeitabhängige Verhalten des Zellstoffwechsels als Reaktion auf äußere Reize zu analysieren. Eine Einzelpunktmessung liefert tatsächlich nur eine Momentaufnahme des zellulären Zustands, in dem nicht alle dynamischen Informationen erfasst werden. In dieser Hinsicht wird erwartet, dass die Inhaltsstoffanalysen von hochaufgelösten Zeitprofilen in der Phase der Datenanalyse weit mehr Informationen liefert als eine Reihe von Messungen, die nur zu wenigen Zeitpunkten gesammelt wurden. Das Hauptziel dieser Arbeit ist die Untersuchung der metabolischen Reaktion von Weinzellkulturen nach Gabe eines externen Stimulus, der die Interaktion mit Umweltfaktoren nachahmt.

Die vorliegende Doktorarbeit beschreibt zunächst die Optimierung der Probenvorbereitungsmethode, des Extraktionsprotokolls und der gezielten Inhaltsstoffanalyse der aus den Traubenzellen isolierten Verbindungen. Dieses optimierte Protokoll wurde dann angewendet, um die langfristige dynamische Antwort von aus Weinreben (*Vitis vinifera* cv. Gamay) gewonnenen Zellsuspensionskulturen zu untersuchen. Das biologische System wurde unter der Behandlung von Methyljasmonat oder Natriumnitroprussid untersucht. Generalisierte additive Modelle wurden angewendet, um das zeitabhängige Profil von Sekundärmetaboliten in den Suspensionskulturen zu untersuchen. Die Ergebnisse deuten darauf hin, dass der Ansatz, generalisierter additiver Modelle und Inhaltsstoffanalysen anzuwenden, eine effektive Methode sein könnte, um die vollständigen zeitabhängigen Veränderungen von Sekundärmetaboliten und deren Reaktionen auf externe Stimuli in Pflanzenzellen zu untersuchen und so die Grundlagen für weitere integrative Omics-Studien zu legen.

Outline of the thesis

This research study highlights that statistical analysis of time course metabolomics data gains power if it moves to approaches able to compare the 'trends' of the different metabolites. In particular, we show how generalized additive models (GAMs) can be used to model the time-dependent profile of polyphenols in grapevine cell suspension cultures (*Vitis vinifera* cv. Gamay) elicited by methyl jasmonate (MeJA) and sodium nitroprusside (SNP).

In Chapter 1 we review the importance of time course metabolomics experiments, rather than single point measurements, to gain insight into the nature of complicated dynamic biological processes. We describe the different types of challenges and limitations in the current analyses of metabolome dynamics using mass spectrometry, and introduce GAM as an appropriate data analysis approach to overcome these bottlenecks.

Chapter 2 describes the sample preparation methods of grape cell cultures suitable for targeted metabolomics analysis. It consists of the optimization of sample collection, extraction solvent mixtures and comparative analysis of the secondary metabolites in two different grape cell cultures. It was found that the extraction with chloroform/methanol/water mixture gave much higher yields of phenolic compounds as compared to that with pure methanol. Moreover, *V. vinifera* cv. Gamay (VGR) cell cultures showed the wider range of polyphenol classes with higher concentration than those in *V. vinifera cv.* Pinot Noir (VPN) cell cultures.

Chapter 3 focuses on the detailed analysis of the time-course targeted metabolomics analysis from VGR cell suspension cultures in response to MeJA elicitation by using generalised additive models.

Chapter 4 describes the modeling of time-course targeted metabolomics data from VGR cell suspension cultures subjected to stimulation with SNP. Furthermore, the experimental conditions and production of secondary metabolites in grape cell cultures grown in flask cultures and bioreactors are compared.

Chapter 1

General introduction

1.1. The importance of moving from single point measure to time courses for the understanding of complex biological phenomena

Metabolites are assumed as basic parts in natural frameworks because of their inclusion in cell and physiological energetics, structure, and flagging (Vinayavekhin, Homan, and Saghatelian 2010). They are the intermediates or end products of cellular processes and the fluctuations of their levels can be regarded as the responses of the biological systems to genetic and/or environmental challenges (Fiehn 2002). Performing metabolite profiling under exposure to stressors is an important approach towards the understanding of the chemistry of stress responses, since it identifies by-products of the stress metabolism, stress signal transduction molecules or molecules that are involved in adaptive responses (Huang et al. 2012). Metabolites are one of the fastest-responding cellular components and the changes of their concentration can occur on a timescale going from seconds to days. Due to this dynamic nature, it is important to analyse the time-dependent behaviour of the cell metabolism in order to understand and model such a complex biological phenomenon in a comprehensive way. Investigating metabolic response in static experiments can indeed only give a snapshot of the cellular state with a substantial loss of information. The entire response process can be captured only by sampling at multiple time points (Gasch et al. 2000). Time-course experiments can also profit of the autocorrelation between successive time points to reduce variability. These correlations make it possible to use one time point to overcome noise in nearby time points by assigning continuous representation to the measured temporal data (Simon et al. 2005).

1.2. Metabolomics, the analytical tools and statistical modelling

Metabolome survey using time series can provide comprehensive information on the composition of a metabolite pool and it provides a functional screen of the cellular state which gains a perspective of how metabolic networks are dynamically regulated (Oliver et al. 1998). Metabolomics techniques are designed to measure rapidly thousands of metabolites simultaneously from only minimal amounts of sample. Despite these desirable characteristics in

metabolomics studies, many factors play a relevant role, including the sample preparation, extraction procedures, careful planning and execution of designed experiments, the choice of analytical tools and procedures employed and the quality of the acquired data.

There are two general analytical approaches which can be applied to perform metabolic profiling: untargeted and targeted. Untargeted metabolomics methods aim to simultaneously measure as many metabolites as possible from biological samples, without requiring previous knowledge of the compounds under investigation (Vinaixa et al. 2012). Due to its inherent complexity, untargeted metabolomics can be used to attain relative quantification (Shulaev 2006, and Patti, Yanes, and Siuzdak 2012). If absolute quantification is required, targeted metabolomics have to be preferred, since the initial definition of the compound of interest results in a more reproducible analytical workflow (Dudley et al. 2010).

A variety of analytical platforms have been used for metabolomics studies, which include nuclear magnetic resonance (NMR) and mass spectrometry (MS) coupled with gas chromatography (GC), capillary electrophoresis (CE), liquid chromatography (LC) and supercritical fluid chromatography (SFC) (Zhou and Yin 2016). Most metabolites can be detected by liquid chromatography followed by mass spectrometry (LC-MS) and it has therefore been the technique of choice for global metabolite profiling efforts. Untargeted metabolomics data are most frequently collected on high resolution MS instruments like time-of-flights (QTOF) or orbitrap (Patti, Yanes, and Siuzdak 2012). In targeted metabolomics studies, triple quadrupole mass spectrometry (QqQ) is often preferred, because it provides reliable quantitative information over a wide concentration range. With these instruments, simultaneous analysis of multiple compounds can be performed by multiple reaction monitoring (MRM) and a targeted lists of phenolic compounds can be screened as potential metabolic signatures in fruits and beverages by applying QqQ mass spectrometry based methods (Vrhovsek et al. 2012).

Metabolomics data usually include hundreds of complex metabolites, and their complexity requires the use of statistical and bioinformatics approaches in the data analysis step. In general, statistical methods used in metabolomics studies consist of univariate and multivariate methods to generate an overview of the considered data set and to identify the metabolites that show

significant changes under studied conditions followed by data mining (Sugimoto et al. 2012). Unfortunately, these data analysis approaches can have some limitations when applied to time-course metabolomics studies and it would be extremely useful to develop new data analysis tools for this type of experiments, in particular because many of the existing methods for time series analysis are not suited to the relatively small numbers of sampling events available in metabolomics experiments. Among the possible solutions, nonlinear statistical modelling approaches would be particularly promising because they could account for the complex evolution of metabolite concentration over time.

1.3. Challenges in metabolomics

In principle, the concept of performing time-course metabolomics combined with tailored statistical modelling may appear straightforward, but in practice, it contains many challenges. Many biological processes are expected to have variability in the timing, which further complicates the analysis of time-course data and encountered difficulties.

1.3.1. Biological challenges

One of the major challenges in metabolomics analysis is biological variability within a given developmental or physiological context. Metabolome measurement may vary between individuals, especially in multicellular organisms, even under approximately constant experimental conditions. For instance, the amount of metabolites detected in urine samples, depend on the amount of water they have had prior to obtain samples (Temmerman et al. 2012). Remarkably, in *in vivo* experiments the biological variability in mice cannot be controlled sufficiently to achieve identical measurements (Altman and Krzywinski 2015). Considering this level of natural variability, an important aspect of a good experimental design is to determine how many biological replicates are needed to provide adequate statistical power. As in many other cases, the number of possible biological replicates is often limited by the cost and feasibility of collecting data. Another important aspect is to minimize the perturbations during sampling and extraction procedures. Especially in the experiments of cell cultures, the media volume and height

of media in the culture vessels may have a significant impact on the cell's metabolism. It was shown that altering culture media volume has a dramatic influence on metabolic rates in renal epithelial cell cultures (Gstraunthaler, Seppi, and Pfaller 1999). In this type of experiment, the amount of biomass should be sufficient to measure efficiently the metabolites, but is required to support the optimum media volume.

1.3.2. Analytical challenges

Besides these biological factors, analytical variation affects the outcomes of metabolomics experiments. Unwanted analytical variability can arise from human error, inadequate planning and control during sample preparation and unavoidable instrumental variability. The non-consistency of sample measurement (in the weight or volume of the samples or the number of cells) during sample collection may introduce to the considerable bias in the results (De Livera et al. 2015). In large-scale studies, the analysis of large batches of samples has to be performed and potentially takes several hours of continuous analysis. More often than not, batch-to-batch variation occurs as a technical source of variation when it is not possible to allocate all the samples in the same batch in large-scale NMR and LC-MS metabolomics studies (Chen et al. 2014). Where possible, randomization should be applied to minimize possible confounding effects, but this is not always possible for large studies and complex experimental designs. Signal intensity drift of metabolites over time and across different batches is another major confounding factor in large-scale metabolomics studies (Shen et al. 2016). Good analytical stability is also important in such analyses due to sample degradation over time in long runs of samples and temperature changes within instruments that may lead unwanted variation in the results (De Livera et al. 2015).

1.3.3. Data analysis challenges

In the case of metabolomics time-course experiments, data analysis remains challenging in particular if the objective is to characterize the temporal pattern of all metabolites throughout the experiment. The most common data analysis approach for time course analysis assumes that the trend could be modelled by a linear function. Nonlinear trends could be used, but they require the researcher to know how to specify the nonlinearity correctly. In many biological processes,

however, it cannot be specified “a priori”. One possible modelling approach in this case relies in non-parametric fitting by, for example, smoothing splines, which use a piecewise polynomial function with a penalty term (Déjean et al. 2007). In time-course gene expression experiments, smoothing based methods have been widely used by assuming that the level for the log-transformed expression of a gene at a time point under a condition is equal to the smooth function of time under the same condition (Ruan and Yuan 2011). The B-spline basis function evolves possibly nonlinearity smoothly but this smoothness is governed by the number of basic functions used in modelling (Yuan 2006). This leads to the main challenge in modelling of actual time-course gene expression profile described by a smooth function. These basis functions and their respective locations control how well the temporal profile is captured (Storey et al. 2005, F. Hong and Li 2006 and Straube et al. 2015). Therefore, a statistical analysis is required which moves to approaches able to compare the “trends” of the different metabolites in order to model the profile of a large pool of metabolites in an efficient manner.

1.4. Plant cell cultures and secondary metabolites

Plants are a tremendous source of diverse metabolites that have been traditionally classified as primary and secondary metabolites. The secondary metabolites play important roles in plant growth, development, and response to environment conditions (J. Hong et al. 2016). Numerous biochemical components vary at different cell levels or even at subcellular levels in the plant and various kinds of metabolites in the plant have organ/tissue-specific characteristics (J. Hong et al. 2016). In plant metabolomics, it remains challenging to identify, quantify and localize every metabolite because of their diverse characteristics and molecular abundance. Plant cell cultures can therefore be a suitable system to investigate the properties and potentialities of plant cells, which may help in understanding their functions in particular tissues or organs (Rao and Ravishankar 2002). Plant cell cultures represent a simplified cell system when compared with a whole plant, due to their limited number of cell types and relatively undifferentiated state (Gould, Davies, and Winefield 2008). In addition, they have a rapid growth rate and higher rate of metabolism than whole plants, making the study of biosynthetic pathways considerably easier

(Deroles 2008). Developments in molecular analysis has improved our understanding of the metabolic pathways responsible for production of specific compounds that can be used for commercial manufacturing of secondary metabolites (Hussain et al. 2012). The utility of plant cell culture techniques derives from the fact of many plant cells being biosynthetically totipotent, meaning that culturing cells preserves their genetic information as in the parent cells and thus are able to produce the range of chemicals found in the parent plant (Rao and Ravishankar 2002). Plant cells, tissues or organs can be grown in isolation from original plants under sterile conditions on culture medium, with the required nutrients and plant hormones and the desired metabolite product can be extracted from the cultured cells (Smetanska 2008). Advantages of plant cell cultures includes that uniform quantity can be available all the time independent of seasons and that they can be employed for *in vitro* production of secondary metabolites, some of which are products of metabolic responses to the stress factors. The plant cells can be grown on a solid surface as friable, pale-brown lumps (called callus), or as individual or small clusters of cells in a liquid medium (called suspension culture) (Chadha, Ravindran, and Sahijram 2000). This second method of culture offers the advantages in terms of feasibility in manipulation of culture conditions for higher productivity, and the use of simpler and more economical downstream processes for the recovery of desired products (Narayani and Srivastava 2017). For all the above mentioned reasons, *in vitro* cultures have been most importantly used to produce valuable bioactive secondary substances under economically viable conditions (Zhong 2001) for commercial purposes (Zárate et al. 2001). In addition, plant cell culture is an ideal system for studying and monitoring the responses to external stress factors as opposed to studies on whole plants in nature which can be a technical challenge for the study.

Plant secondary metabolites are the products of metabolic responses and play a major role in the adaptation of plants to the environment and in overcoming stress conditions. They are synthesized in specific biosynthetic pathways whereby substrate specific enzymes play an important key role in their synthesis (Dewick 1998). Despite their structural diversity, secondary plant metabolites can be divided into three large families: phenolic, terpenes, and alkaloids

(Crozier, Jaganath, and Clifford 2006), of which the phenolic are the most widespread family of metabolites (Cheynier et al. 2013).

1.4.1. Biosynthesis of phenolic compounds

Phenolic compounds or polyphenols are synthesised by plants during development and in response to conditions such as infections, wounding, UV irradiation, exposure to ozone and pollutants (Dixon and Paiva 1995). There are more than 8000 phenolic structures currently known (Stalikas 2007). Phenolic compounds share a common structural feature, an aromatic ring bearing of one or more hydroxyl substituents, and range from simple phenolic molecules to highly polymerised compounds (Balasundram, Sundram, and Samman 2006). Overall, they can be divided into two groups based on their carbon skeleton: flavonoids (including anthocyanins, flavanols, flavonols) and non-flavonoids (including phenolic acids and stilbenoids such as resveratrol) (Shi et al. 2003).

Plant phenolic compounds are mainly synthesized from phenylalanine, which is derived from the shikimic acid pathway in primary metabolism (Özeker 1999). The general phenylpropanoid pathway starts with phenylalanine, which is converted to cinnamic acid by the activity of the enzyme phenylalanine ammonia lyase (PAL). The scheme of general phenylpropanoid pathway is shown in Figure 1 (Nanda et al. 2016). The deamination of L-phenylalanine by the enzyme phenylalanine ammonia lyase (PAL), forms cinnamic acid. It is then converted to p-coumaric acid by the cinnamate-4-hydroxylase (C4H) and finally to caffeic acid through the enzyme 4-coumarate 3-hydroxylase (C3H) (Espíndola et al. 2019). Caffeic acid can further be methylated by caffeic acid/5-hydroxyferulic acid–*O*-methyltransferase (COMT) to form ferulic acid (Nair et al. 2004). Hydroxybenzoic acids like gallic acid can be derived directly from the shikimic acid pathway especially from dehydroshikimic acid (Haddock et al. 1982) or they can be produced by the degradation of hydroxycinnamic acids, in a similar way to the (beta)-oxidation of fatty acids (Macheix and Fleuriet 1990). 4-Coumaric acid is converted to 4-coumaroyl-CoA with the help of 4-coumarate-CoA-ligase (4CL).

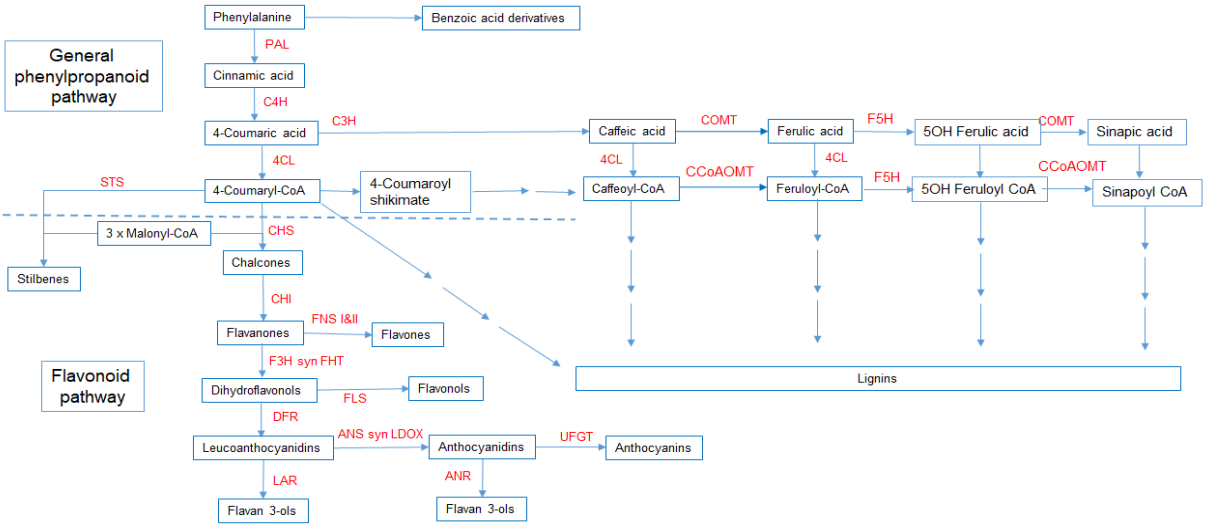

Figure 1 General phenylpropanoid and flavonoids pathway (Boss, Davies, and Robinson 1996, Nanda et al. 2016 and Barros et al. 2019). Enzymes that function in multiple or specific pathways are indicated. Abbreviations are as follows: PAL, phenylalanine ammonia lyase; C4H, cinnamate 4-hydroxylase; C3H, 4-coumarate 3-hydroxylase; COMT, caffeic acid/5-hydroxyferulic acid–*O*-methyltransferase; F5H, ferulate 5-hydroxylase/coniferaldehyde 5-hydroxylase; 4CL, 4-coumarate-CoA ligase; CCoAOMT, caffeoyl CoA 3-*O*-methyltransferase; CHS, chalcone synthase; CHI, Chalcone isomerase; FNS I, II, Flavone synthase I and II; F3H syn FHT, Flavanone 3ß-hydroxylase; FLS, Flavonol synthase; DFR, Dihydroflavonol-4-reductase; ANS syn LDOX, anthocyanidin synthase syn leucoanthocyanidin dioxygenase; UFGT, UDP glucose: flavonoid-3-*O*-glucosyltransferase; LAR, Leucoanthocyanidin reductase; ANR, Anthocyanidin reductase; STS, stilbene synthase;

The schematic presentation of flavonoid biosynthesis pathway leading to anthocyanins is shown in Figure 2. It includes two sections: the basic flavonoid upstream pathway and the specific anthocyanin branch (He et al. 2010). The flavonoid upstream pathway starts with 4-coumaroyl-CoA from the general phenylpropanoid pathway. Generally, by the action of chalcone synthase (CHS), the condensation of 4-coumaroyl-CoA and three molecules of malonyl-CoA produce naringenin chalcone (Raja Abdul Rahman et al. 2012). In the next step, chalcone isomerase (CHI) converts stereospecifically the naringenin chalcone to *S*-naringenin (NAR) which consists of the basic three rings of the general C6-C3-C6 flavonoid skeleton (He et al. 2010). NAR is at a crucial point to give rise to different final products including other flavanones, flavonols, flavones, isoflavones, flavan-3-ols, proanthocyanidins and anthocyanins. Flavones (e.g. apigenin and luteolin) are synthesized from flavanones such as NAR or eriodictyol (ERI) by catalysis of flavone synthase I or II (FNSs). The next step is catalysed by flavanone 3ß-hydroxylase (F3H syn. FHT) which hydroxylates NAR to yield dihydrokaempferol (DHK). Dihydroquercetin (DHQ) and

dihydromyricetin (DHM) are formed by hydroxylation of DHK with flavonoid 3’-hydroxylase (F3'H) and flavonoid 3’5’-hydroxylase (F3’5’H), respectively. NAR may also be directly hydroxylated by F3’H or F3’5’H to deliver ERI and pentahydroxyflavanone, respectively, which can again be hydroxylated to DHQ and DHM by F3H and activity. Flavonol synthase (FLS) catalyzes the dehydrogenation of dihydroflavonols (DHK, DHQ and DHM) to the corresponding three major flavonols, kaempferol (Km), quercetin (Qu) and myricetin (My). Further downstream, dihydroflavonol 4-reductase (DFR) provides leucoanthocyanidins which are the entry steps for anthocyanin production by using dihydroflavonols as substrates. In this step, leucopelargonidin (LPg) is formed from DHK while leucocyanidin (LCy) and leucodelphinidin (LDp) are formed from DHQ and DHM, respectively. After this, leucoanthocyanidins are converted to their corresponding anthocyanidins by catalysis of anthocyanidin synthase (ANS syn. LDOX). Anthocyanidins are further glycosylated to anthocyanins by UDP-glucose: flavonoid-3-*O*-glucosyltransferase (UFGT) enzymes. Methylation of cyanidin (Cy) and delphinidin (Dp) result in the methylated anthocyanidins (peonidin (Pn), petunidin (Pt) and malvidin (Mv)) by the enzyme *O*-methyltransferase (OMT). Further acylation of produced anthocyanins is possible by the action of different anthocyanin acyltransferases (AAT) (Sparvoli et al. 1994 and Ananga et al. 2013). Leucoanthocyanidins and anthocyanidins besides being used to synthesize the corresponding anthocyanins can also be reduced to their corresponding (2,3)-trans-flavan-3-ols (e.g. catechin) by the action of leucoanthocyanidin reductase (LAR) and (2,3)-cis-flavan-3-ols (e.g. epicatechin) by anthocyanidin reductase (ANR), respectively, which are the direct substrates for proanthocyanidin polymerization (Bogs et al. 2005). Proanthocyanidins (PAs) are oligomers of flavan-3-ols, and result from the sequential addition of starter and extension units of e.g. catechin and epicatechin monomers (Dixon and Paiva 1995).

Similarly to chalcones, stilbenes including resveratrol, are also synthesized via the phenylpropanoid pathway. In the first step, the condensation of 4-coumaroyl-CoA with three molecules of malonyl-CoA is performed through the activity of stilbene synthase (STS) leading to the production of *trans*-resveratrol (Shumakova, Manyakhin, and Kiselev 2011).

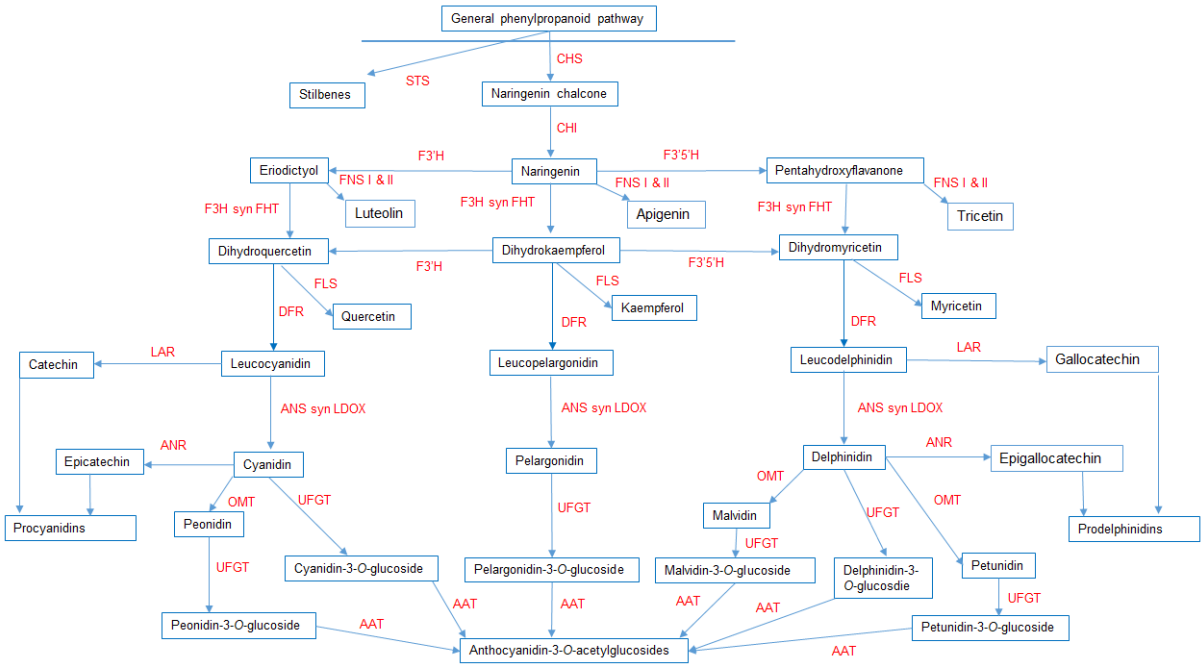

Figure 2 Flavonoid/stilbene biosynthesis pathway (Ananga et al. 2013, Brugliera et al. 2013, Mu et al. 2014 and Saw et al. 2017). Enzymes that function in multiple or specific pathways are indicated. Abbreviations are as follows: AAT, Anthocyanin acyltransferases; ANR, Anthocyanidin reductase; ANS syn LDOX, Anthocyanidin synthase syn leucoanthocyanidin dioxygenase; CHI, Chalcone isomerase; CHS, Chalcone synthase; DFR, Dihydroflavonol-4-reductase; F3H syn FHT, Flavanone 3ß-hydroxylase; F3'H, Flavonoid 3'-hydroxylase; FLS, Flavonol synthase; F3'5'H, Flavonoid 3',5'-hydroxylase; FNS I, II, Flavone synthase I and II; LAR, Leucoanthocyanidin reductase; OMT, O-methyltransferase; STS, Stilbene synthase; UFGT, UDP glucose:flavonoid-3-*O*-glucosyltransferase

1.4.2. Grape cell cultures as a model system and elicitor treatment

Grapes, as food and as the major ingredients of wine, are one of the main fruit crops in the world. Due to various nutrient elements in grape, such as vitamins, minerals, carbohydrates, edible fibers and phytochemicals, substantial scientific attention has been focused on the potential biomedical effect of grapes and grape products (X. Zhang et al. 2013). Furthermore, grapes are rich in phenolic compounds which are accumulated in its skins, short stems, seeds, leaves, and are even present in industrial grape pomace after wine-making (Poudel et al. 2008 and Rodríguez Montealegre et al. 2006/9). Anthocyanins, flavan-3-ols, proanthocyanidins, flavonol glycosides, stilbenes and phenolic acids are the principal phenolic constituents found in grape polyphenol fraction (Hernández-Jiménez et al. 2009). Therefore, cultivation of grape cell suspension cultures became particularly interesting for the production of secondary metabolites such as stilbenes and pigments.

In recent decades, extensive studies have focused on the application of elicitors to grapevine cell cultures, mostly to improve and enhance the synthesis and yield of secondary metabolites. When the plant cells are exposed to external stress factors, they have evolved complex mechanisms that consequently cause modifications in signalling components, gene transcription, non-coding RNAs, proteins, and metabolites (Long 2011). Elicitors are factors from different natural and artificial sources, which can trigger the physiological and morphological reactions and the synthesis of phytoalexins in plants (Zhao, Davis, and Verpoorte 2005). Depending on their origin, type and structure, elicitors can be classified as biotic and abiotic which can be chemical or physical factors. A variety of molecules can act as biological elicitors, including oligo- and polysaccharides derived from microorganisms and plant cell walls, peptides, proteins and lipids or hormones (Boller 1995). Abiotic elicitors are non-biological compounds that can be of chemical or physical nature. The chemical variants can be inorganic salts, fungicides, herbicides, heavy metals and some chemicals that disturb membrane integrity such as detergents. Physical factors, instead, include mechanical wounding, ultraviolet irradiation, high or low salinity, osmolality and temperatures, high-pressure and pulsed electric fields (Smetanska 2008 and Dörnenburg 2008). In studies that aimed at optimizing the *in vitro* production of secondary metabolites, several elicitors have been shown to induce and/or modify polyphenol biosynthesis and metabolism in grape cell cultures. The elicitors used to enhance the *in vitro* production of secondary metabolites by grape cell cultures are summarized in Table 1. Of all these elicitors, MeJA has been widely used as exogenous elicitor in the concentration range from 10 to 200 µM inducing an increased production of secondary metabolites such as anthocyanins as well as extra- and intra-cellular stilbenes. Apart from enhancing the productivity, MeJA modulates biochemical and metabolic pathways. For example, the exogenous addition of MeJA induced the accumulation of pathogenesis-related proteins in *V. vinifera* cv Monastrell cell cultures, which suggests that these signaling molecules could play a role in mediating defense-related gene product expression (Belchí-Navarro et al. 2013). Furthermore, it was observed that the expression of genes involved in the biosynthesis of polyphenols was increased, and associated with a strong stilbene and anthocyanin accumulation (Belhadj et al. 2008). These results revealed that elicitation

experiments can be implemented for characterizing and understanding the role of stress factors on plants using plant cell/tissue culture as model systems.

Table 1 Production of secondary metabolites in grape cell cultures by elicitation

Plant	Type of culture	Elicitor/Inducer	Target compound	Reference
rootstock 41B (*V. vinifera* cv. Chasselas × *V. berlandieri*)	Cell suspension culture	MeJA	Extracellular resveratrol and viniferins	Donnez et al. 2011
V. amurensis	Callus	MeJA, salicylic acid, calcium ionophore, 4-coumaric acid	Resveratrol	Shumakova, Manyakhin, and Kiselev 2011, Dubrovina and Kiselev 2012 and Kiselev et al. 2012
V. vinifera cv. Alphonse Lavallée	Cell suspension culture	MeJA, Ultrasound	Viniferins	Santamaria et al. 2012
V. vinifera cv Barbera	Cell suspension culture, fed-batch bioreactors	Chitosan, MeJA, red light, Na-orthovanadate, jasmonic acid, sucrose	Polyphenols, stilbenes, anthocyanin	Ferri et al. 2011, Ferri, Righetti, and Tassoni 2011, Tassoni, Durante, and Ferri 2012 and Tassoni et al. 2005
V. vinifera cv. Cabernet Sauvi-gnon	Callus, Cell suspension culture	UV-C, chitosan, MeJA, jasmonic acid, salicylic acid, ethephon	*Trans*-resveratrol, stilbenes, total flavonoids, sesquiterpene, PAs	Keskin and Kunter 2008, Krisa et al. 1999, Bertrand Faurie, Cluzet, and Mérillon 2009, D'Onofrio et al. 2009, B. Faurie et al. 2009 and Xu, Zhan, and Huang 2016

V. vinifera cv. Gamay Fréaux	Cell suspension cultures	N-linolenoyl-L-glutamine, indanoyl isoleucine, malonyl CoA, insect saliva, jasmonic acid, light irradiation, salicylic acid, ethephon, high hydrostatic pressure, streptomycin, activated charcoal, pulsed electric fields, polysaccharides, cobalt chloride, silver nitrate, cadmium chloride	Phenolic acids, anthocyanin	W. Zhang et al. 2002, Cai, Riedel, Saw, et al. 2011, Cai, Riedel, Thaw Saw, et al. 2011, Saw et al. 2012, Heidi Riedel et al. 2012, H. Riedel et al. 2012, Cai et al. 2012 and Cai et al. 2013
V. vinifera cv. Gamay Fréaux var. Teinturier	Cell suspension culture	MeJA, sucrose, jasmonic acid, salicylic acid, 3-methyl salicylic acid, betaine, β-glucan, methyl-β-cyclodextrin, Extracts of *Aspergillus niger* and *Fusarium orthoceras*	Gene expression of PR proteins, stilbene and anthocyanin	Krisa et al. 1999, Belhadj et al. 2008, Martinez-Esteso et al. 2009, Martinez-Esteso et al. 2011, Qu, Zhang, and Yu 2011, Yue, Zhang, and Deng 2011, and Vuong, Franco, and Zhang 2014
V. vinifera cv. Isabelle	Callus	*Fusarium oxysporum* extract, mannitol, abscisic acid, jasmonic acid	*Trans*-resveratrol	Mihai et al. 2011
V. vinifera *cv* Italia	Cell suspension culture	MeJA, jasmonic acid, chitosan	Resveratrols, viniferins	Santamaria et al. 2011

V.vinifera cv. Kyoho	Cell suspension cultures	MeJA, 2, 3-dihydroxypropyl jasmonate	*Trans*-resveratrol	Lu et al. 2012
V. vinifera cv. Monastrell	Cell suspension cultures	Cyclodextrins, MeJA, UV irradiation, salicylic acid, ethephon, coronatine	Extracellular *trans*-resveratrol	Lijavetzky et al. 2008, Belchí-Navarro et al. 2012, Belchí-Navarro et al. 2013, and Almagro et al. 2015
V.vinifera cv. Negramaro	Cell suspension culture	MeJA, chitosan, 12-oxo-phytodienoic acid, coronatine	Stilbenes	Taurino et al. 2015
V. vinifera cv. Pok Dum	Cell suspension cultures	ammonium nitrate	Total phenolics, *trans*-resveratrol	Sae-Lee, Kerdchoechuen, and Laohakunjit 2014
V. vinifera cv. Pinot Noir *V. vinifera* cv. Merzling *V. amurensis* *V. riparia* x *V. berlandieri*	Cell suspension cultures	Dimethyl-β-cyclodextrin	*Trans*-resveratrol and *trans*-piceid in cells and the medium	Zamboni et al. 2006
Vitis rotundifolia cv. Fry	Hairy root culture	MeJA , hydrogen peroxide	Stilbenes	Nopo-Olazabal et al. 2014
V. vinifera cv. St. Laurent	Cell suspension cultures	*Phaeomoniella chlamydospora*	Polyphenols	Sák et al. 2014

1.5. Generalized additive models as a potential approach to modelling time series metabolome data

In time series metabolomics experiments, the true levels of metabolites, as a representative of underlying biological processes, vary smoothly over time as part of normal biological cycles and in response to stimuli (Berk, Ebbels, and Montana 2011). A standard data analysis looking for significant differences at each individual time point for each metabolite would not be suitable to capture this 'longitudinal' structure of the data (Saw et al. 2017) and the assessment of statistical significance could also suffer from multiplicity issues (Franceschi, Giordan, and Wehrens 2013). A method of performing data analysis at the level of trends is therefore the right data analysis approach. Since biological interactions is also expected to produce complex time-dependent behaviors, these trends should be fitted with flexibility and in this respect generalized additive models (GAMs) represent an ideal tool (Saw et al. 2017).

GAMs are regression models originally proposed by Trevor Hastie and Robert Tibshirani (Hastie and Tibshirani 1987), which allow for the inclusion of non-parametric smoothing allowing to model nonlinear relationships in the data (Wood 2006). GAMs can be applied to the modeling of time-course metabolomics data in order to address

(i) if these data describe a trend over time,

(ii) if the trend is a straight or non-linear when it exists,

(iii) if non-linear, what the shape of the curve is.

Suppose we have a response variable (y) and predictors $x_1,\ldots, x_n$. A simple linear model takes the form

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \ldots + \beta_n x_n + \varepsilon$$

Where, β_0 is intercept,

$\beta_1, \beta_2, \ldots, \beta_n$ are regression coefficients and

ε is residual error.

The predictors are multiplied by the coefficients (β) and summed, giving us the linear predictor, which in this case also directly provides us the estimated fitted values. Linear models (LMs) are

very limited in assumptions such as the residuals are (i) normally distributed, (ii) with constant variance and (iii) uncorrelated (Faraway 2014). Unfortunately, they are not suitable to capture what is going on in many real data situations.

In generalized linear models (GLMs), a transformed version response variable is modeled instead of the observations. The objective of this type of modeling is to account for non-normally distributed response variables (Faraway 2016). A GLM can be written as

$$g(\mu) = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \ldots + \beta_n x_n + \varepsilon$$

Where $g(\mu)$ is the link function, which links the measured response with is transformed form and $\beta_1, \beta_2, \ldots, \beta_n$ are parameters to be estimated (coefficients).

GAMs extend the GLMs with a linear predictor involving a sum of smooth functions of covariates (Wood 2006). As in GLM, GAMs are not restricted only to normally distributed responses, but can be used for any probability distribution from the exponential family. As it can be easily expected, a GAM has substantially more flexibility, because the relationship between response and predictor variable is not assumed to be linear.

To see the similarities to the GLM, formally describe the GAM as:

$$g(\mu) = \beta + s_1(x_1) + \ldots. + s_n(x_n) + \varepsilon$$

The terms $s_1(x_1), \ldots., s_n(x_n)$ denote smooth, nonparametric functions.

The main advantage of using GAM is flexibility. By nature GAMs are data-driven instead of model-driven. This can allow for more flexible estimation of the underlying predictive patterns without determining the functional form of the relationship in beforehand.

Moreover, their additive structure helps the interpretation phase since each predictor term enters the model separately as illustrated in Fig. 3. This allows to study the effect of each predictor separately.

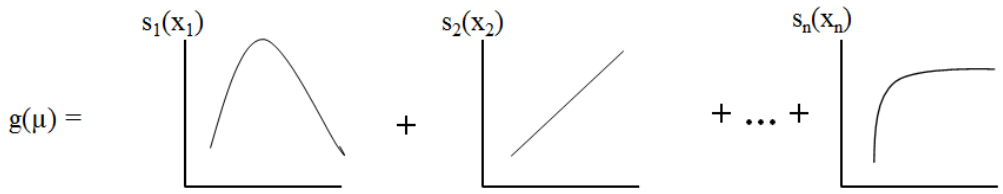

Figure 3 Illustration of the additive terms from a GAM structure (Larsen 2015)

In addition, an important feature of GAM is the ability to control the smoothness of the predictor functions that helps avoid overfitting (Larsen 2015).

Flexibility, however, comes with a price, which have to be paid in terms of experimental points: GAMs are indeed not suitable for investigations dealing with only a small number of time points. In this thesis, GAM is used as a tool of advances in data analysis of the time-course metabolomics data to capture the dynamic view of responses in plant cell cultures.

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Chapter 2

Optimization of sample preparation method for targeted metabolomics analysis of grape cell suspension cultures

2.1. Introduction

Targeted metabolomics is widely used to identify and analyze concentrations of a selected list of metabolites, quantitatively or semi-quantitatively (Shulaev 2006). This approach takes advantage of the comprehensive understanding of a vast array of metabolic enzymes, their kinetics, end products, and the known biochemical pathways to which they contribute (Roberts et al. 2012). The choice of an efficient and reproducible metabolite extraction and sample preparation method is extremely important in metabolomics studies, because it affects both the observed metabolite content and the biological interpretation of the data. Different strategies for metabolite sampling can be performed. The sampling method should be as simple and fast as possible to provide a real snapshot of the metabolome at a certain time point as well as to avoid possible enzymatic or chemical degradation during the procedure.

Grapevine (*Vitis* sp.) is known to synthesize a wide range of secondary metabolites especially polyphenols, which are composed besides others of anthocyanins, proanthocyanidins, flavonols and stilbene derivatives. These classes of compounds display peculiar characteristics which play important roles in plant metabolism (Flamini 2003). Various studies have evaluated the effect of solvents on extraction of polyphenols from grape and its products. For instance, Vrhovsek et al. (2012) developed a sample preparation and extraction method for a targeted metabolomics analysis of 135 phenolics in fruits and tea extracts using UPLC/QqQ-MS/MS. Nonetheless, the set of proposed extraction methods and choice of solvents for secondary metabolites in grapevine cell cultures are still limited. Mewis et al. (2011) described the extraction of specific polyphenolic compounds in cell culture of *V. vinifera* L. cv. Gamay Fréaux in a methanol/water solution, but this method is mainly for phenolic acid derivatives and ineffective for extracting other classes of polyphenols.

The first phase of the study aimed to optimize the sample collection method and solvent conditions for polyphenol extraction of grape cell cultures, with the purpose of metabolite profiling studies by obtaining as many features as possible with the best analytical repeatability and maximising the metabolite coverage provided by UPLC-MS/MS. The comparison of two extraction methods (mixture of chloroform/methanol/water and pure methanol) was performed

in order to establish the most efficient solvent mixture for extraction of as many as possible classes of polyphenols in the grapevine cell cultures.

2.2. Materials and methods

2.2.1. Plant cell cultures

a) *V. vinifera* cv. Pinot Noir

The cell line of *V. vinifera* cv. Pinot Noir (referred to as VPN) (Fig 4) was established at the Fondazione Edmund Mach in San Michele all'Adige (Italy) (Zamboni et al. 2015) and has been maintained as callus cultures. The cell culture was cultivated on B5 medium (Gamborg B5 Medium B5VIT, Duchefa B.V., The Netherlands) supplemented with 30 g/l sucrose, 250 g/l casein hydrolysate (Merck, Darmstadt), 0.1 mg/l α-naphthaleneacetic acid (NAA) and 0.2 mg/l kinetin (K) and 0.8% agar. Callus cultures were transferred every 28 days onto fresh solid sterile medium. Liquid cell cultures were established by growing 2-3 g grape callus in a 250 ml Erlenmeyer flask with 50 ml of a liquid B5 medium, continuously agitated on a rotary shaker at 110 rpm at 25 ± 2 ˚C under continuous fluorescent light (approximately 3,000 lux). The initial pH of the medium was adjusted to 5.5 with 0.1 M KOH before autoclaving. Cells were subcultured every seven days with an inoculum dilution of 1:3.

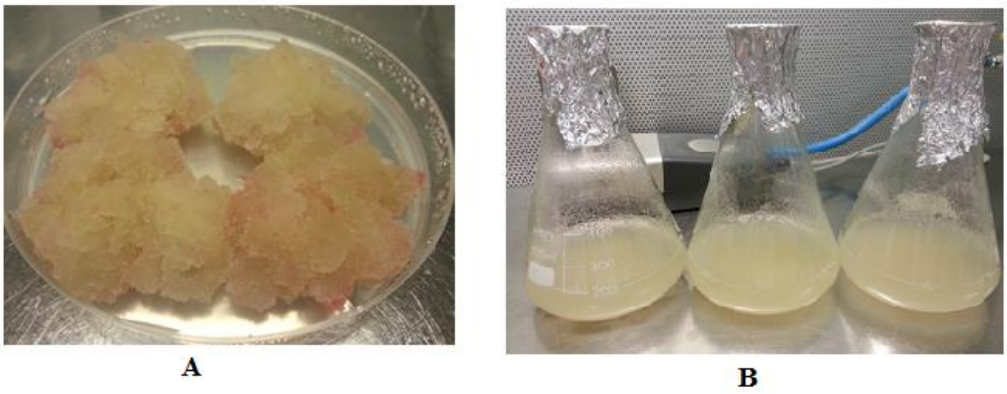

Figure 4 Callus culture (A) and suspension cultures (B) of VPN.

b) *V. vinifera* cv. Gamay Fréaux

The cell line of *V. vinifera* cv. Gamay Fréaux (referred to as VGR) (Fig 5), was a gift of Dr. Francosis Cormier's group who originally isolated the cell line (Cormier, Do, and Nicolas 1994). It has been maintained at the Fondazione Edmund Mach and the cell culture was cultivated as previously described for VPN. Callus cultures were transferred every 28 days onto fresh solid sterile medium. The most pigmented cell aggregates were selected for growth in liquid suspension cultures, obtained by transferring cell aggregates into 50 ml of liquid B5 medium in 250 ml Erlenmeyer flasks, continuously agitated on a rotary shaker at 110 rpm at 25 ± 2 ˚C under continuous fluorescent light (approximately 3,000 lux). The initial pH of the medium was adjusted to 5.5 with 0.1 M KOH before autoclaving. Cells were subcultured every seven days with an inoculum dilution of 1:3.

Figure 5 Suspension cultures of VGR.

2.2.2. Sample collection

10 ml cell suspension cultures from each flask were harvested by pipetting, filtered by vacuum filtration and weighed to obtain fresh cell weight. These filtered fresh cells were flash frozen in liquid nitrogen and stored at -80'C until further analysis. For the time-course experiment, sample collection was performed at the same time of every harvesting day throughout the experiment.

2.2.3. Determination of cell growth

The cell growth was determined as fresh cell weight and/or dry cell weight on every harvesting day. The vacuum filtered cells of 10 ml cell suspension cultures from each flask were determined as fresh cell weight (FW). Dry cell weight (DW) was determined when 100 mg of the weighed fresh cells were dried at 60°C in a vacuum oven for 24 hours until no further change and place it for 1 hour at room temperature.

2.2.4. Extraction of polyphenols (non-anthocyanins)

The filtered frozen cells were ground under liquid nitrogen using a mortar and pestle to obtain a homogenous powder. The protocol used for the extraction of the phenolic metabolites from these matrices was adapted from Vrhovsek et al. (2012). Briefly, 100 mg powder from each frozen sample was extracted in sealed glass vials using 1 mL of a mixture of water/methanol/chloroform (20:40:40). After vortexing for 1 min, the samples were placed in an orbital shaker for 15 min at room temperature. Samples were centrifuged at 13000 rpm and 4°C for 10 min, and the upper phases constituted of aqueous methanol extract were collected. Extraction was repeated by adding another 600 µL of water/methanol (1:2) to the pellet and chloroform fractions and shaking for another 15 min. After centrifugation, the upper phases from the two extractions were combined, brought to 1 mL, and filtered through a 0.2 µm PTFE filter prior to analysis.

As an alternative method, pre-cooled (-20°C) absolute methanol was used as an extraction solvent. Briefly, 100 mg of powder from each frozen sample was extracted in sealed glass vials using 1 mL of pure cold methanol. After vortexing for 1 min, the samples were put in an orbital shaker for 15 min at room temperature. Samples were centrifuged at 13000 rpm and 4°C for 10 min and the supernatant was collected and filtered through a 0.2 µm PTFE filter prior to analysis.

In order to identify the minimal initial sample weight needed for the extraction of polyphenols, 100 mg and 300 mg of fresh filtered samples, 30 mg and 50 mg of freeze-dried samples of seven days old VPN cell cultures were analysed by two extraction methods in a preliminary experiment.

2.2.5. UPLC analysis and identification and quantification (MS/MS) of polyphenols (non-anthocyanins)

The targeted metabolite analysis of polyphenols was performed by using the UPLC/QqQ-MS/MS method published before (Vrhovsek et al. 2012). UPLC was performed on a Waters Acquity UPLC system (Milford, MA) consisting of a binary pump, an online vacuum degasser, autosampler, and a column compartment. Separation of phenolic compounds was achieved on a Waters Acquity HSS T3 column 1.8 µm, 100 mm × 2.1 mm (Milford, MA, USA), kept at 40 °C. Mobile phase A was water containing 0.1% formic acid; mobile phase B was acetonitrile containing 0.1% formic acid. The flow was 0.4 mL/min, and the gradient profile was 0 min, 5% B; from 0 to 3 min, linear gradient to 20% B; from 3 to 4.3 min, isocratic 20% B; from 4.3 to 9 min, linear gradient to 45% B; from 9 to 11 min, linear gradient to 100% B; from 11 to 13 min, wash at 100% B; from 13.01 to 15 min, back to the initial conditions of 5% B. The injection volume of both the standard solutions and the samples was 2 µL. After each injection, the needle was rinsed with 600 µL of weak wash solution (water/methanol, 90:10) and 200 µL of strong wash solution (methanol/water, 90:10). Samples were kept at 6°C during the analysis. Mass spectrometry detection was performed on a Waters Xevo TQMS (Milford, MA, USA) instrument equipped with electrospray (ESI) source. Capillary voltage was 3.5 kV in positive mode and −2.5 kV in negative mode; the source was kept at 150°C; desolation temperature was 500°C; cone gas flow, 50 L/h; and desolation gas flow, 800 L/h. Unit resolution was applied to each quadrupole. Flow injections of each individual metabolite were used to optimize the multiple reaction monitoring (MRM) conditions. For the majority of the metabolites, this was done automatically by the Waters Intellistart software, whereas for some compounds the optimal cone voltages and collision energies were identified during collision-induced dissociation (CID) experiments and manually set. A dwell time of at least 25 ms was applied to each MRM transition. Data processing was done using Waters MassLynx 4.1 and TargetLynx software.

2.2.6. Data analysis

All statistical analyses were performed using the R statistical environment (Team 2015), and “ggplot2” package was used for visualization (Hadley 2015).

Non-supervised approaches such as principal component analysis (PCA) are generally applied to explore the overall sample variance with the goal of clustering the samples, according to the different metabolic features, and detecting outliers. PCA was performed on mean-centred and scaled data. To compare quantitative measurements at the 95% confidence interval, Student’s t-test and analysis of variance (ANOVA) were employed followed by Bonferroni multiple comparison and the post hoc test Tukey’s honestly significant difference (HSD), respectively.

2.3. Results

2.3.1. Comparison of different extraction solvent mixtures for characterization of phenolic compounds in *V. vinifera* cv. Pinot Noir cell suspension cultures

2.3.1.1. Sample collection and growth kinetics of cell cultures

The accurate and rapid measurement of cell growth and assessment of growth-related bioprocess kinetics are essential to evaluate the production of secondary metabolites in the cells. In our experiment, 10 ml of the homogeneous suspension cell culture from each flask were harvested by pipetting. Pipetting accuracy is very important and careful monitoring of handling pipette is necessary. Plant cell suspensions may contain cell aggregates that cause irregular flow depending on the types of pipettes. Using a single calibrated pipette showed minimal error in comparison to using either two pipettes or an uncalibrated pipette. For instance, Fig 6 shows the typical error of alternative usage of different types of pipettes during the growth curve measurement, leading to erroneous conclusions that the cell growth was sudden increased 230 mg to 420 mg in a day.

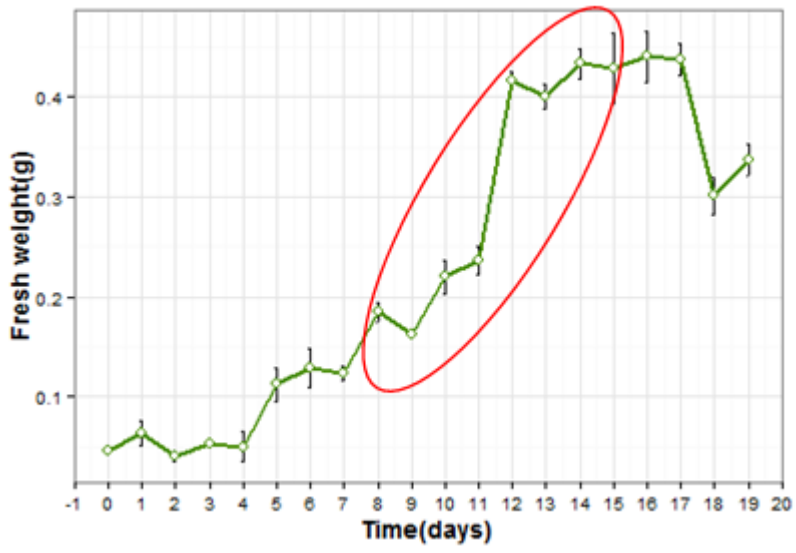

Figure 6 Erratic growth kinetics of the VPN cell cultures by using different type of pipette during the experiment (bars represent standard deviation of the mean of 3 biological replicates)

After eliminating this sampling error, the growth kinetics of the suspension cultures of VPN was monitored by measuring fresh and dry cell weight in mg/l. As shown in Fig 7 the cells are rapidly adapted to the fresh medium after inoculation and displayed a dynamically linear growth up to day 13, followed by a short stationary phase. The cultures started to propagate from time zero until they reached their maximum cell densities approximately 220 mg/l before dropping. After 15 days of culture period, the fresh and dry weight showed a decline in growth indicating that the number of viable cells was gradually decreased due to the exhausting of nutrients in the culture media. The ratio of FW and DW is an index of cell water content. Growth kinetics of the cell culture can be used as a tool to determine the concentration of cells in suspension cultures. These factors are important to determine the optimal amount of cell culture to use in metabolite extraction.

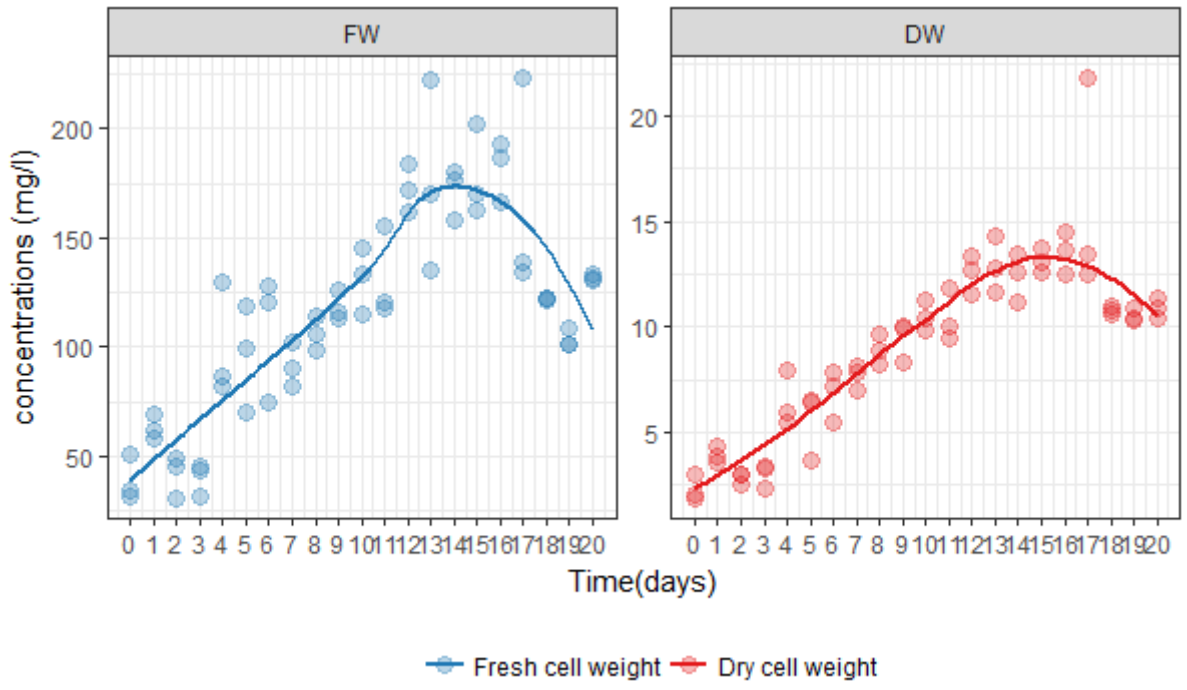

Figure 7 Growth kinetics of the VPN cell cultures

2.3.1.2. Determination of optimal initial sample weight and extraction solvents

Before the time-course experiment was performed, the seven days old VPN cell cultures were analysed to investigate the minimal weight needed for targeted metabolomics analysis. This parameter is of great importance to minimize the effects of the sampling on the cell culture dynamics. In this preliminary experiment, 100 mg and 300 mg of fresh frozen samples and 30 mg and 50 mg of freeze-dried samples were used with two extraction solvent mixtures. The results indicated that the concentration of total polyphenols were not different among fresh frozen and freeze dried samples by extraction with cold methanol. Nonetheless, a significantly higher total polyphenol concentration was found in freeze-dried samples extracted by chloroform/methanol/water mixture. Total polyphenols in the fresh frozen samples were not different between the two extraction methods, regardless of the initial sample weight (Fig 8). It was decided that 100 mg fresh frozen samples could be the minimal initial weight of cell cultures to be able to extract metabolites and allow multiple sample collections from the same culture flask.

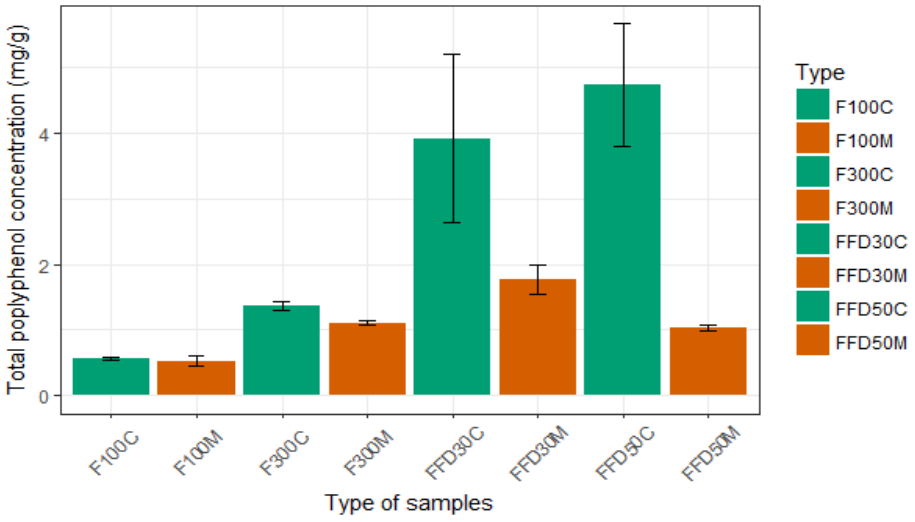

Figure 8 Total polyphenol concentrations in VPN cell cultures with different initial sample weight and extraction methods
F100C- 100 mg fresh frozen cells extracted by chloroform/methanol/water
F100M- 100 mg fresh frozen cells extracted by cold absolute methanol
F300C- 300 mg fresh frozen cells extracted by chloroform/methanol/water
F300M- 300 mg fresh frozen cells extracted by cold absolute methanol
FFD30C- 30 mg freeze-dried cells extracted by chloroform/methanol/water
FFD30M- 30 mg freeze-dried cells extracted by cold absolute methanol
FFD50C- 50 mg freeze-dried cells extracted by chloroform/methanol/water
FFD50M- 50 mg freeze-dried cells extracted by cold absolute methanol

To evaluate the effects of the two extraction methods, 100 mg of fresh frozen cells of *VPN* were collected at 12 time points (between day 0 to day 14). Each sample consisted of three biological replicates. The UPLC-MS/MS based targeted metabolomics analysis was already proven suitable to analyse polyphenols in grape berries (Vrhovsek et al. 2012). Using this optimized protocol of chromatography and mass spectrometric detection, 16 (poly)phenolic compounds in VPN cell suspension cultures could be identified. To obtain the first overview of the analysis of all detected phenolic acids, stilbenes, flavonols and flavan-3-ols in the grape cells, heat maps are created for all 16 UPLC-MS/MS metabolite profiles from 12 time points (Fig 9). The overall profiles of polyphenols appeared to be higher in the ternary mixture of solvent chloroform/methanol/water than in absolute methanol. In terms of absolute concentrations, the concentration of total phenols during the 15 days of growing period ranged between 0.13 mg/g and 3.95 mg/g with the mixture containing chloroform. In the same period, the extraction with absolute methanol yielded only 0.06-0.87 mg/g.

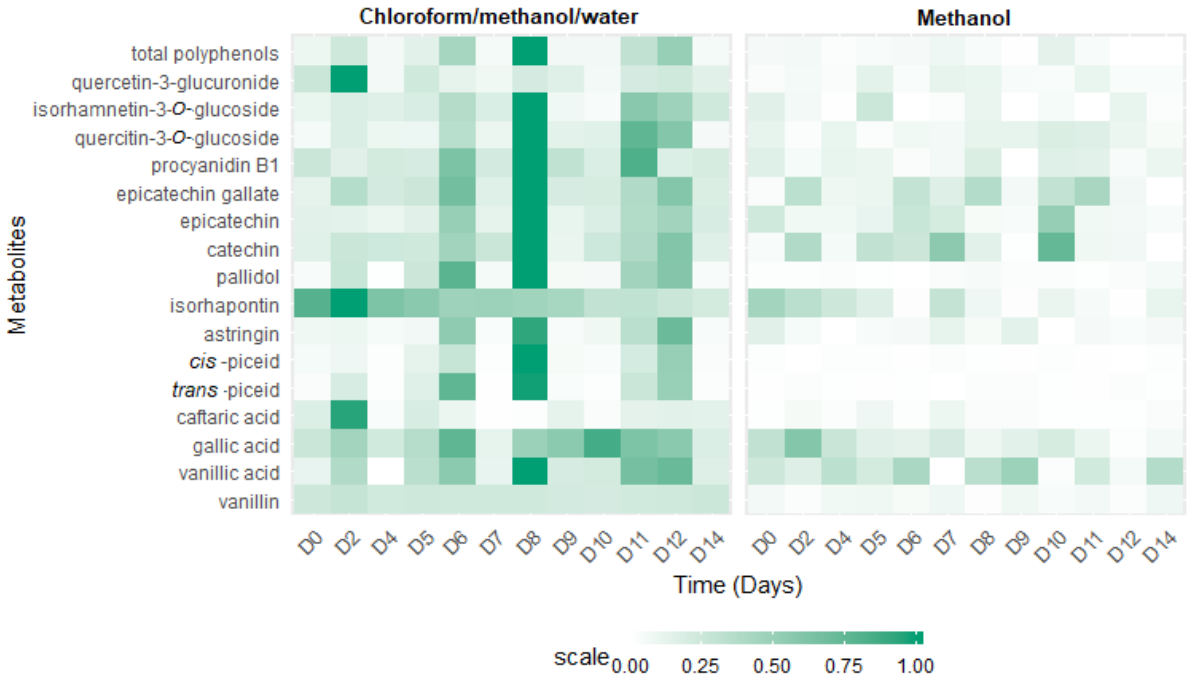

Figure 9 Heatmap visualising the concentrations (in logarithmic scale) of the metabolites in the VPN cell suspension cultures by two extraction methods.

To get an immediate overview of the patterns in the data, and to see whether these patterns are associated with extraction methods, PCA was performed. PCA biplot (Fig 10) revealed obvious clustering patterns of two extraction methods for grape cells, suggesting good analytical resolution that can distinguish different extracted metabolic profiles in the cells. The variables related to these two extraction methods are shown as loadings in the biplot. The samples extracted with chloroform method appears to be separated due to the high concentrations of isorhapontin, flavonoids such as quercetin 3-*O*-glucuronide, phenylpropanoid derivatives such as caftaric acid as well as vanillin.

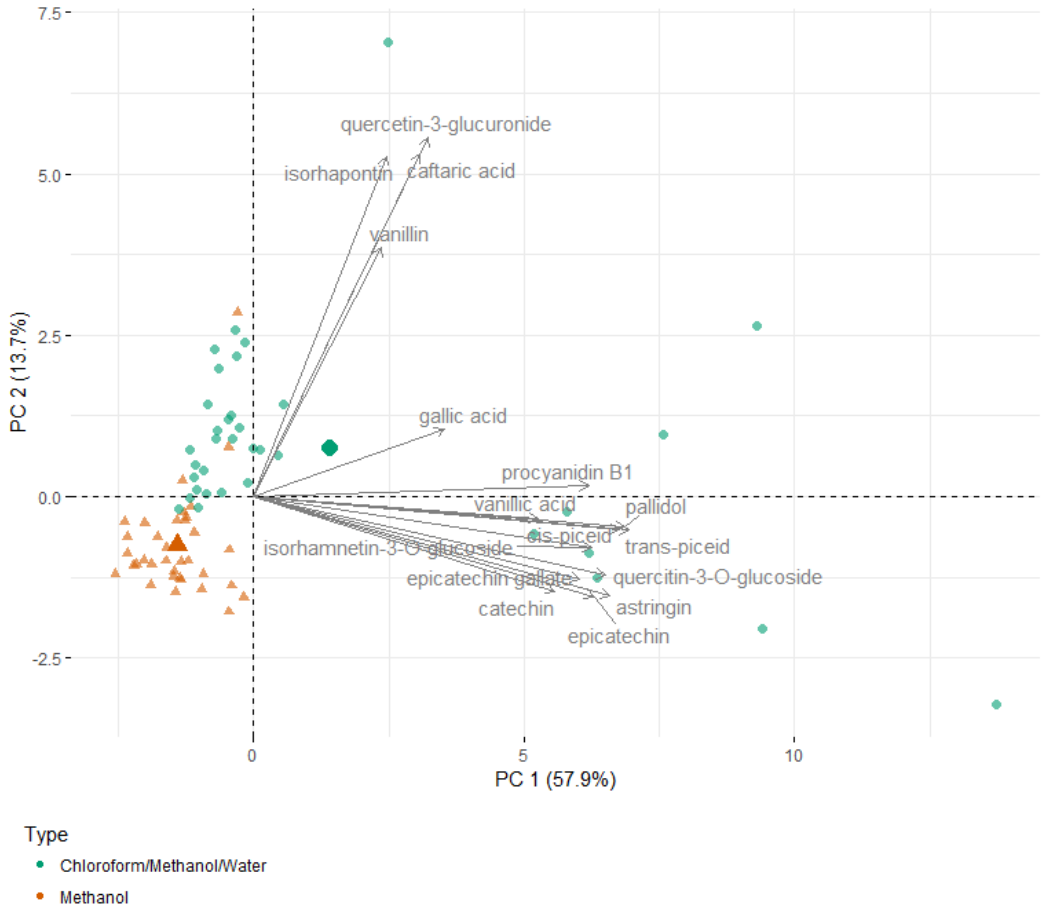

Figure 10 PCA biplot shows the projection of the samples from two extraction solvents in the PC1 and PC2 plane. The loadings show the contribution of the different metabolites. The same color shows the extraction methods.

A more clear view of the yield of phenolic compounds determined by UPLC-MS/MS in the extracts is presented in Fig 11. The graphical presentation clearly indicates the differences between two solvents used, which is also supported by a statistical analysis (two samples t-test at $p < 0.05$). Chloroform/methanol/water mixture gave much higher yields of phenolic compounds than pure methanol, and this difference can mainly be attributed to the higher quantities of stilbene compounds for all time points.

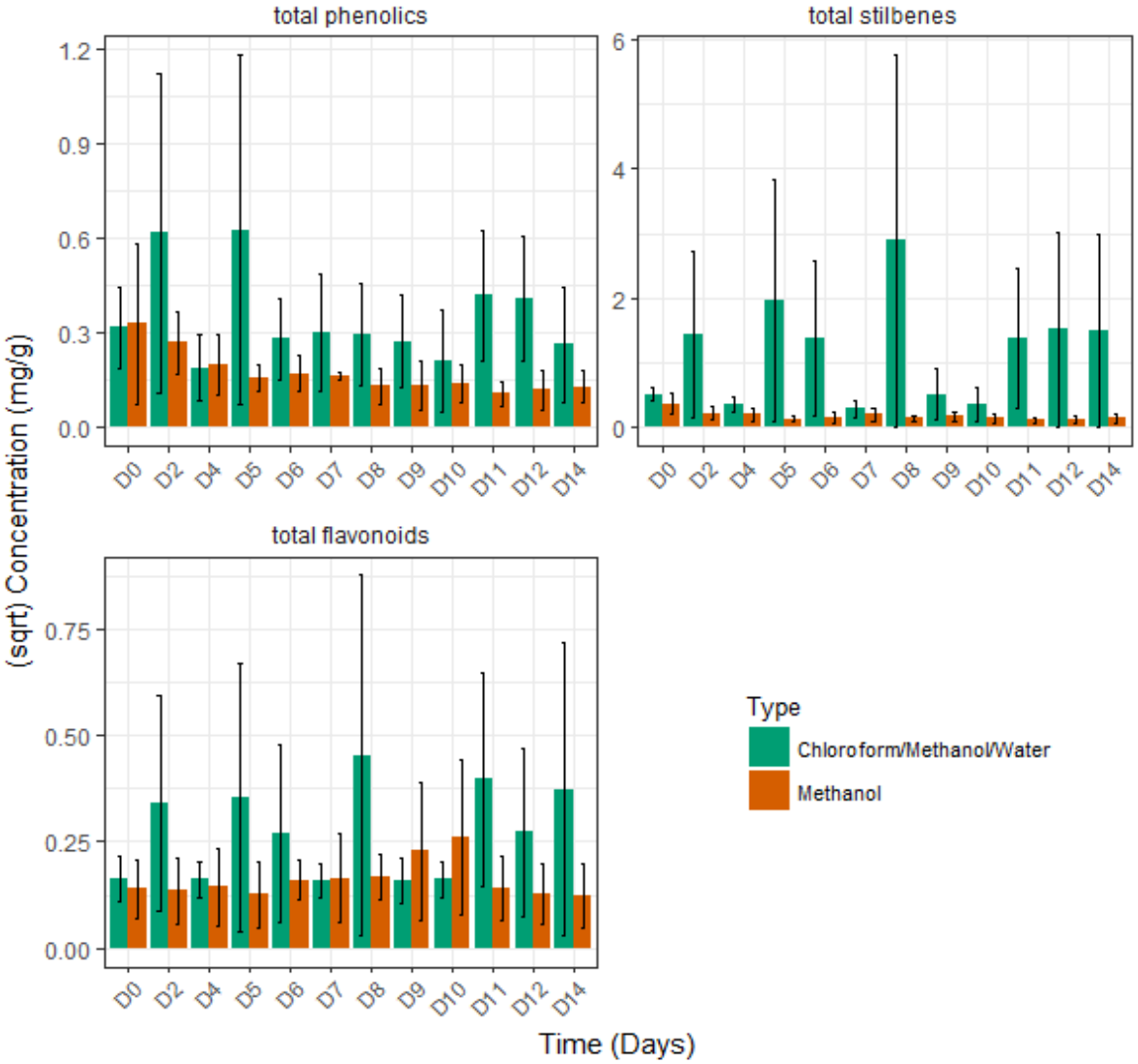

Figure 11 Total content (in square-root scale) of polyphenols in VPN cell cultures (expressed as mg/g fresh cells) in the extracts of chloroform/methanol/water and methanol.

Aiming to evaluate the repeatability of the sample preparation procedure and measurements, the relative standard deviation (RSD) of the concentration of metabolites were measured. These values are determined using the same biological samples for the two extraction methods. The comparison of the distribution of the RSD between the extraction mixtures is presented as histograms in Fig 12. The compounds extracted by methanol were detected in lower and varying amounts; most of them are lower than the limit of quantification (LOQ). The majority of the metabolites extracted by methanol displayed a RSD of the area between 50-100%. The metabolites extracted with chloroform/methanol/water mixture have lower RSD than those extracted with methanol, suggesting that samples with chloroform/methanol/water mixture gave the more precise metabolite abundance.

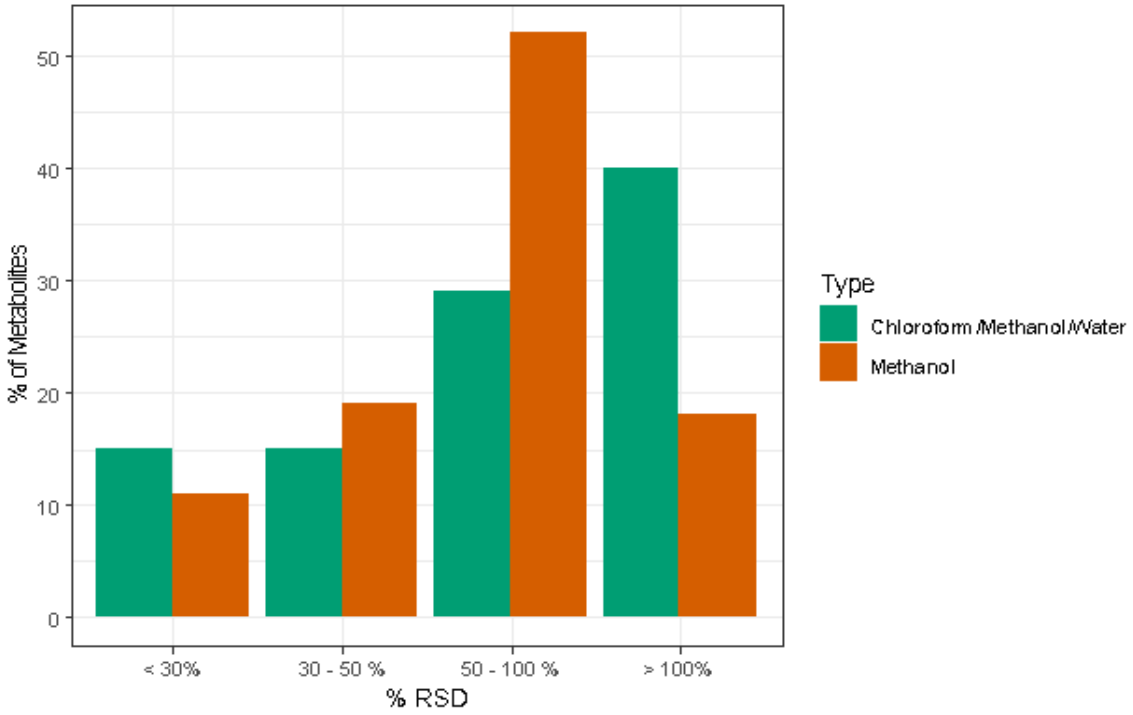

Figure 12 Histograms of relative standard deviations (RSDs) of metabolite abundancy for two extraction methods.

2.3.2. Comparative analysis of secondary metabolites in *V. vinifera* cv. Pinot Noir and *V. vinifera* cv. Gamay cell cultures

The next objective of the study was to model the time dependent behaviour of polyphenols in the grape cells during the growing period. In the previous section, the sample collection method, initial sample weight and solvent mixture for polyphenol extraction was optimized. However, the RSD plots and total polyphenol content plot (Figure 11) indicated high variability in the extractions even with the chloroform extraction. The main reason for this suboptimal behavior was that most of the detected metabolites were under (or very close to) their LOQ due to the fact that their content in VPN cell cultures was very low. In order to solve this problem, keeping the experimental and sampling conditions unchanged was to choose another grape variety known to produce a higher amount of metabolites in liquid cell culture. Therefore a comparative analysis of polyphenol production in VPN and VGR cell cultures was performed to assess if this second cell line was more suitable to develop a modelling strategy for the analysis of time course changes of secondary metabolites and their responses to external stimuli.

Seven days old cell cultures from the two grape cell lines were used in order to determine and compare their polyphenol content and significant differences were observed between them. Fig 13 presents the results related to the contents of polyphenols present both in VPN and VGR cell cultures. Anthocyanins were only detected in VGR cells that displayed the highest content regarding both total and individual compounds. When comparing the average contents of each polyphenol in studied materials, it was obvious that remarkably more of these compounds were recorded in VGR cells vs. those from VPN cells.

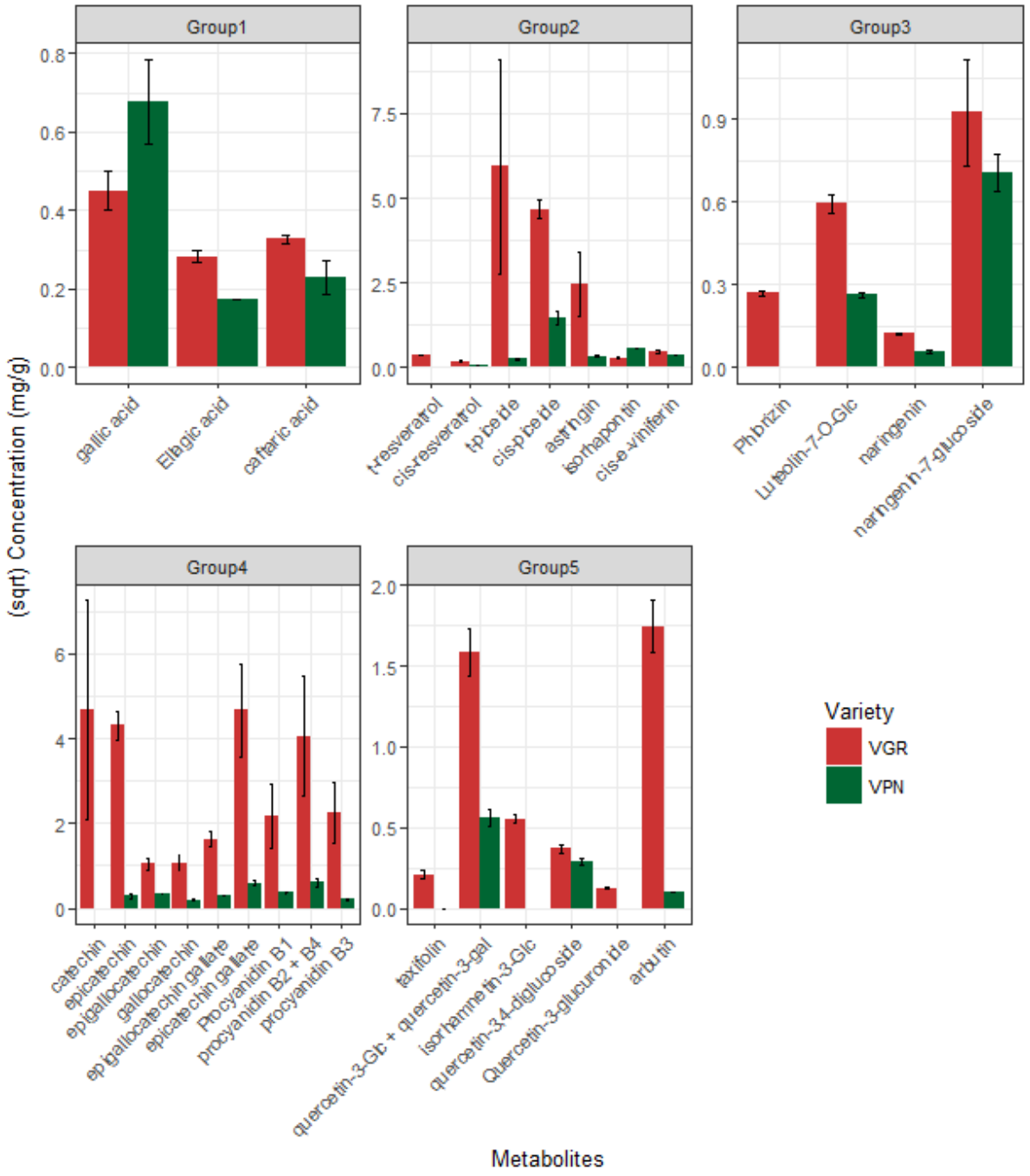

Figure 13 Concentration (in square-root scale) of polyphenols (expressed as mg/g fresh cells) present in VPN and VGR cell cultures.

In relation to the benzoic acid derivatives, VGR cells showed a higher content than VPN cells which are 0.44 in gallic acid and 2.67 folds in ellagic acid. Caftaric acid is the only phenylpropanoid identified in both cell lines which is double in VGR cells than VPN. A significant higher content of stilbene compounds was observed in VGR compared to VPN cell cultures. Among 10 stilbenes, *trans*-resveratrol, *trans*-ε-viniferin and pallidol were not detected and the others showed lower concentration in VPN cells. Phloridzin is the only dihydrochalcone found in VGR cells. Besides catechin, taxifolin, isorhamnetin-3-*O*-glucoside and quercetin-3-*O*-glucoside, which were not detected in VPN cells, it was observed that VGR cells possess a significantly higher amount of flavones, flavanones, flavan-3-ols and flavonols, respectively. One hydroquinone compound, arbutin, was identified in all samples with high contrasting amount in VGR and VPN cells. Hence, data provided by the present work showed strong differences between two cell lines and favoured to use the VGR cell cultures in future experiments.

2.4. Discussion

In metabolomics experiments, sample preparation plays an important role as the choice of respective strategies are fundamental and have important consequences for the accuracy of results (Teahan et al. 2006). In this chapter, different parameters to be considered in sample preparation procedures were explored and an optimized protocol for targeted metabolomics analysis of grape cell cultures was developed. First of all, the cell growth was determined to optimize the growing conditions of the cells, to minimize the possible sources of contamination and variability, to assess and optimize the method of sample collection. The density of cells in the suspension cultures should be enough to allow multiple collections from the same flask cultures and volume of suspension cultures to grow in that flask. The less or higher density of cells in the suspension culture can be harmful to the growth and the production of secondary metabolites. To eliminate the effect of multiple sample collection from the cell cultures, the initial sample weight should be minimum, but the biomass to extract should be sufficient to allow a reliable quantification of the metabolites in the cells. To determine minimum initial sample weight, 100 mg, 300 mg of fresh frozen samples and 30 mg and 50 mg of freeze-dried samples were extracted by two different solvent mixtures. Although higher total polyphenol content was present in freeze-dried samples, in practical, the harvesting of such an amount of biomass from the cell suspension was impossible. That is why 100 mg of fresh frozen samples which could be similar to 10 mg of freeze-dried samples according to the ratio of FW and DW was the optimal initial sample weight for metabolomics analysis.

Another point to consider in time-course experiments is the time of sample collection (of the day) since the levels of plant metabolites vary throughout the day. It is a very important factor to be aware in time series experiments to observe the metabolic changes over time. H. K. Kim and Verpoorte (2010) described that the content of primary and secondary metabolites in *Cannabis sativa* varied between the samples harvested in the morning and afternoon, and that this could be especially observed in the chloroform fraction (extracted with the mixture of chloroform/methanol/water 2:1:1). Although the metabolite changes of plant cell cultures in

different harvesting time has not been described elsewhere, we have taken into account this fact in our experiment in order to reduce the possible confounding effect of these phenomenon. After determining the optimal sample collection procedure, time-course analysis of targeted metabolites in the VPN cell cultures was performed to compare the effect of two different solvent mixtures. The extraction solvents can be selected based on their physicochemical properties such as polarity and selectivity, and their toxicity and inertness (H. K. Kim and Verpoorte 2010). The extraction is generally performed by the disruption of cell walls and subsequent distribution of metabolites into polar (methanol, water) and non-polar (chloroform, hexane, ethyl acetate) solvents followed by the removal of the cellular residue. In our experiment, we have compared two solvents (mixture of chloroform/methanol/water and cold absolute methanol) for the extraction of polyphenols from VPN cell cultures. According to D.-O. Kim and Lee (2002), polyphenols are more soluble in organic solvents such as methanol, ethanol and acetone which are less polar than water. Cold methanol extraction solvent is used to simultaneously quench and extract metabolites which is quick and recovers a large variety of metabolites. It has been widely used for extraction of intracellular metabolites of animal cells (Dettmer et al. 2011), bacteria (Maharjan and Ferenci 2003) and yeast cells (Villas-Bôas et al. 2005). Tatli, Nikerel, and Özdemir (2015) described that methanol extraction of *Brachypodium distachyon* generally yielded higher detectable metabolite levels than methanol/chloroform extraction. Unlikely to these reports, we have observed that the levels of metabolites in VPN cell cultures extracted by cold methanol method were showing critically low levels. Stalikas (2007) also reported that gallic acid cannot be fully extracted in pure organic solvents and other authors (Yilmaz and Toledo 2006, Spigno, Tramelli, and De Faveri 2007) showed that moderate amounts of water mixed with organic solvents are highly effective for extracting polyphenols in *V. vinifera* seeds and skins. Water increases polarity of these mixtures and yields a broader extraction range (Zhang et al. 2007). The highest recovery of phenolics from grapes has been obtained with ethanol/water and acetone/water (Vatai, Škerget, and Knez 2009). On the other hand, it was reported that methanol/water and acetone/water provided a higher yield of grape seed proanthocyanidins (Sun and Spranger 2005). Polyphenols in grapes are highly polar and

hydrophilic and thus methanol/water mixtures facilitate the extraction of polyphenols because the hydroxyl groups of both protic solvents in the mixture may participate as hydrogen bond-disrupting agents (Izquierdo-Hernández et al. 2016). Our results indicated that methanol/chloroform/water mixture may result better fractionation in metabolites with high level of concentrations. For this reason, methanol/chloroform/water mixture was chosen as the mixture for extraction of polyphenols from the cell cultures with minimum initial sample weight. However, it is important to remark that also with this mixture of solvents the RSD for VPN cells suggest that the overall biomass extracted was to low to obtain reliable results.

After the sample preparation method has been optimized, the polyphenol content in VPN and VGR cell cultures were compared. VGR cell cultures showed a wider range of polyphenol classes with higher concentration than those in VPN cell cultures. According to these results, VGR cell cultures will be used in next experiments of metabolomics study.

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Chapter 3

Time-course targeted metabolomics analysis of *V. vinifera* cv. Gamay cell suspension cultures in response to methyl jasmonate elicitation

Part of the work reported in this chapter is published in Saw *et al* "Applying generalized additive models to unravel dynamic changes in anthocyanin biosynthesis in methyl jasmonate elicited grapevine (*Vitis vinifera* cv. Gamay) cell cultures" in *Horticulture Research* **4**: 17038 (2017), doi:10.1038/hortres.2017.38

3.1. Introduction

Plant cell cultures are model systems for the study of plant biosynthetic pathways and to aid the investigation of which factors influence metabolism. Unlike whole organisms, they can be grown under controlled and reproducible conditions and this makes them ideal to collect robust datasets and to develop new methods of data analysis.

The treatment of plant cell cultures with biotic or abiotic elicitors often induces the production of secondary metabolites, which under natural conditions are synthesized in response to pathogen attack or environmental stimuli (Delaunois et al. 2014). A variety of molecules can act as elicitor (Boller 1995), among them, JA and MeJA own a place of merit. Both are fatty acid derived plant hormones that occur ubiquitously in the plant kingdom and act as regulators of defence responses and other plant processes. They trigger a signal transduction chain, which in turn activates multiple secondary biosynthetic pathways (M. I. R. Khan et al. 2012). When exogenously applied to plant cell cultures in the concentration range from 10 to 200 µM, MeJA can lead to an increased production of secondary metabolites including alkaloids, volatile terpenes and anthocyanins (Uppalapati et al. 2005, Rischer et al. 2006 and Wasternack and Hause 2013). Anthocyanins are a class of phenylpropanoids responsible for the red, bluish or purple colour of grapes and thus of wine. They are widely studied for their antioxidant properties, free radical scavenging potential and for their possible suppression of proliferation of human cancer cells (Xia et al. 2010). All known grape anthocyanins derive from five main anthocyanidin skeletons, cyanidin (Cy), delphinidin (Dp), peonidin (Pn), petunidin (Pt) and malvidin (Mv), and might undergo further chemical modifications such as glycosylation or acylation (Mazza and Francis 1995). Several studies aiming to optimize the *in vitro* production of anthocyanins in *V. vinifera* cell cultures used MeJA as elicitor (Belhadj et al. 2008 and Qu, Zhang, and Yu 2011) and considerable progress has been made in the study of the anthocyanin biosynthetic pathway in grapevine (Belhadj et al. 2008). As far as non-anthocyanin polyphenols are concerned, a significant research effort has been dedicated to the effects of MeJA on stilbenes in different grape cell cultures of diverse genotypes/cultivars of *V. vinifera* such as cv. Gamay Fréaux var. Teinturier (Krisa et al. 1999), Barbera (Tassoni et al. 2005, Laura et al. 2007 and Tassoni,

Durante, and Ferri 2012), Monastrell albino calli (Lijavetzky et al. 2008), Cabernet Sauvignon (Faurie, Cluzet, and Mérillon 2009 and Xu, Zhan, and Huang 2015), rootstock 41B (*V. vinifera* cv. Chasselas × *V. berlandieri*) (Donnez et al. 2011), Italia (Santamaria et al. 2011), Alphonse Lavallee (Santamaria et al. 2012) and Negramaro (Taurino et al. 2015).

Although all these studies focused on the enhanced production of polyphenols in grape cells, a deep knowledge of the time-dependent production of the individual polyphenols in response to MeJA elicitation is still lacking. The recent advances in analytical technologies, however, make it now possible to use high throughput metabolomics to investigate the cellular metabolic response over time. Therefore, in this study targeted metabolomics was used to obtain a detailed metabolic characterization over twenty days of MeJA elicited Gamay cell cultures. To fully capture the whole information within this type of data, a new approach was implemented using GAM (S. Wood 2006). This study illustrates how GAM can be applied to the modeling of targeted metabolomic data in order to address the following questions:

- Are there specific trends in the synthesis of anthocyanins and non-anthocyanin polyphenols over time?
- Is the addition of MeJA responsible for a change in the production of these polyphenols?
- Is MeJA affecting the synthesis of anthocyanin derivatives differently?

3.2. Materials and methods

3.2.1. Plant cell cultures

The cell suspension line of *V. vinifera* cv. Gamay Fréaux (VGR) was prepared as described in section 2.2.1.

3.2.2. MeJA treatment of the cell cultures

For the elicitation experiment, seven-day old cells were transferred to the 1L Erlenmeyer flasks containing fresh B5 medium with the dilution of 1:3 and the total volume for each flask was 450 ml. The cultures were grown under the same conditions as described in Section 2.2.1 (b). MeJA (Sigma, Italy) was dissolved in 100% EtOH and added at 100 µM final concentration to the 5-days old cultures. Control cultures were given the same volume of a blank solution of ethanol to disentangle the effect of MeJA from the one of ethanol; in all flasks ethanol concentration did not exceed 0.05% (v/v). Triplicate biological replicates were prepared for both control and elicited samples. For the time course effect of MeJA, the cells were harvested every day for a culture period of 20 days from the same flasks.

3.2.3. Determination of total anthocyanin concentration

For determining anthocyanin accumulation, 10 ml of cell suspension cultures from each flask were harvested daily, filtered by vacuum filtration through filter paper and weighed to obtain cell fresh weight. All samples were flash frozen by liquid nitrogen and stored at -20°C. Anthocyanins were extracted from 100 mg fresh cells. To each sample, 750 µL of 79% (v/v) ethanol with 1% (v/v) glacial acetic acid (extraction solvent) was added, and the samples were incubated in a heat block at 85°C for 20 min. After centrifugation at 13000 rpm for 5 min, the supernatants were collected, and the pellets were re-extracted with 600 µl of extraction solvent twice. Supernatants were combined, and 50 µL of 37% (v/v) hydrochloric acid was added to stabilise the anthocyanins. After 10-min incubation in the dark at room temperature, the sample was diluted 1:1 (v/v) with the extraction solvent. Total anthocyanin content was determined by measuring absorbance at 535 nm using ε = 98.2 (dilution factor = 2) (Mewis et al. 2011). For compound

identification, the extracts were analysed by UPLC-MS/MS. To minimize possible analytical artifacts, all the samples were extracted and analyzed at the end of the experiment in a single batch.

3.2.4. UPLC analysis and identification and quantification (MS/MS) of anthocyanin compounds

Analytical separation of anthocyanin compounds was performed in an Acquity Ultra Performance Liquid Chromatography (UPLC) system (Waters, UK). The system was coupled to a Waters Xevo TQ MS (Milford, MA, USA) equipped with an electrospray (ESI) source. All samples were analysed on a reverse phase Acquity UPLC BEH C18, 1.7 µm, 2.1 × 150 mm column (Waters), protected with an Acquity UPLC BEH C18, 1.7 µm, 2.1 × 5 mm precolumn (Waters) at 40°C and under mobile phase flow rate of 0.4 mL/min. Water was used as weak eluting solvent (A) and methanol as strong eluting solvent (B); formic acid 5% v/v was used as additive in both eluents. The multistep linear gradient used was as follows: from 95 to 60% A for the first 4 min, from 60 to 45% A from 4 to 9 min, from 45 to 5% A from 9 to 11 min, and an isocratic hold for 3 min to clean the column. The equilibration time was 4 min, and the injection volume was 2µl. A quality control standard mixture was injected periodically. All the anthocyanin compounds were detected by multiple reaction monitoring (MRM) by screening the MS/MS transitions and using the parameters earlier optimized for grape wine (Arapitsas et al. 2012). For quantification, external calibration curves were prepared by injecting authentic standards of each compound at different concentrations.

3.2.5. Extraction of polyphenols (non-anthocyanins)

The extraction of non-anthocyanin polyphenols in VGR cell cultures was performed as described in section 2.2.3.

3.2.6. UPLC analysis, identification and quantification (MS/MS) of polyphenols (non-anthocyanins)

UPLC analysis, identification and quantification of non-anthocyanin polyphenols were performed as described in section 2.2.4.

3.2.7. Data analysis and statistical modelling

All statistical analyses were performed using the R statistical environment (R Core Team 2015), and “ggplot2” package was used for visualization (Hadley 2015).

PCA was performed on mean-centred and scaled data. Pearson correlation was used to assess the similarity between the accumulation patterns of different anthocyanins and the results were visualized using the “corrplot” package (Wei and Simko 2016). GAMs were fitted by using the “mgcv” package (S. N. Wood and Wood 2017) and with a log link with the Gamma family by using the restricted maximum likelihood (REML) algorithm because REML estimates are more nearly unbiased (Pinheiro and Bates 2006) when using small sample sizes. We applied log transformation to stabilize the variance of concentration data. All models were inspected for the presence of heteroscedasticity in the residuals by using the diagnostic plots provided by mgcv. The diagnostic plots are included in the Appendix.

To highlight the differences in the time courses induced by the treatment, in all cases the time dependent curves were modelled as a common smoother accounting for the “general” time trend, plus a second smoother fitted only for the MeJA treated cultures. An additional treatment factor was used to account for possible constant shifts.

The structure of such type of GAM is the following:

$C_{Polyphenol}(t) = Treatment + s_{cm}(t) + s_{MeJA}(t) + \varepsilon$

Where,

- $C_{Polyphenol}(t)$ is concentration of polyphenols over time,
- $s_{cm}(t)$ is a smoother that accounts for a common trend in the concentration of polyphenols over time, both in control and treated cell cultures,

- $s_{MeJA}(t)$ is a smoother that accounts for the “difference” in trend for the samples which are treated with MeJA,
- ε is the error.

This type of model is optimal to highlight the effect of the treatment on the concentration of a metabolite, because the specific effect of the elicitation will be captured by the $s_{MeJA}(t)$ term. Nonetheless, it is not the most favourable to compare the trends of two metabolites. To do that the same approach was used to model the *ratios* between the measured concentrations.

3.3. Results

3.3.1. Effect of methyl jasmonate on the anthocyanin biosynthesis in the cell cultures

3.3.1.1. Anthocyanin profile of the grapevine cell cultures

A total of fifteen anthocyanins were identified in all the samples of VGR cells by LC-MS/MS. All anthocyanins were present as their 3-*O* monoglucosides: delphinidin (Dp-glu), malvidin (Mv-glu), petunidin (Pt-glu), cyanidin (Cy-glu) and peonidin (Pn-glu), as the corresponding acetyl derivatives (Dp-ac-glu, Mv-ac-glu, Pt-ac-glu, Cy-ac-glu, Pn-ac-glu, respectively), and as coumaroyl derivatives (Dp-pc-glu, Mv-pc-glu, Pt-pc-glu, Cy-pc-glu, Pn-pc-glu, respectively). An example of a typical chromatogram of the principal anthocyanins in the VGR cell cultures is shown in Fig 14 and their structures can be seen in Fig 15.

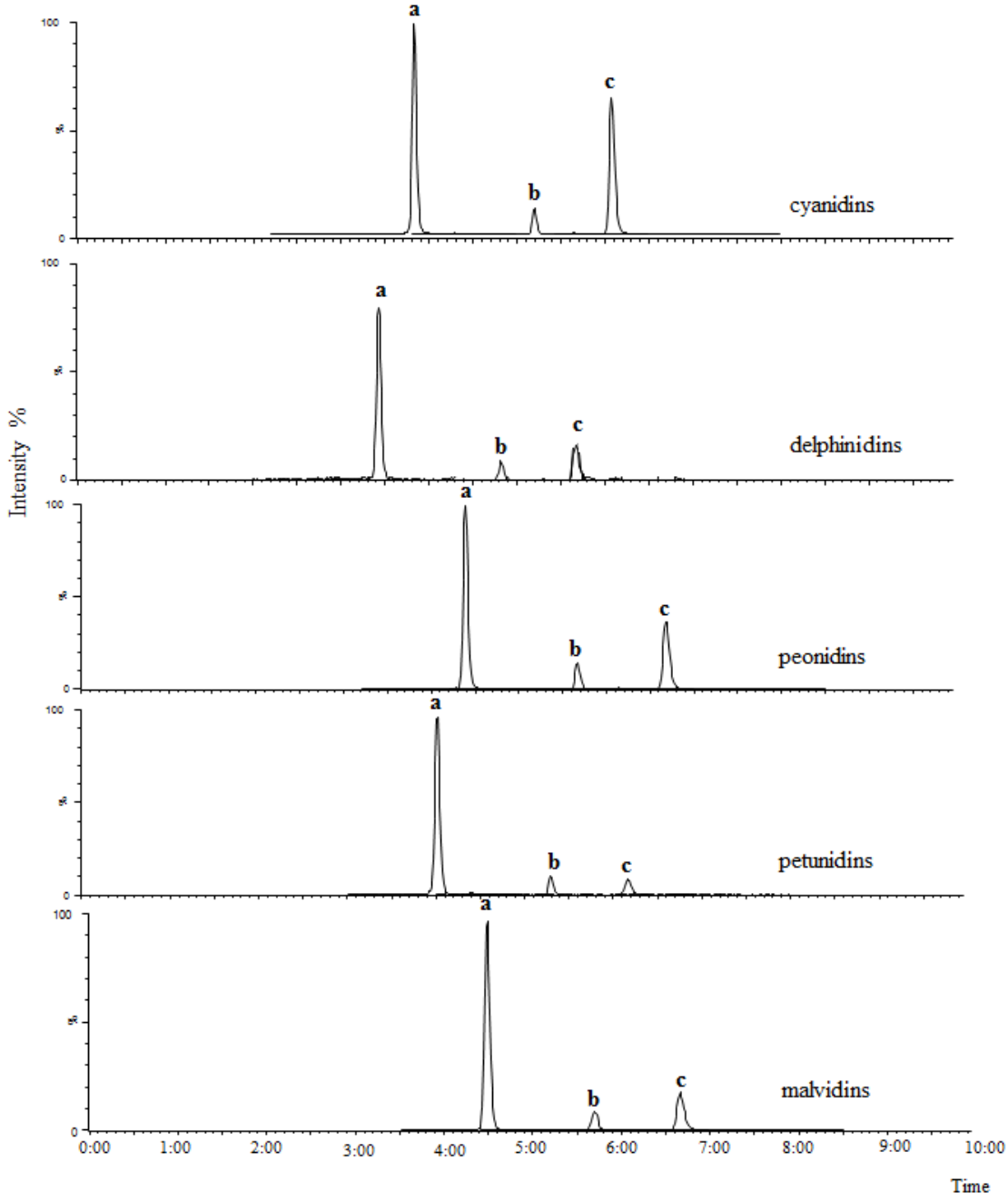

Figure 14 UPLC/QqQ-MS/MS chromatograms of anthocyanins; monoglucosides (a), acetyl glucosides (b) and coumaroyl glucosides (c) obtained from grape cells extract.

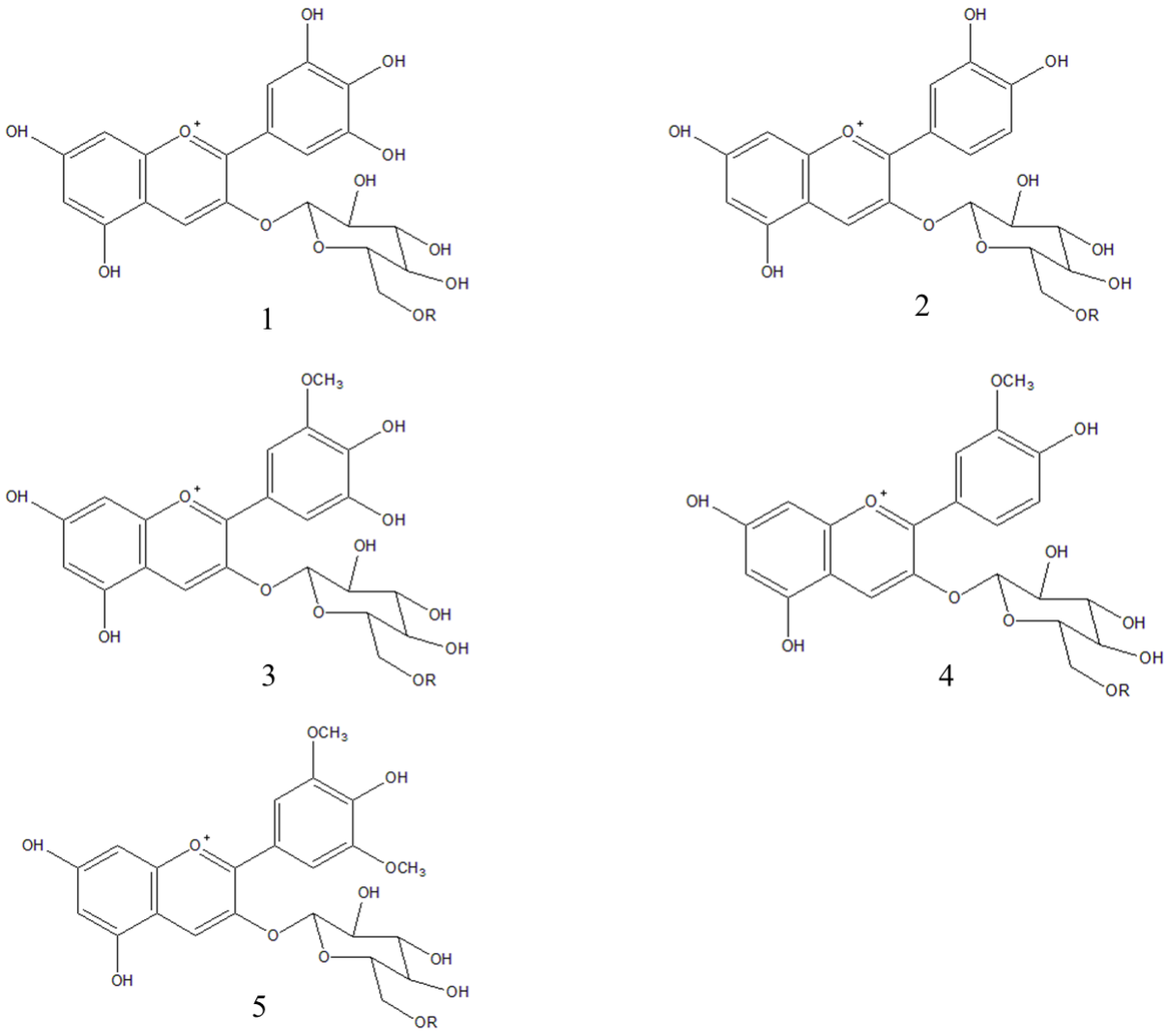

Figure 15 Principal anthocyanins in VGR cell suspension cultures
R: H, acetyl, or *p*-coumaroyl;
(1) delphinidin 3-*O*-glucoside,
(2) cyanidin 3-*O*-glucoside,
(3) petunidin 3-*O*-glucoside,
(4) peonidin 3-*O*-glucoside,
(5) malvidin 3-*O*-glucoside

In terms of relative concentrations, Pn-glu and its derivatives were the dominant anthocyanin type in all samples, accounting for 46 – 62% of the total in the control and 43 – 68% in the MeJA treated cultures. The second most abundant pigment is Cy-glu and its derivatives with 35 – 52% and 35 – 53% respectively, which is typical for suspension cultures of this grape variety. The other three anthocyanins, Dp-glu, Pt-glu, Mv-glu and their derivatives, each ranged from 0.4 to 7% in all the samples. The *p*-coumaroyl derivatives represented around 44 – 80% while non-acylated anthocyanins were 18 – 50% of all anthocyanins. The contribution of the different classes of pigments varied over the growing period. Cy based anthocyanins (Cy and Pn) were dominant at the beginning of the culture (lag phase) while Dp based anthocyanins (Dp, Mv, Pt) accumulated rapidly from the beginning of the exponential growth phase in both control and MeJA treated cell cultures Fig 16.

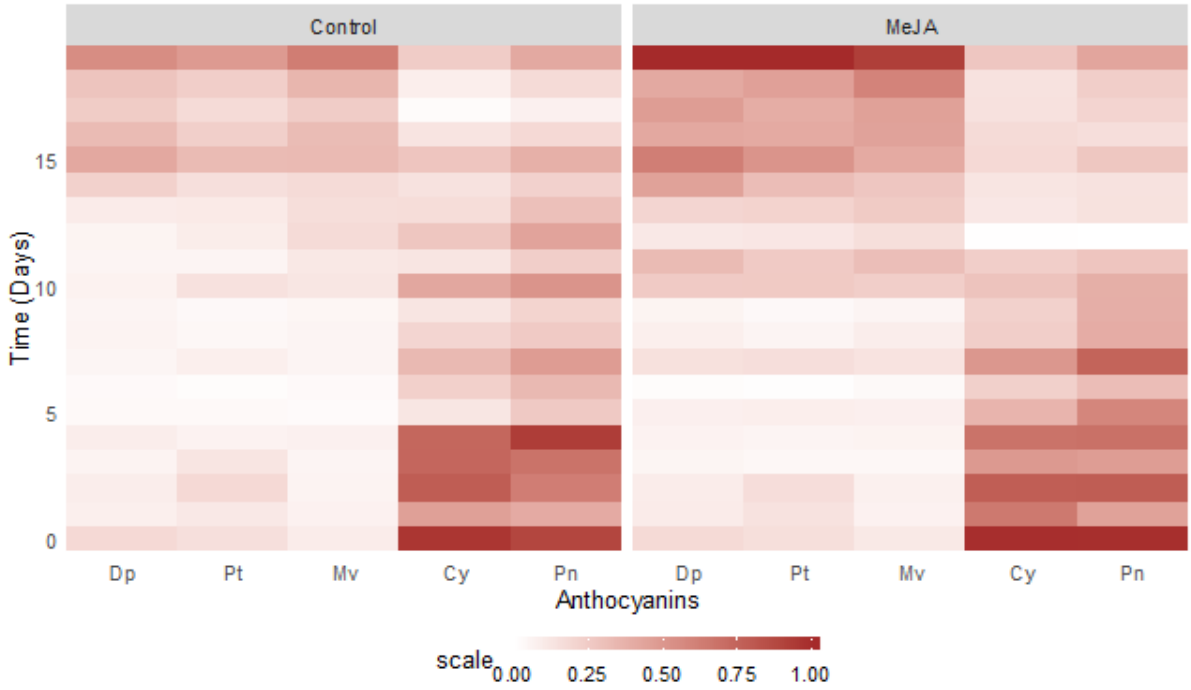

Figure 16 Anthocyanin composition in percentage of total anthocyanins of VGR cell suspensions (for control and for MeJA treatment). Dp: Delphinidins, Pt: Petunidins, Mv: Malvidins, Cy: Cyanidins, Ph: Peonidins

3.3.1.2. Time course changes of anthocyanin biosynthesis and MeJA treatment

PCA was performed to get a global overview of the effects of MeJA on the anthocyanin profile. PCA biplot (Fig 17) shows the projection of the data on the lane formed by the first two PC scores, accounting for 81% of the total variance and the distribution of individual anthocyanins. It does not show a clear separation between treatment and control at the earlier stages. However, the treated samples start to diverge after elicitation, indicating that the effect of elicitation on the anthocyanin biosynthesis could be appreciated only after few days from the treatment. PC1 is the direction characterizing time evolution, which then accounts for the bigger part of the total variability.

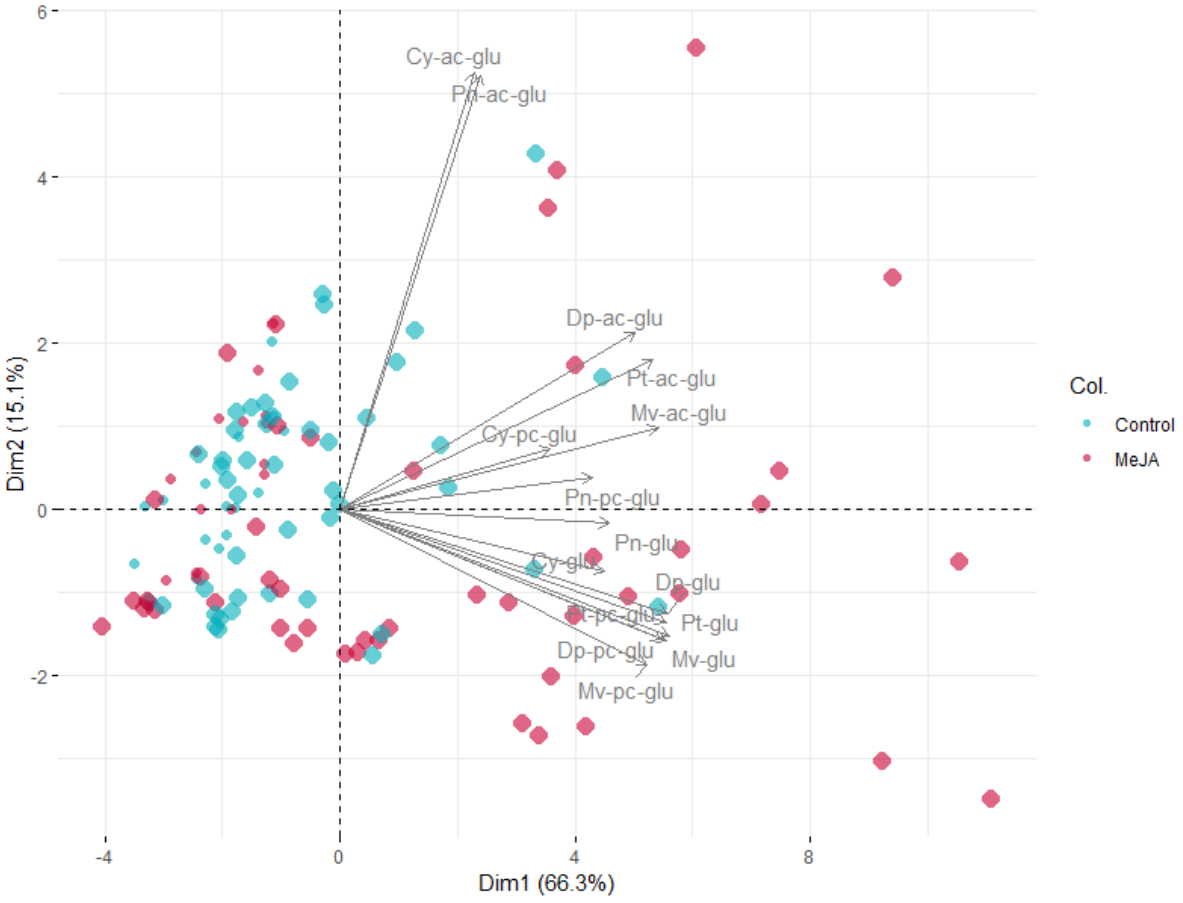

Figure 17 PCA biplot on the autoscaled data showing the projection of the anthocyanin data set in the PC1 × PC2 plane. The smaller points indicate the anthocyanin concentration before elicitation and the bigger points indicate those after elicitation in control and MeJA treated cell cultures.

Pairwise correlation analysis was performed to compare the trends of the fifteen anthocyanins and to identify the ones to be included into statistical modelling. Pearson correlation coefficients for anthocyanins in control cell cultures are shown in Fig 18**.** Dp-glu shows a strong and highly significant positive correlation with Mv-glu, Pt-glu and their acetyl and coumaroyl derivatives, in agreement with the fact that Dp is the precursor of Pt and Mv (Holton and Cornish 1995). In both cases, monoglucosides were positively correlated with their acetyl and *p*-coumaroyl glucosides within the pathway too. Cy and Pn seem to be independent from Dp. Cy, as a precursor of Pn, was strongly correlated with Pn-glu and the same is true for their acetyl and *p*-coumaroyl derivatives. Remarkably, no strong correlations were found between monoglucosides and their respective acetyl and *p*-coumaroyl glucosides for both Cy and Pn. Correlation analysis shows that anthocyanin production followed two distinct pathways: one typical of Dp and the other typical of Cy. Based on the correlation analysis Dp-glu, Cy-glu and their derivatives were taken as “representative” for the subsequent statistical modeling.

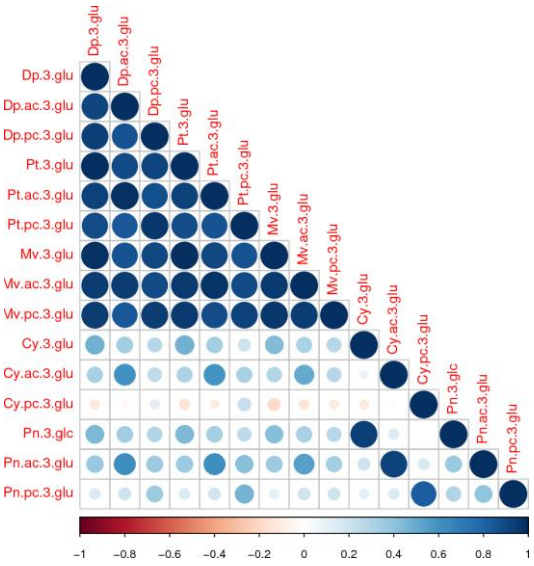

Figure 18 Correlation matrix of all anthocyanins in the control VGR cell cultures. Pearson correlation coefficient (positive or negative) between pairs of anthocyanins is represented by virtual colour as indicated in the colour key. Positive strong correlations stand out in dark blue. Lighter colours indicate weaker relations between the metabolites.

The model showing the effect of MeJA treatment on the total anthocyanin synthesis is depicted in Fig 19-A. The common trend line (edf=3.293, P<0.0001) indicates a continuous increase of total anthocyanin content in the cells during the first 7 days of growth, followed by a stable anthocyanin concentration. The bottom model component shows instead the “additional” effect of MeJA. The two cultures start to diverge after day 9 and the difference in concentration stabilizes around day 13. A slight decrease in the anthocyanin concentration seems to be present between day 5 and day 9.

The same modeling approach was applied to investigate the Dp and Cy behaviour, by considering the production of Dp-glu, Cy-glu, and their ratios (Fig 19-B and 19-C). The plots indicate that during the culture period, the relative contribution of the two metabolites evolves. The upward trend of the common smoother (edf=4.793, F=61.58, p< 0.001) in Fig 19-B indicates an increase in the concentration of Dp-glu at later stages, as confirmed by the trend for the two metabolites displayed in Fig 19-C. The global smoother accounting for the production of Dp-glu in the cells shows an upward trend after 10 days (edf=3.701, F=26.42, p < 0.0001) and consequently, the maximum accumulation of Dp-glu was found at the later growth phase (17-19 days). However, the situation for Cy-glu is different and the global trend (edf=1.08, F=4.24, p=0.0284) is indeed barely significant. Considering that the *p*-values calculated for GAMs are only approximate, the model does not clearly indicate a significant change on the overall concentration for this metabolite. These results show the different accumulation patterns of the two anthocyanins and the alteration of their ratio over time, which is mainly dependent on Dp-glu biosynthesis. GAMs clearly indicate that MeJA influenced the biosynthesis of anthocyanin glucosides. In Fig 19-B, the differential smoother of the Dp-glu/Cy-glu ratio shows the highest peak at the stationary phase (between day 10-15) and decreases thereafter (edf=5.355, F= 17.89, p < 0.001). This is likely due to the higher production of Dp-glu in the MeJA treated cells clearly visible in Fig 19-C. The effect of elicitation on this metabolite started soon after elicitation as demonstrated by the differential smoother (edf=4.88, F=11.85, p < 0.0001). The maximum concentration of Dp-glu in the MeJA cell cultures reached 94.74 ±7.11 µg/mg (mean ± sd), and was three times higher than in the control culture at day 20. Considering the other branch of the

pathway, the differential smoother (edf=2.00, F=10.08, p <0.001) of Cy-glu suggests a positive, even if small, effect of MeJA also there. The maximum concentration of Cy-glu was indeed found in MeJA treated cell with 198.81 ± 11.46 (mean ± sd), which is 1.63 times higher than those in the control cultures at day 19.

Moving a step forward in the pathway, we have investigated the effects of growth and elicitation on the production of acetylated and coumaroylated anthocyanins (Fig 19-D and 19-E). In terms of absolute concentrations, acetylated anthocyanins were present in lower amounts in both, control and treated, cell cultures as compared to coumaroylated ones. The average Dp-ac-glu concentration ranged from 0.04 to 1.1 mg/g and 0.02 to 1.85 mg/g fresh cell weight in the control and MeJA treated cells, respectively. The global trend of Dp-ac-glu biosynthesis in the cells follows a significant nonlinear increase over time (edf=3.48, F=3.82, p< 0.001). However, the differential smoother is not significant (edf=3.28, F=0.603, p=0.55) with large 95% confidence interval bands, indicating that MeJA elicitation had no effect on the production of Dp-ac-glu. This result was confirmed for Cy-ac-glu. The concentration of this metabolite is indeed neither changing with time (global, edf=2.973, F=1.625, p=0.161), nor affected by MeJA treatment (differential, edf=2.000, F=0.687, p=0.505). On the contrary, MeJA elicitation had a much greater effect on the coumaroyl derivatives. The differential smoother for Dp-pc-glu biosynthesis, indeed, is significant (edf = 8.42, F=16.07, p < 0.0001) and demonstrates a steep increase of concentration at the exponential growth phase (7-10 days) from 9.14±0.61 mg/g (mean ± sd) to 36.27±0.08 mg/g (mean ± sd). Thereafter, the concentration remained stable (at 10-15 days) showing only a slight decrease at the end of the growth phase. The effect of MeJa on this class of derivatives was confirmed by the models built on Cy-pc-glu. In this case, the global trend (edf= 2.106, F=4.740, p=0.005) showed a descending trend of Cy-pc-glu until day 9 after inoculation and remained nearly unchanged afterwards. During the same period, the differential smoother (edf= 4.752, F=8.167, p<0.001) showed a significant increase starting from the day 10 until the end of the growth curve.

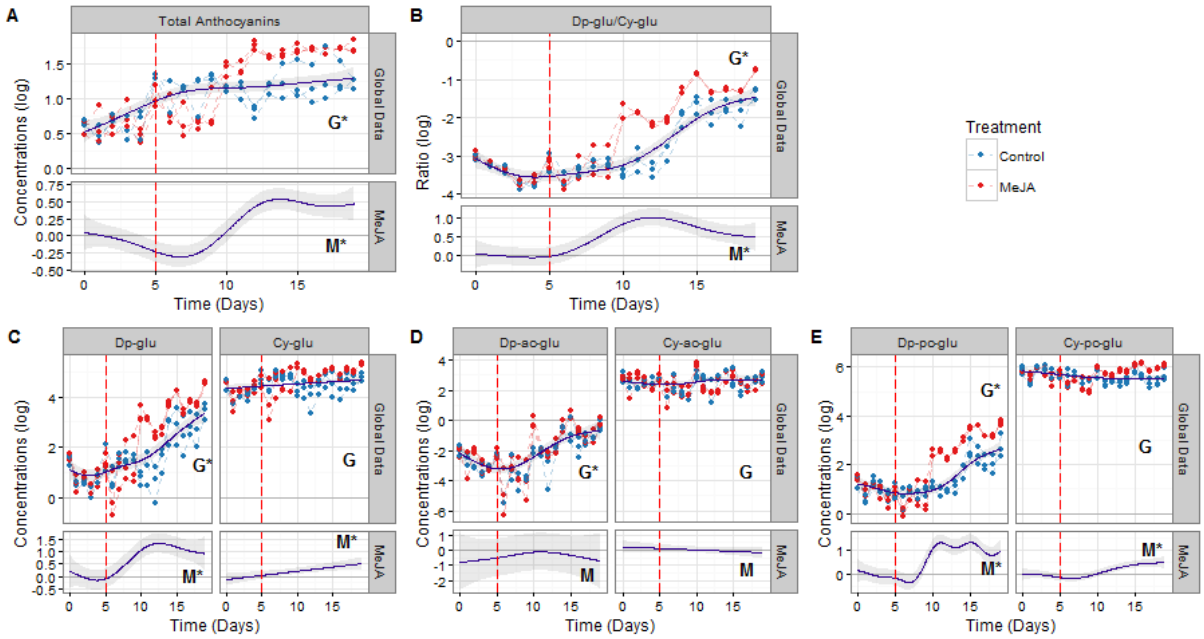

Figure 19 Time course accumulation of anthocyanins in VGR cell suspensions elicited with MeJA : total anthocyanins (A), Dp-glu/Cy-glu ratio (B), total Dp-glu and Cy-glu (C), total Dp-ac-glu and Cy-ac- glu (D), total Dp-pc- glu and Cy-ac- glu (E). In each panel, the upper graph (global data) shows the fitted smooth of the global trend ($s_{cm}(t)$), while the lower graph (MeJA) shows the fitted smooth of the MeJA term ($s_{MeJA}(t)$). The shaded areas of the trends are 95% confidence intervals on the fitted smoothers. The day of MeJA elicitation is indicated by a red dashed line. G = global trend, M = trend of MeJA term, * indicates the significance ($p < 0.01$). The concentrations and ratios of the global data are log transformed.

As before, the effect of MeJA can be highlighted considering the ratios between the different anthocyanin derivatives. The results of this analysis are shown in Fig 20.

For the Dps, the global trend of Dp-ac-glu/Dp-glu remained unchanged all over the growth period (edf=3.327, F=1.837, p=0.1310), but the effect of MeJA was significant (edf =2.000, F=9.372, p=0.0002) (Fig 20-A (Dp-ac-glu/Dp-glu)). On the contrary, the global trend of Dp-pc-glu/Dp-glu (edf=7.065, F=4.917, p<0.001) was significant, speaking of a time dependent effect on this metabolic conversion and of a non-significant effect of the elicitation (edf=2.854, F=0.777, p=0.477) Fig 20-A (Dp-pc-glu/Dp-glu). The general smoother shows that the ratio between Dp-pc-glu and Dp-ac-glu for all the samples was high at the earlier stage of the growth while declining progressively over time (edf=3.184, F=3.515, p=0.01) due to the increased concentration of Dp-ac-glu as described in Fig 19 D (Dp-ac-glu). MeJA treatment led to an increment of Dp-pc-glu/Dp-ac-glu ratio linearly but it did not show a significant change (edf=2.00, F=2.26, p=0.11) Fig 20 A (Dp-pc-glu/Dp-ac-glu).

For the Cy branch we have found similar results. The global smoother for Cy-ac-glu/Cy-glu was not significant (edf=2.954, F=2.298, p=0.09779), while MeJA had a significant negative effect (edf=1.000, F=10.783, p=0.00135) Fig 20-B (Cy-ac-glu/Cy-glu). As in the case of Dp, the global trend of the ratio between Cy-pc-glu and Cy-glu is significantly depending on time (edf=5.153, F=5.4161, p<0.001), but no significant change is induced by the treatment with MeJA as shown by the trend of MeJA elicited samples (edf=2.410, F=0.532, p=0.566) of Fig 20-B (Cy-pc-glu/Cy-glu). Unlike Dps, the ratio between Cy-pc-glu and Cy-ac-glu was shown by the global trend (edf= 1.23, F=2.61, p=0.07) which did not change over time. However, the smoother of MeJA treatment (edf=2.00, F=3.90, p=0.02) showed a significant linear increment along the growing period of the cells (Fig 20 B (Cy-pc-glu/Cy-ac-glu). Our results of the ratio of acylated and non-acylated anthocyanins suggested that MeJA primarily enhances the synthesis of non-acylated glucosides which can lead to an increase in concentration of several anthocyanins.

The anthocyanin profiles in grape cells are also generally characterized in terms of non-methylated (Cy and Dp) and methylated anthocyanins (Pn, Pt and Mv). In this study it was of interest to investigate if the balance between these two classes of pigments is time dependent

and/or can be affected by MeJA. This can be done by modeling the ratio of methylated/non-methylated anthocyanins. The results are presented in Fig 20-C. In this type of modeling, the amount of anthocyanin characterized by a specific “backbone” (Cy, Pn, Dp, Pt and Mv) was calculated by summing up the amounts of their respective derivatives (glu, ac-glu, pc-glu). From these aggregated values it was possible to evaluate the relation among methylated and non-methylated forms. In the case of di-hydroxylated anthocyanins, the global trend (edf=5.727, $F=36.91$, $p<0.001$) shows that the proportion of Pn/Cy increased at the exponential phase and then remained almost unchanged along the stationary phase. The differential smoother (edf=2.00, $F=15.90$, $p<0.001$) instead shows a decreasing trend along the growth. The same analysis performed on the tri-hydroxylated anthocyanins, considering the ratio of ([Pts + Mvs]/Dps), shows less clear results for the global trend (edf=5.365, $F=7.69$, $p<0.001$) but here MeJA favors non-methylated forms in the later growth phase (edf=4.150, $F=6.721$, $p<0.001$) Fig 20-C.

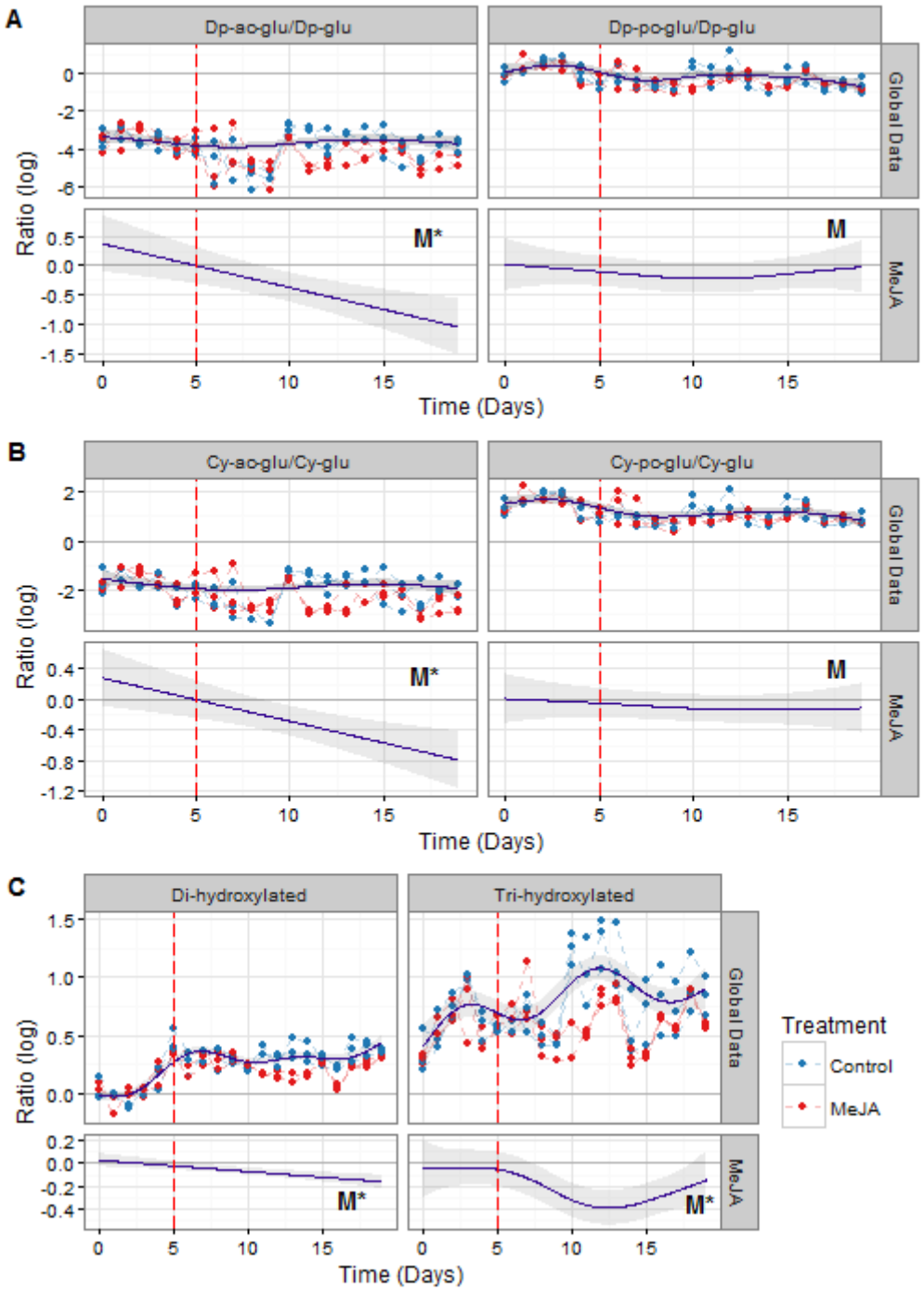

Figure 20 Changes of the ratio of Dp (A) and Cy derivatives (B) and di-hydroxylated (Pns/Cys) and tri-hydroxylated anthocyanins (Pts+Mvs)/Dps (C) in VGR cell suspensions elicited with MeJA. In each figure, panel (global data) shows the fitted smooth for global trend ($s_{cm}(t)$) and panel (MeJA) shows the fitted smooth for MeJA term ($s_{MeJA}(t)$). The shaded areas of the trends are 95% confidence intervals on the fitted smoothers. The day of MeJA elicitation is indicated by red dash line. G = global trend, M = trend of MeJA term, * indicates the significance ($p < 0.001$). The concentrations and ratios of the global data are log transformed.

3.3.2. Effect of methyl jasmonate on the non-anthocyanin polyphenols in the cell cultures

3.3.2.1. General profile of non-anthocyanin polyphenols in grapevine cell cultures

The targeted UPLC-MS/MS method revealed the presence of 39 polyphenolic compounds in VGR cell suspension cultures. They are a total of twelve stilbenes, 19 flavonoids, four benzoic acid derivatives, three phenylpropanoids and one hydroquinone. A first overview of the composition of all detected non-anthocyanin polyphenols in all samples is shown in the form of a heatmap. The class specific concentrations in Fig 21 were obtained by summing the individual compounds belonging to each chemical class at each time point. The metabolic patterns of control and MeJA treated cell cultures are not clearly different, but from the plot it is clear that the biosynthesis of different classes of non-anthocyanin polyphenols vary over growing period. The contribution of stilbenes, benzoic acid derivatives and hydroquinones were dominant at the beginning of the culture period (lag phase) while the accumulation of flavonoids started rapidly from the beginning of the exponential growth phase in both control and MeJA treated cell cultures. In terms of relative concentrations, stilbenes were the most abundant polyphenol in all samples ranging from 24 - 86 % of the total in control and 22 - 86% in MeJA treated cultures. Both in control and MeJA cell cultures, flavonoids followed with 11 - 77% and benzoic acid derivatives represented around 0.8 – 4% while phenylpropanoids were 0.1 – 0.75% of all non-anthocyanin polyphenols. Hydroquinone ranged only in a trace amount of 0.1 - 1% of total in all samples.

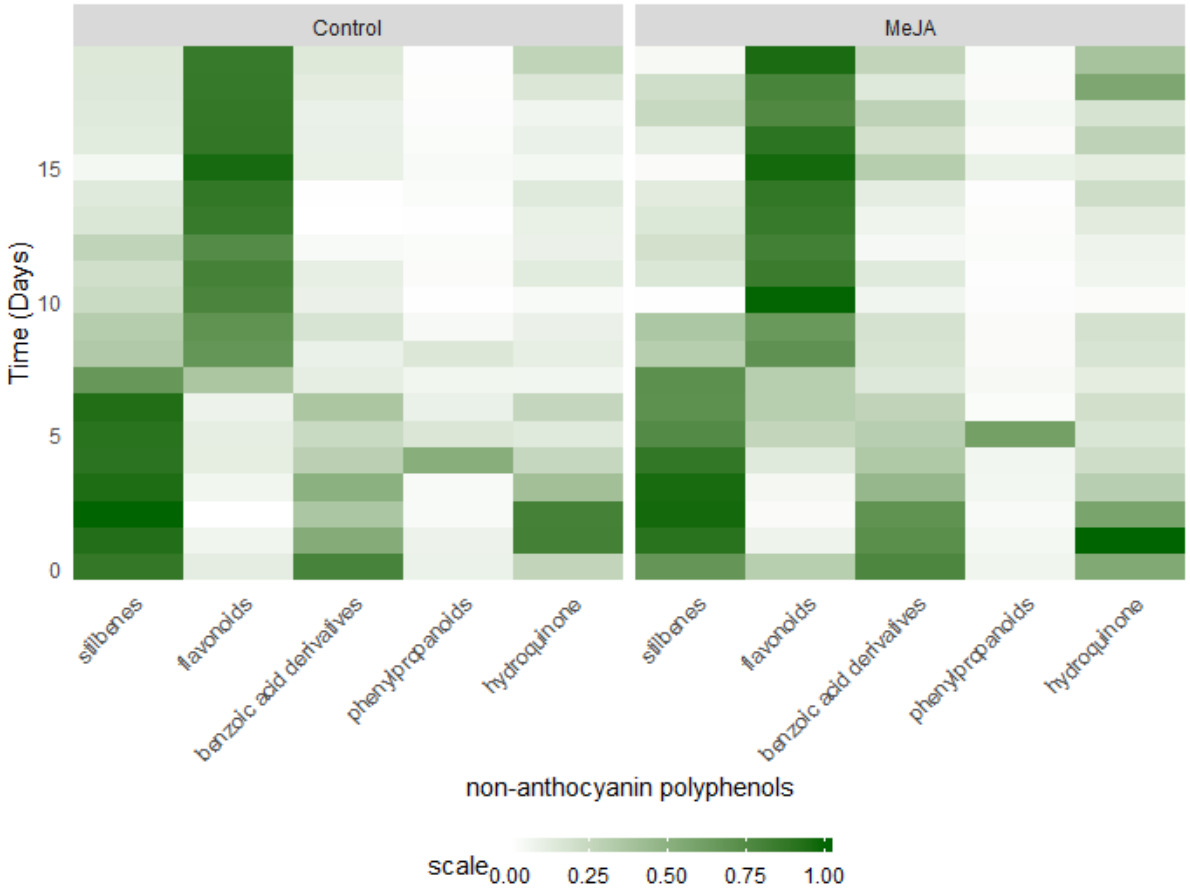

Figure 21 Composition of non-anthocyanin polyphenols in percentage of of VGR cell suspensions (control and MeJA treated) cultures.

PCA was performed to get the overview of the time-course evolution of non-anthocyanin polyphenols, and to analyse variation among the samples by elicitation with MeJA. PCA biplot (Fig 22) shows the projection of the data set on the PC1 × PC2 plane accounting for 59% of the total variance. The evolution over time is highlighted by the size of each time point. PC1 is the direction characterizing time evolution, which then accounts for the bigger part of the total variability. PCA scores plot demonstrated a defined reproducible trajectory of non-anthocyanin polyphenols during the growing period of cell cultures but does not show clearly the alteration of polyphenols by elicitation.

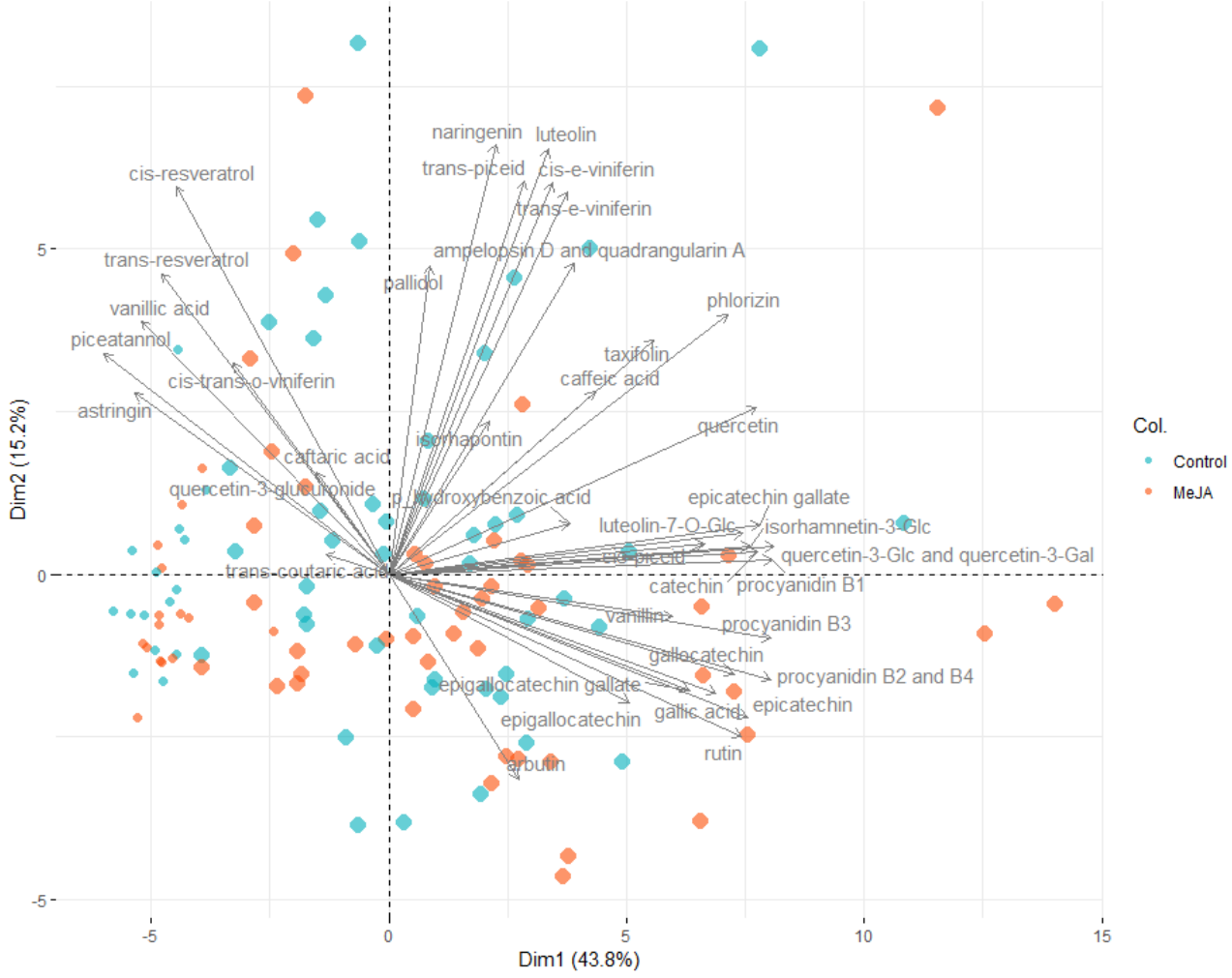

Figure 22 PCA biplot on the autoscaled data showing the projection of the non-anthocyanin data set in the PC1 × PC2 plane. The smaller points indicate the non-anthocyanin polyphenol concentrations before elicitation and the bigger points indicate those after elicitation in control and MeJA treated cell cultures.

3.3.2.2. Time course changes of stilbenes biosynthesis and MeJA treatment

Stilbenes are considered as the most important group of phytoalexins within the class of polyphenols. Metabolites of this group identified and quantified in this analysis include *trans*- and *cis*-resveratrol (3,4'5-trihydroxystilbene), pallidol (a *trans*-resveratrol dimer), piceatannol (trans-3,3', 4,5'-tetrahydroxystilbene), astringin (piceatannol-3-*O*-*β*-D-glucopyranoside), *trans*- and *cis*- glycosylated forms of resveratrol named piceid (resveratrol-3-*O*-*β*-D-glucopyranoide), *trans*- and *cis*-ε-viniferin (*trans*-resveratrol dimers), *trans*- and *cis*-δ-viniferin (resveratrol dehydrodimers, isomers of ε-viniferin), isorhapontin, ampelopsin D and quadrangulanin A, which are resveratrol dimers. Their structures are shown in Fig 23. Among these stilbene compounds, *cis*-piceid was the most abundant in all samples which belong to 20 - 60% of total stilbenes, followed by *trans*-piceid with 15 - 30% in both, control and MeJA treated cultures.

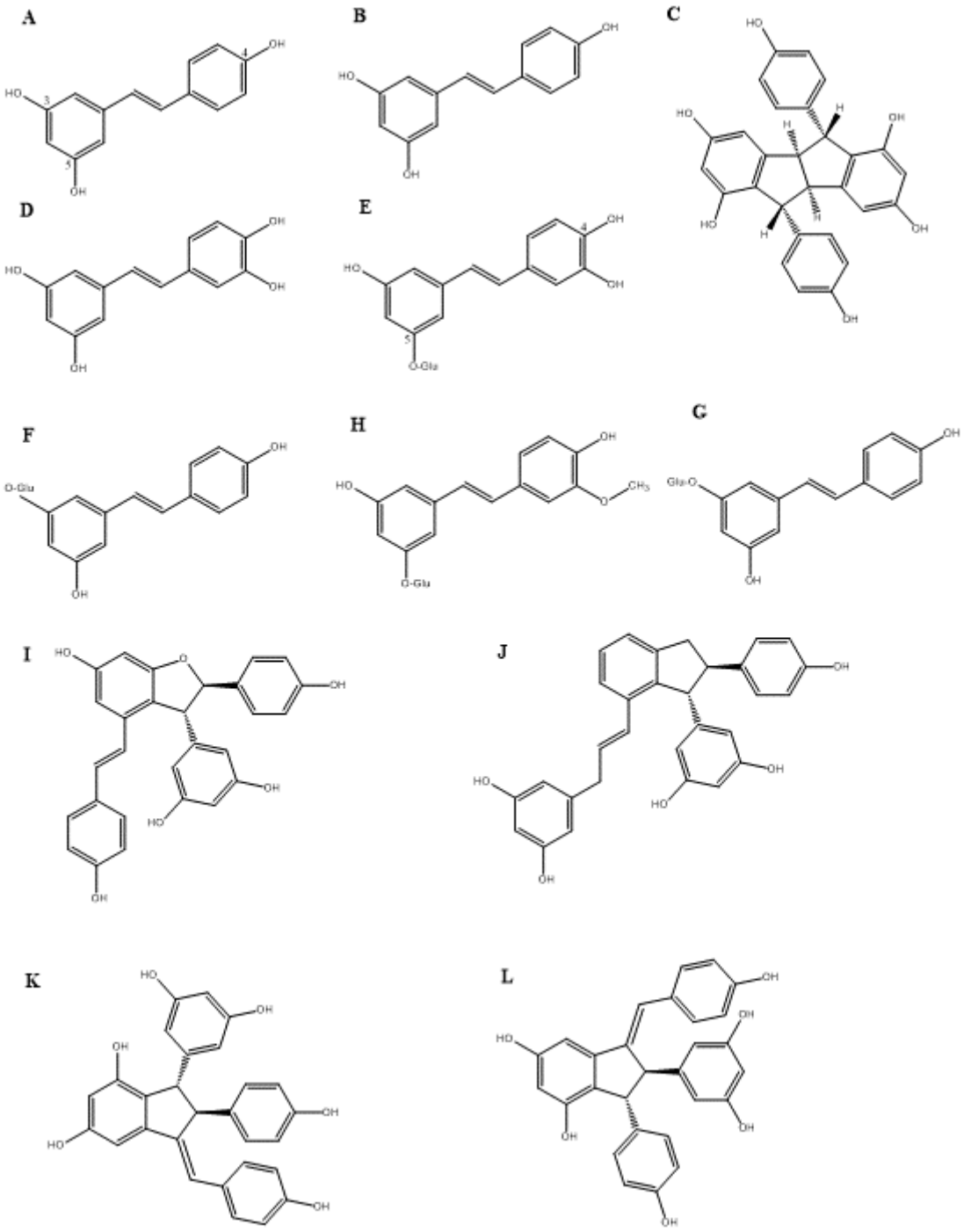

Figure 23 Chemical structures of stilbene compounds in VGR cell suspension cultures; *trans*-resveratrol (A), *cis*-resveratrol (B), pallidol (C), piceatannol (D), astringin (E), *trans*-piceid (F), *cis*-piceid (G), isorhapontin (H), *ε*-viniferin (I), *δ*-viniferin (J), ampelopsin D (K), quadrangularin A (L)

Table 2 Summary statistics of GAMs applied for stilbene compounds in VGR cell cultures

Metabolites	smoother terms	edf	F	p-value
trans-resveratrol	Global	2.483	7.425	0.0001***
	MeJA	2.309	3.307	0.0300*
cis-resveratrol	Global	2.957	18.257	0.0000***
	MeJA	2.029	4.307	0.0142*
piceatannol	Global	4.332	36.04	< 2e-16***
	MeJA	4.896	6.095	0.0000***
trans-piceid	Global	4.081	11.54	0.0000***
	MeJA	3.223	0.78	0.6360
cis-piceid	Global	3.473	12.218	0.0000***
	MeJA	2.634	1.621	0.1910
astringin	Global	4.704	52.144	< 2e-16***
	MeJA	3.57	4.208	0.0028**
isorhapontin	Global	1.001	49.34	0.0000***
	MeJA	4.783	17.21	0.0000***
trans-ε-viniferin	Global	4.01	6.831	0.0000***
	MeJA	2	1.995	0.1410
cis-ε-viniferin	Global	4.86	16.227	0.0000***
	MeJA	2.339	2.497	0.0837
trans- and *cis*-δ-viniferin	Global	3.925	6.016	0.0001***
	MeJA	4.89	10.363	0.0000***
pallidol	Global	2.743	5.757	0.0008***
	MeJA	5.854	11.152	0.0000***
ampelopsin D + quadrangulanin A	Global	3.349	6.935	0.0000***
	MeJA	2	0.12	0.8870

* =p-value < 0.05, ** = p-value < 0.01, *** =p-value < 0.001

GAMs were applied to model their time dependent behaviour during the culture period both in control and MeJA treated cell cultures. The approximate significance of smoothing terms of the stilbene compounds are presented in Table 3.1. Our statistical analysis showed the different accumulation patterns of stilbene compounds over the time course and that, in comparison to what was observed for anthocyanins, the elicitation of MeJA did not modulate the trend of some compounds belonging to this chemical class. GAMs described the significant changes of *trans*- and *cis*-resveratrol over time. The concentration of *trans*-resveratrol in the cells did not change until day 10, but slightly decreased thereafter as shown by the global trend. However, the trend of MeJA indicates that the biosynthesis of *trans*-resveratrol is not affected after the elicitation of MeJA (Fig 24-A). The global trend of *cis*-resveratrol shows the similar behavior as *trans*-resveratrol, but the addition of MeJA built up a slight increment of *cis*-resveratrol after 5 days of elicitation and shows the significance from the global trend (Fig 24-B). The concentration of pallidol steadily increased during the growing period as shown by the global trend. In contrast, MeJA demonstrated a different pattern of pallidol content in the cells. The differential smoother shows a descending trend and a slight decrease in the pallidol concentration seems to be present between day 10 and 15 (Fig 24-K).

The concentration of piceatannol is stabilized only during the first five days of culture and declined gradually until the end of the growth curve as shown by the global trend of Fig 24-C. However, the differential smoother of the piceatannol shows an increment at the stationary phase (between day 10 -15) meaning that of MeJA influenced the biosynthesis of piceatannol (Fig 24-C). The general common smoother of astringin, a glucoside of piceatannol, shows the descending trend after five days of inoculation. The addition of MeJA had a negative effect in astringin biosynthesis which can be seen by the differential trend in Fig 24-F.

Concerning piceid, the time course changes of both, *trans*- and *cis*-piceid, in the cells followed a significant increase along the growth of the cells as shown by the global trend. However, MeJA elicitation had no effect on the production of piceid. It was evident by differential smoothers of both piceid which are not significant with large 95% confidence interval bands (Fig 24-D and E).

The changes of viniferin derivatives in the grape cells can be seen by the global trends of *trans*- and *cis*-ε-viniferin. They show a steep increase of concentration at the exponential growth phase and thereafter the concentration remained stable (at 5-15 days) showing only a slight decrease at the end of the growth phase. However, the non-significant differential smoother indicates that MeJA elicitation had no effect on the production of ε-viniferin (Fig 24-I and H). At odd, the biosynthesis and occurrence of *trans*- and *cis*-δ-viniferin, respectively, was altered by MeJA elicitation. A slight decrease of concentration of this metabolite seems to be present in MeJA treated samples between day 10 and 15, but increased again thereafter. The two cultures start to diverge after day 9 and the difference in concentration can be seen after day 15 (Fig 24-J).

In the case of isorhapontin, the global trend showed a descending trend along the growth of cells. The effect of elicitation on this metabolite started soon after treatments demonstrated by the differential smoother with a significant increase starting from the day 10 until the end of the growth curve (Fig 24-G).

Ampelopsin D and quadrangulanin A were detected in lower and varying amounts in the cells. According to the global trend, the concentrations of these metabolites significantly depend on time but no significant change is induced by the treatment with MeJA as shown by the trend of elicited samples (Fig 24-L).

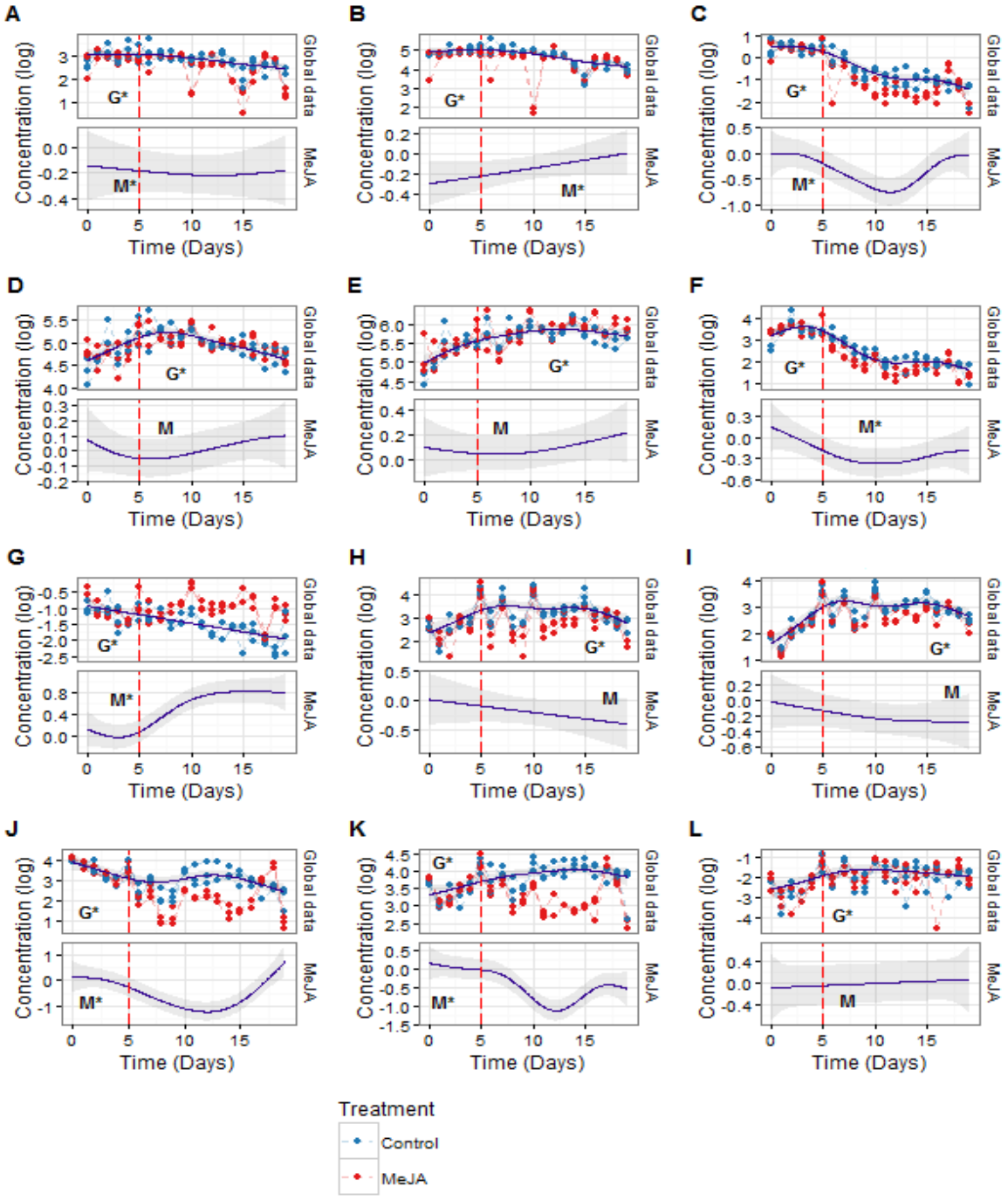

Figure 24 Time course accumulation of stilbenes in VGR cell suspensions elicited with MeJA: *trans*-resveratrol (A), *cis*-resveratrol (B), piceatannol (C), *trans*-piceid (D), *cis*-piceid (E), astringin (F), isorhapontin (G), *cis*-ε-viniferin (H), *trans*-ε-viniferin (I), *trans*- and *cis*-δ-viniferin (J), pallidol (K), ampelopsin D and quadrangulanin A (L). In each panel, the upper graph (global data) shows the fitted smooth of the global trend ($s_{cm}(t)$), while the lower graph (MeJA) shows the fitted smooth of the MeJA term ($s_{MeJA}(t)$). The shaded areas of the trends are 95% confidence intervals on the fitted smoothers. The day of MeJA elicitation is indicated by a red dashed line. G = global trend, M = trend of MeJA term. * indicates the significance ($p < 0.01$). The concentrations of the global data are log transformed.

3.3.2.3. Time course changes of flavonoid biosynthesis and MeJA treatment

Flavonoids and other polyphenolics represent a huge portion of the soluble phenolics occurring in grape cell cultures belonging to the classes of dihydrochalcones, flavones, flavanones, flavan-3-ols and flavonols. The structures of identified metabolites are shown in Fig 25. Our GAM results showed that, in general, the accumulation of these compounds in the cells significantly changed over time, but the addition of MeJA could vary only the trend of some phenolics concentrations in the cells. The approximate significance of smoothing terms of these compounds is presented in Table 3.2.

Table 3 Summary statistics of GAMs applied for flavonoids and other polyphenolic compounds in VGR cell cultures.

Metabolites		smoother terms	edf	F	p-value
Dihydrochalcones	Phloridzin	Global	4.745	15.414	<0.0001 ***
		MeJA	3.267	1.821	0.176
Flavones	Luteolin	Global	5.881	9.684	<0.0001 ***
		MeJA	2.641	1.393	0.258
	Luteolin-7-*O*-glucoside	Global	7.568	12.573	<0.0001 ***
		MeJA	2	0.506	0.604
Flavanones	Naringenin	Global	5.046	9.584	<0.0001 ***
		MeJA	4.049	4.044	0.00225 **
Flavan-3-ols	Catechin	Global	7.837	117.398	<0.0001 ***
		MeJA	2.837	4.405	0.00546 **
	Epicatechin	Global	6.608	94.85	<0.0001 ***
		MeJA	2.003	1.53	0.222
	Epigallocatechin	Global	7.222	17.872	<0.0001 ***
		MeJA	7.168	5.929	<0.0001 ***
	Gallocatechin	Global	6.021	32.15	<0.0001 ***
		MeJA	2.77	1.113	0.345
	Epigallocatechin gallate	Global	4.549	21.542	<0.0001 ***
		MeJA	5.357	3.579	0.00295 **
	Epicatechin gallate	Global	4.75	41.31	<0.0001 ***
		MeJA	2.605	0.94	0.394

	Procyanidin B1	Global	5.79	50.393	<0.0001 ***
		MeJA	2.001	4.737	0.0105 *
	Procyanidin B2+B4	Global	5.103	64.09	<0.0001 ***
		MeJA	2.001	1.215	0.3
	Procyanidin B3	Global	5.305	57.433	<0.0001 ***
		MeJA	2.001	9.486	0.000152 ***
Flavonols	Quercetin	Global	4.519	12.985	1.18e-10 ***
		MeJA	2.085	1.431	0.219
	Quercetin-3-*O*-glucoside and Quercetin-3-*O*-galactoside	Global	4.969	23.324	<2e-16 ***
		MeJA	2.935	1.449	0.272
	Quercetin-3-*O*-glucuronide	Global	4.178	3.643	0.00429 **
		MeJA	7.758	2.396	0.38960
	Quercetin-3-*O*-rutinoside (syn. Rutin)	Global	4.845	42.996	< 2e-16 ***
		MeJA	4.485	3.547	0.00404 **
	Isorhamnetin-3-*O*-glucoside	Global	6.859	22.274	<2e-16 ***
		MeJA	3.014	2.172	0.0849
Dihydroflavonol	Taxifolin (syn. Dihydroquercetin)	Global	6.13	12.733	6.29e-13 ***
		MeJA	2.342	3.189	0.0431*

* =p-value < 0.05, ** = p-value < 0.01 , *** =p-value < 0.001

Phloridzin (Pz) is the only one dihydrochalcone (per definition not a true flavonoid) identified in the control and MeJA treated cell cultures. According to the global trend as shown in Fig 26-A, the concentration of Pz started to increase after five days of inoculation and remained stable during the exponential and stationary phase of growth despite the non-significant differential trend indicated that this metabolite has no response to the elicitation of MeJA in the grape cell cultures (Fig 26-A).

Flavones identified in the grape cell cultures were luteolin and luteolin-7-*O*-glucosides. The global trends of both metabolites make evident their time dependent behavior in the cells. However, their biosynthesis in the cells was not affected by the MeJA (Fig 26 B and C).

NAR is the only flavanone detected in the cell cultures. The global trend presented the raising concentration of NAR along with the growth of the cells and it decreased after ten days of the

culture. In contrast, the elicitation of MeJA declined the concentrations of NAR after elicitation but indicates a continuous increase after day 10 (Fig 26-D).

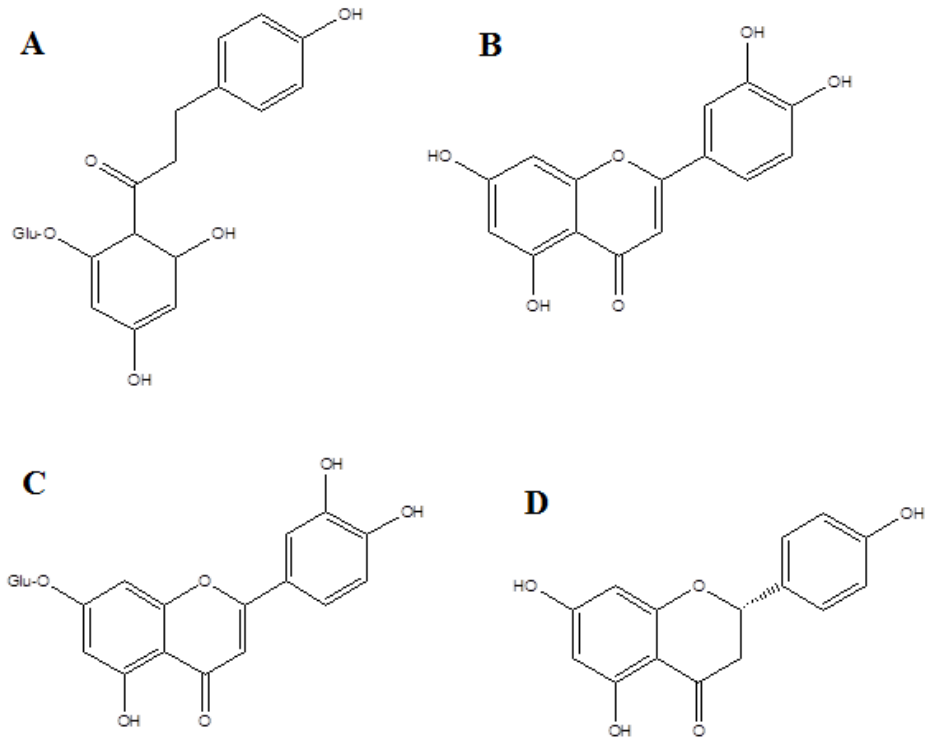

Figure 25 Chemical structures of dihydrochalcones; phloridzin (A), flavones; luteolin (B), luteolin-7-O-glucoside (C) and flavanone; naringenin (D) identified in VGR cell cultures.

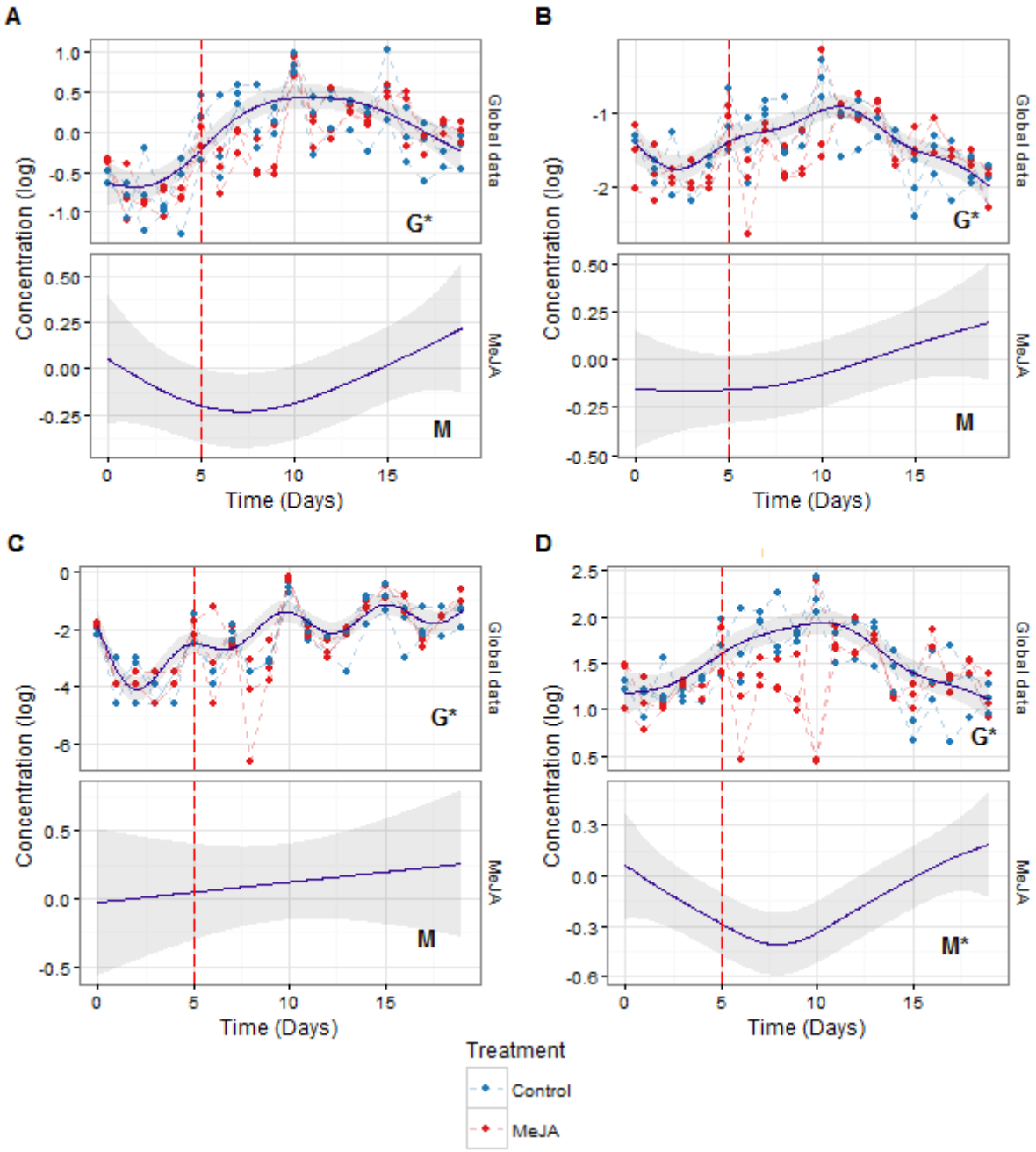

Figure 26 Time course accumulation of phloridzin (A), luteolin (B), luteolin-7-O-glucoside (C) and naringenin (D) in VGR cell suspensions elicited with MeJA. In each panel, the upper graph (global data) shows the fitted smooth of the global trend ($s_{cm}(t)$), while the lower graph (MeJA) shows the fitted smooth of the MeJA term ($s_{MeJA}(t)$). The shaded areas of the trends are 95% confidence intervals on the fitted smoothers. The day of MeJA elicitation is indicated by a red dashed line. G = global trend, M = trend of MeJA term. * indicates the significance ($p < 0.01$). The concentrations of the global data are log transformed.

Regarding flavan-3-ols (Fig 27), their time course changes and the effect of MeJA is shown in Fig 28. The global trends of all these metabolites were significant, indicating a time dependent effect on these metabolic conversions. Nonetheless, no significant change is induced by the treatment with MeJA as shown by the trend, except for epicatechin, gallocatechin, epicatechin gallate and procyanidin B2 and B4. Induction of MeJA has a short-term significant increment on the biosynthesis of epigallocatechin and epigallocatechin gallate with a peak on day 10 of the MeJA treated cultures. For catechin, procyanidin B1 and procyanidin B3, the upward trend of concentrations can be seen by the differential smoothers respectively.

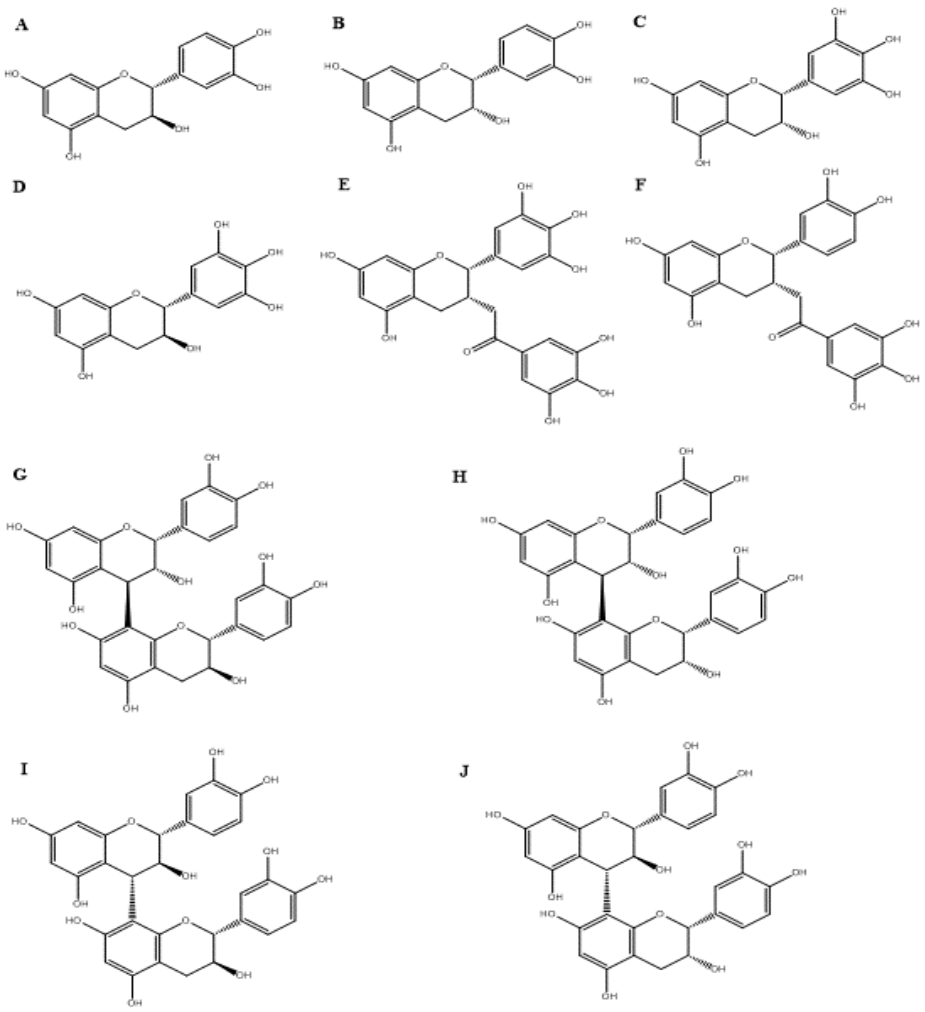

Figure 27 Chemical structures of flavan-3-ols; catechin (A), epicatechin (B), epigallocatechin (C), gallocatechin (D), epigallocatechin gallate (E) and epicatechin gallate (F), procyanidin B1 (G), procyanidin B2 (H), procyanidin B3 (I) and procyanidin B4 (J) identified in VGR cell cultures.

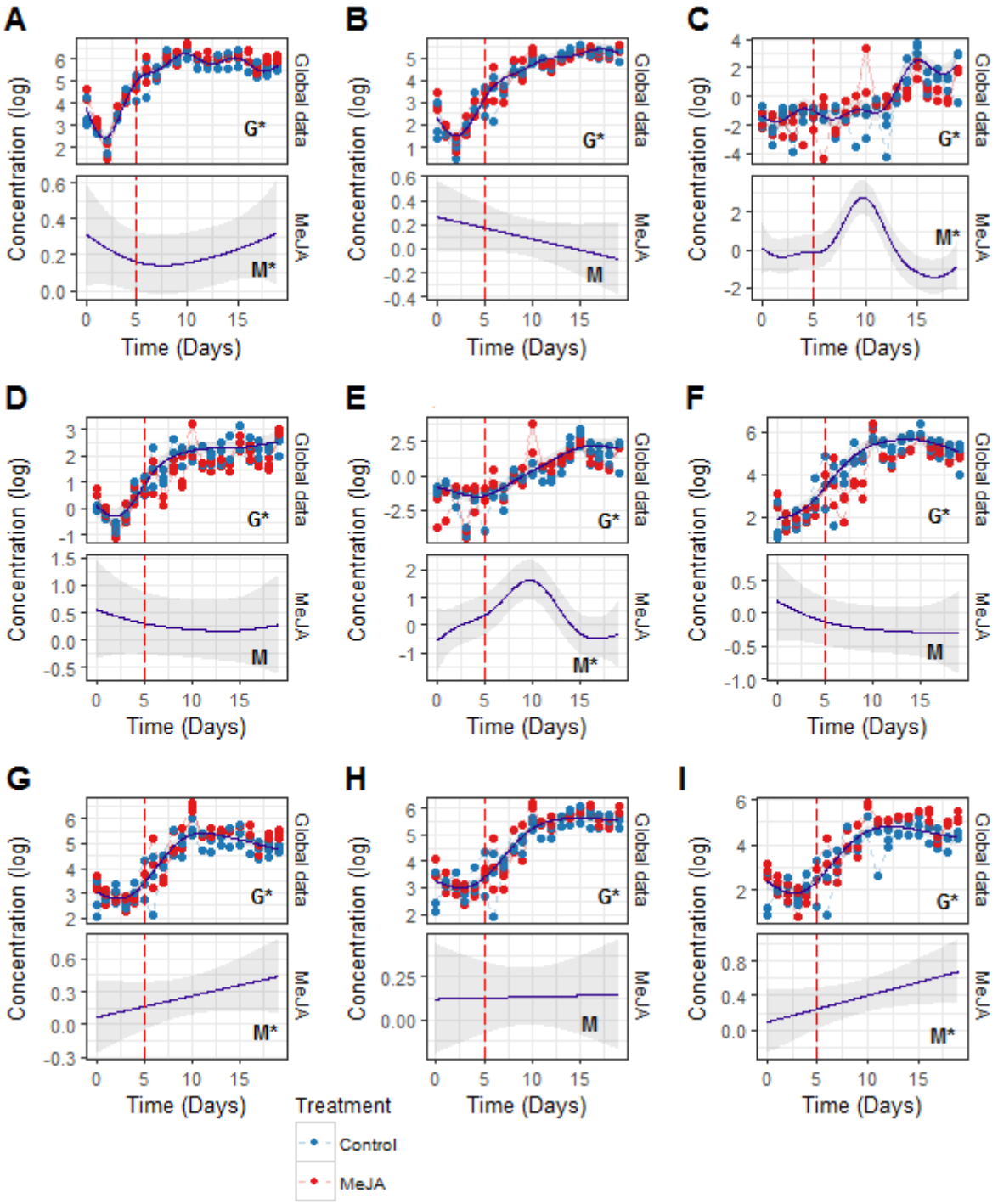

Figure 28 Time course accumulation of flavan-3-ols in VGR cell suspensions elicited with MeJA; catechin (A), epicatechin (B), epigallocatechin (C), gallocatechin (D), epigallocatechin gallate (E), epicatechin (F), procyanidin B1 (G), procyanidin B2 and B4 (H) and procyanidin B3 (I). In each panel, the upper graph (global data) shows the fitted smooth of the global trend ($s_{cm}(t)$), while the lower graph (MeJA) shows the fitted smooth of the MeJA term ($s_{MeJA}(t)$). The shaded areas of the trends are 95% confidence intervals on the fitted smoothers. The day of MeJA elicitation is indicated by a red dashed line. G = global trend, M = trend of MeJA term. *indicates the significance ($p < 0.01$). The concentrations of the global data are log transformed.

The main flavonols found in grape cell cultures include quercetin, quercetin-3-*O*-glucoside, quercetin-3-*O*-galactoside, quercetin-3-*O*-glucuronide, isorhamnetin-3-*O*-glucoside, quercetin-3-*O*-rutinoside (syn. rutin). Taxifolin (syn. dihydroquercetin) is the only dihydroflavonol found in grape cell cultures. The structures of flavonols and taxifolin are shown in Fig 29. The concentrations of all flavonols significantly changed all over the time, as can be seen by global trends and MeJA treated samples. For the dihydroflavonol taxifolin, MeJA treated samples showed with a positive increment during the growing period (Fig 30).

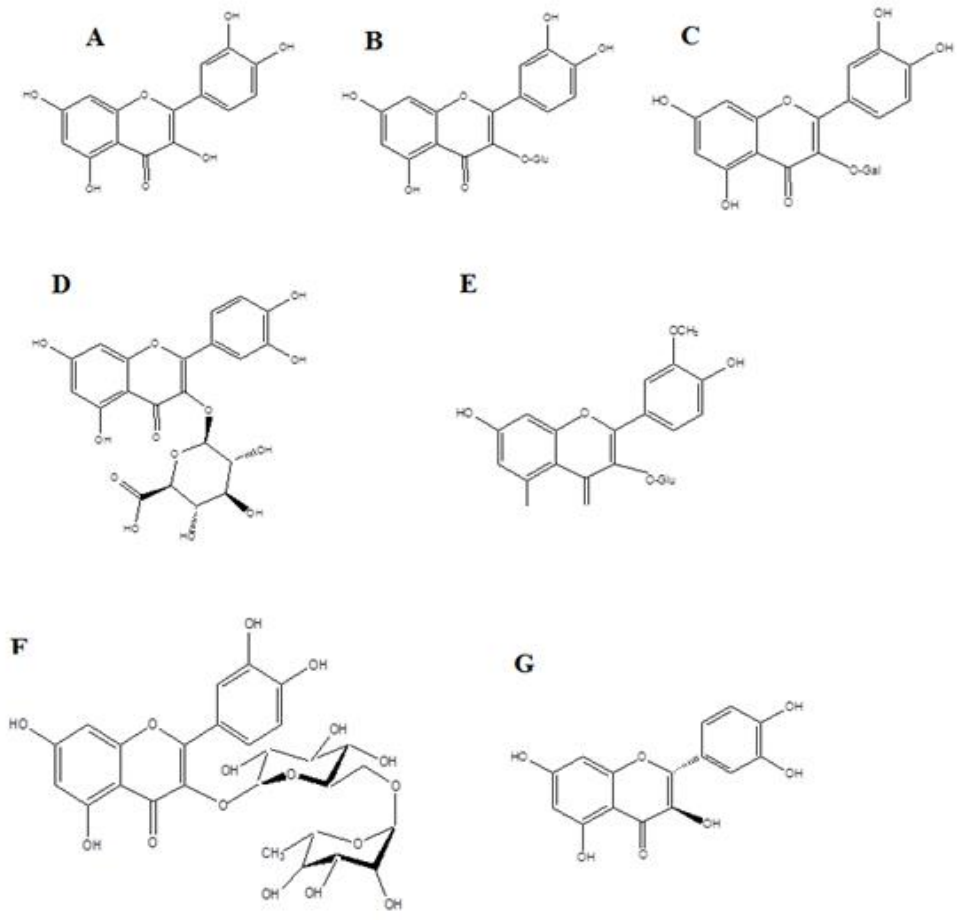

Figure 29 Chemical structures of flavonols; quercetin (A), quercetin-3-*O*-glucoside (B), quercetin-3-*O*-galactoside (C), quercetin-3-*O*-glucuronide (D), isorhamnetin-3-*O*-glucoside (E) and quercetin-3-*O*-rutinoside (syn. rutin) (F) and dihydroquercetin (taxifolin) (G) identified in VGR cell cultures.

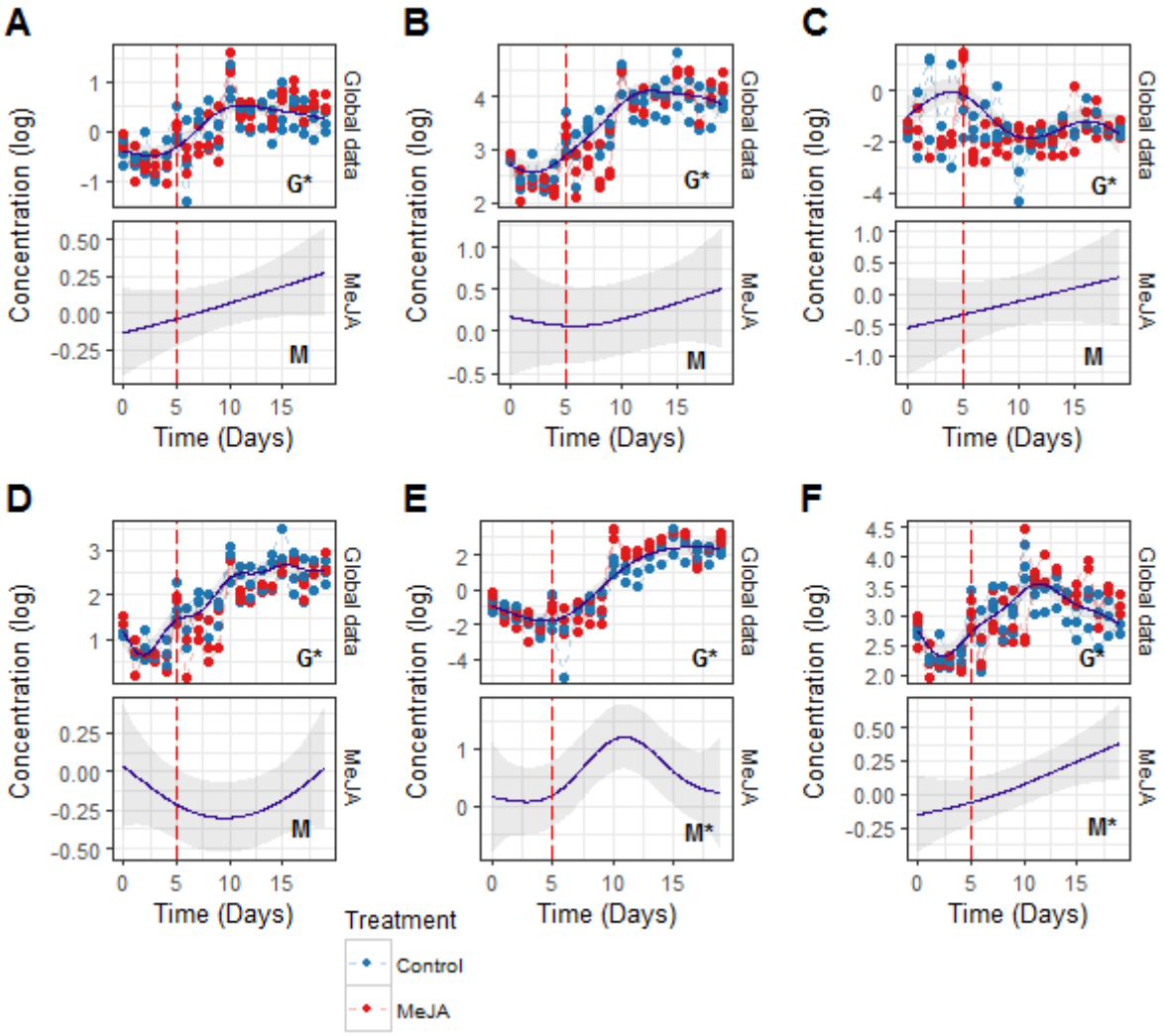

Figure 30 Time course accumulation of flavonols in VGR cell suspensions elicited with MeJA; quercetin (A), quercetin-3-*O*-glucoside and quercetin-3-*O*-galactoside (B), quercetin-3-*O*-glucuronide (C), isorhamnetin-3-*O*-glucoside (D) and quercetin-3-*O*-rutinoside (E) and dihydroflavonol dihydroquercetin (F). In each panel, the upper graph (global data) shows the fitted smooth of the global trend ($s_{cm}(t)$), while the lower graph (MeJA) shows the fitted smooth of the MeJA term ($s_{MeJA}(t)$). The shaded areas of the trends are 95% confidence intervals on the fitted smoothers. The day of MeJA elicitation is indicated by a red dashed line. G = global trend, M = trend of MeJA term. * indicates the significance ($p < 0.01$). The concentrations of the global data are log transformed.

3.3.2.4. Time course changes of benzoic acid derivatives biosynthesis and MeJA treatment

Benzoic acid derivatives identified in all grape cell cultures consist of 4-hydroxybenzoic acid, vanillin, vanillic acid and gallic acid. Their structures are shown in Fig 31. Due to their trace amounts, the sum of individual derivatives was used in order to investigate their time dependent behavior and the influence of MeJA treatment. The model showing the effect of MeJA treatment on the total benzoic acid derivatives is depicted in Fig 32. The common trend line (edf=2.480, P=0.027) indicates a slight increase of total benzoic acid derivatives in the cells along the growing period. The bottom model component shows instead the “additional” effect of MeJA (edf=5.359, P<0.001). The two cultures start to diverge after day 7 and the difference in concentration stabilizes around day 10. A slight decrease in the concentration of total benzoic acid derivatives seems to be present at day 5.

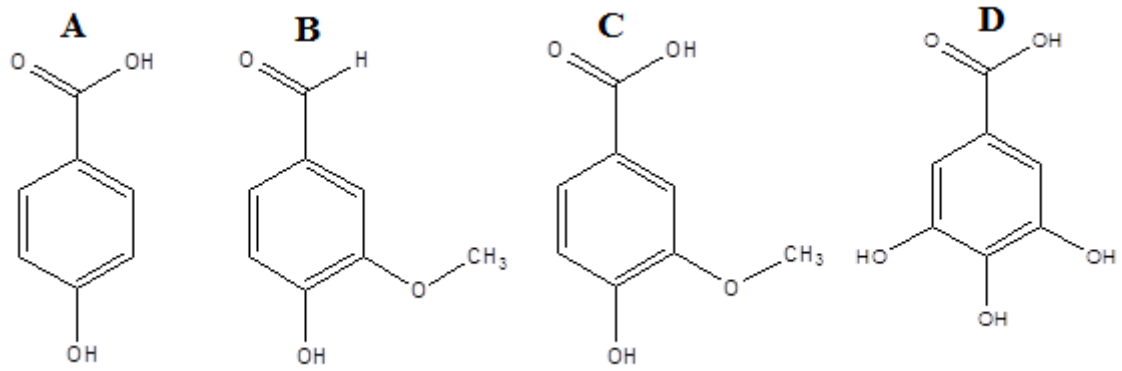

Figure 31 Chemical structures of benzoic acid derivatives; 4-hydroxybenzoic acid (A), vanillin (B), vanillic acid (C) and gallic acid (D) identified in VGR cell cultures.

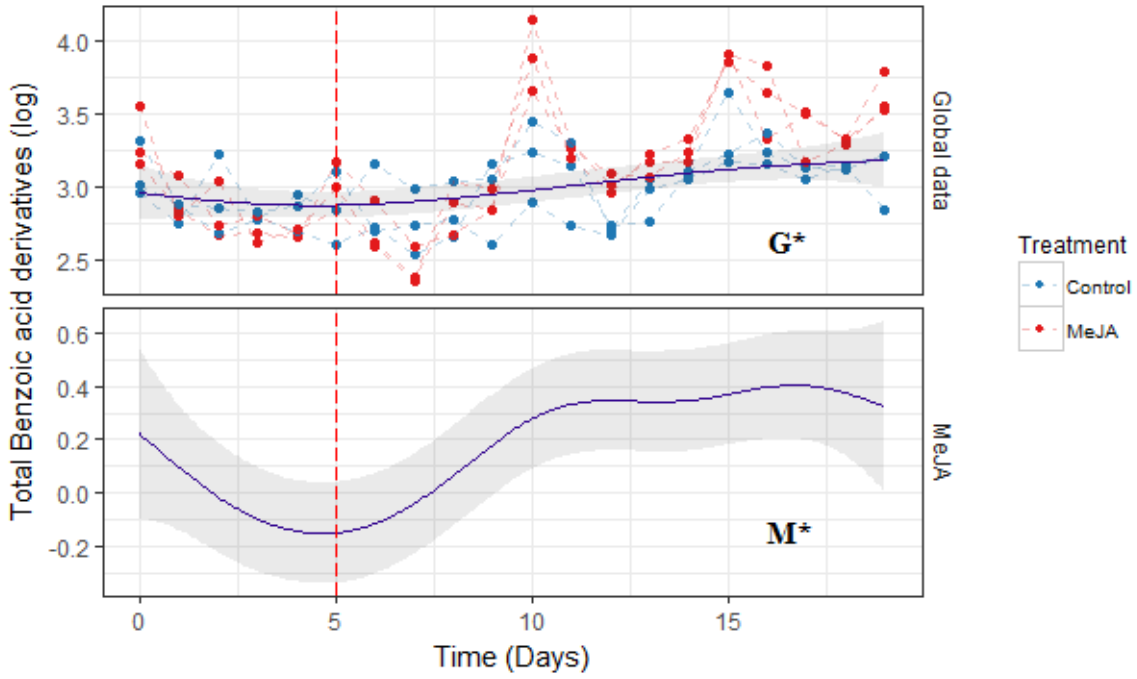

Figure 32 Time course accumulation of total benzoic acid derivatives in VGR cell suspensions elicited with MeJA. In each panel, the upper graph (global data) shows the fitted smooth of the global trend ($s_{cm}(t)$), while the lower graph (MeJA) shows the fitted smooth of the MeJA term ($s_{MeJA}(t)$). The shaded areas of the trends are 95% confidence intervals on the fitted smoothers. The day of MeJA elicitation is indicated by a red dashed line. G = global trend, M = trend of MeJA term. * indicates the significance ($p < 0.01$). The concentrations of the global data are log transformed.

3.3.2.5. Time course changes of phenylpropanoid biosynthesis and MeJA treatment

Caffeic, caftaric and *trans*-coutaric acid are phenylpropanoids (Fig 33) identified in the grape cell cultures. In terms of absolute concentrations, phenylpropanoids were present in lower amounts in both control and treated cell cultures. Similarly to benzoic acid derivatives, the sum of all phenylpropanoids was used to model the changes over time and the effect of MeJA. The global trend (edf=4.159, P=0.005) of total phenylpropanoids indicate a higher concentration at the beginning of the culture period and a decrease in the cells over time. In contrast, the non-significant differential smoother (edf=2.377, P=0.16301) demonstrated that MeJA did not affect the total phenylpropanoids (Fig 34).

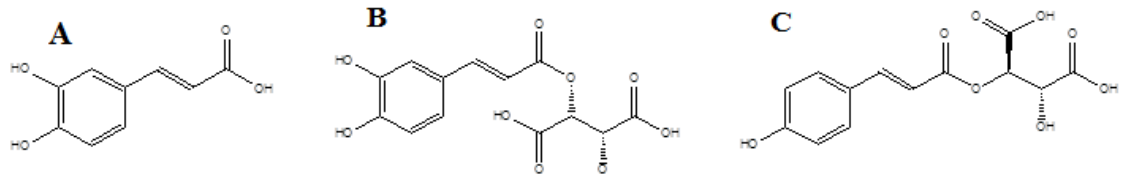

Figure 33 Chemical structures of phenylpropanoids; caffeic acid (A), caftaric acid (B), trans-coutaric acid (C) identified in VGR cell cultures.

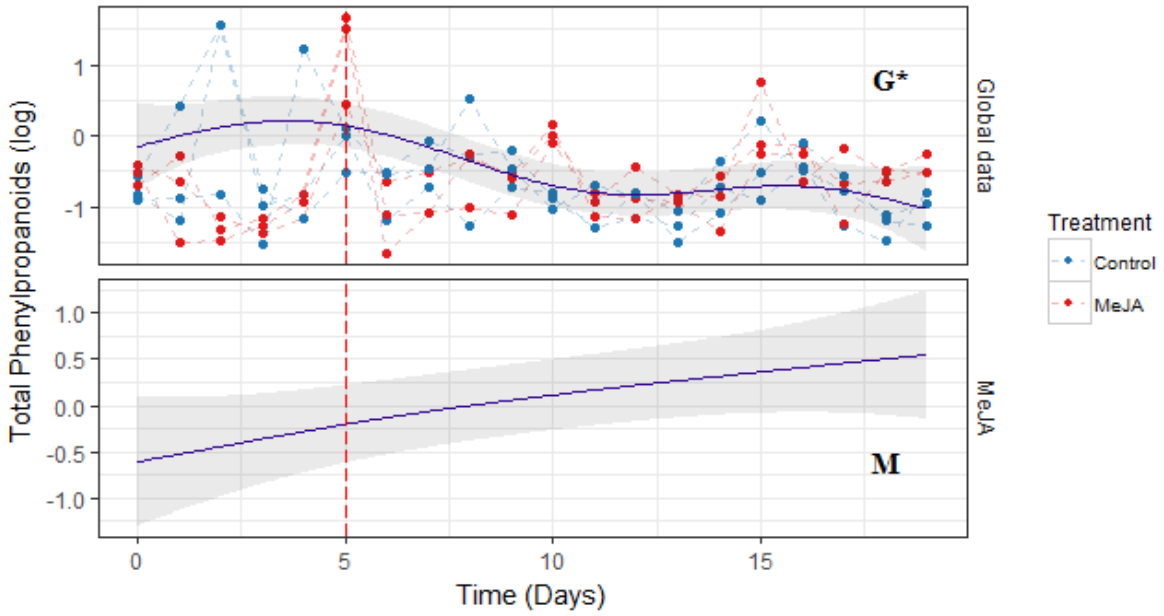

Figure 34 Time course accumulation of total phenylpropanoids in VGR cell suspensions elicited with MeJA. In each panel, the upper graph (global data) shows the fitted smooth of the global trend ($s_{cm}(t)$), while the lower graph (MeJA) shows the fitted smooth of the MeJA term ($s_{MeJA}(t)$). The shaded areas of the trends are 95% confidence intervals on the fitted smoothers. The day of MeJA elicitation is indicated by a red dashed line. G = global trend, M = trend of MeJA term.* indicates the significance ($p < 0.01$). The concentrations of the global data are log transformed.

3.3.2.6. Time course changes of other polyphenols biosynthesis and MeJA treatment

Arbutin (Fig 35) is the only hydroquinone identified ranging from 1.25 - 15.23 mg g^{-1} in all cell cultures. The concentration of arbutin gradually increased over time as can be seen by global trend (edf=6.164, P<0.001). However, the differential smoother is not significant (edf=2.924, p=0.132) with large 95% confidence interval bands, indicating that MeJA elicitation had no influence on the biosynthesis of arbutin (Fig 36).

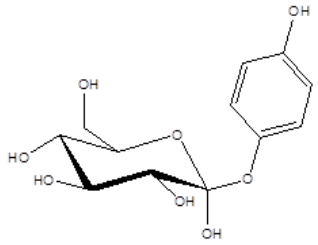

Figure 35 Chemical structures of arbutin identified in VGR cell cultures

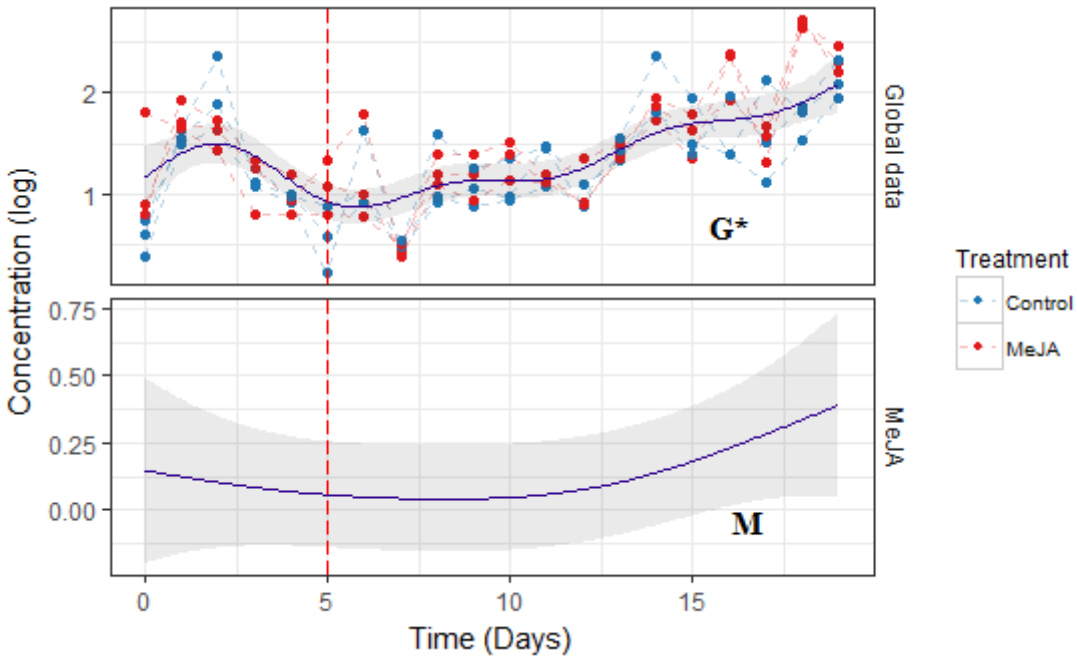

Figure 36 Time course accumulation of arbutin in VGR cell suspensions elicited with MeJA. In each panel, the upper graph (global data) shows the fitted smooth of the global trend ($s_{cm}(t)$), while the lower graph (MeJA) shows the fitted smooth of the MeJA term ($s_{MeJA}(t)$). The shaded areas of the trends are 95% confidence intervals on the fitted smoothers. The day of MeJA elicitation is indicated by a red dashed line. G = global trend, M = trend of MeJA term. * indicates the significance ($p < 0.01$). The concentrations of the global data are log transformed.

3.4. Discussion

The composition of polyphenol pool in grapes play an important role in plant metabolism and is highly affected by differences in genotype/cultivar, geographic origin, and environmental conditions (Flamini et al. 2013). The main polyphenol compounds in grapes are anthocyanins and other flavonoids and related metabolites, which include stilbenes, flavonols, flavan-3-ols but also phenolic acids (including hydroxycinnamic acids and hydroxybenzoic acids) (Zhang et al. 2015). The dynamic changes of polyphenols in VGR cell cultures over time and in relation to MeJA elicitation was studied by targeted metabolomics approach and statistical modeling. The general finding of our investigation is that the concentration and pattern of polyphenols are changing in time to a different extent depending on their position in the biosynthetic pathway and that the elicitation by MeJA selectively changes these trends.

This study shows that total benzoic acid accumulation has slightly increased over time at the global scale but remarkably increased in MeJA treatment. It was found that MeJA application had no effect on production of gallic acid, the only detected phenolic acid in *Vitis vinifera* cv. Tempranillo (Portu et al. 2016, 2015). Nevertheless, many studies have been reported in literature that phenolic acid accumulation was promoted in radish sprouts (Kim et al. 2006), hairy roots of *Daucus carota* (Sircar et al. 2012) and red raspberries (Flores and Ruiz del Castillo 2014) with application of MeJA, as a consequence of PAL activity. It is believed that MeJA can promote PAL activity resulting in an increase of total benzoic acid derivatives.

In contrast to these metabolites, no influence of MeJA on total phenylpropanoids was found assuming that it favored the downstream flavonoid metabolism.

The next interesting class of polyphenols in *V. vinifera* cell cultures are stilbene compounds. Several authors have undertaken studies to investigate the factors that are able to induce and/or modify stilbenes biosynthesis, regulation and metabolism (Waffo Teguo et al. 1998, Krisa et al. 1999 and Decendit et al. 2002). In contrast to anthocyanins, stilbene compounds in this study demonstrated distinct accumulation patterns in response to MeJA during the growing time. While *trans* and *cis*-resveratrol, piceatannol, *trans*-piceid, astringin, isorhapontin and *trans*- and *cis*-δ-viniferin showed descending trends over time, *cis*-piceid, *trans*- and *cis*-ε-viniferin, pallidol and

the sum of ampelopsin D and quadrangulanin A content were increasing along the cell growth. Many studies reported that MeJA treatment of grape cell suspension produces a direct effect on the stilbene synthesis (Tassoni, Durante, and Ferri 2012, Belhadj et al. 2008, Krisa et al. 1999, Donnez et al. 2011 and Vezzulli et al. 2007). In disagreement with these studies, our results indicated, in terms of trends, only isorhapontin concentration was enhanced by the MeJA elicitation. The concentrations of piceid (both *trans*- and *cis*-piceid) and ε-viniferins (both *trans*- and *cis*-ε-viniferin) remained unchanged. The concentrations of *trans*- and *cis*-δ-viniferin is significantly reduced after MeJA elicitation but increased again at the ending phase of the growth. In the cell cultures of *V. vinifera* cv Italia, MeJA stimulates preferentially δ-viniferin production other than ε-viniferins (Santamaria et al. 2011). The non-significant influence of MeJA on the accumulation of stilbene compounds might be assumed due to many factors. Firstly, concentration of MeJA used in this study might not be optimal for the stilbene production in the cell cultures. Krisa et al. (Krisa et al. 1999) applied 25-50 µM MeJA which was optimal for piceid production in *V. vinifera* cv. Gamay cell cultures. Secondly, stilbenes and anthocyanins may compete with each other for the potential substrates (Jeandet et al. 1995). It is already stated that application of MeJA in combination with carbohydrates to grape cell suspension cultures stimulated the gene expression of CHS and UFGT, resulting in flavonoid/anthocyanin biosynthesis enhancement (Belhadj et al. 2008). It can be suggested that CHS may compete with STS and the decrease of the stilbene content in response to MeJA may be a consequence of the concomitant rise of anthocyanin accumulation in the cells. Another factor may also likely be considering that as reported by Tassoni et al. (2012), stilbenes are mainly accumulated into the culture medium which was not analysed in this study.

In the present study, a total number of 19 flavonoids were firstly reported in the VGR cell cultures. The composition of flavonoids can be changed over time and it has been found that those in grape skin during the different stages of berry maturation (Conde et al. 2007) and (Bogs et al. 2006). Similar behaviour has been found in grape cell cultures as well and at the global scale, the accumulation of flavonoids demonstrated time-dependent changes during the culture period of the cell cultures.

The time-dependent behaviours of flavanone, flavones and dihydrochalcones, which are at the upper stream of flavonoid biosynthesis pathway, are similar to the typical growth curve of cell cultures (Section 2.3.1.1). Initially, they describe the slow growth followed by a rapid increment where the maximum production can be seen in a short time and declined, suggesting that their production was correlated with the aging of the cells. The production of flavonols, flavan-3-ols and dihydroflavonol at the global scale are higher at the later phase of the cell growth, providing that NAR is the main substrate in flavonoid biosynthesis pathway. The treatment of MeJA altered specific flavonoid compounds in terms of time trends. Less information is available concerning the effect of these treatments on flavonoids in grape cell cultures, and only Ruiz-Garcia et al. (2012) described an increase of flavonols and PAs in *V. vinifera* L. Monastrell grapes with the use of MeJA. In our study, although MeJA did not affect the biosynthesis of flavones and dihydrochalones, it may have a greater promoting effect on CHS activity, which regulates the formation of NAR and, consequently, to larger contents of anthocyanins. In MeJA treated samples, it is indicated that NAR is consumed rapidly to synthesize further flavonoids, which is proved by the increment of anthocyanin at the same time range. However, our results indicated no significant accumulation of flavonols over time in MeJA treated samples. This may be due to the inhibitory effect of MeJA on the activity of FLS which is involved in the formation of flavonols from dihydroflavonols (de la Peña Moreno, Blanch, and Ruiz del Castillo 2010) but modulation of the activity of DFR which is a key downstream regulator for the production of PAs and anthocyanins (Chen et al. 2007). Regards to flavan-3-ols, it can be seen that MeJA preferentially elicits the 2,3-*cis*-flavan-3-ols, i.e. epigallocatechin and epicatechin gallate, which lead to the further increment of PAs over time. These compounds are very close to anthocyanins in the biosynthetic pathway; indeed, they share most of the pathway, so that an increase in the activity of enzymes upstream in the flavonoid biosynthetic pathway may also affect their concentration. (Belhadj et al. 2008) demonstrated that grape cells responds to MeJA application by activating genes encoding enzymes responsible also for UFGT with subsequent accumulation of anthocyanins.

The composition of anthocyanin compounds found in this work at the global scale is in agreement with other studies (Sinilal et al. 2011 and Curtin, Zhang, and Franco 2003). Anthocyanin biosynthesis in *V. vinifera* species is indeed genetically controlled and involves two main pathway branches, namely the delphinidin-based (3',5' OH) and the cyanidin-based (3'OH) branch (Holton and Cornish 1995 and He et al. 2010). In terms of time trends, this study demonstrates that the total anthocyanin content increased over time mainly due to an increased concentration of Dp-glu and its derivatives (Dp-ac-glu and Dp-pc-glu) and that the elicitation by MeJA further enhanced this behaviour. The increase in anthocyanin content was expected as it has been reported in several studies with grapes and grape cell cultures (Tassoni, Durante, and Ferri 2012, Tassoni et al. 2005, Qu, Zhang, and Yu 2011, Ruiz-García et al. 2013 and Portu et al. 2016). For example, MeJA increased the levels of phenolic compounds including anthocyanins in the six selected clones of Monastrell grape variety, although the extent of the response differed among different clones from the same variety (Tassoni, Durante, and Ferri 2012). It could elicit a significant increase in anthocyanin production, whether used alone or in combination with other elicitors or precursor in suspension cultures of *V. vinifera* (Qu, Zhang, and Yu 2011).

Looking at the individual anthocyanins, Dp-glu and its derivatives (Dp-ac-glu and Dp-pc-glu) increased over time whereas Cy-glu and its derivatives remained constant. The non-significant changes of Dp-pc-glu/Dp-glu and Cy-pc-glu/Cy-glu ratios might be the consequence of coumaroylated glucosides being produced at the same rate of non-acylated glucosides. However, a different rate of consumption of the two molecules cannot be ruled out and it would lead to the same result. A higher reactivity has been previously reported in a fermentation study, where the rate of synthesis of *p*-coumaroyl-vitisins resulted higher than that of non-acylated vitisins (Morata et al. 2006).

It is interesting to focus on the effect of MeJA and on the "balance" between the four acylated anthocyanins and their relative glycosylated precursors. After treatment, indeed, coumaroylated derivatives "were following" the trend of their glycosylated precursor, while their acetylated counterparts were not. Remarkably, this was happening both in the Dp and in the Cy branch of

the pathway. These results suggest that the treatment with MeJA could selectively modulate the activity of specific acyltransferases that can use both glycosylated forms as substrate. These findings are interesting because acylation is one of the most common modifications of anthocyanins (Mazza and Francis 1995), but the mechanism controlling the balance between the pool of non-acylated and acylated forms is still unknown. The presence and the specific activities of the enzymes involved in these reactions are strongly related to the genetic background of the different grape varieties (Núñez et al. 2004/1) and indeed anthocyanin patterns are variety specific in grapes.

According to this, the relation between coumaroyl and acetyl glucosides was another parameter considered in characterizing the effect of MeJA (Burns et al. 2002, Otteneder, Marx, and Zimmer 2008 and González-Neves et al. 2006). The global trends resulting from modelling the ratio between coumaroyl and acetyl glucosides in the two branches revealed that there are no significant changes over time in this grape variety. The smoothers of MeJA showed upward trends of this ratio both in Dps and Cys based anthocyanins due to the high concentration of coumaroyl glucosides, as described above. As the ratios are noisier in variance, only barely statistical significance could be seen in Cy based anthocyanins. These results confirmed that MeJA preferentially elicits the synthesis of coumaroyl-glucosides in the cells.

Anthocyanin methylation in grapes is mainly affected by genetic factors (Mattivi et al. 2006), as well as by environmental and culture conditions (Downey, Dokoozlian, and Krstic 2006). In our study, the ratio of methylated and non-methylated anthocyanins showed a descending trend in MeJA-treated samples for the Cy-based anthocyanins, indicating the preferential production of Cys. A similar behavior was found in Dp-based anthocyanins, as indicated by the lower production of methylated anthocyanins (Pts + Mvs). According to these results, despite anthocyanin methylation in grape cell cultures being time dependent, the treatment of MeJA did not affect the methylation of anthocyanin in the cell culture.

From this study, it can be suggested that MeJA is involved only in the activities of specific enzymes and it makes a complete understanding of the influence of MeJA in general phenylpropanoid pathway and stilbene/flavonoid biosynthesis. However, a complete

understanding of the changes in the metabolic flux of the pathway is still needed. The use of GAMs is an effective tool to model complete time dependent profiles of secondary metabolites biosynthesis in plant cells as shown in results, providing valuable information on their specific dynamic interaction. The flexibility of GAMs to fit the data closely is advantageous characteristics that make a better view of how plants respond to stress factors. This lead in the next chapter, to extend applying GAM to reveal the response of grape cells cultures to nitric oxide (NO) signalling pathway in the flask and bioreactor cultures.

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Chapter 4

Time-course targeted metabolomics analysis of sodium nitroprusside induced *V. vinifera* cv. Gamay teinturier cell suspension in flask and bioreactor cultures

4.1. Introduction

As described in chapter 3, we have studied the time-course changes of polyphenols in grape cell cultures and our results indicated that MeJA has an influence mainly on anthocyanins and some flavonoids, but the elicitor seems to have a negligible effect on the production of stilbenoids (at least as far as intracellular medium is concerned). Among the polyphenols in grapes, however, the stilbene compounds are non-flavonoid polyphenols which are very popular due to their role in grapevine fungal resistance and multiple health benefits. Many studies have been focused on their nutritional and medicinal functions along with biosynthetic pathway analysis, as well as the effect of biotic and abiotic stresses on the synthesis and accumulation of stilbenes in plant cell cultures. It would be interesting to investigate if other signaling molecules, at odd with what happened for MeJA, can induce a modulation in the production of this class of compounds.

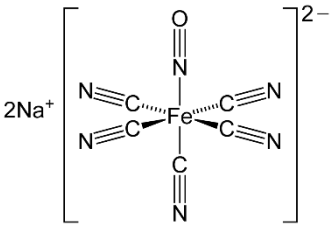

Figure 37 Chemical structure of sodium nitroprusside

Nitric oxide (NO) is a signalling molecule, which plays an important role in a variety of physiological processes, such as growth and adaptive responses to environmental stress (Arasimowicz and Floryszak-Wieczorek 2007), leading to the biosynthesis of plant secondary metabolites. Hence, the elicitor-induced NO production is tought to be essential for triggering the biosynthesis of pharmaceutically important secondary metabolites in plants (M. Xu and Dong 2007). Many previous studies have shown that NO is involved in elicitation induced production of metabolites, such as ginseng saponin (Hu et al. 2003), hypericin (M.-J. Xu, Dong, and Zhang 2005), catharanthine (M. Xu and Dong 2005), puerarin (M. Xu, Dong, and Zhu 2006), taxanes (Wang et al. 2006) and artemisinin (L.-P. Zheng et al. 2008) in the respective plant cell and tissue cultures. Artificial NO donors are widely used as tools to study the role of NO in plants. Sodium nitroprusside (SNP) (Figure 37), for example, is widely used as NO donor and its

addition directly into the culture medium can promote the accumulation of plant biomass and enhance secondary metabolite production (B. Zhang, Zheng, and Wang 2012). Different routes can be involved in the mechanism of NO release from SNP *in vivo*. The key step would be an electron transfer process to form the reduced SNP radical which leads to NO as the final product (Grossi and D'Angelo 2005). It has also been reported that the treatment of *V. amurensis* Rupr. callus cultures with SNP produced four-fold increase of resveratrol content, indicating the importance of NO signalling in resveratrol production (Kiselev et al. 2007). As in the case of MeJA, the effects of NO on the metabolic profiling of grape cell cultures along full time-course experiment that allows to analyse the time-dependent behavior of cell metabolism is still limited. Here an investigation on the SNP induced modulation of the production of stilbene compounds in *V. vinifera* cv. Gamay Fréaux var. Teinturier (referred as GT) cell suspension culture is reported. GT cell suspension cultures have been shown to be particularly suitable because they are able to produce various stilbenes including piceid and other *trans*-resveratrol glucoside derivatives (Larronde et al. 1998).

From an industrial point of view, large-scale cultures in bioreactors are necessary to obtain rapidly sufficient quantities of desired secondary metabolites like polyphenols (Decendit et al. 1996, Hiroyuki et al. 2002 and Aumont et al. 2004). For these reasons the experiments performed in flasks were also scaled-up in a 5 litre bioreactor which allowed, for the first time, a time course analysis of the metabolic changes occurring in this type of systems. The present study should add to the understanding of biosynthesis of stilbene compounds and accumulation under the exposure of NO as signalling molecule.

4.2. Materials and methods

4.2.1. Plant cell cultures

A cell suspension culture of GT was established (Krisa et al. 1999) and has been maintained at the Institute of Vine and Wine Science, University of Bordeaux. The culture media (ME) has been prepared as previously described in (Decendit et al. 1996). Cell lines were maintained under continuous fluorescent light (5000 Lux) at 25°C in 250 ml Erlenmeyer flasks containing 50 ml cell suspension on an orbital shaker (100 rpm). The initial pH of the medium was adjusted to 5.8 with 0.1 M KOH before autoclaving. Cells were subcultured every seven days with an inoculum dilution of 1:5.

For the experiments with bioreactor, cells were grown in a 5 litre stirred glass bioreactor (4l of culture) (Fig 38). The agitation rate was adjusted to 60 rpm. The aeration rate was between 0.075 - 0.15 vvm. The bioreactor cultures were initiated by inoculating a 7-day-old cell suspension into ME medium at 1:4 ratio.

Figure 38 Suspension cultures of GT in the bioreactor.

4.2.2. Sodium nitroprusside treatment of the cell cultures

For the elicitation experiment in flasks, seven-day old GT cells were transferred to the 1l Erlenmeyer flasks containing fresh ME medium with the dilution of 1:5. The total volume for each flask was 450 ml. For bioreactor experiments, seven-day old GT cells were transferred to the 5l stirred glass bioreactor with the ratio of 1:4 in total culture volume 4l. The cultures were grown under the same conditions as described above. SNP (Sigma, Italy) was dissolved in water, sterilized by filtration and added at 100 µM final concentration to the flasks and bioreactor cultures. Control cultures were given the same volume of a blank solution of sterilized water. Three biological replicates were prepared for both, control and elicited samples for a total of 15 days of growth. For the time course effect of SNP, the cells were harvested at timely on day 0, 3, 5, 6, 7, 8, 9, 10, 11, 12, 13 and 14. In order to investigate the immediate effect of SNP, the cells were harvested at 10 min, 30 min, 1 hour, 2 hour and 6 hours after elicitation on day 7 from the same flasks and bioreactors.

4.2.3. Extraction of polyphenols (non-anthocyanins)

The extraction of non-anthocyanin polyphenols in GT cell cultures was performed as described in section 2.2.3.

4.2.4. UPLC analysis and identification and quantification (MS/MS) of polyphenols (non-anthocyanins)

UPLC analysis, identification and quantification of non-anthocyanin polyphenols were performed as described in section 2.2.4.

4.2.5. Data analysis and statistical modeling

The same approach of data analysis and statistical modelling was conducted as previously described in Section 3.2.7.

4.3. Results

4.3.1. General profile of stilbenes in grape cell cultures

To investigate the effect of SNP on non-anthocyanin polyphenol accumulation, GT cell cultures were treated with 100 µM SNP in the flask cultures and bioreactors on the 7th day of culture. A total of 12 anthocyanins and 40 non-anthocyanin polyphenolic compounds in GT cell cultures were detected by targeted UPLC-MS/MS method. The general profile of anthocyanins and non-anthocyanin polyphenols from the flask and bioreactor cultures are described in Appendix.

Among the non-anthocyanin polyphenols, a total of 11 stilbenes were identified in GT cell cultures from the flasks and bioreactors. These stilbenes include *trans*-resveratrol, *cis*-resveratrol, *trans*-piceid, *cis*-piceid, *trans*-ε-viniferin, *cis*-ε-viniferin, *trans*- and *cis*-δ-viniferin, pallidol, astringin, isorhapontin, and the sum of ampelopsin D and quadrangulanin A. The contribution and composition of stilbene compounds in GT cell cultures are visualized by a heatmap in Fig 39. Generally, a higher concentration of stilbenes can be seen in SNP treated cell cultures, however, the distribution of stilbenes during the culture period cannot be found as clear pattern. Piceids are the most abundant stilbenes found in all samples, which possess 71% of total stilbenes in *cis*-piceid and 20% in *trans*-piceid in flasks and 62% in *cis*-piceid and 22% in *trans*-piceid in bioreactor cultures, respectively. Isorhapontin followed accounting for 4% and 8% in flasks and bioreactors, respectively. The other stilbenes cover only a small percentage with less than 1% of total stilbene compounds. In order to identify the general trends in the relations between the growing period of cell cultures and any possible culture type or SNP treatment or patterns in the data, PCA analysis was performed. Fig. 40 shows the projection of the cell culture samples defined by the first and second principal components. The PC1 describes 35.3% of the variability in the data and the second (PC2) 18.8%. PCA allowed a clear separation of the stilbenes from samples upon the treatment with SNP to the cells. Concerning the immediate effect of SNP on the stilbene biosynthesis, the stilbene concentration was different according to the culture type (flasks and bioreactors). For the long-term influence of SNP addition to the cells, the separation of PCA scores can be seen starting from one day after

treatment (day 8 of the culture) both in bioreactors and flasks. Therefore, SNP is an important factor in the changes of stilbene accumulation in GT cells both in flasks and bioreactor cultures.

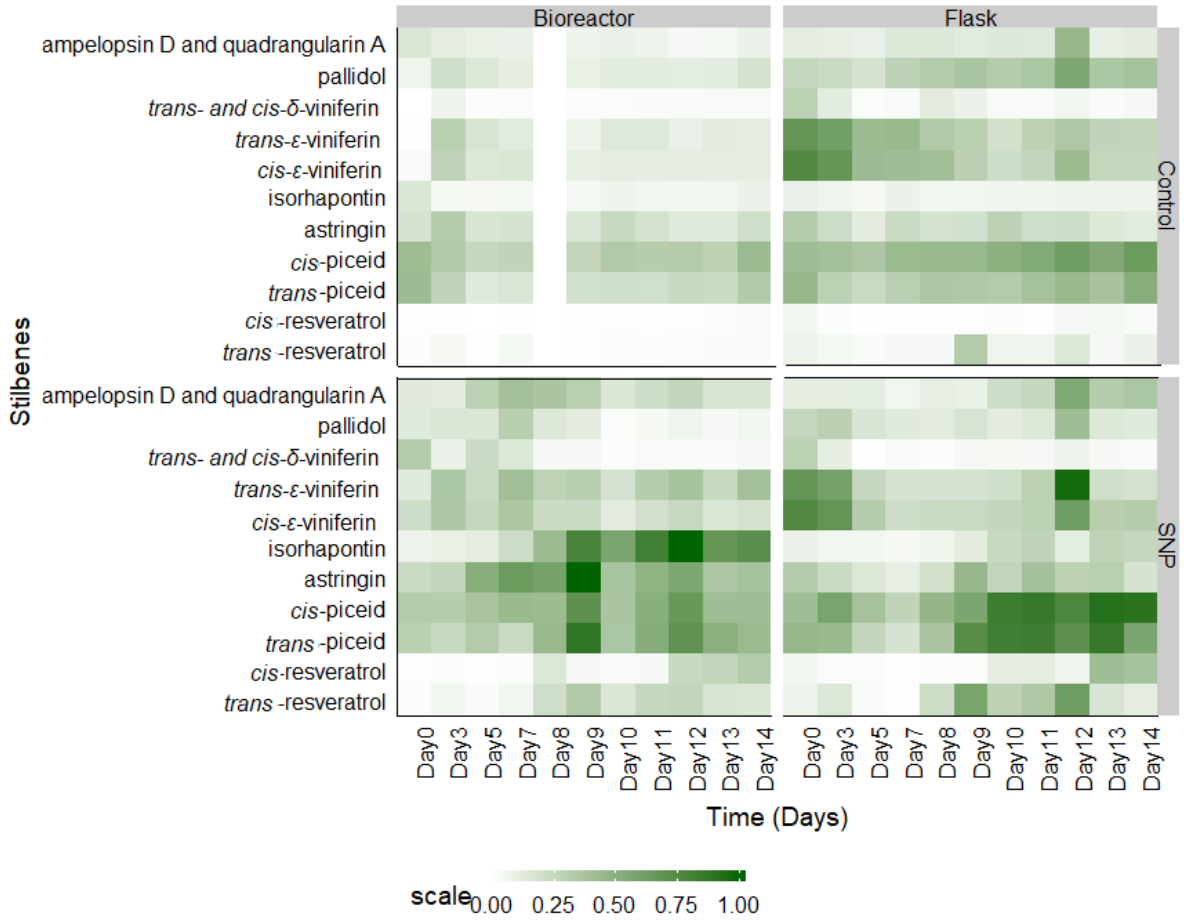

Figure 39 Composition of stilbenes in percentage of total stilbenes of GT cells in the flasks and bioreactor cultures for control and SNP treatment.

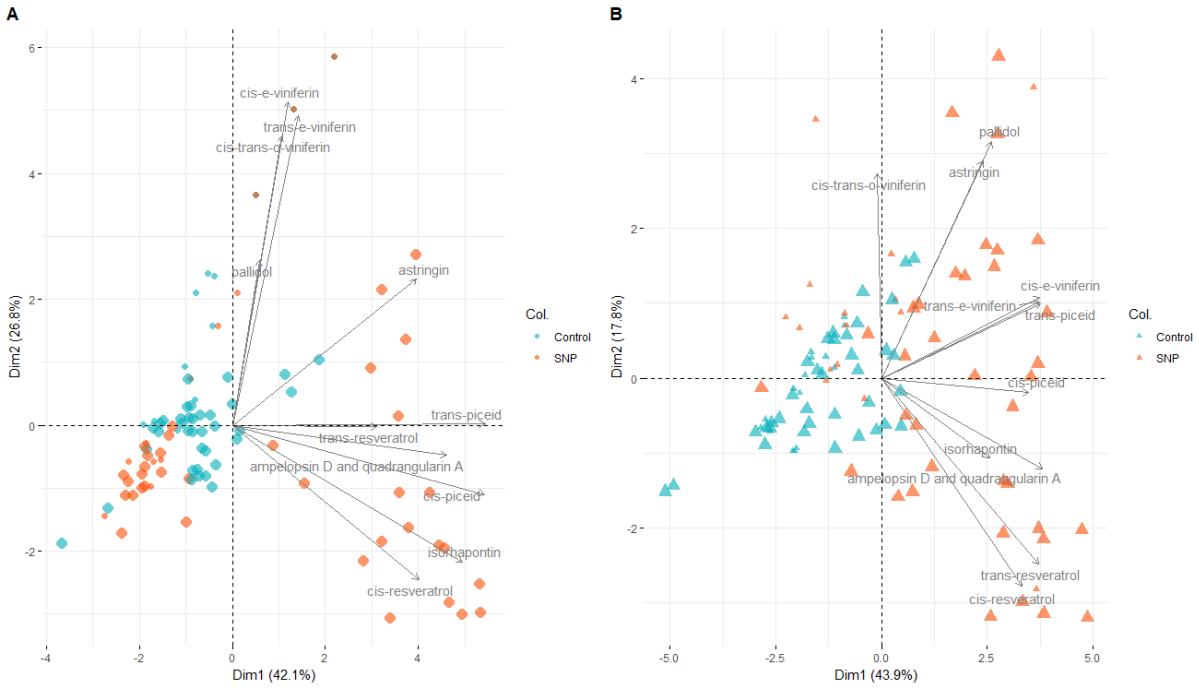

Figure 40 PCA biplot on the auto-scaled data showing the projection of the stilbene data set in the PC1 × PC2 plane for the GT cell cultures in flasks (A) and in bioreactors (B). The smaller points indicate the stilbene concentrations before elicitation and the bigger points indicate those after elicitation in control and SNP treated cell cultures both in flasks and bioreactors.

4.3.2. Effect of SNP elicitation on the stilbene biosynthesis of GT cells

4.3.2.1. Flask cultures

GAMs were applied in order to investigate the long-term effect of SNP on the stilbene biosynthesis in GT cells from both flasks and bioreactor cultures. In this experiment, the cells were harvested daily after elicitation for 7 days. In the flask cultures of GT cells the production of stilbene compounds, except isorhapontin, were dependent on the growing time. The approximate significance of smoothing terms of the stilbene compounds resulting from GAM are presented in Table 4.

Table 4 Summary statistics of GAM applied for stilbene compounds in GT cell flask cultures.

Metabolites	smoother terms	edf	F	p-value
trans-resveratrol	Global	6.574	13.34	0.0001***
	SNP	3.919	15.21	0.0001***
cis-resveratrol	Global	4.285	15.27	0.0001***
	SNP	2..659	49.05	0.0001***
trans-piceid	Global	4.227	17.00	0.0001***
	SNP	7.599	34.36	0.0001***
cis-piceid	Global	4.089	16.19	0.0001***
	SNP	6.619	25.34	0.0001***
trans-ε-viniferin	Global	2.431	17.454	0.0001***
	SNP	6.856	6.387	0.0001***
cis-ε-viniferin	Global	2.465	32.285	0.0001***
	SNP	5.182	4.945	0.0001***
trans- and *cis*-δ-viniferin	Global	6.197	27.050	0.0001***
	SNP	2.499	1.501	0.237
pallidol	Global	4.789	4.775	0.0000***
	SNP	2.000	26.679	0.0001***
astringin	Global	4.833	8.883	0.0001***
	SNP	7.552	6.183	0.0001***
isorhapontin	Global	1.663	2.256	0.107
	SNP	6.703	45.762	0.0001***
ampelopsin D + quadrangulanin A	Global	5.058	5.140	0.0001***
	SNP	4.028	9.074	0.0001***

* =p-value < 0.05, ** = p-value < 0.01 , *** =p-value < 0.001

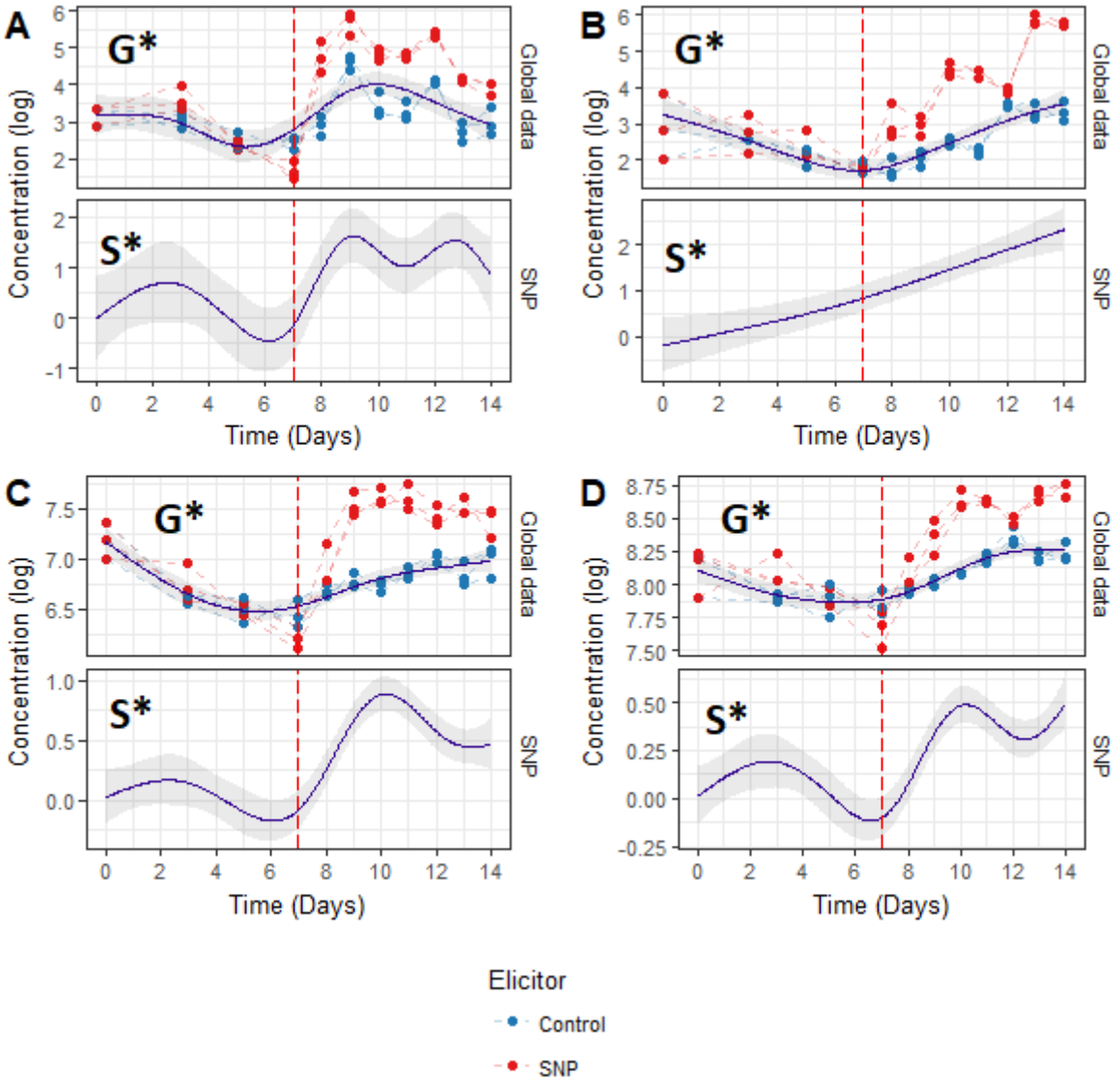

Figure 41 Time course accumulation of *trans*-resveratrol (A), *cis*-resveratrol (B), *trans*-piceid (C), *cis*-piceid (D) in GT cell suspensions in flasks elicited with SNP. In each panel, the upper graph (global data) shows the fitted smooth of the global trend ($s_{cm}(t)$), while the lower graph (SNP) shows the fitted smooth of the SNP term ($s_{SNP}(t)$). The shaded areas of the trends are 95% confidence intervals on the fitted smoothers. The day of SNP elicitation is indicated by a red dashed line. G = global trend, S = trend of SNP term. * indicates the significance ($p < 0.01$). The concentrations are log transformed.

As seen by the global trend, the accumulation of resveratrols (*trans*- and *cis*-) is relatively low in the GT cell cultures during early days of the growing period. However, it increased in the later phase of growth. The elicitation by SNP has produced a similar pattern of resveratrols with higher concentration in the cells (Fig. 41 A and B). Both *trans*- and *cis*-piceid showed a similar behaviour in the flask cultures of GT cells. Their global trends demonstrated the higher accumulation of piceid at the end of the culture period. The elicitation of SNP increased the concentration of piceid with the maximum peak at Day 10 and a decrease afterward (Fig. 41 C and D).

Concerning viniferins, the concentration of all classes of viniferin (*trans*- and *cis*-ε-viniferin and *trans*- and *cis*-δ-viniferin) showed descending trends over time. Nonetheless, SNP induced significant changes of *trans*- and *cis*-ε-viniferin and non-significant increment of *trans*- and *cis*-δ-viniferin in the cells (Fig. 42 A, B and C).

Pallidol remained stable along the growth curve with a slight increment before the end of the culture while it negatively responded to the SNP treatment (Fig. 43 A). The global trend of astringin illustrated its reduction over time, whereas SNP elicited a significant increment of astringin and stabilized until the growth curve ended is obvious (Fig. 43 B).

Unlikely to other stilbenes, the concentration of isorhapontin in the cells did not change over time but it responded to the SNP elicitation with a significant increment (Fig. 43 C). The concentration of ampelopsin D and quadrangularin A remained stable until 7th day of growth with a slightly raise from day 10 to 12 and declined afterwards whereas it continued in the SNP treated cells until the growing period ended (Fig. 43 D).

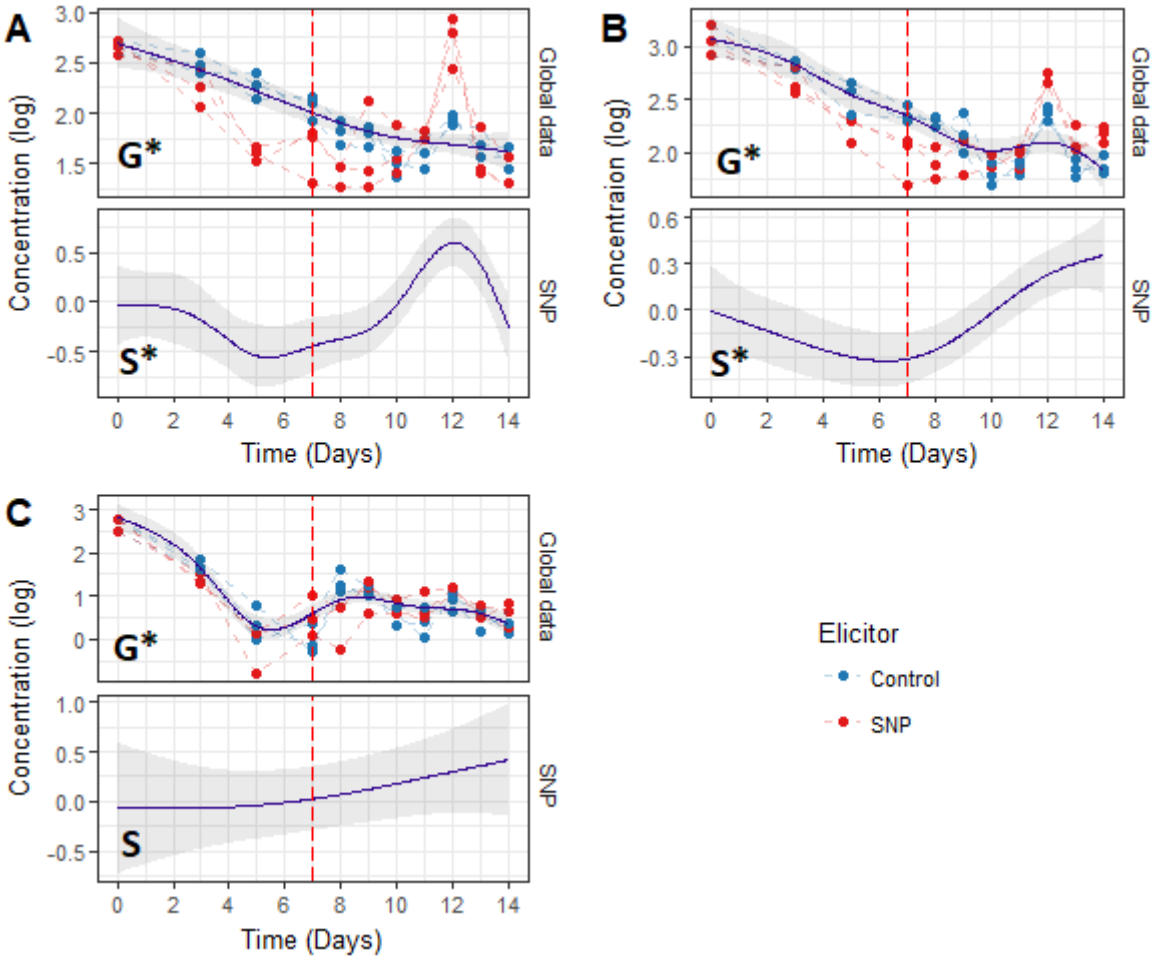

Figure 42 Time course accumulation of *trans*-ε-viniferin (A), *cis*-ε-viniferin (B), *trans*- and *cis*-δ-viniferin (C) in GT cell suspensions in flasks elicited with SNP. In each panel, the upper graph (global data) shows the fitted smooth of the global trend ($s_{cm}(t)$), while the lower graph (SNP) shows the fitted smooth of the SNP term ($s_{SNP}(t)$). The shaded areas of the trends are 95% confidence intervals on the fitted smoothers. The day of SNP elicitation is indicated by a red dashed line. G = global trend, S = trend of SNP term. * indicates the significance ($p < 0.01$). The concentrations are log transformed.

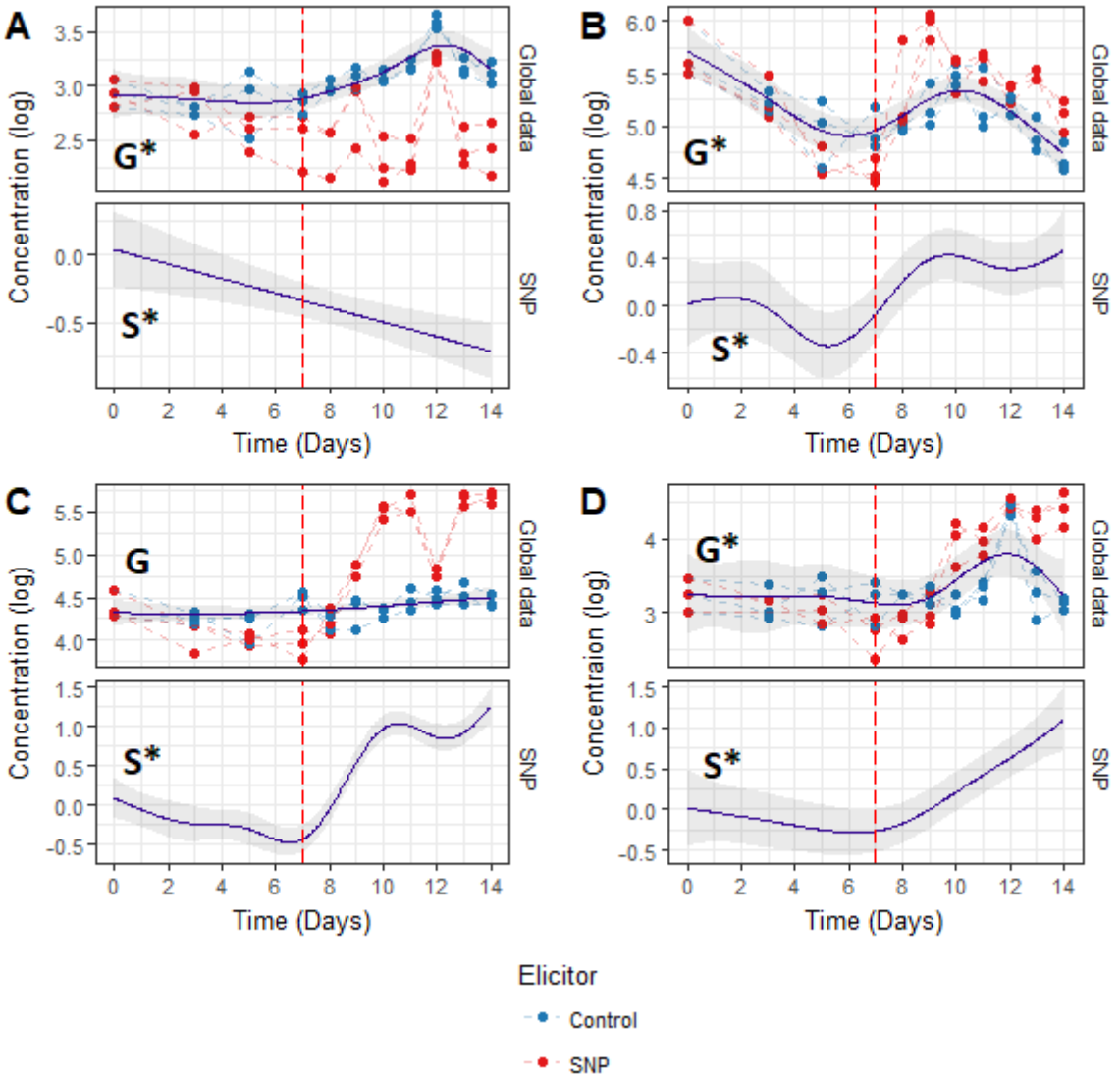

Figure 43 Time course accumulation of stilbenes in GT cell suspensions in flasks elicited with SNP: pallidol (A), astringin (B), isorhapontin (C), and ampelopsin D and quadrangulanin A (D). In each panel, the upper graph (global data) shows the fitted smooth of the global trend ($s_{cm}(t)$), while the lower graph (SNP) shows the fitted smooth of the SNP term ($s_{SNP}(t)$). The shaded areas of the trends are 95% confidence intervals on the fitted smoothers. The day of SNP elicitation is indicated by a red dashed line. G = global trend, S = trend of SNP term. * indicates the significance ($p < 0.01$). The concentrations are log transformed.

4.3.2.2. Bioreactor cultures

GAM was also used to analyse the long-term effect of SNP and the variation among the different classes of stilbene compounds in the bioreactor cultures. The approximate significance of smoothing terms of the stilbene compounds in bioreactor cell cultures are presented in Table 5. Both, *trans*- and *cis*-resveratrol demonstrated similar upward global trends and same response to the SNP treatment. The concentration of resveratrols reached their peak at day 8 and dropped thereafter (Fig. 44 A and B).

Concerning piceids, the synthesis and accumulation of *trans*- and *cis*-piceid in all cell cultures was quite similar. In response to SNP treatment, the amount of *trans*- and *cis*-piceid peaked at day 8 (Fig. 44 C and D).

The accumulation of viniferins showed that their biosynthesis follows the same pattern of accumulation to each other as shown by global trends. However, their responses to SNP treatment were different to each other. The increase of *trans*-ε-viniferin showed an upward trend in the SNP treated cell cultures (Fig. 45 A). In the case of *cis*-ε-viniferin, the concentration rapidly decreased from day 0 to day 7 of the culture and remained stable later on (Fig. 45 B). It is demonstrated that the content of the *trans*- and *cis*-δ-viniferin was decreasing along the growing period (Fig. 44 C).

In the bioreactor cultures, in contrast to the cells in flasks, the content of pallidol remained stable along the growing period. However, pallidol concentration showed an increment only at day 2 after SNP elicitation with a descending trend after that (Fig. 46 A). It can be seen that astringin steadily increased in the cells as shown by the global trend. In contrast, SNP did not induce the astringin synthesis or accumulation in the cells and the concentration remained constant throughout the growth period (Fig. 46 B). The global trend in Fig. 46 C shows that the content of isorhapontin in the cells started after 7 days of the inoculation with a steady increment during the growing period while addition of SNP can enhance the considerable production of isorhapotin in the bioreactor cultures. In the bioreactor cultures, the global trend indicated non-significant changes of ampelopsin D and quadrangularin A biosynthesis over time. Our GAM

results present considerable quantitative changes of these secondary metabolites as a result of SNP treatment (Fig. 46 D).

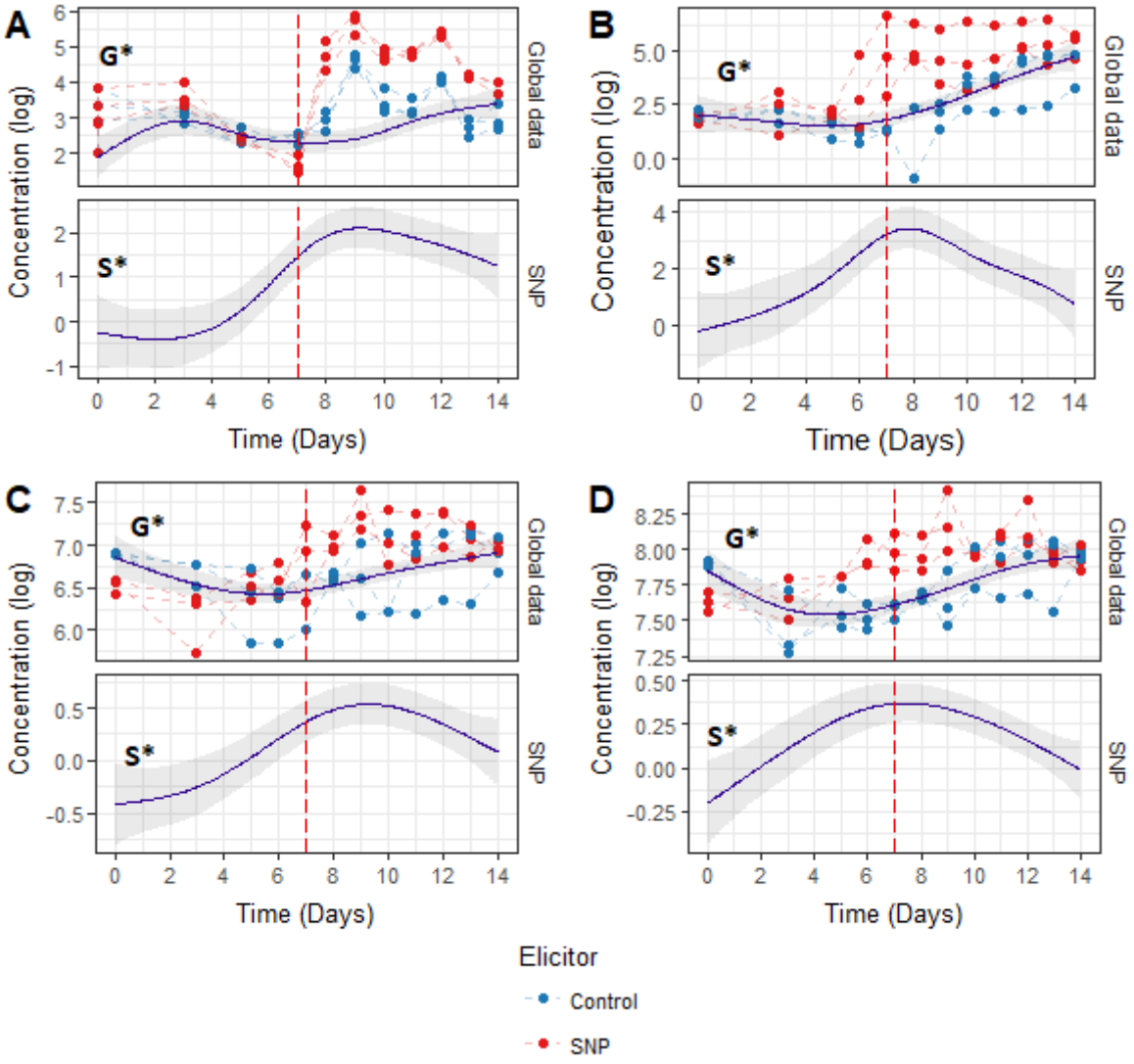

Figure 44 Time course accumulation of *trans*-resveratrol (A), *cis*-resveratrol (B), *trans*-piceid (C), *cis*-piceid (D) in GT cell suspensions in bioreactors elicited with SNP. In each panel, the upper graph (global data) shows the fitted smooth of the global trend ($s_{cm}(t)$), while the lower graph (SNP) shows the fitted smooth of the SNP term ($s_{SNP}(t)$). The shaded areas of the trends are 95% confidence intervals on the fitted smoothers. The day of SNP elicitation is indicated by a red dashed line. G = global trend, S = trend of SNP term. * indicates the significance ($p < 0.01$). The concentrations are log transformed.

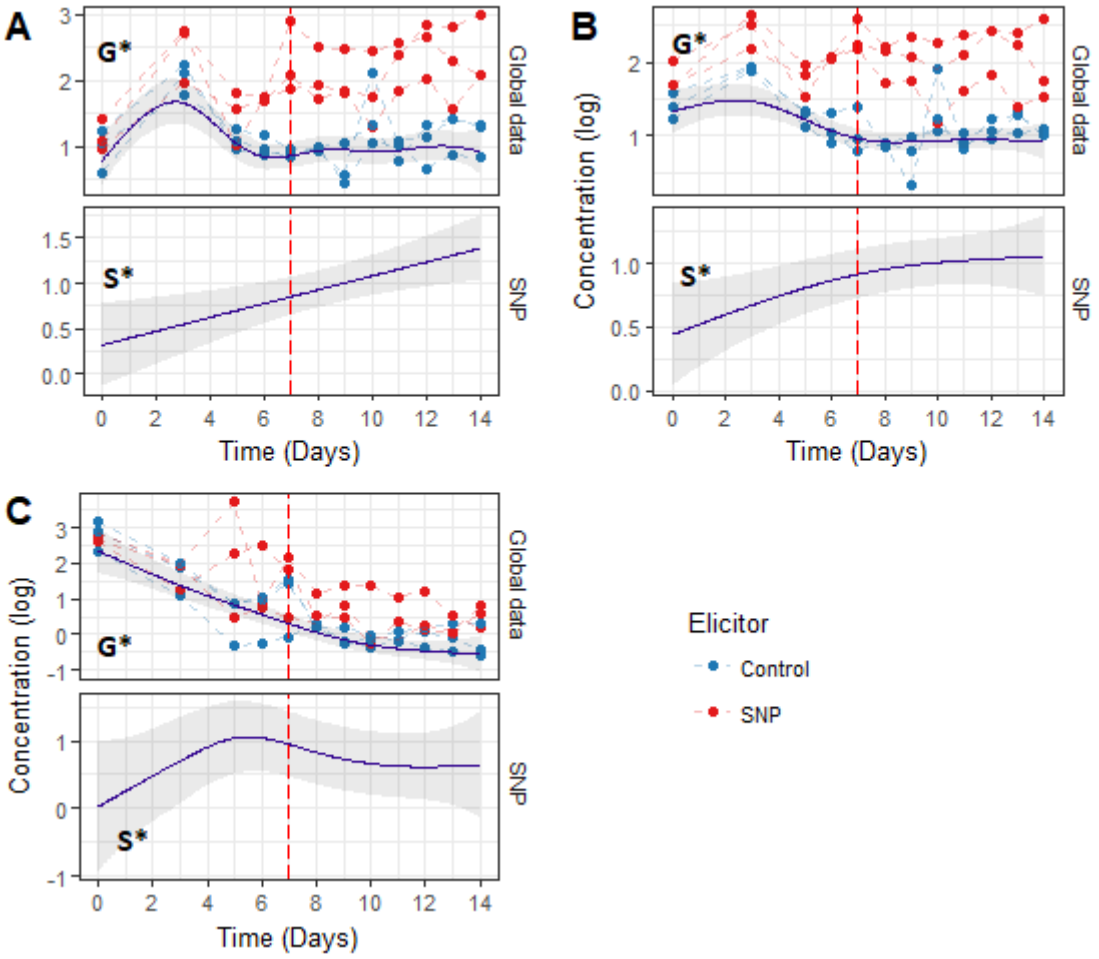

Figure 45 Time course accumulation of *trans*-ε-viniferin (A), *cis*-ε-viniferin (B), *trans*- and *cis*-δ-viniferin (C) in GT cell suspensions in bioreactors elicited with SNP. In each panel, the upper graph (global data) shows the fitted smooth of the global trend ($s_{cm}(t)$), while the lower graph (SNP) shows the fitted smooth of the SNP term ($s_{SNP}(t)$). The shaded areas of the trends are 95% confidence intervals on the fitted smoothers. The day of SNP elicitation is indicated by a red dashed line. G = global trend, S = trend of SNP term. * indicates the significance ($p < 0.01$). The concentrations are log transformed.

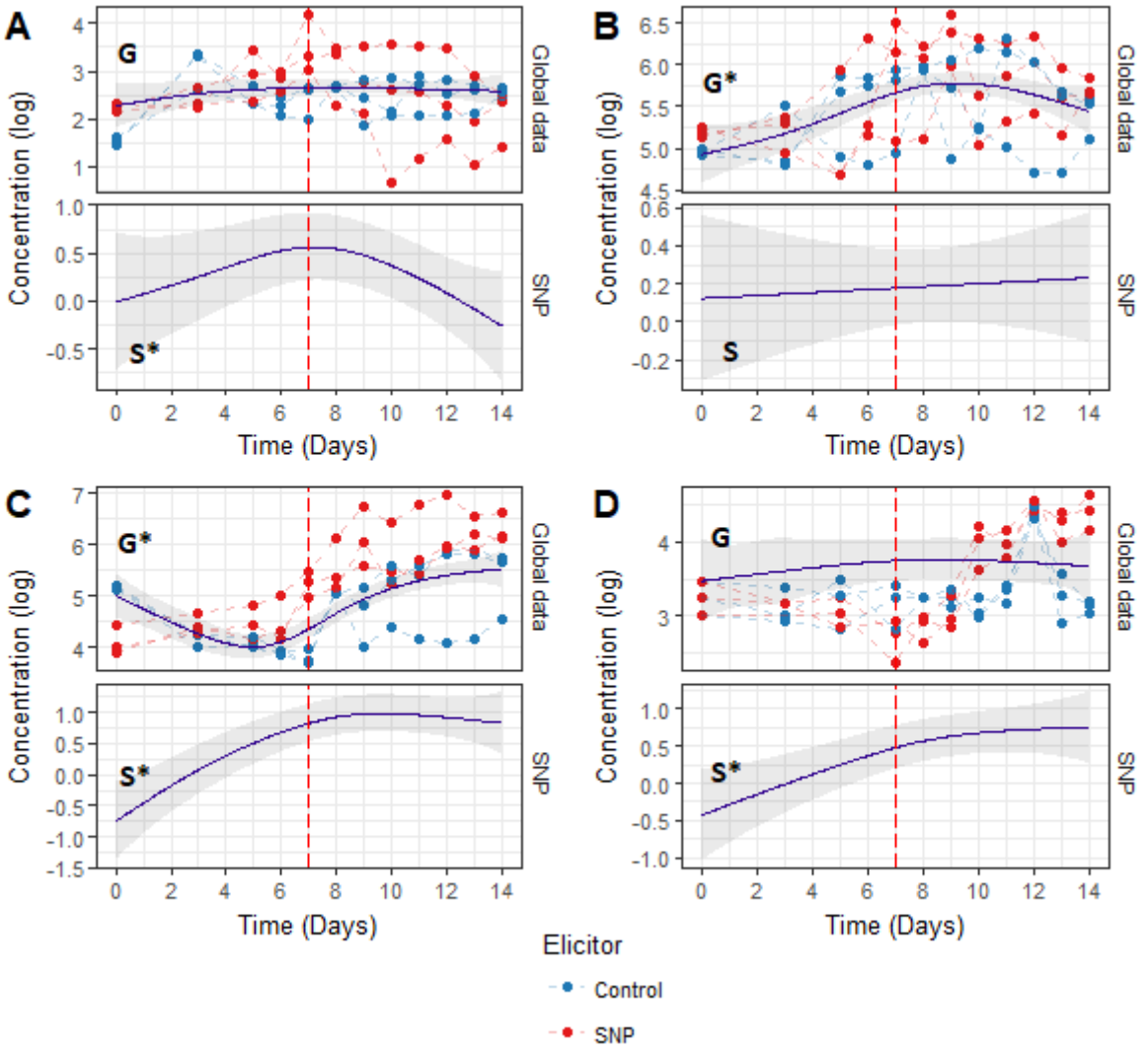

Figure 46 Time course accumulation of stilbenes in GT cell suspensions in bioreactors elicited with SNP: pallidol (A), astringin (B), isorhapontin (C), and ampelopsin D and quadrangulanin A (D). In each panel, the upper graph (global data) shows the fitted smooth of the global trend ($s_{cm}(t)$), while the lower graph (SNP) shows the fitted smooth of the SNP term ($s_{SNP}(t)$). The shaded areas of the trends are 95% confidence intervals on the fitted smoothers. The day of SNP elicitation is indicated by a red dashed line. G = global trend, S = trend of SNP term. * indicates the significance ($p < 0.01$). The concentrations are log transformed.

Table 5 Summary statistics of GAMs applied for stilbenes compounds in GT cell bioreactor cultures.

Metabolites	smoother terms	edf	F	p-value
trans-resveratrol	Global	4.500	4.954	0.0001***
	SNP	5.036	22.720	0.0001***
cis-resveratrol	Global	3.126	14.40	0.0001***
	SNP	5.180	18.54	0.0001***
pallidol	Global	1.003	0.203	0.0000***
	SNP	3.652	4.273	0.0001***
astringin	Global	2.581	4.882	0.0001***
	SNP	2.001	2.275	0.0001***
trans-piceid	Global	2.725	5.009	0.0001***
	SNP	3.075	4.749	0.0001***
cis-piceid	Global	2.725	5.009	0.0001***
	SNP	3.075	4.749	0.0001***
isorhapontin	Global	3.412	6.161	0.107
	SNP	4.116	9.130	0.0001***
trans-ε-viniferin	Global	3.883	12.44	0.0001***
	SNP	3.527	15.49	0.0001***
cis-ε-viniferin	Global	6.151	20.39	0.0001***
	SNP	2.00	45.12	0.0001***
trans- and *cis*-δ-viniferin	Global	4.711	7.687	0.0001***
	SNP	4.310	15.117	0.237
ampelopsin D + quadrangulanin A	Global	1.002	0.325	0.0001***
	SNP	3.104	7.559	0.0001***

* =p-value < 0.05, ** = p-value < 0.01, *** =p-value < 0.001

4.3.2.3. Effect of SNP on the stilbene derivatives

Stilbenes are synthesised via the phenylpropanoid pathway and STS catalyses formation of simple monomeric stilbenes (e.g. resveratrol) and they can be glycosylated, methylated or prenylated by the action of specific enzymes to form multiple complex oligomeric stilbene structures (Dubrovina and Kiselev 2017). In this study, it was of interest to investigate if the balance between these modifications of stilbenes is time dependent and/or can be affected by SNP. It can be done by modeling the ratio of monomeric resveratrols and modified stilbenes which are glycosylated, methoxylated and oligomeric stilbenes. The results of these models from the flask cultures are presented in Fig 47. For glycosylated stilbenes, the concentrations of all glycosylated stilbenes (piceid and astringin) are summed regardless of their isomers. Isorhapontin is the only methoxylated stilbene and the sum of viniferin, pallidol, ampelopsin D and quadrangularin A are used as oligomeric stilbenes. It was found that relation between the modified stilbenes and monomeric stilbenes are similar. The global trends of glycosylated (edf=5.30, F=5.568, p<0.0001), methoxylated (edf=6.582, F=13.73, p<0.0001) and oligomeric (edf=6.754, F=18.12, p<0.0001) stilbenes increased at the exponential phase and then decreased along the stationary phase of the cell growth. Instead, the differential smoothers of glycosylated (edf=2.78, F=24.683, p<0.0001), methoxylated (edf= 2.327, F=63.43, p<0.0001) and oligomeric (edf=2.000, F=71.25, p<0.0001) stilbenes shows a clear decreasing trend along the growth.

The same approach of modelling was performed for the bioreactor cultures and the results are shown in Fig 48. In contrast to the flask cultures, the global trend of the ratio of glycosylated and monomeric stilbenes (edf=1, F=18.11, p<0.0001) in bioreactors was linearly reduced and similarly the differential smoother also significantly dropped over time. Regards to the ratios of methoxylated and oligomeric stilbenes with monomeric stilbenes, the similar pattern can be found in global trends which show no changes until the exponential phase but descending thereafter. The smoothers of SNP (edf= 2.767, F=9.593, p<0.001) presented that the methoxylated ratio was decreased before elicitation but remained constant after elicitation with

SNP. Concerning with oligomeric ratio, the smoother of SNP (edf=4.799, F=10.64, p<0.001) indicated a reduction soon after elicitation with a slight increment before the end of the growth.

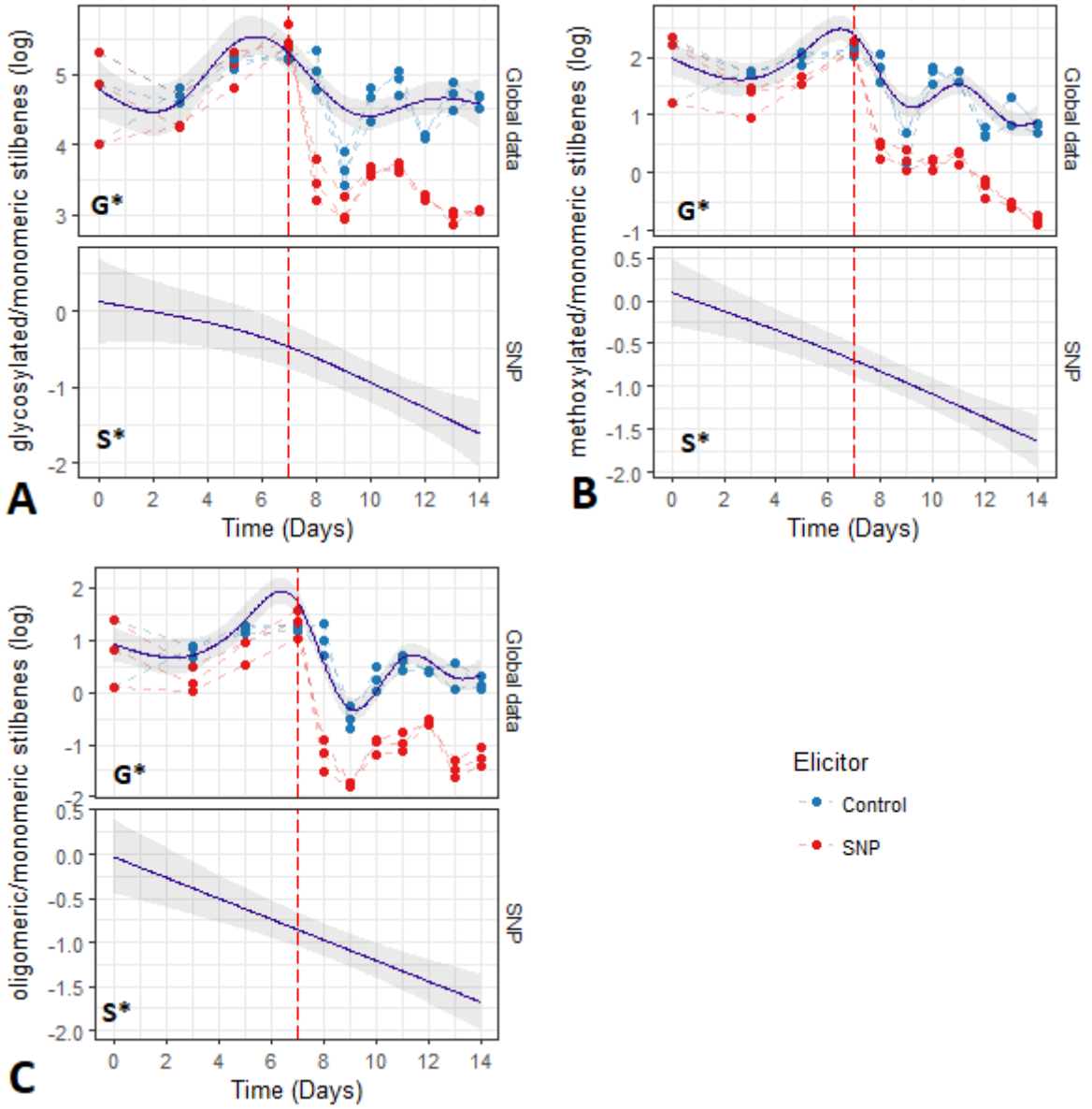

Figure 47 Changes of the ratio of glycosylated and monomeric stilbenes (A), methoxylated and monomeric stilbenes (B) and oligomeric and monomeric stilbenes (C) in GT cell cultures in flasks elicited with SNP. In each figure, panel global data shows the fitted smooth for global trend ($s_{cm}(t)$) and panel SNP shows the fitted smooth for SNP term ($s_{SNP}(t)$). The shaded areas of the trends are 95% confidence intervals on the fitted smoothers. The day of SNP elicitation is indicated by red dash line. G = global trend, S = trend of SNP term, * indicates the significance ($p < 0.001$). The ratios are log transformed.

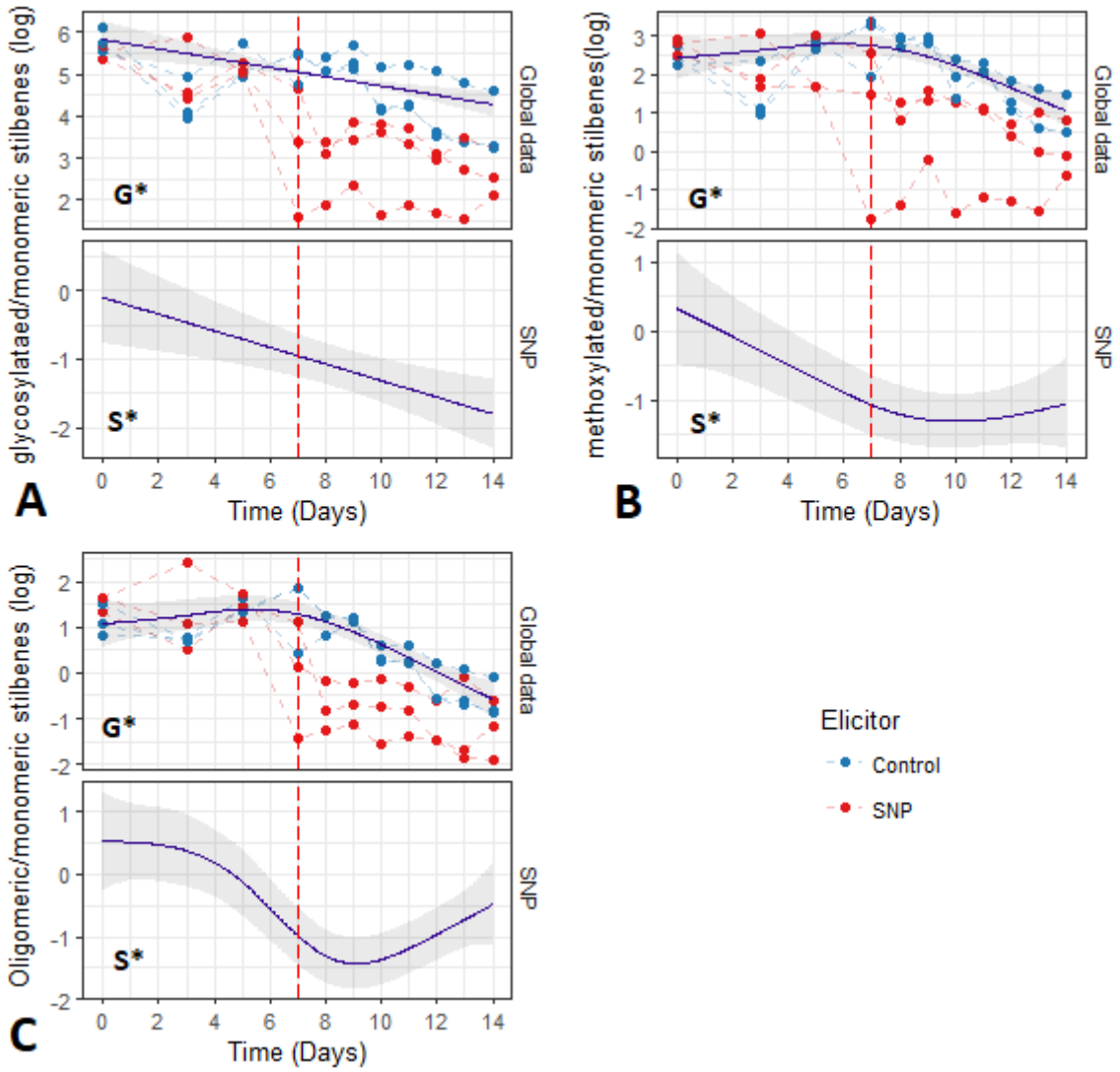

Figure 48 Changes of the ratio of glycosylated and monomeric stilbenes (A), methoxylated and monomeric stilbenes (B) and oligomeric and monomeric stilbenes (C) in GT cell cultures in bioreactors elicited with SNP. In each figure, panel global data shows the fitted smooth for global trend ($s_{cm}(t)$) and panel SNP shows the fitted smooth for SNP term ($s_{SNP}(t)$). The shaded areas of the trends are 95% confidence intervals on the fitted smoothers. The day of SNP elicitation is indicated by red dash line. G = global trend, S = trend of SNP term, * indicates the significance ($p < 0.001$). The ratios are log transformed.

4.4. Discussion

Plant secondary metabolites are regulated by multiple signalling pathways and NO is one of the key signaling molecules in elicitor-induced secondary metabolite biosynthesis in plants. SNP is the most commonly used NO donor in plant tissue culture studies because of its longest measured half-life (Floryszak-Wieczorek et al. 2006). Due to the plant species-dependent effect of NO, the used concentration of SNP ranged from 10 µM to 100 mM (B. Zhang, Zheng, and Wang 2012). In the present study the effects of SNP has been investigated on time course accumulation of stilbene compounds in GT cell cultures in flasks and bioreactors. In our experiments, we found that the supply of SNP at 100 µM revealed a long term effect on stilbene production, both in flask and bioreactor cultures.

In term of global trends, most of the stilbene production in suspension cultures of GT cells in bioreactor was similar to those obtained from flask cultures due to their same accumulation behavior over time. Indeed the high variability among biological replicates of bioreactor cultures can be considered responsible for different production rate of some stilbenes (e.g. *cis*-resveratrol) at the later stage of the cell growth. Remarkable, these accumulation patterns of stilbene compounds are different from those found in the global trends of VGR cells from the experiment with MeJA. Moreover, piceatannol was not found in GT cells as in VGR cells, while the production of resveratrols, for instance, was increased over time in this experiment, but decreasing in the experiment with MeJA. Obviously, a declined concentration of viniferins was found in SNP experiments, whereas increment of those in MeJA ones. These different patterns can be explained by the different nutrient media used in the experiments and the original source of the cell cultures. It was already demonstrated that the production of stilbene compounds can be altered by using the same cell line but in different media (Aumont et al. 2004). Nevertheless, the results in this study indicate that the stilbene production in the GT cells was not influenced by scaling-up to larger volumes in bioreactors by using the same culture medium.

Concerning SNP treatment, the biosynthesis of stilbene compounds is highly induced both in flask and bioreactor cultures, indicating that SNP could be used as a potential elicitor on the production of stilbenoids in the GT cells, even if the bioreactor data show a higher variability,

most likely due to the fact that, in this case, experiments were performed sequentially and not in parallel. The data presented in this study indicate that SNP increased and altered the accumulation of stilbenes in flask and bioreactor cultures over long time, starting from 2 days after elicitation. As compared to MeJA treatment, the different mode of action of SNP can be highlighted considering the significance of the differential smoothers. Fig 49 summarized the significant effects of MeJA in flask cultures and SNP on flask and bioreactor cultures. The detailed description of the long-term effect of MeJA in VGR cell cultures is discussed in chapter 3. Most of the stilbene compounds are not affected by MeJA treatment in VGR cell cultures. Only *cis*-resveratrol and *trans*- and *cis*- δ-viniferin showed an increasing accumulation over time while pallidol and astringin showed a decreasing trend. The time-course profiles of stilbene compounds were different in flask and bioreactor cultures after the SNP treatment. Surprisingly, the production of *trans*- and *cis*- δ-viniferin did not change over time in SNP flask cultures whereas it shows an upward trend in bioreactor cultures same as in MeJA treatment. The concentration of pallidol is decreased in the SNP treated flask cultures as opposed to the bioreactor cultures. Astringin has different accumulation patterns for all three types of cultures with reduction in MeJA treated cultures, but raising trend in SNP treated flask cultures and no significant changes in SNP treated bioreactor cultures. Although MeJA treatment has no influence on the accumulation of isorhapontin, SNP could give an opposite profile of it in the flasks and bioreactor cultures. These findings indicate that the treatment with MeJA and SNP modulate at different levels grape cells in terms of stilbene production.

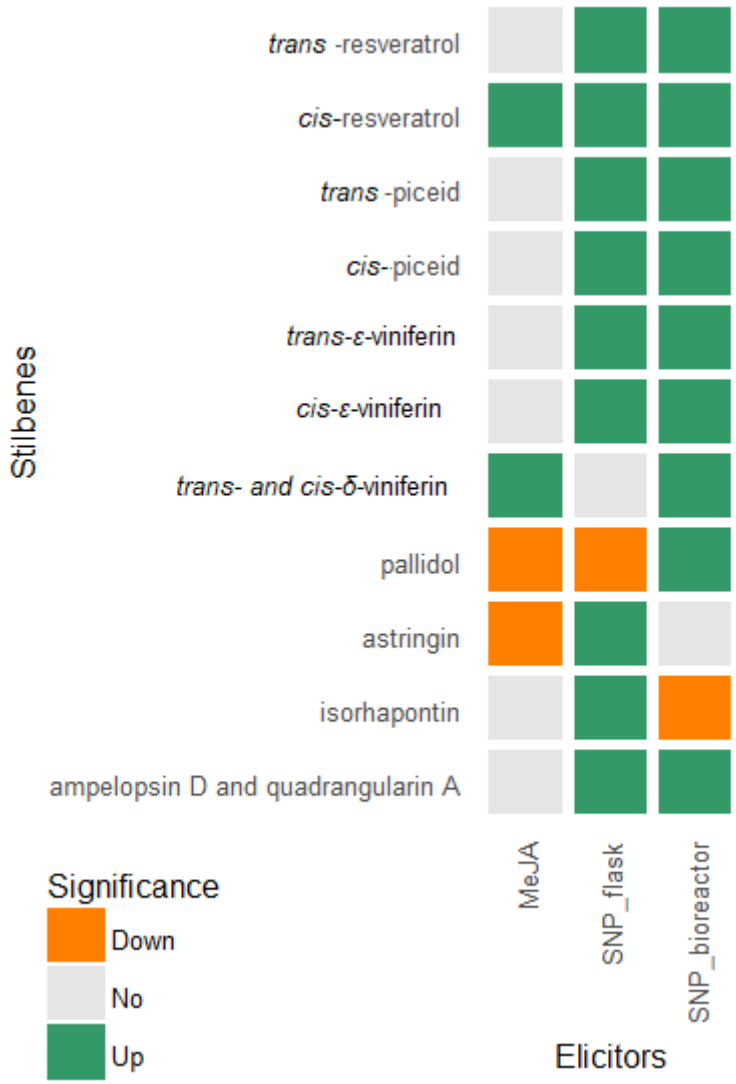

Figure 49 Summary of the significance of the differential smoother produced by GAM in MeJA and SNP treated GT cells flask and bioreactor cultures.

Stilbenes are organic phenolic compounds derived from the general phenylpropanoid pathway and the effect of SNP on this pathway towards polyphenols has been described by many researchers. The findings reported by (Modolo et al. 2002) showed that there is a close dependence between the activation of phenylpropanoid pathway and the effect of SNP in soy seedlings. Moreover, the results of studies conducted by (Floryszak-Wieczorek et al. 2006) show that the activity of PAL was activated by SNP only in the light which also plays an important role in anthocyanin biosynthesis (W. Zhang et al. 2002 and Tassoni, Durante, and Ferri 2012). It has been reported that SNP stimulated flavonoid biosynthetic enzymes responsible for phytoalexin and anthocyanin biosynthesis in soybean (*Glycine max* L.)(Suita et al. 2009). PAL initiates phenylpropanoid metabolism and CHS and CHI both are key enzymes in early

flavonoid/anthocyanin pathway (Dixon and Paiva 1995). Due to the fact that the treatment of SNP has a large impact on the PAL activity and as a consequence STS activity for stilbene production in our study was increased. However, in the study of (Duan et al. 2007) the treatment with SNP significantly inhibited PAL activity of longan fruit during storage.

SNP has been used not only to promote anthocyanins and flavonoids but also to enhance the production of phenolic compounds. When *rolB* transformed *V. amurensis* cells were treated with SNP, production of resveratrol was enhanced, indicating the importance of NO signaling pathway in resveratrol synthesis (Kiselev et al. 2007). W. Zheng et al. (2009) found that the exogenous addition SNP could increase the total mycelial phenolic (TMP) compounds accumulation as NO generation was an early response to the fungal elicitor in submerged cultures of *Inonotus obliquus* to accumulate TMP. Esringu et al. (2016) revealed that foliar spraying of 100 µM of SNP concentration to lettuce (*Lactuca sativa* L.) seedlings also resulted in a significant increase of concentration in total phenolic as compared to the control. The pre-treatment of cotton plants with SNP caused an enhancement of total phenols to alleviate the drought stress in cotton plant (Shallan et al. 2012).

The modification of monomeric stilbenes, including glycosylation, methoxylation, oligomerization and some other modifications are an important process in the regulation of stilbenes in plants. For instance, glycosylation can alter the stability of monomeric stilbenes by protecting them from enzymatic oxidation (Regev-Shoshani et al. 2003) and maintain their biological properties. That is why it is interesting to focus on the effect of SNP on the balance between monomeric and modified stilbenes. Remarkably, in the SNP treated samples, the accumulation of stilbene derivatives were not the same as the trends of monomeric stilbenes, suggesting that SNP could modulate only the main enzymes which catalyses the formation of the monomeric stilbenes that can be used as a substrate for other modified stilbenes. This phenomenon was observed both, in flasks and bioreactor cultures. Less is known about the enzymes catalysing various stilbene modifications (Dubrovina and Kiselev 2017) and the mechanism controlling the balance between monomeric stilbenes and modified stilbene derivatives are yet to be explored for understanding the signal transduction leading to stilbene production. Indeed, using SNP as NO donor can be

a promising potential for the industrial application on the production of desired secondary metabolites.

4.5. Grape cells in flasks and bioreactor cultures

In this study, the main challenge in data analysis of bioreactor experiments was the variability of the inoculum cell cultures. Due to the limited numbers of bioreactors, it was impossible to perform the experiment of control and SNP treated cultures at the same time. Since the samples were collected batch by batch, the characteristics and composition of polyphenols in the inoculum of cell culture might be different every time. This fact introduced a huge variability in the data which affected the downstream data analysis and statistical modelling, with the results that some polyphenols cannot be modelled as in flask cultures. This fact, highlights the very important point that should be taken into consideration in this type of experiments: to maximize reproducibility, the inoculum should be from the same source of cell cultures. In this case, it can be seen that the data set from the flask cultures are more reproducible.

Scaling-up plant cell cultures from flasks to bioreactor is always a challenging matter that depends on several parameters such as the type of bioreactors, aeration rate, the speed of agitation, supply of nutrients and the size of inoculum etc. (Jeandet et al. 2016). Our study described the successful production of polyphenols in GT cell cultures in stirred-tank bioreactors and the distinct time-course profiles of stilbenes were found in bioreactor cultures as compared to flask cultures.

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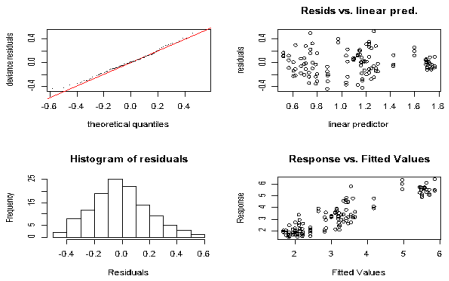
Conclusion

The main focus of this thesis was the application of statistical analysis approaches able to compare the ‘trends’ of the different metabolites. In particular, it has been shown how generalized additive models can be used to model the time-dependent profile of secondary metabolites in grapevine cell suspension cultures, following treatment with methyl jasmonate and sodium nitroprusside. Although this type of data analysis strategy has strong potential for the interpretation of the data coming from a wide class of -omic time-resolved experiments, more investigations and statistical methods, such as integration of multi-omics data, need to be made to obtain better understanding of the more complex biological mechanism and to characterize the biochemical interactions of interest organisms.

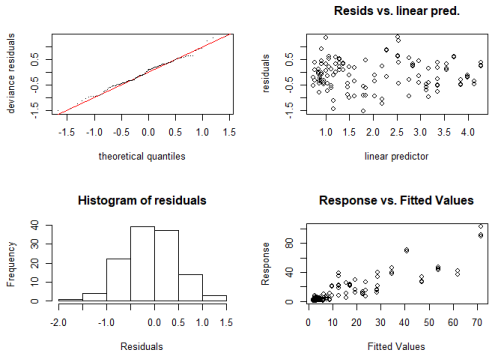
Appendix

1. Diagnosis plots of GAM models for anthocyanins in VGR cell cultures

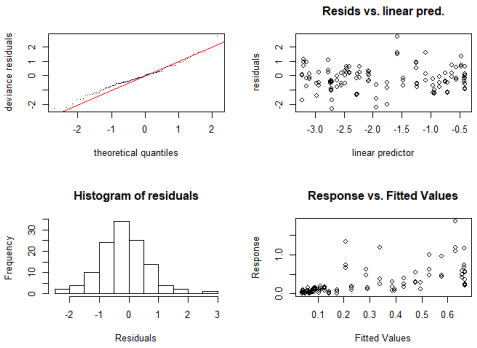
1.1 Total anthocyanins



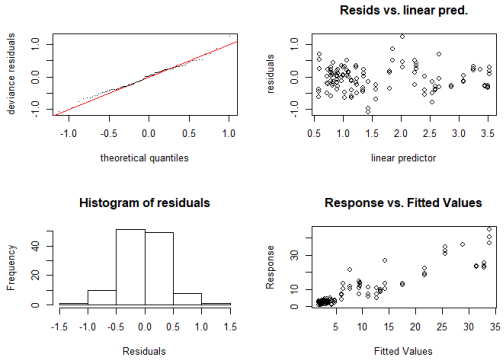
1.2 Dp-glu



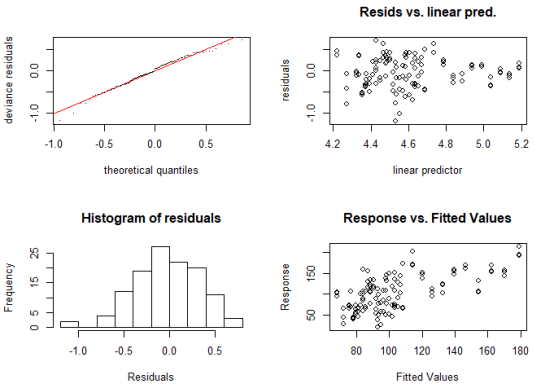
1.3 Dp-ac-glu



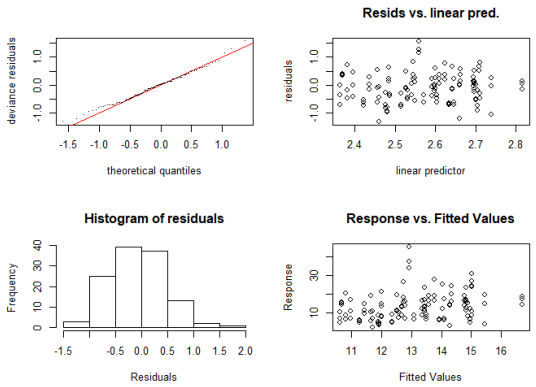
1.4 Dp-pc-glu



1.5 Cy-glu



1.6 Cy-ac-glu



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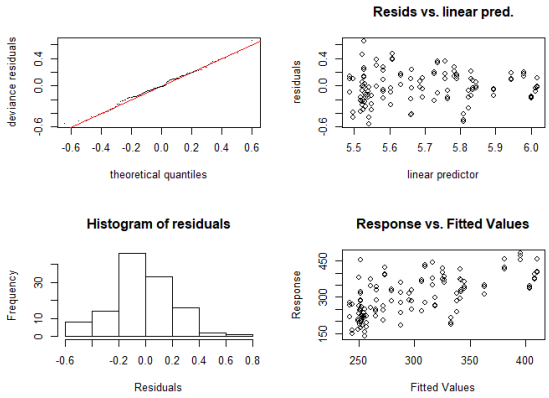
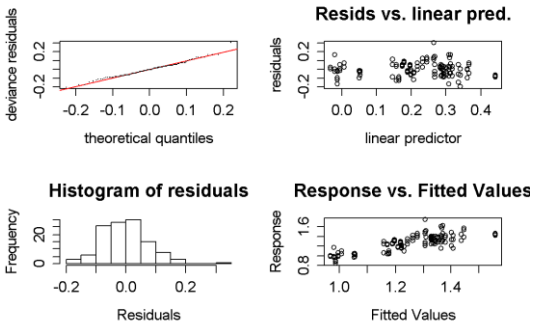
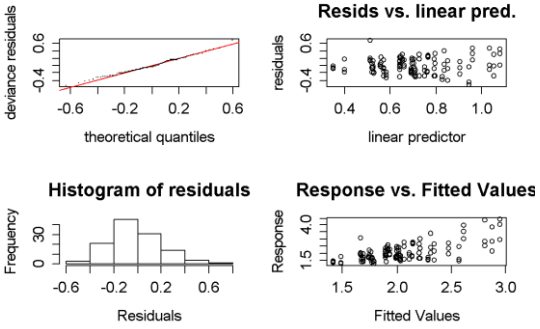


1.8 Di-hydroxylated anthocyanins (Pns/Cys)

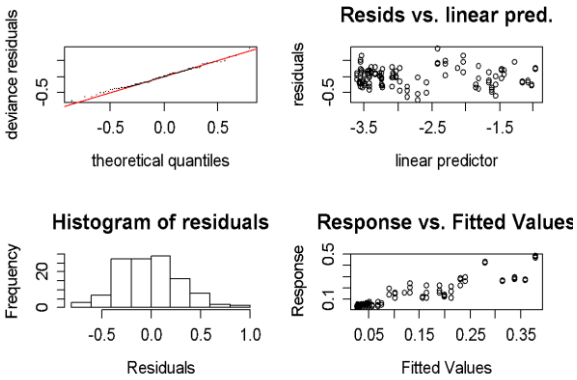


1.9 Tri-hydroxylated anthocyanins (Pts+Mvs)/Dps)

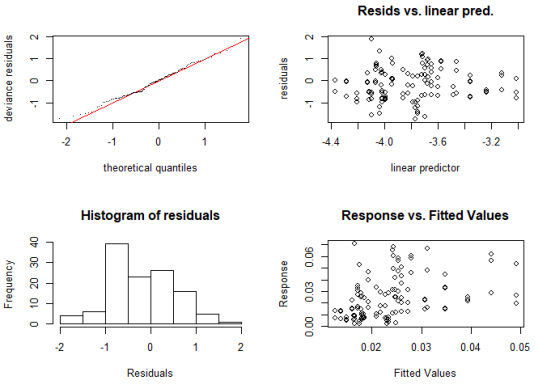


2. Diagnosis plots of GAM models for ratios of anthocyanins in VGR cell cultures

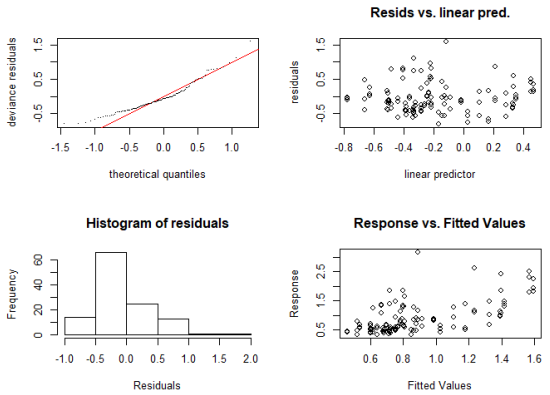
2.1 Dp-glu/Cy-gluu



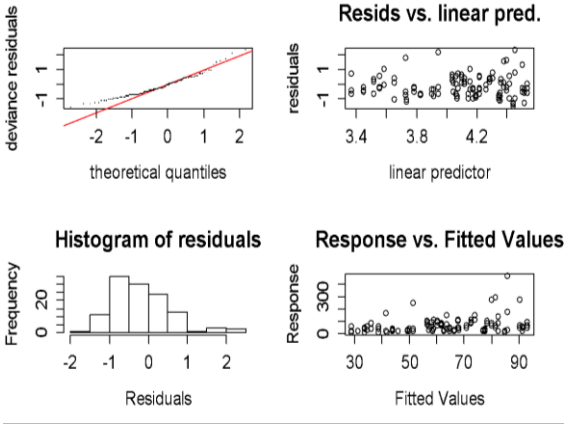
2.2 Dp-ac-glu/Dp-glu



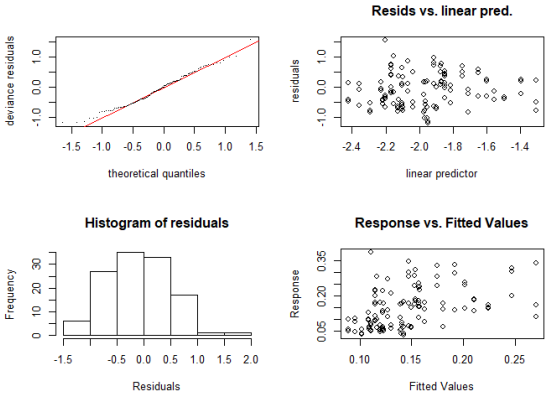
2.3 Dp-pc-glu/Dp-glu



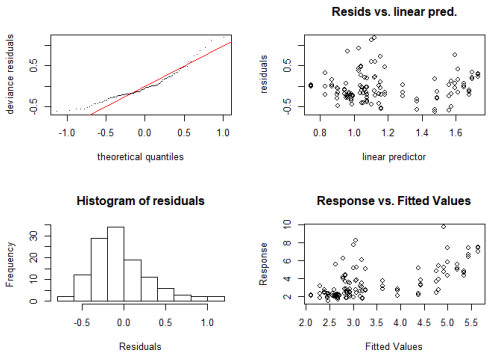
2.4 Dy-pc-glu/Dy-ac-glu



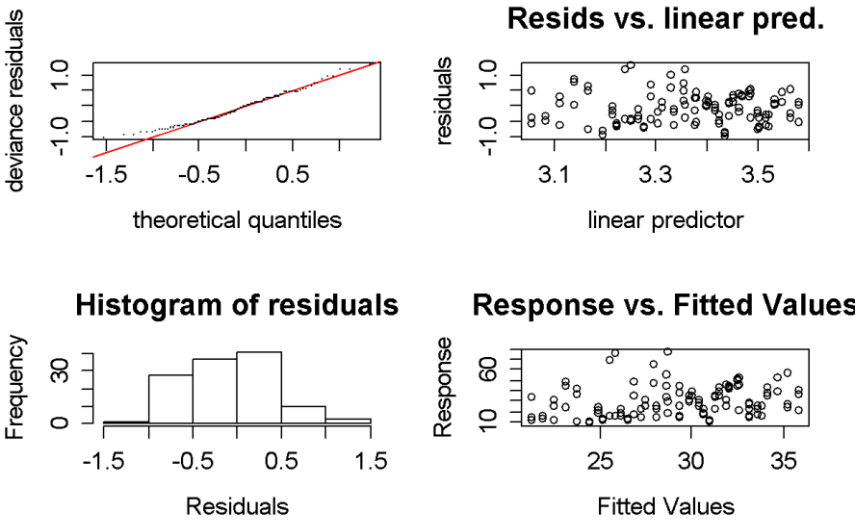
2.5 Cy-ac-glu/Cy-glu



2.6 Cy-pc-glu/Cy-glu

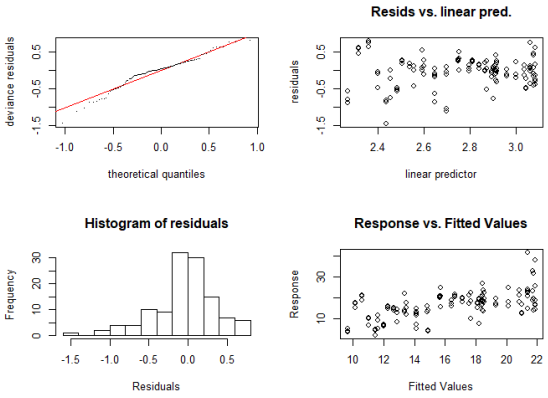


2.7 Cy-pc-glu/Cy-ac-glu

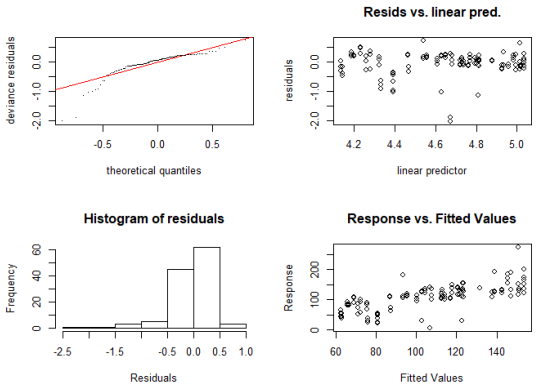


3. Diagnosis plots of GAM models for stilbenes in VGR cell cultures

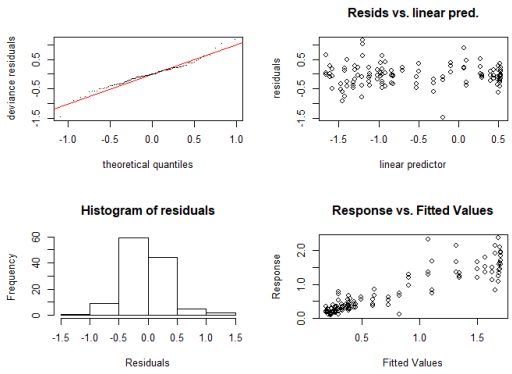
3.1 *trans*-resveratol



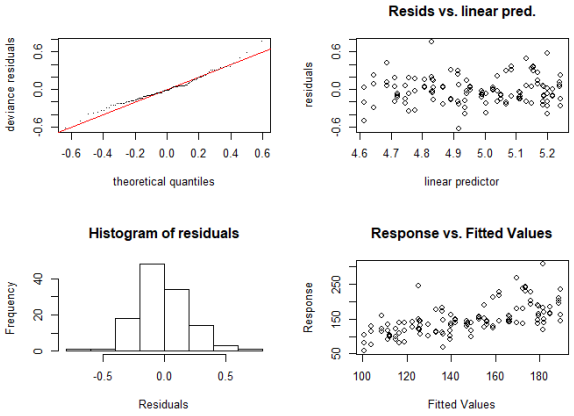
3.2 *cis*-resveratrol



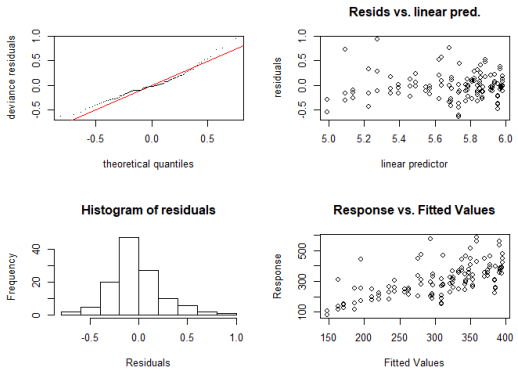
3.3 Piceatannol



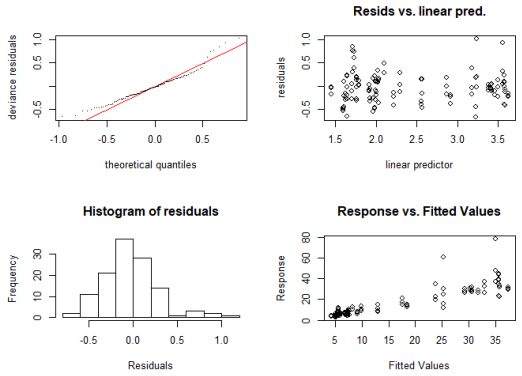
3.4 *trans*-piceid



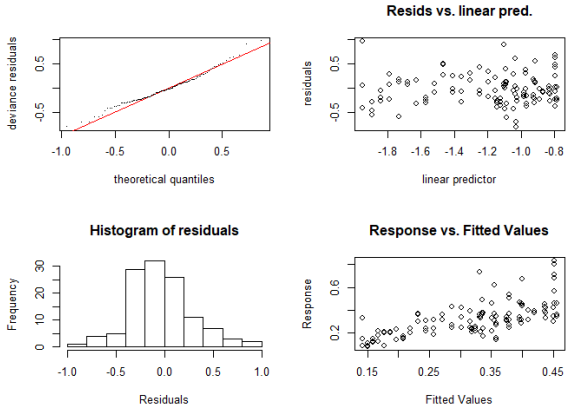
3.5 *cis*-piceid



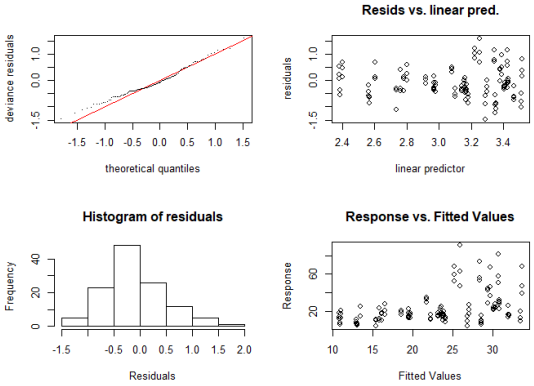
3.6 Astringin



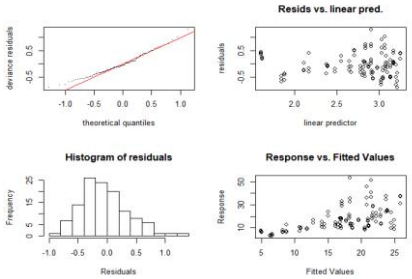
3.7 Isorhapotin



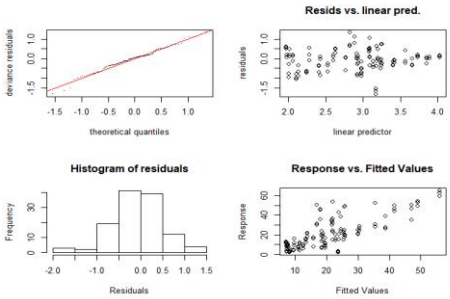
3.8 *cis*- ε-viniferin



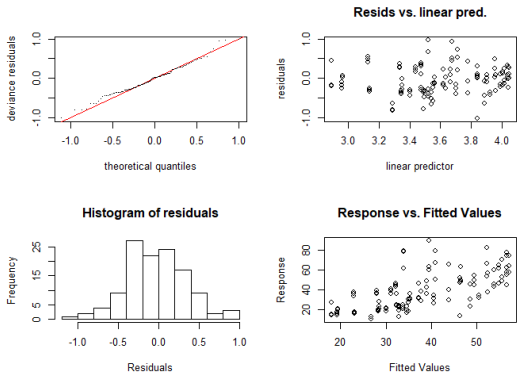
3.9 *trans*- ε-viniferin



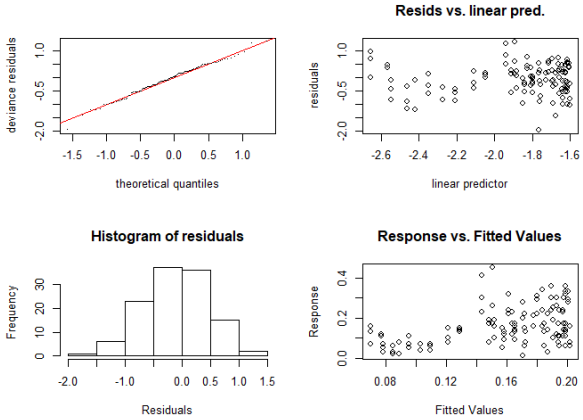
3.10 *trans*- and *cis*- *δ*-viniferin



3.11 Pallidol

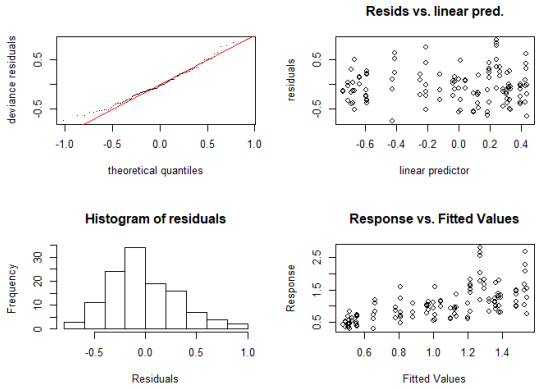


3.12 Ampelopsin D and quadrangulanin A

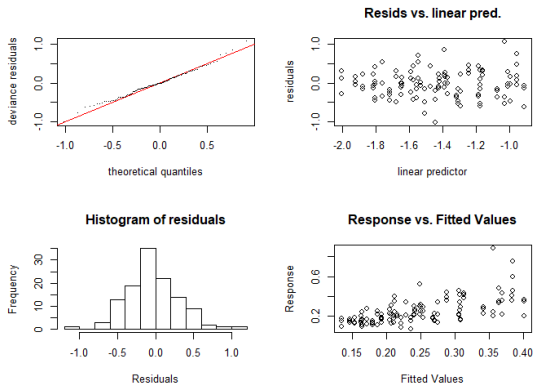


4. Diagnosis plots of GAM models for flavonoids and other polyphenols in VGR cell cultures

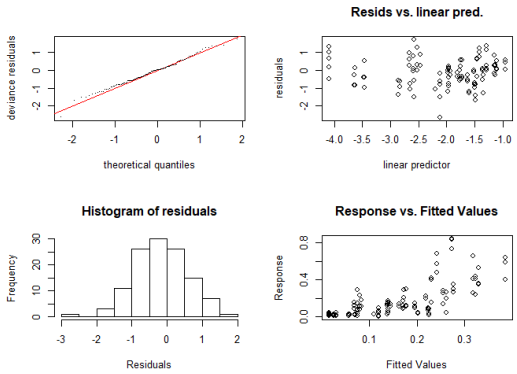
4.1 Phloridzin



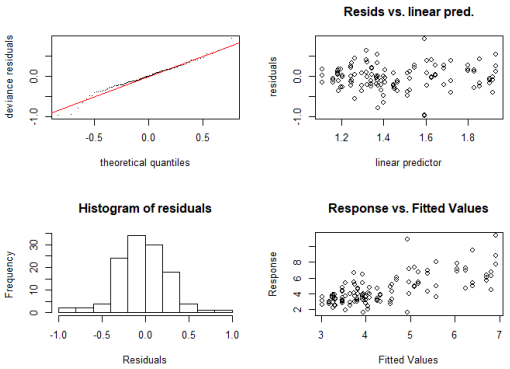
4.2 Luteolin



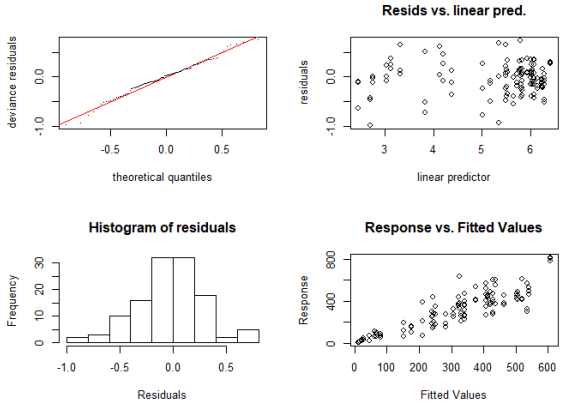
4.3 Luteolin-7-*O*-glucoside



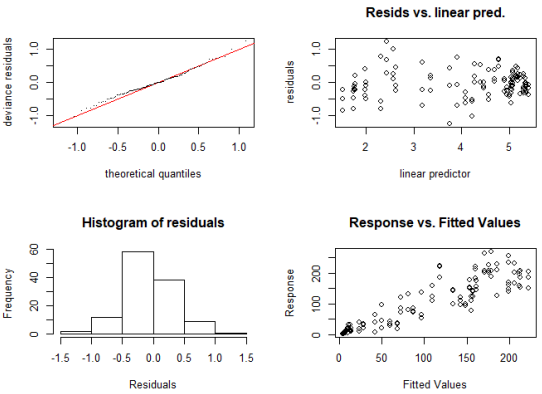
4.4 Naringenin



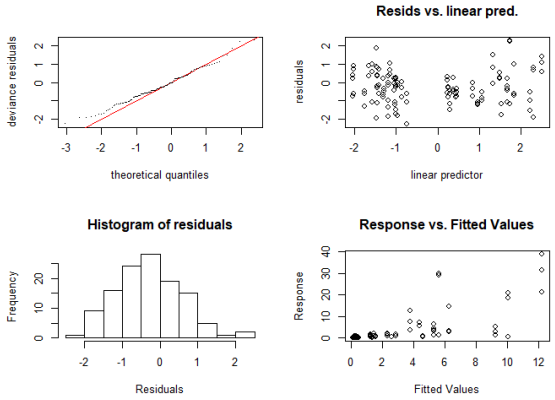
4.5 Catechin



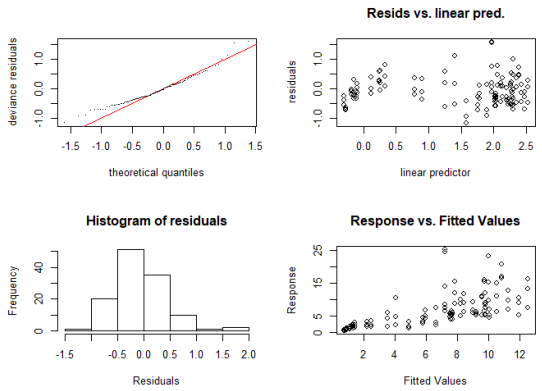
4.6 Epicatechin



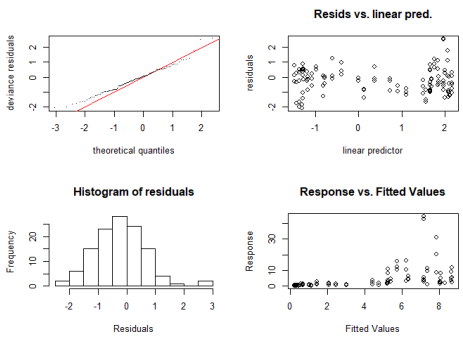
4.7 Epigallocatechin



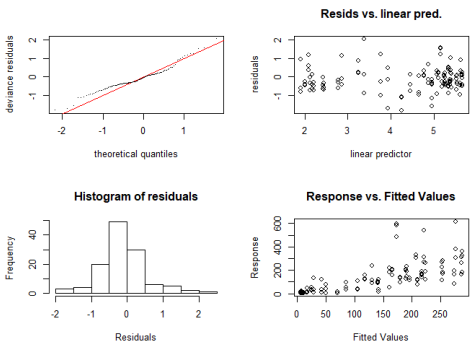
4.8 Gallocatechin



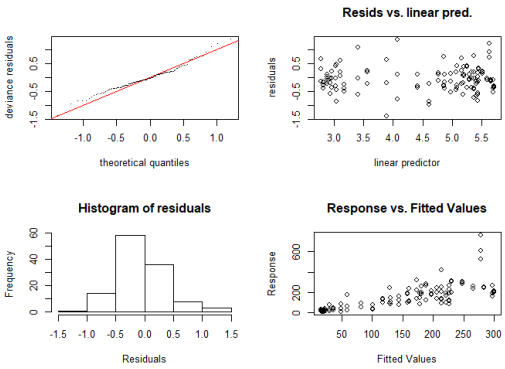
4.9 Epigallocatechin gallate



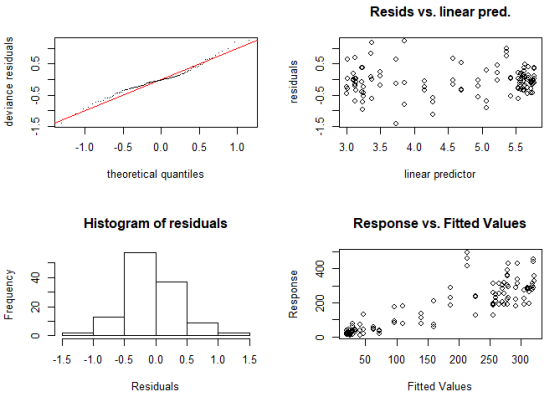
4.10 Epicatechin gallate



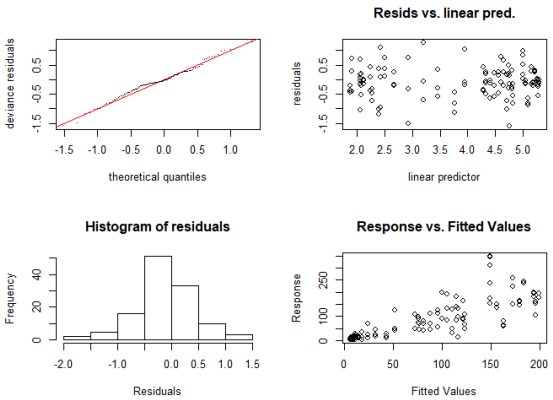
4.12 Procyanidin B1



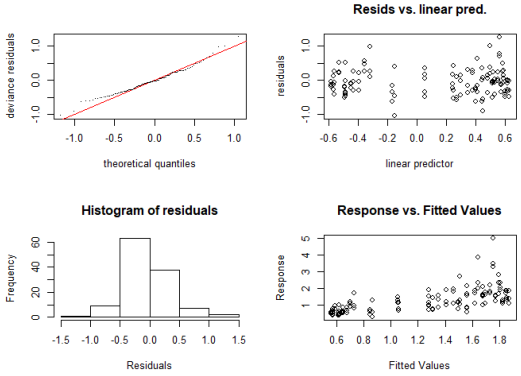
4.13 Procyanidin B2+ B4



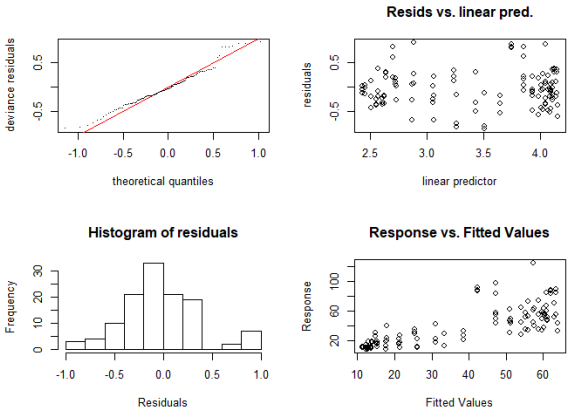
4.14 Procyanidin B3



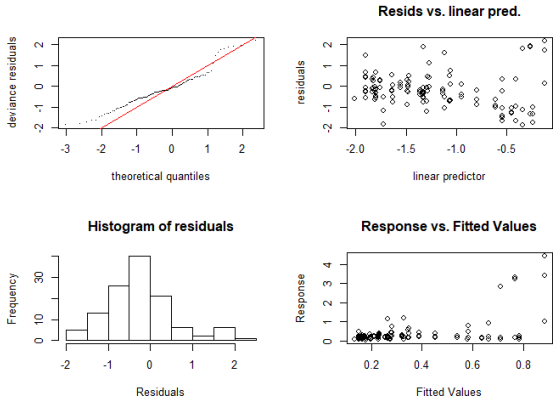
4.14 Quercetin



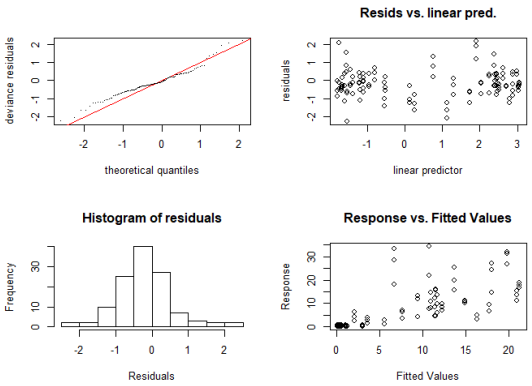
4.15 Quercetin-3-O-glucoside and quercetin-3-O-galactoside



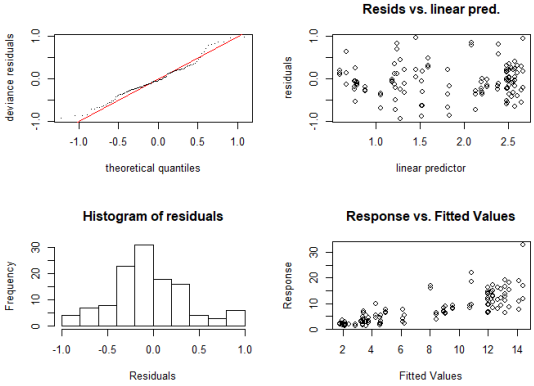
4.16 Quercetin-3-O-glucuronide



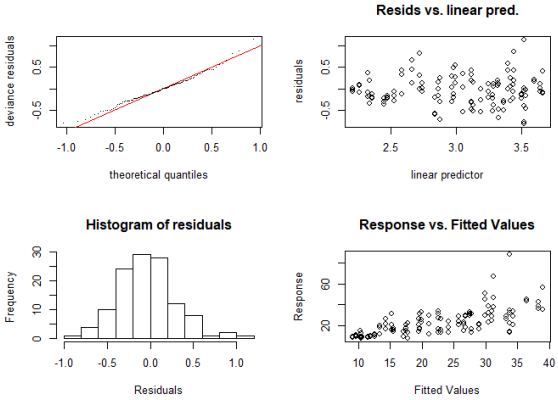
4.17 Quercetin-3-O-rutinoside (rutin)



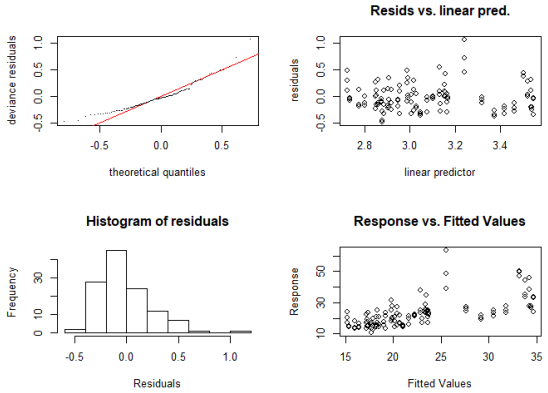
4.18 Isorhamnetin-3-O-glucoside



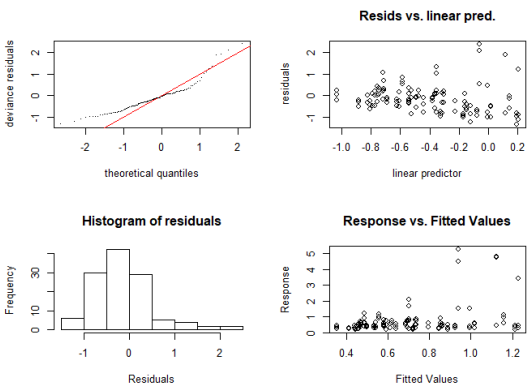
4.19 Taxifolin



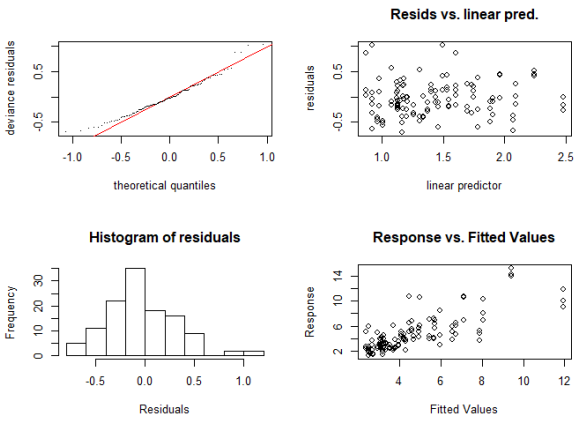
4.20 Total benzoic acid derivatives



4.21 Total phenylpropanoids

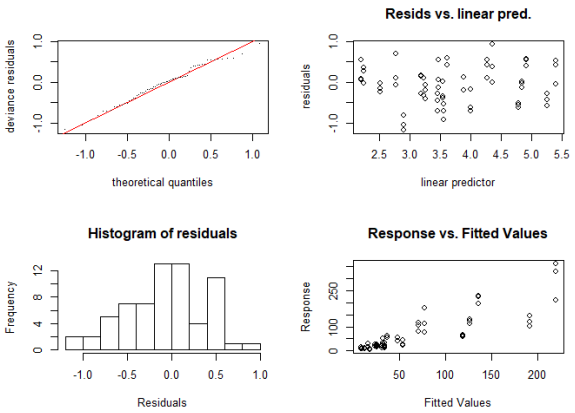


4.22 Arbutin

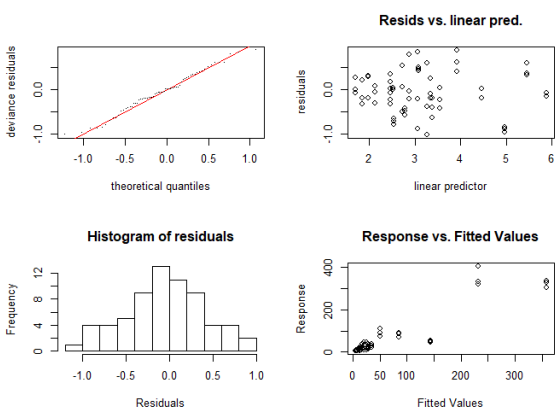


5. Diagnosis plots of GAM models for stilbenes in GT cell flask cultures

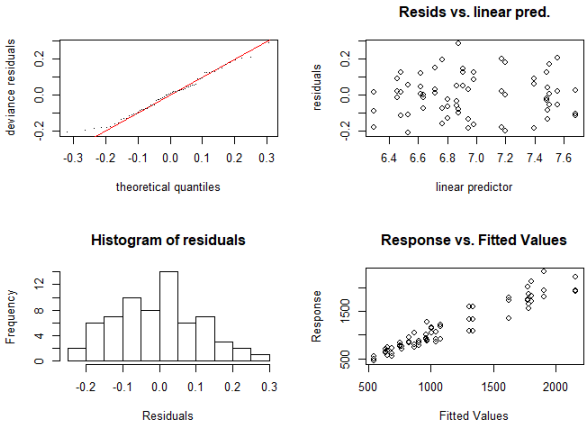
5.1 *trans*-resveratrol



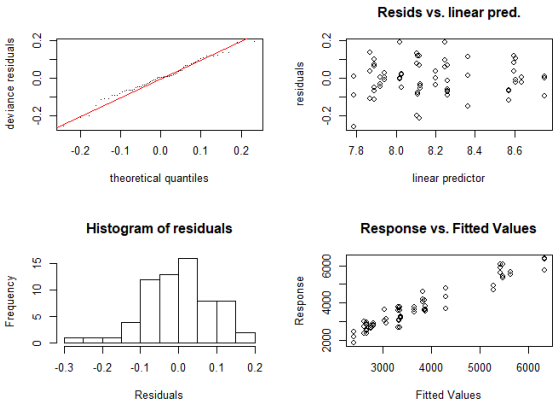
5.2 *cis*-resveratrol



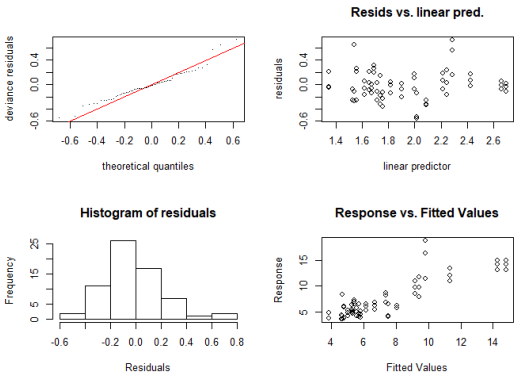
5.3 *trans*-piceid



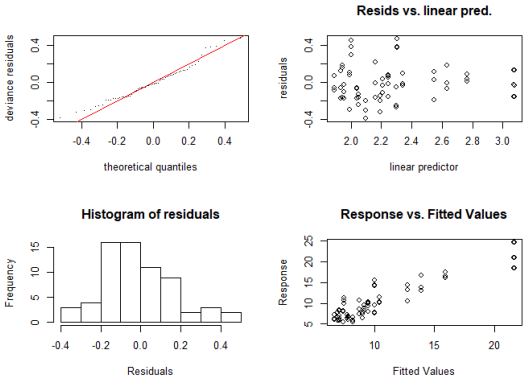
5.4 *cis*-piceid



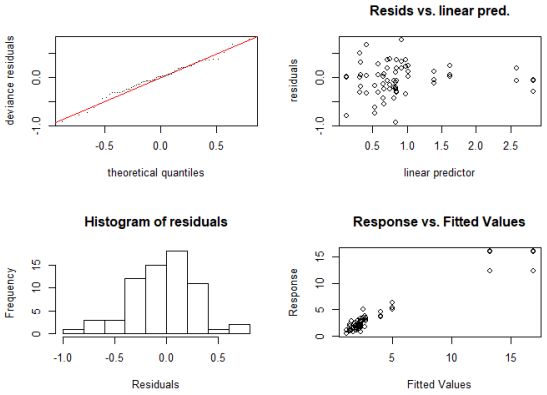
5.5 *trans*- ε-viniferin



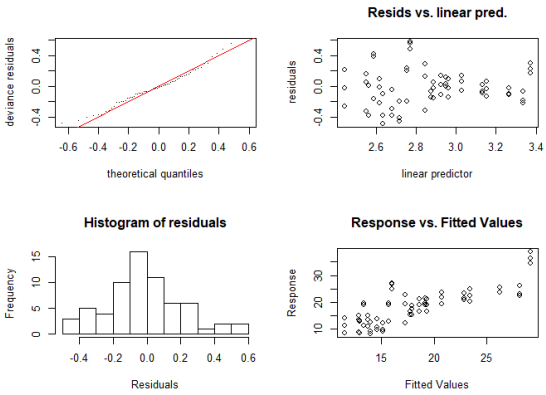
5.6 *cis*- ε-viniferin



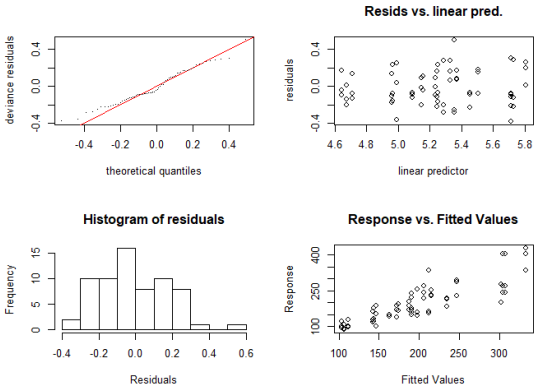
5.7 *trans*- and *cis*- *δ*-viniferin



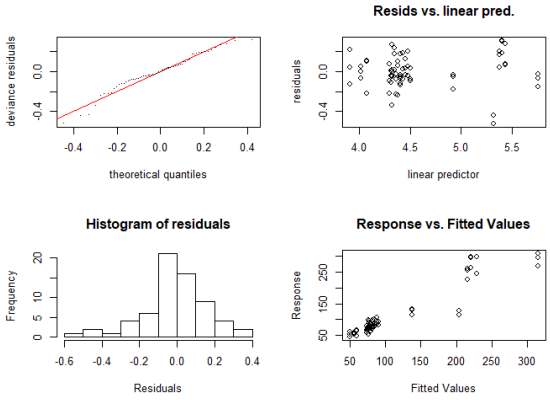
5.8 pallidol



5.9 Astringin

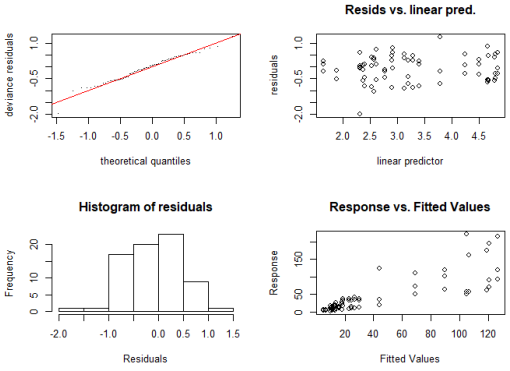


5.10 isorhapotin

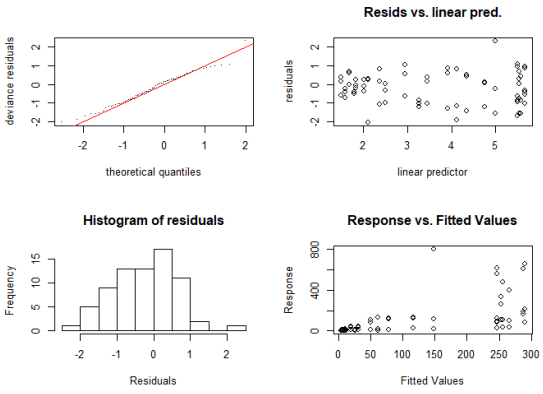


6. Diagnosis plots of GAM models for stilbenes in GT cell bioreactor cultures

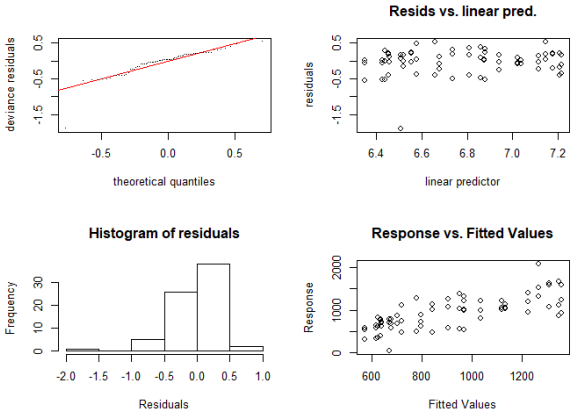
6.1 *trans*-resveratrol



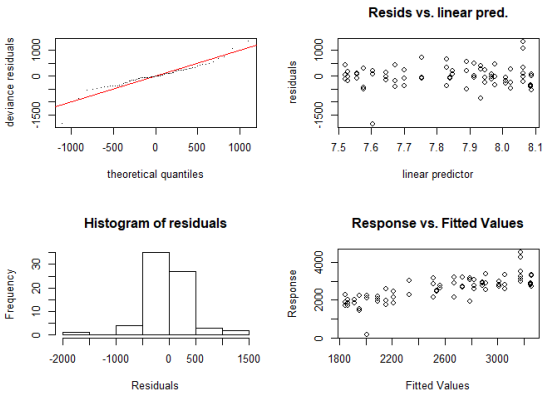
6.2 *cis*-resveratrol



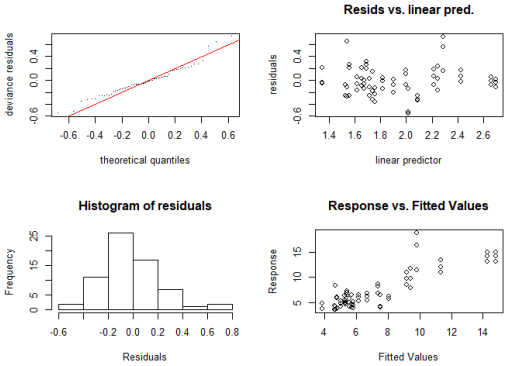
6.3 *trans*-piceid



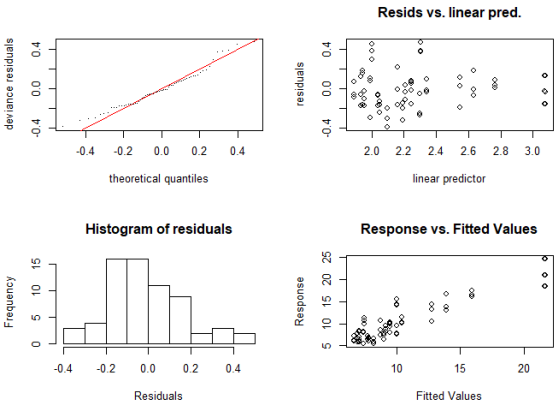
6.4 *cis*-piceid



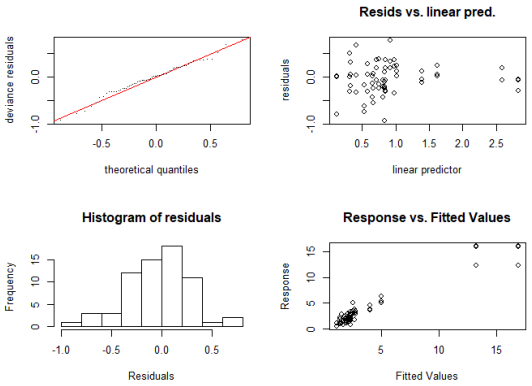
6.5 *trans*- ε-viniferin



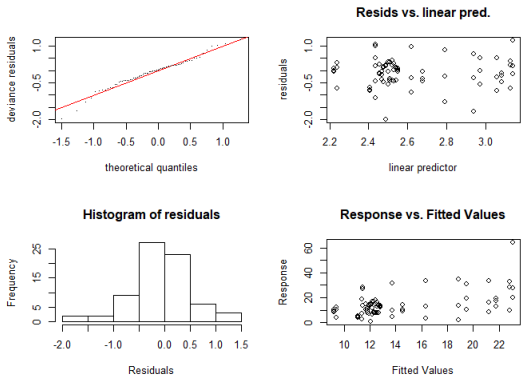
6.6 *cis*- ε-viniferin



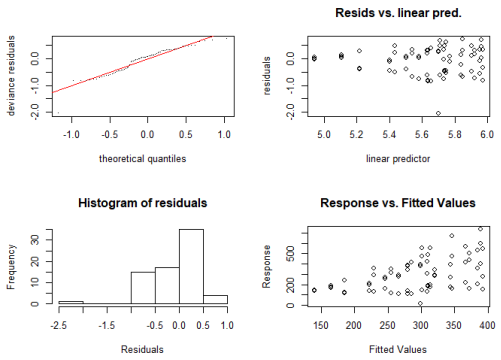
6.7 *trans*- and *cis*- *δ*-viniferin



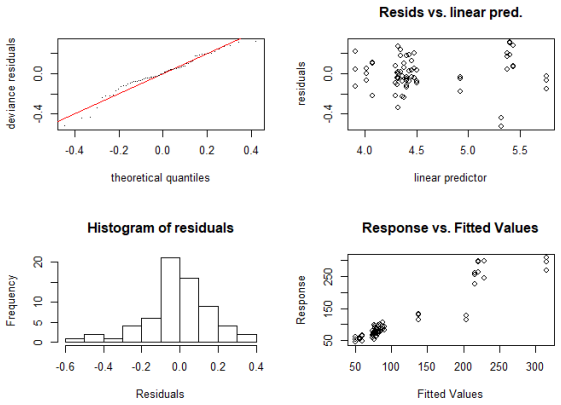
6.8 pallidol



6.9 Astringin

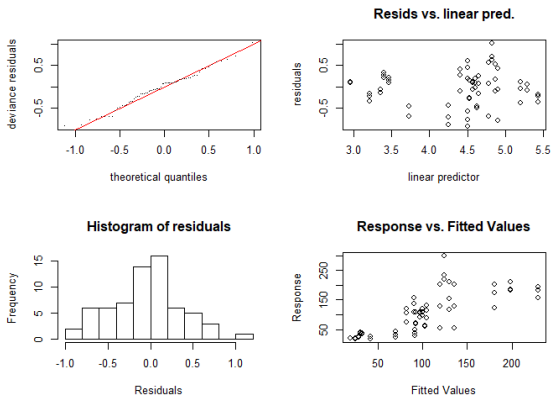


6.10 isorhapotin

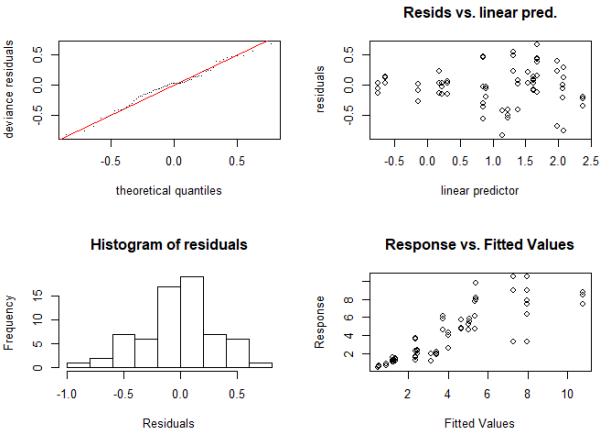


7. Diagnosis plots of GAM models for stilbenes derivatives in GT cell flask cultures

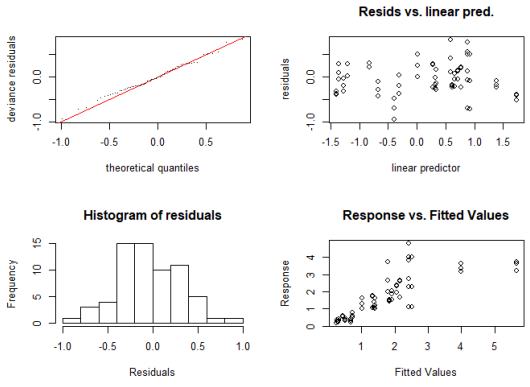
7.1 Ratio of glycosylated and monomeric stilbenes



7.2 Ratio of methoxylated and monomeric stilbenes

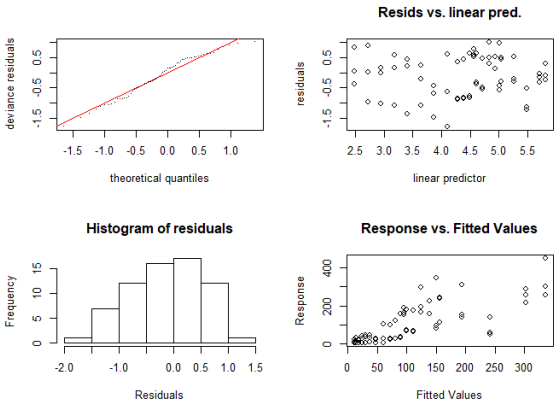


7.3 Ratio of oligomeric and monomeric stilbenes

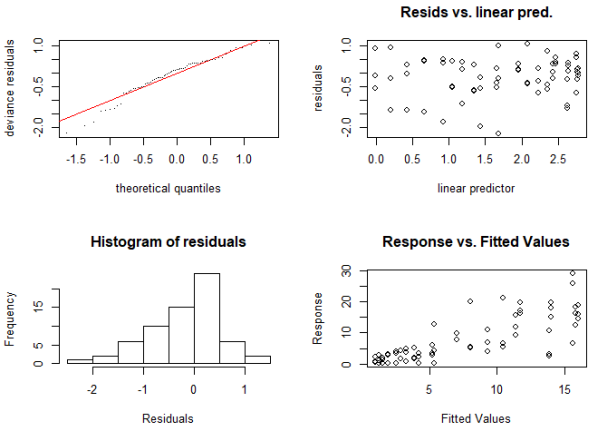


8. Diagnosis plots of GAM models for stilbenes derivatives in GT cell bioreactor cultures

8.1 Ratio of glycosylated and monomeric stilbenes



8.2 Ratio of methoxylated and monomeric stilbenes



8.3 Ratio of oligomeric and monomeric stilbenes